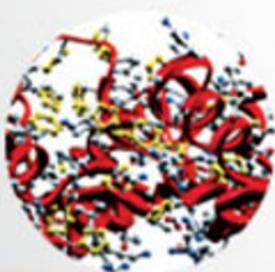


SECOND EDITION

Genomics, Proteomics and Metabolomics in Nutraceuticals and Functional Foods

Edited by
Debasis Bagchi, Anand Swaroop
and Manashi Bagchi



WILEY Blackwell

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Second Edition

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and

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Dedicated to my beloved and respected Baba Maulana Abdul Subhan

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Preface

Approximately two and half millennium ago the Father of Medicine, Hippocrates, proclaimed “Let food be thy medicine and medicine be thy food.” Hippocrates also prophesied the importance of individualized nutrition that “if we could give every individual the right amount of nourishment and exercise, not too little and not too much, we would have found the safest way to optimal health.” Today’s nutrition and nutritional sciences have repeatedly proved his immortal words and hypothesis. In the recent past, Thomas Edison concurred with Hippocrates by stating that “the doctors in future will no longer treat the human diseases with drugs, but rather will prevent diseases with nutrition.”

Nutraceuticals and functional foods have received considerable interest in the past decade, largely due to increasing consumer awareness of the health benefits associated with food and nutrition. A functional food is a fortified food material that provides medical or pharmacological benefits beyond the basic nutrients. When a functional food facilitates the prevention of certain diseases or disorders, it is a nutraceutical. The founder of the Foundation for Innovation in Medicine, Dr. Stephen DeFelice, coined the term “nutraceutical”, which combines the words nutrition and pharmaceutical emphasizing its therapeutic properties.

According to market statistics, the global functional food and nutraceutical market is growing at a rate that is outpacing the traditional processed food market. In 2012, the Council for Responsible Nutrition (CRN) reported that 68% of Americans take nutritional or dietary supplements based on the data released from its annual consumer survey. CRN further reported that this data is consistent with previous years’ statistics of 69% in 2011, 66% in 2010, and 65% in 2009. According to the results from 2012 CRN Consumer Survey on Dietary Supplements, approximately 76% users classify themselves as “regular” users, while 18% are occasional users and 6% as “seasonal” users. According to a new report by Global Industry Analysis, global nutraceutical market will cross US\$ 243 bn by 2015.

Successful completion of the Human Genome Project and advances in genomics technologies have revolutionized the field of nutrition research. Nutritional genomics or nutrigenomics provides the means for a high-throughput platform for simultaneously evaluating the expression of thousands of genes at the mRNA (transcriptomics), protein (proteomics), and metabolites (metabolomics) levels. A significant expansion has taken place in the field of genomics, proteomics and metabolomics. Today, the science of genomics has expanded to functional genomics, evolutionary genomics, comparative genomics, nutrigenomics, epigenetics, and transcriptomics, while the integrated field of proteomics has developed into great detail of protein expression profiling, peptidomics, protein complexes in terms of structure, function, properties and interactions, and metabolomics. Nutritionists also coined an interesting terminology “Foodomics” by combining functional foods and omics technologies.

The field of bioinformatics has expanded in the depth of genome analysis, sequence analysis, genetic and population analysis, phylogenetics, gene expression, database, web server, algorithms, tools, and software, with emphasis on large-scale data analysis based on high-throughput sequencing techniques. Some interesting findings were observed in the science of nutrigenomics and nanotechnology especially bio-nanotechnology, which will further expand the area to a greater extent.

The second Edition of this book has 10 major sections including (1) Introduction, (2) Genomics, (3) Proteomics, (4) Metabolomics, (5) Epigenetics, (6) Peptidomics, (7) Nutrigenomics and Human Health, (8) Transcriptomics, (9) Nutrigenomics, and (10) Nanotechnology, and a total of 48 chapters in this publication. Scientists coined a new terminology “Foodomics” and a couple interesting publications also came out in the recent past co-bonding Food and Omics technologies. The introduction section has three chapters (Chapters 1–3) explaining the key omics technology in food nutrition and applications of foodomics in seafood authentication and use of red microalgae in hypercholesterolemic activity. The second section has 14 chapters highlighting the diverse disease scenario including obesity, diabetes, arthritis and cancer with intricate aspects of genomics (Chapters 4–17). A chapter on nutrigenomics highlights the promise of a new discipline in nutrigenomics. The third section on proteomics has eight dedicated chapters discussing the diverse features and applications of proteomics in human health and nutrition science (Chapters 18–25). The aspects of Metabolomics are discussed in six dedicated chapters in the fourth section highlighting the diverse applications of metabolomics in nutrition science (Chapters 26–31). The fifth section highlights the salient features on epigenetics with nutrition, omics, and human health. There are three extensive

chapters on epigenetics (Chapters 32–34). It is really the time to explore the nutriepigenomic studies. The sixth section on peptidomics highlights the novel detection techniques of food-derived peptides in human blood and its possible application in human health (Chapter 35). The seventh section narrates down the salient features of nutrigenomics in human health in five diverse dedicated chapters on gut health, anti-inflammatory pathways, and blood glucose regulation (Chapters 36–40). The features of transcriptomics have been discussed in six chapters in the eighth section (Chapters 41–46). The ninth section covers a very important area on nutriethics (Chapter 47), while the tenth section highlights the aspects of nanotechnology (Chapter 48). The objectives of the editors and publisher are to bring out a cutting-edge book with the latest developments in the Omics field. The comprehensive reviews on nutritional genomics, proteomics, metabolomics, peptidomics, transcriptomics, epigenetics, and nutriethics were gathered together by a panel of experts from around the globe, with emphasis on the approach of these novel technologies to functional foods and nutraceuticals. We sincerely hope that the eminent readers will be greatly benefited from this second edition of the book.

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Part I

Introduction

1

Novel Omics Technologies in Food Nutrition

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1.1 Introduction

Many nutrients and non-nutrient components of foods have multiple functions. For example, fatty acids not only function as constituents of cell membrane phospholipids but also participate in numerous biochemical processes in a cell-specific and tissue-specific fashion, involving hundreds of genes, many signal transduction pathways, and a large number of biomolecules, such as transcription factors, receptors, hormones, apolipoproteins, enzymes, and so on. Hence, the measurements of single genes, single proteins, or single metabolites are not enough to provide us sufficient thorough information to understand the mechanisms that underlie the beneficial or adverse effects induced in the human body by the uptake of dietary nutrients or components. In recent years, novel omics technologies, including transcriptomics, proteomics, metabolomics, and systems biology, have received increased attention due to their power in addressing complex issues related to human health, disease, and nutrition.

Currently, in order to study the molecular basis of health effects of specific components of the diet, nutritionists are making increasing use of these state-of-the-art omics technologies (Zhang *et al.*, 2008). The term “genomics” refers to the study of all nucleotide sequences in the genome of an organism. Nutrigenomics refers to the study of the impact of specific nutrients or diets on gene expression. Note that it should not be confused with another closely related discipline “nutrigenetics”, which investigates how genetic variability influences the body’s response to a nutrient or diet. Thus, nutrigenomics and nutrigenetics approach the interplay of diet and genes from opposing start points. Transcriptomics measures the relative amounts of all messenger RNAs (mRNAs) in a given organism for determining the patterns and levels of gene expression. Proteomics is the study of all proteins expressed in a cell, tissue, or organism, including all protein isoforms and post-translational modifications. Metabolomics is defined as the comprehensive analysis of all metabolites generated in a given biological system, focusing on the measurements of metabolite concentrations and secretions in cells and tissues. It is not to be confused with “metabonomics”, which investigates the fingerprint of biochemical perturbations caused by disease, drugs, and toxins (Goodacre, 2007). Systems biology aims for simultaneous measurement of genomic, transcriptomic, proteomic, and metabolomic parameters in a given system under defined conditions. The vast amount of data generated with such omics technologies requires the application of advanced bioinformatics tools, to obtain a holistic view of the effects of the nutrients or non-nutrient components of foods, and to identify a system of biomarkers that can predict the beneficial or adverse effects of dietary nutrients or components. The ultimate goals are to understand how nutrients/foods interact with the body and the related mechanisms of action and hence to enhance health and treat diet-related diseases (Norheim *et al.*, 2012).

1.2 Transcriptomics in Nutritional Research

The classical gene analysis approach, such as Northern blotting and real-time RT-PCR, can only analyze gene expression for a limited number of candidate genes at a time. DNA microarray technology allows us to measure the expression level of thousands of genes, or even entire genomes, simultaneously. A typical DNA microarray experiment includes a number of characteristic steps:

1. RNA extraction from a sample;
2. reverse transcription of the RNA to obtain complementary DNA (cDNA) and labeling of the cDNA with specific dyes (usually fluorophores like Cyanine 3 and 5), or reverse transcription of the cDNA to obtain cRNA and labeling of the cRNA;
3. hybridization of the labeled cDNA or cRNA onto the microarray under given conditions;
4. washing the slides to remove non-hybridized labeled oligonucleotides;
5. using an appropriate scanning device to detect signal; and
6. data analysis by bioinformatics tools.

There are more and more examples of DNA microarray technology being performed in cell culture systems or laboratory animals to identify the cellular responses to dietary constituents and their molecular targets. For example, green tea catechins (McLoughlin *et al.*, 2004; Vittal *et al.*, 2004), soy isoflavones (Herzog *et al.*, 2004), polyunsaturated fatty acids (Kitajka *et al.*, 2004; Lapillonne *et al.*, 2004; Narayanan *et al.*, 2003), vitamins D and E (Johnson and Manor 2004; Lin *et al.*, 2002), quercetin (Murtaza *et al.*, 2006), arginine (Leong *et al.*, 2006), anthocyanins (Tsuda *et al.*, 2006), and hypoallergenic wheat flour (Narasaka *et al.*, 2006).

For example, Lavigne *et al.* (2008) used a DNA oligo microarray approach to examine effects of genistein on global gene expression in MCF-7 breast cancer cells. They found that genistein altered the expression of genes belonging to a wide range of pathways, including estrogen- and p53-mediated pathways. At physiologic concentrations (1 or 5 μ M), genistein elicited an expression pattern of increased mitogenic activity, while at pharmacologic concentrations (25 μ M), genistein generated an expression pattern of increased apoptosis, decreased proliferation, and decreased total cell number. Park *et al.* (2008) performed a comprehensive analysis of hepatic gene expression in a rat model of an alcohol-induced fatty liver using the cDNA microarray. It was found that chronic ethanol consumption regulated mainly the genes related to the processes of signal transduction, transcription, immune response, and protein/amino acid metabolism. For the first time, this study revealed that five genes (including beta-glucuronidase, UDP-glycosyltransferase 1, UDP-glucose dehydrogenase, apoC-III, and gonadotropin-releasing hormone receptor) were regulated by chronic ethanol exposure in the rat liver.

Furthermore, the number of microarray-based transcriptomics analysis for assessing the biological effects of dietary interventions on human nutrition and health is steadily increasing. van Erk *et al.* (2006) investigated the effect of a high-carbohydrate (HC) or a high-protein (HP) breakfast on the transcriptome of human blood cells with RNA samples taken from eight healthy men before and 2 h after consumption of the diets. About 317 genes for the HC breakfast and 919 genes for the HP breakfast were found to be differentially expressed. Specifically, consumption of the HC breakfast resulted in differential expression of glycogen metabolism genes, and consumption of the HP breakfast resulted in differential expression of genes involved in protein biosynthesis. Using GeneChip microarrays, Schäuber *et al.* (2006) examined the effect of regular consumption of the low-digestible and prebiotic isomalt and the digestible sucrose on gene expression in rectal mucosa in a randomized double-blind crossover trial with 19 healthy volunteers over 4 weeks of feeding. They revealed that dietary intervention with the low digestible isomalt compared with the digestible sucrose did not affect gene expression in the lining rectal mucosa, although gene expression of the human rectal mucosa can reliably be measured in biopsy material. Mangravite *et al.* (2007) used expression array analysis to identify the molecular pathways responsive to both caloric restriction and dietary composition within adipose tissue from 131 moderately overweight men. They found that more than 1000 transcripts were significantly downregulated in expression in response to acute weight loss. The results demonstrated that stearoyl-coenzyme A desaturase (SCD) expression in adipose tissue is independently regulated by weight loss and by carbohydrate and saturated fat intakes, and SCD and diacylglycerol transferase 2 (DGAT2) expression may be involved in dietary regulation of systemic triacylglycerol metabolism. Kallio *et al.* (2007) assessed the effect of two different carbohydrate modifications (a rye-pasta diet characterized by a low postprandial insulin response and an oat-wheat-potato diet characterized by a high postprandial insulin response) on subcutaneous adipose tissue (SAT) gene expression in 47 people with metabolic syndrome. They detected that there are rye-pasta diet downregulated 71 genes (linked to insulin signaling and apoptosis) and

oat-wheat-potato diet up-regulated 62 genes (related to stress, cytokine-chemokine-mediated immunity, and the interleukin pathway). Using microarray analysis, Niculescu *et al.* (2007) investigated the effects of dietary soy isoflavones on gene expression changes in lymphocytes from 30 postmenopausal women. They indicated that isoflavones had a stronger effect on some putative estrogen-responsive genes in equol producers than in nonproducers. In general, the gene expression changes caused by isoflavone intervention are related to increased cell differentiation, increased cAMP signaling and G-protein-coupled protein metabolism and increased steroid hormone receptor activity.

Recently, using transcriptomics, Marlow *et al.* (2013) investigated the effect of a Mediterranean-inspired diet on inflammation in Crohn's disease patients. They observed significant changes in gene expression, totally, 1902 genes were up-regulated and 1649 genes were downregulated, after a 6-week diet intervention. By Ingenuity Pathway Analysis (IPA), key canonical pathways affected by diet intervention were identified, including EIF2 signaling, B-cell development, T-helper cell differentiation, and thymine degradation. Rosqvist *et al.* (2014) performed transcriptomics to investigate liver fat accumulation and body composition after overfeeding saturated (SFA) (palm oil) or n-6 polyunsaturated (PUFA) (sunflower oil) for 7 weeks in 39 young and normal-weight individuals. The results revealed that SFA markedly increased liver fat compared with PUFA, and PUFA caused an almost three-fold increase in lean tissue than SFA. The differentially regulated genes were involved in regulating energy dissipation, insulin resistance, body composition, and fat cell differentiation.

However, there are some problems or limitations for transcriptomics approaches in nutritional research. One major problem is non-reproducibility of gene expression profiles. Different conclusions could be drawn from the same experiment but performed at different times or different labs or different platforms. Fortunately, for reducing errors or variations, standards for reporting microarray data have been established under MIAME (minimum information about a microarray experiment) (Brazma *et al.*, 2001). Barnes *et al.* (2005) evaluated the reproducibility of microarray results using two platforms, Affymetrix GeneChips and Illumina BeadArrays. The results demonstrated that agreement was strongly correlated with the level of expression of a gene, and concordance was also improved when probes on the two platforms could be identified as being likely to target the same set of transcripts of a given gene. Another major issue is the analysis of the data sets and their interpretation. Analyses only providing gene lists with significant p-values are insufficient to fully understand the underlying biological mechanisms, a single gene that is significantly upregulated or downregulated does not necessarily have any physiological meaning (Kussmann *et al.*, 2008). The combination of statistical and functional analysis is appropriate to facilitate the identification of biologically relevant and robust gene signatures, even across different microarray platforms (Bosotti *et al.*, 2007). An additional and more specific limitation in human nutritional applications is that microarray studies require significant quantities of tissues material for isolation of the needed RNA, while access to human tissues is obviously limited, although it is not impossible to obtain biopsies from a control subjects involved in a nutrition research. If using human blood cells instead of tissue material, large inter-individual variation exists in gene expression profiles of healthy individuals (Cobb *et al.*, 2005), this makes it challenging to identify robust gene expression signatures in response to a nutrition intervention. On the other hand, sample handling and prolonged transportation significantly influences gene expression profiles (Debey *et al.*, 2004), the highly standardized protocol across different labs is needed. In particular whole-blood samples require the depletion of globin mRNA for enabling detection of low-abundance transcripts. Shin *et al.* (2014) showed that the experimental globin depletion removed approximately 80% of globin transcripts, and allowed for reliable detection of thousands of additional transcripts. However, a concern is that globin depletion leads to the significant reduction in RNA yields.

1.3 Proteomics in Nutritional Research

In the last two decades, proteomics has developed into a technology for biomarker discovery, disease diagnosis, and clinical applications (Beretta, 2007; Lescuyer *et al.*, 2007; Zhang *et al.*, 2007a, b). The workflow for the proteomics analysis essentially consists of sample preparation, protein separation, and protein identification.

For the gel-based proteomics experiments, proteins are extracted from cell or tissue samples, separated by two-dimensional polyacrylamide gel electrophoresis (2D-Gel), and stained. In order to identify differences in protein content between protein samples, images of the spots on the gels can be compared. Subsequently, the protein spots of interest are excised and the proteins are digested. Last, the resulting peptides can be identified by mass spectrometry (MS). However, 2D-gel technology has many inherent drawbacks (Corthesy-Theulaz *et al.*, 2005; Kussmann *et al.*, 2005): (1) bias towards the most abundant changes, giving poor resolution for low abundant proteins, which might generate erroneous conclusions due to the fact that subtle variation may lead to important changes in metabolic pathways; (2) inability to detect proteins with extreme properties (very small, very large, very hydrophobic, and very acidic or basic proteins); and (3) difficulty in identification of the proteins, time-consuming and costly.

Instead of the gel approaches, chromatography-based techniques have been developed for protein/peptide separation, such as gas chromatography (GC), liquid chromatography (LC). When these separation technologies are combined with MS or tandem MS (MS/MS), the superior power of MS in the proteomic analysis is greatly enhanced. The mostly used MS instruments for proteomics experiments are ESI-MS (electrospray ionization MS), MALDI-TOF-MS (matrix-assisted laser desorption ionization with a time-of-flight MS) and its variant SELDI-TOF-MS (surface-enhanced laser desorption ionization with a time-of-flight MS). In addition, FTICR-MS (Fourier transform ion cyclotron resonance MS) is an increasingly useful technique in proteomic research, which provides the highest mass resolution, mass accuracy, and sensitivity of present MS technologies, although its relatively expensive (Bogdanov and Smith, 2005).

In recent years, there have been exponentially increasing numbers of publications on the application of proteomic techniques to nutrition research (Griffiths and Grant, 2006), but many investigations were performed in animal models (Breikers *et al.*, 2006; de Roos *et al.*, 2005; Kim *et al.*, 2006). Limited proteomics analysis in humans was involved in identifying the molecular target of dietary components in human subjects. For example, proteomic analysis of butyrate-treated human colon cancer cells (Tan *et al.*, 2002), and identification of molecular targets of quercetin in human colon cancer cells (Wenzel *et al.*, 2004), the identification of cellular target proteins of genistein action in human endothelial cells (Fuchs *et al.*, 2005). Smolenski *et al.* (2007) applied 2D-gel and MALDI-TOF-MS identified 15 proteins that are involved in host defense. Batista *et al.* (2007) employed 2D-gel and the MS method to identify new potential soybean allergens from transgenic and non-transgenic soy samples. Similarly, a proteomic analysis method based on 2D-gel and MALDI-TOF-MS was used to characterize wheat flour allergens and revealed that nine subunits of glutenins are the most predominant IgE-binding antigens (Akagawa *et al.*, 2007). Fuchs *et al.* (2007) conducted the proteomic analysis of human peripheral blood mononuclear cells (PBMC) from seven healthy men after a dietary flaxseed-intervention. The results showed that flaxseed consumption affected significantly the steady-state levels of 16 proteins, including enhanced levels of peroxiredoxin, reduced levels of the long-chain fatty acid beta-oxidation multienzyme complex and reduced levels of glycoprotein IIIa/II. PBMCs are an important sample for monitoring dietary interventions and are accessible with little invasive means. Vergara *et al.* (2008) have established a public 2-DE database for human peripheral blood mononuclear cells (PBMCs) proteins, which have the potentiality of PBMCs to investigate the proteomics changes possibly associated with food or drug interventions.

Recently, Bachmair *et al.* (2012) evaluated the effect of supplementation with an 80:20 *cis*-9,*trans*-11 conjugated linoleic acid blend on the human platelet proteome. Forty differentially regulated proteins were identified by LC-ESI-MS/MS, which participate in regulation of the cytoskeleton and platelet structure, as well as receptor action, signaling, and focal adhesion. Keeney *et al.* (2013) examined the effect of vitamin D (VitD) on brain during aging from middle to old age. Proteomics analysis revealed that several brain proteins were significantly elevated in the low-VitD group compared to the control and high-VitD groups, such as 6-phosphofructokinase, triose phosphate isomerase, pyruvate kinase, peroxiredoxin-3, and DJ-1/PARK7. This demonstrates that dietary VitD deficiency contributes to significant nitrosative stress in brain and may promote cognitive decline in middle aged and elderly adults. Qiu *et al.* (2013) applied quantitative proteomics to investigate the effects of lycopene on protein expression in human primary prostatic epithelial cells. The proteins that were significantly upregulated or downregulated following lycopene exposure were identified, which were involved in antioxidant responses, cytoprotection, apoptosis, growth inhibition, androgen receptor signaling, and the Akt/mTOR cascade. This suggests the preventive role of lycopene in prostate cancer.

In any proteomic study aiming for biomarker discovery a critical question is “how much of a given protein is present at a given time in a given condition?” Now a number of quantitative proteomic techniques have been developed, such as 2D DIGE (difference gel electrophoresis), ICAT (isotope-coded affinity tag), iTRAQ (isobaric tags for relative and absolute quantification), and proteolytic O-18-labeling strategies (Chen *et al.*, 2007a; Miyagi *et al.*, 2007). Wu *et al.* (2006) conducted the comparative study of three methods (DIGE, ICAT, and iTRAQ) and demonstrated that all three techniques yielded quantitative results with reasonable accuracy, although iTRAQ is most sensitive than DIGE and ICAT. Due to the fact that these methods displayed limited overlapping among the proteins identified, the complementary information obtained from different methods should potentially provide a better understanding of biological effects of dietary intervention. However, there are still some potential problems: the protein comigration problem for DIGE, cysteine-content bias for ICAT and susceptibility to errors in precursor ion isolation for iTRAQ. It is noted that all quantification approaches discussed so far deliver relative quantitative information. Moreover, absolute or stoichiometric quantification of proteome is becoming feasible, in particular, with the development of strategies with isotope-labeled standards composed of concatenated peptides. On the other hand, remarkable progress has also been made in label-free quantification methods based on the number of identified peptides (Gerber *et al.*, 2003; Kito and Ito, 2008; Old *et al.*, 2005). To date, few sample of quantitative proteomics analysis in nutritional research is available. For example, using DIGE and MALDI-MS/MS,

Alm *et al.* (2007) performed proteomic variation analysis within and between different strawberry varieties. They found that biological variation was more affected by different growth conditions than by different varieties, the amount of strawberry allergen varied between different strawberry varieties, and the allergen content in colorless (white) strawberry varieties was always lower than that of the red ones. However, only three proteins were the same among the proteins correlated with allergen and the color and this means that it is possible to breed a strawberry with low amount of allergen. Thus, the proteomic-based method has the potential to be used for variety improvement of fruit and vegetables.

Furthermore, protein microarray technology is a promising approach for proteomics, which can be used to detect changes in the expression and post-translational modifications of hundreds or even thousands of proteins in a parallel way. Its advantages include high sensitivity, good reproducibility, quantitative accuracy, and parallelization. The details of protein microarray method are described in recent review (Kricka *et al.*, 2006). Protein microarray platforms should open new possibilities to gain novel insight into the molecular mechanisms underlying nutrient-gene or nutrient-drug interactions (such as grapefruit-cyclosporine interaction). Puskas *et al.* (2006) applied the Panorama protein microarray to analyze the cholesterol diet-induced protein expression and found that a different phosphorylation pattern could be detected as well. Lin *et al.* (2007) showed that coupling the diversity of protein array with the biological output of basophilic cells was able to detect allergic sensitization. This is of great interest in nutrition research.

1.4 Metabolomics in Nutritional Research

Changes in mRNA concentration do not necessarily result in changes in cellular protein levels, and changes in protein levels may not always cause changes in protein activity. Metabolites represent the real endpoints of gene expression. Thus, alterations in the concentrations of metabolites may be better suited to describe the physiological regulatory processes in a biological system and may be a better measure of gene function than the transcriptome and proteome. Biological effects in nutrition cannot be reduced to the action of a single molecule but actually result from the modulation of many metabolic pathways at the same time, which is the product of a complex interplay between multiple genomes represented by the mammalian host and its gut microflora, and environmental factors (e.g., food habits, diet composition, and other lifestyle components) (Nicholson *et al.*, 2004; Rezzi *et al.*, 2007a). Metabolomics in nutrition has already delivered interesting insights to understanding the metabolic responses of humans or animals to dietary interventions.

The workflow for metabolomics involves a tandem use of analytical chemistry techniques to generate metabolic profiles and various bioinformatics tools to extract relevant metabolic information. Currently, the widely used tool for metabolomics experiments in nutrition research is proton nuclear magnetic resonance (NMR) technology. For example, the determination of metabolic effect of vitamin E supplementation in a mouse model of motor neuron degeneration (Griffin *et al.*, 2002); the evaluation of biochemical effects following dietary intervention with soy isoflavones in five healthy premenopausal women (Solanky *et al.*, 2003); the detection of human biological responses to different diets (e.g., chamomile tea, Wang *et al.*, 2005; or vegetarian, low meat, and high meat diets, Stella *et al.*, 2006); the characterization of the metabolic variability due to different populations (e.g., American, Chinese, and Japanese – Dumas *et al.*, 2006a; or Swedish and British populations – Lenz *et al.*, 2004). Bertram *et al.* (2007) employed a NMR-based metabolomic method to investigate biochemical effects of a short-term high intake of milk protein or meat protein on 8-year-old boys; this was the first report to demonstrate the capability of proton NMR-based metabolomics in identifying the overall biochemical effects of consumption of different animal proteins. They found that the milk diet increased the urinary excretion of hippurate, while the meat diet increased the urinary excretion of creatine, histidine, and urea. Moreover, based on NMR analysis of serum, the results demonstrated that the milk diet slightly changed the lipid profile of serum, but the meat diet had no effect on the metabolic profile of serum. Fardet *et al.* (2007) investigated the metabolic responses of rats fed whole-grain flour (WGF) and refined wheat flour (RF) using a NMR-based metabolomic approach. The results showed that some tricarboxylic acid cycle intermediates, aromatic amino acids, and hippurate were significantly increased in the urine of rats fed the WGF diet. Moazzami *et al.* (2011) evaluated the effects of a whole grain rye and rye bran diet on the metabolic profile of plasma in prostate cancer patients using ¹H NMR-based metabolomics. They found that five metabolites were increased after rye bran product (RP), including 3-hydroxybutyric acid, acetone, betaine, N,N-dimethylglycine, and dimethyl sulfone. This suggests a shift in energy metabolism from anabolic to catabolic status. Rasmussen *et al.* (2012) assessed the effect of high or low protein diet on the human urine metabolome by ¹H NMR and chemometrics. The results showed that citric acid was increased by the low (LP) protein diet, while urinary creatine was increased by the high (HP) protein diet.

Another exciting and powerful tool for metabolomics is MS-based technology. The main advantage of MS technique is its high sensitivity and rapid determination of mass or structure information. MS instruments in combination with

some separation technologies (such as gas or liquid chromatography, GC or LC, or capillary electrophoresis, CE) can quantitatively profile molecular entities like lipids, amino acids, bile acids, and other organic solutes at high sensitivity (Fiehn *et al.*, 2000; Watkins and German, 2002). A typical MS-based metabolomics system is the HPLC system using sub-2- μ m packing columns combined with high operating pressures (UPLC technology). Compared with conventional HPLC-TOF-MS systems using 3–5- μ m packing columns, UPLC-TOF-MS systems allow a remarkable decrease of the analysis time, higher peak capacity, and increased sensitivity. Recently, a number of applications of MS-based metabolomics to nutritional research have been reported. For example, a HPLC-TOF-MS-based study of changes of urinary endogenous metabolites associated with aging in rats (Williams *et al.*, 2005); a noninvasive extractive ESI-Q-TOF-MS for differentiation of maturity and quality of bananas, grapes, and strawberries (Chen *et al.*, 2007b); and combined GC-MS and LC-MS metabolic profiling for comprehensive understanding of system response to aristolochic acid intervention in rats (Ni *et al.*, 2007).

Recently, Tulipani *et al.* (2011) examined urinary changes in subjects with metabolic syndrome following 12-week nut consumption by an HPLC-Q-TOF-MS-driven nontargeted metabolomics approach. Twenty potential markers of nut intake were identified, including fatty acid conjugated metabolites, microbial-derived phenolic metabolites, and serotonin metabolites. Through employing urinary metabolic-profiling analysis based on UPLC coupled with quadrupole time-of-flight tandem mass spectrometry, Wang *et al.* (2013) identified reliable biomarkers of calcium deficiency from the rat model. In particular, significant correlations between calcium intake and two biomarkers, pseudouridine and citrate, were further confirmed in 70 women. Astarita *et al.* (2014) applied a multi-platform lipidomic approach to compare the plasma lipidome between WT and fat-1 mice, which can convert omega-6 to omega-3 PUFAs and protect against a wide variety of diseases including chronic inflammatory diseases and cancer. Fat-1 mice exhibited a significant increase in the levels of omega-3 lipids (unesterified eicosapentaenoic acid [EPA], EPA-containing cholesteryl ester, and omega-3 lysophospholipids), and a significant reduction in omega-6 lipids (unesterified docosapentaenoic acid [omega-6 DPA], DPA-containing cholesteryl ester, omega-6 phospholipids, and triacylglycerides). These lipidomic biosignatures may be used to monitor the health status and the efficacy of omega-3 intervention in humans.

However, a major problem for metabolomics is that the experimental metabolic profile is influenced not only by the genotype but also by age, gender, lifestyle, nutritional status, drugs, stress, physical activity, and so on. To minimize the variations in studies with humans, some attempts were made, such as using standardized diet, avoiding any vigorous activity, excluding smokers, and so on. Unfortunately, even under the consumption of standard diet, the metabolic variability remains. Using 1 H NMR spectroscopy, Walsh *et al.* (2006) investigated the acute effects of standard diet on the metabonomic profiles of urine, plasma and saliva samples from 30 healthy volunteers. There are important biochemical variabilities to be observed for all biofluids at both intra- and inter-individual levels, significant variations in creatinine and acetate for urine and saliva, respectively, exist. After the consumption of standard diet, a reduction in inter-individual variation was observed in urine, but not in plasma or saliva. Indeed, different diets consumption in different populations leads to different metabolic profiles (Rezzi *et al.*, 2007a): higher urinary levels in creatine, creatinine, carnitine, acetylcarnitine, taurine, trimethylamine-N-oxide (TMAO), and glutamine are the metabolic signature of high-meat diet; higher urinary excretion of p-hydroxyphenylacetate, a microbial mammalian co-metabolite, and a decreased level in N,N,N-trimethyllysine are associated with the vegetarian diet; elevated β -aminoisobutyric acid and ethanol in Chinese urinary samples; increased urinary excretion in TMAO in the Japanese and Swedish populations due to the high dietary intake of fish; and usually high level of urinary taurine in the British population as a consequence of the Atkins diet. It is noted that a report reveals a “natural”, stable over time, and invariant metabolic profile for each person, although the existence of human metabolic variations resulting from various dietary patterns (Assfalg *et al.*, 2008). This provides the possibility of eliminating the day-to-day “noise” of the individual metabolic fingerprint and opens new perspectives to metabolomic studies for personalized therapy and nutrition.

Another important issue in nutritional metabolomics is gut microbiota-host metabolic interactions, such as the interaction between the microbiome and the human, which makes the human become a “superorganism” (Goodacre, 2007). More than 400 microbial species exist in the large-bowel microflora of healthy humans, which produce significant metabolic signals so that the true metabolomic signals of nutrients in the diet could be “swamped” and the metabolome of biofluids in human nutrition is altered. Dumas *et al.* (2006b) investigated the metabolic relationship between gut microflora and host co-metabolic phenotypes using the plasma and urine metabolic NMR profile of the mouse. They found that the urinary excretion of methylamines from the precursor choline was directly related to microflora metabolism, demonstrating significant interaction between the mammalian host and microbiota metabolism. Rezzi *et al.* (2007b) performed the NMR analysis of plasma and urine metabolic profiles in 22 healthy male volunteers with

behavioral/psychological dietary preference (chocolate desire or chocolate indifference). The results revealed that chocolate preference was associated with a specific metabolic signature, which is imprinted in the metabolism even in the absence of chocolate as a stimulus. Marcobal *et al.* (2013) applied the UPLC technique to investigate the effects of the human gut microbiota on the fecal and urinary metabolome of a humanized (HUM) mouse. They found that the vast majority of metabolomic features are produced in the corresponding HUM mice, the metabolite signatures can be modified by host diet, and simplified bacterial communities can drive major changes in the host metabolomic profile. This demonstrates that metabolomics constitutes a powerful avenue for functional characterization of the intestinal microbiota and its interaction with the host.

1.5 Systems Biology in Nutritional Research

In order to better understand the complex interplay between genes, diet, lifestyle, and endogenous gut microflora, and to understand how diet can be modified to maintain optimal health throughout life, the integrative use of various omics technologies-systems biology technology offer exciting opportunities to develop the emerging area of personalized nutrition and healthcare (Naylor *et al.*, 2008; Zhang *et al.*, 2008). Currently, there has been limited work in this arena.

Using an integrated reverse functional genomic and metabolic approach, Griffin *et al.* (2004) identified perturbed metabolic pathways by orotic acid treatment. In the searching for correlations between the 60 most differentially expressed genes and the largest changed metabolite trimethylamine-N-oxide, they found that the most significant negative correlation is stearyl-CoA desaturase 1, which highlights the relationship between transcripts and metabolites in lipid pathways. Herzog *et al.* (2004) performed proteome and transcriptome analysis of human colon cancer cells treated with flavone. About 488 mRNA targets were found to be regulated by flavone at least two-fold. On the other hand, many proteins involved in gene regulation, detoxification, and intermediary metabolism, such as annexin II, apolipoprotein A1, and so on, were found to be altered by flavone exposure. Dieck *et al.* (2005) conducted transcriptome and proteome analysis to identify the underlying molecular changes in hepatic lipid metabolism in zinc-deficient rats. The experimental findings provide evidence that an unbalanced gene transcription control via the PPAR- α , thyroid hormone, and SREBP-dependent pathways could explain most of the effects of zinc deficiency on hepatic fat metabolism. Mutch *et al.* (2005) used an integrative transcriptome and lipid-metabolome approach to understand the molecular mechanisms regulated by the consumption of PUFA. They identified stearoyl-CoA desaturase as a target of an arachidonate-enriched diet and revealed a previously unrecognized and distinct role for arachidonate in the regulation of hepatic lipid metabolism. By combining DNA microarray, proteomics, and metabolomics platforms, Schnackenberg *et al.* (2006) investigated the acute effects of valproic acid in the liver and demonstrated a perturbation in the glycogenolysis pathway after administration of valproic acid.

Recently, by applying transcriptomics, proteomics, and metabolomics technologies to liver samples from C57BL/6J mice, Rubio-Aliaga *et al.* (2011) revealed alterations of key metabolites and enzyme transcript levels of hepatic one-carbon metabolism and related pathways, suggesting the important role of coupling high levels of choline and low levels of methionine in the development of insulin resistance and liver steatosis. Vendel Nielsen *et al.* (2013) investigated the hepatic response to the most abundant trans fatty acid in the human diet, elaidic acid, using a combined proteomic, transcriptomic, and lipidomic approach in HepG2 cells. They found that many proteins involved in cholesterol synthesis and the esterification and hepatic import/export of cholesterol were upregulated. Moreover, at the phospholipid level, there existed a marked remodeling of the cellular membrane. This suggests that trans fatty acids from the diet induce abundance changes in several hepatic proteins and hepatic membrane composition to alter plasma cholesterol levels.

1.6 Conclusions

The main goal of omics-based nutrition research is to understand the relationships between diet and disease and the relationships between diet and health, and finally to make recommendations for personalized nutrition or individualized diets (Figure 1.1, modified from Zhang *et al.*, 2008). In order to better understand the complex interplay that occurs between the individual in terms of genetics, physiology, health, diet, and environment, comparative genetic, transcriptomic, proteomic, and metabolomic analyses for individuals and populations are highly required. In particular, systems biology, more than the

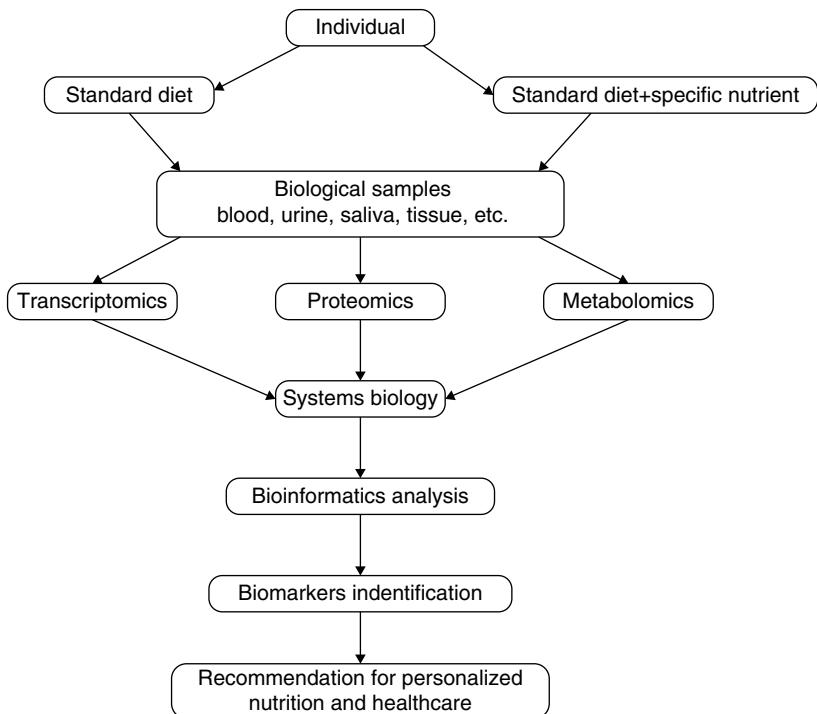


Figure 1.1 Workflow for omics-based nutritional research.

simple merger of various omics technologies (transcriptomics, proteomics, and metabolomics), aims for understanding the biological behavior of a cellular system in response to external stimuli, and opens up a new road to understanding the complex interaction network between nutrients and molecules in biological systems. An era of personalized medicine and nutrition is coming.

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2

Seafood Authentication using Foodomics: Proteomics, Metabolomics, and Genomics

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2.1 Introduction

The term *foodomics* has been defined as a new discipline that studies the food and nutrition domains through the application of advanced omics technologies in order to improve consumer well-being, health, and confidence (Cifuentes, 2009). All areas related to food, from food quality and safety to toxicity and nutrition including food technology, can be potentially covered by foodomics studies. In Figure 2.1 the main areas covered and the tools used by foodomics are shown.

Regarding food quality, the authentication of food products is one of the most relevant issues that has demanded great attention by consumers and the food industry in recent years. Food components may be adulterated, either deliberate or inadvertent, this leads to mislabeling and commercial fraud (Moore *et al.*, 2012). Some examples of common adulterations are the substitution of the species declared in the label of a food product by a similar but lower quality and cheaper one; false information about the geographic origin or production method of a food component; or the presence of an undeclared ingredient in a foodstuff. Apart from the prevention of commercial fraud, food adulteration has also implications related to food safety, since the undeclared introduction of any food ingredient that can be harmful to human health, such as allergenic or toxic ingredients, is a public health issue (Spink and Moyer, 2011).

The increasing awareness of consumers about food composition has led to the implementation of many regulations in order to avoid food adulterations. Examples include, the Federal Food, Drug and Cosmetic Act, Section 403, Misbranded Food (US Food and Drug Administration 2014), and the General Food Law (European Parliament and European Council, 2002) in the USA and Europe, respectively, highlight the requirement of providing complete and truthful information about the food products that are being traded, guaranteeing market transparency and providing consumers with the basis for making informed choices about the food they buy. Some regulations have been promulgated for seafood products in particular. For instance, the Council Regulation (EC) No 104/2000 (European Council, 1999) on the common organization of the markets in fishery and aquaculture products, states the legal requirement of labeling seafood products at each step of the

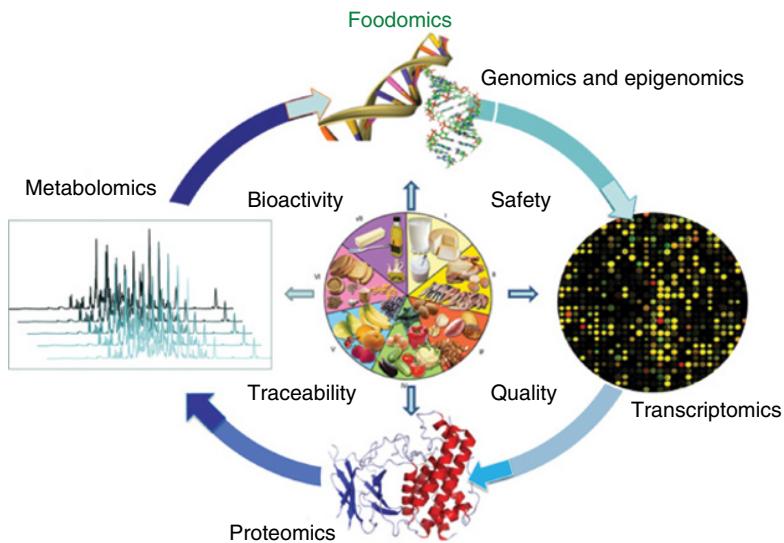


Figure 2.1 Areas covered and tools used by foodomics. Copyright © 2013 by John Wiley & Sons, Inc. Used with permission from Cifuentes (2013), in: *Foodomics: Principles and Applications*, *Foodomics: Advanced Mass Spectrometry in Modern Food Science and Nutrition*, John Wiley & Sons, Inc.

marketing chain with (1) the commercial name of the species, (2) the production method (wild caught or farmed), and (3) the geographic zone where the product has been fished or farmed.

Among fish and shellfish products, the substitution of an appreciated high quality species by another of lower quality is especially frequent (Pascoal *et al.*, 2008a). In addition to the commercial fraud derived from this, sometimes inadvertent, sometimes deliberate, practice, it can affect marine conservation programs that protect overexploited species or populations (Rasmussen and Morrisey, 2008). Morphological identification of fish and shellfish species is complex when the species are phylogenetically close and even impossible when the external features have been removed during processing. Production method (e.g., wild or farmed) is another important element affecting food quality, since organoleptic features, nutritional values, and price are not the same for fish or shellfish that is wild caught and the same species that is farmed in aquaculture facilities. The geographic origin of the food components should also be checked, not only because of the demand for information from consumers, but also to ensure food safety, since seafood is particularly exposed to contaminants such as heavy metals and pathogens, and therefore the authentication of origin and traceability assurance are especially relevant when a contaminated product from a particular area must be withdrawn from the market.

For all these reasons, accurate and reliable analytical tools are needed in order to guarantee the correct and complete labelling of foodstuffs, therefore verifying that food components are what the purchaser is demanding and providing food traceability.

Many different classical instrumental techniques have been used for food authentication, such as liquid and gas chromatography, isoelectric focusing (IEF), capillary electrophoresis, and spectroscopy (Drivelos and Georgiou, 2012). In recent years, new approaches are emerging, namely those compiled under the term omics, which can overcome the drawbacks of those classical techniques in terms of sensitivity, speed, accuracy, and multiplexing capacity.

This chapter is a comprehensive overview of seafood authentication studies where omics-related technologies, namely genomics, metabolomics, and proteomics, have been used as tools to comply with food labeling regulations and fight against food adulteration.

2.2 Proteomic Approaches

Proteomics has been defined as the large-scale study of proteins (Pandey and Mann, 2000). Although currently MS is the method of choice for the analysis of proteins, the first step of proteomics was the development of two-dimensional electrophoresis (2DE), a technique that allows the separation of hundreds to thousands of proteins on a single polyacrylamide

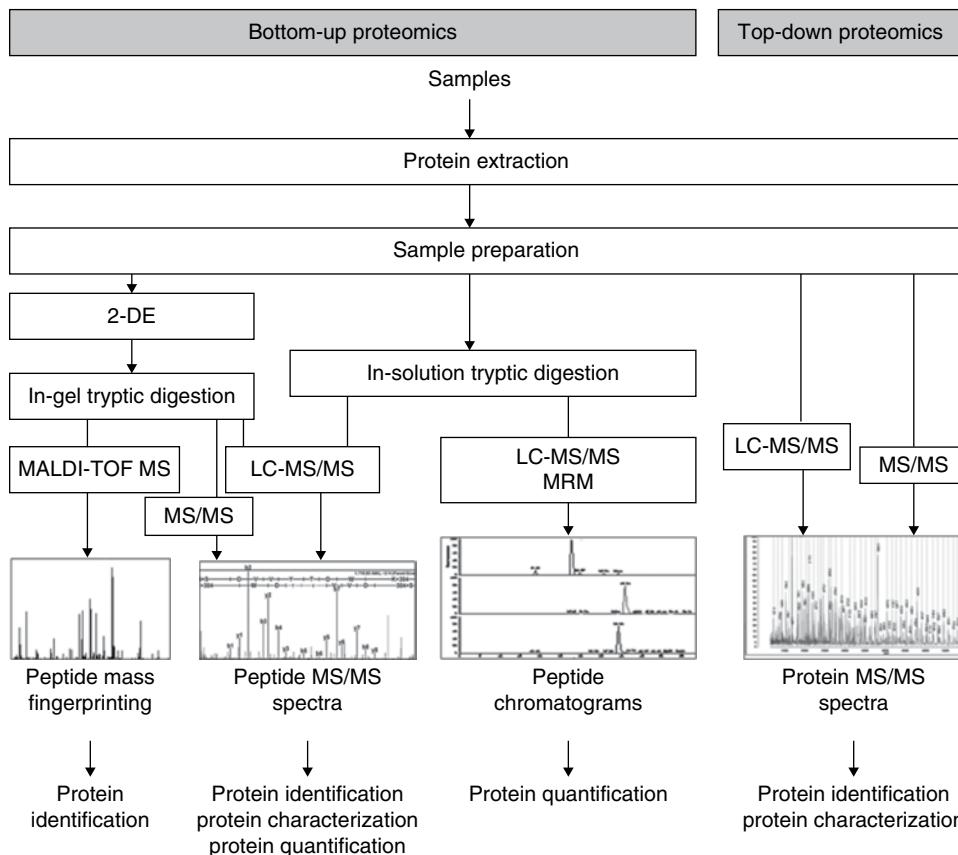


Figure 2.2 Proteomics workflows commonly used for food-technology research. Copyright © 2013 Elsevier Limited. Used with permission from Gallardo *et al.* (2013). *Proteomics and its applications for food authentication and food-technology research*. Trends in Analytical Chemistry 52, 135–141.

electrophoresis gel. In the last years, development of bioinformatics has contributed to the great advance of both gel-based and MS-based proteomic tools. The proteomic workflows commonly used in food-related research are shown in Figure 2.2. All of them consist of some steps of sample preparation to obtain the protein extract, some fractionation steps, usually using electrophoresis or liquid chromatography (LC), and finally analysis by MS. In the gel-based approaches, 2DE (or sometimes one-dimensional electrophoresis techniques like SDS-PAGE or IEF) is the separation step and the analytical technique at the same time, although commonly, the electrophoretically isolated proteins are then identified by MS. Opposite to the so-called *top-down* proteomic approaches, where the whole proteins are analyzed in the mass spectrometer, in the most generally used approach *bottom-up* proteomics, proteins are digested with a protease such as trypsin and then the resulting peptides are analyzed by MS. The produced spectra can be used for protein identification and characterization (Eng *et al.*, 1994; Perkins *et al.*, 1999), commonly by comparison with database entries, and even peptides and proteins can be quantified following different approaches (Panchaud *et al.*, 2008). In Table 2.1, the proteomics applications for the assessment of the authenticity of seafood products, both gel- and MS-based, are shown.

Since the late 1980s, the SDS-PAGE and IEF of muscular proteins have showed their potential for differentiating crustacean and fish species using raw and cooked material (An *et al.*, 1988, 1989; Bossier and Cooreman, 2000; Civera and Parisi, 1991; Etienne *et al.*, 2000; Rehbein, 1995; Renon *et al.*, 2005). The IEF protein profiles of water soluble muscular proteins allowed the unambiguous differentiation of 14 closely-related shrimp and prawn commercial species (Ortea *et al.*, 2010). The species-specific markers were identified by MS as heat-stable sarcoplasmic calcium-binding proteins (SCPs), therefore extending the application of this method to heat-processed products. 2DE has also been extensively used for assessing differences between seafood species, such as gadoid fishes (Piñeiro *et al.*, 1998), flat fishes (Piñeiro *et al.*, 1999),

Table 2.1 Proteomics applications for the authenticity assessment of seafood products.

Main technique	Discrimination between/identify	Target	Reference
SDS-PAGE	Three shrimp species	Muscular proteins	An <i>et al.</i> (1988)
	Shrimp and crab meat	Muscular proteins	Civera and Parisi (1991)
Urea-IEF	Three shrimp species	Muscular proteins	An <i>et al.</i> (1989)
SDS-PAGE, urea-IEF	10 fish species	Muscular proteins	Etienne <i>et al.</i> (2000)
SDS-PAGE, IEF, urea-IEF	Cod, redfish, lobster, shrimp, mussel, squid, salmon, trout	Sarcoplasmic and myofibrillar proteins	Rehbein (1995)
IEF	17 flatfish species	Muscular proteins	Bossier and Cooreman (2000)
	Swordfish, blue marlin and spearfish	Sarcoplasmic proteins	Renon <i>et al.</i> (2005)
	14 shrimp species	Sarcoplasmic calcium- binding proteins	Ortea <i>et al.</i> (2010)
2DE	Gadoid fish species	Parvalbumins	Piñeiro <i>et al.</i> (1998)
	Flat fishes	Parvalbumins	Piñeiro <i>et al.</i> (1999)
	Hake species	Parvalbumins	Piñeiro <i>et al.</i> (2001)
	Two mussel species	Muscular proteome	López <i>et al.</i> (2002a)
	Cod, saithe, haddock, mackerel and capelin; two breeding stocks of Artic charr	Myosin light chains isoforms	Martínez and Friis (2004)
	Five puffer fish species	Muscle proteins	Chen <i>et al.</i> (2004)
	Wild and farmed cod	Muscle proteins	Martínez <i>et al.</i> (2007)
	Tuna species	Triose phosphate isomerase	Pepe <i>et al.</i> (2010)
	Two scallop populations	Mantle proteins	Artigaud <i>et al.</i> (2014)
2DE and PMF	10 hake species	parvalbumins	Carrera <i>et al.</i> (2006)
	Six shrimp species	AK	Ortea <i>et al.</i> (2009a)
PMF and MS/MS <i>de novo</i> sequencing	Three mussel species	Muscular proteome	López <i>et al.</i> (2002b)
MS/MS	11 hake species	Nucleoside diphosphate kinase B	Carrera <i>et al.</i> (2007)
	Seven shrimp species	AK	Ortea <i>et al.</i> (2009b)
	Shrimp species <i>Pandalus borealis</i>	AK	Pascoal <i>et al.</i> (2012)
	Two river fish species	Triose phosphate isomerase	Barik <i>et al.</i> (2013)
pSRM	22 fish species	Muscle proteins	Wulff <i>et al.</i> (2013)
	Seven shrimp species	AK	Ortea <i>et al.</i> (2011)
	11 hake species	Parvalbumins	Carrera <i>et al.</i> (2011)
MALDI-TOF MS protein profiling	25 fish species	Parvalbumins	Mazzeo <i>et al.</i> (2008)
	Differentiation of shrimp species, geographic origin, and fresh/ frozen state	Muscle proteins	Salla and Murray (2013)

Notes: 2DE, two-dimensional electrophoresis; AK, arginine kinase; IEF, isoelectric focusing; MALDI-TOF MS, matrix-assisted laser desorption/ionization mass spectrometry; MS/MS, tandem mass spectrometry; PMF, peptide mass fingerprinting; pSRM, pseudo selected reaction monitoring; SDS-PAGE, sodium dodecyl polyacrylamide gel electrophoresis.

hake (Piñeiro *et al.*, 2001), mussels (López *et al.*, 2002a), puffer fishes (Chen *et al.*, 2004), and shrimps (Ortea *et al.*, 2009a); for the differentiation of cod, saithe, haddock, mackerel, and capelin (Martínez and Friis, 2004); for the identification of the tuna species *Thunnus thynnus* (Pepe *et al.*, 2010); and even to discriminate between populations of the same species, such as the Great Scallop (Artigaud *et al.*, 2014), and to differentiate between wild and farmed cod (Martínez *et al.*, 2007).

Peptide mass fingerprinting (PMF), a MS-based technique where the spectrum of the masses of the tryptic peptides from a previously isolated protein acts as a fingerprint for that protein, and tandem mass spectrometry (MS/MS), where the spectra obtained reflect the amino acid sequence of the peptides, provide some advantages over 2-DE, since the identification and characterization of the analysed proteins are obtained. Moreover, the MS spectra can be used for a direct comparison of the samples. PMF and MS/MS have been used for species differentiation in mussels (López *et al.*, 2002b), hake (Carrera *et al.*, 2006, 2007), shrimps (Ortea *et al.*, 2009a, b; Pascoal *et al.*, 2012), and *Sperata* fish species

(Barik *et al.*, 2013). Recently, Wulff *et al.* (2013) built a MS/MS spectral library for the identification of 22 fish species in an automated and standardized workflow.

Once marker peptides are identified, fast and easy-to-use methods for the sensitive detection of the species, such as immunoassays or targeted MS assays, can be developed. Ortea *et al.* (2011) described a targeted MS method, namely pseudo Selected Reaction Monitoring (pSRM), which was able to identify the seven most commercial shrimp species in less than 90 min, and Carrera *et al.* (2011) developed a similar method for hake species. Selected reaction monitoring (SRM), sometimes called multiple reaction monitoring (MRM), is the golden standard for quantifying peptides using LC-MS. Figure 2.3 shows the different proteomic approaches used at our laboratory for the identification, characterization, and monitoring of species-specific peptides from shrimps with food authentication purposes.

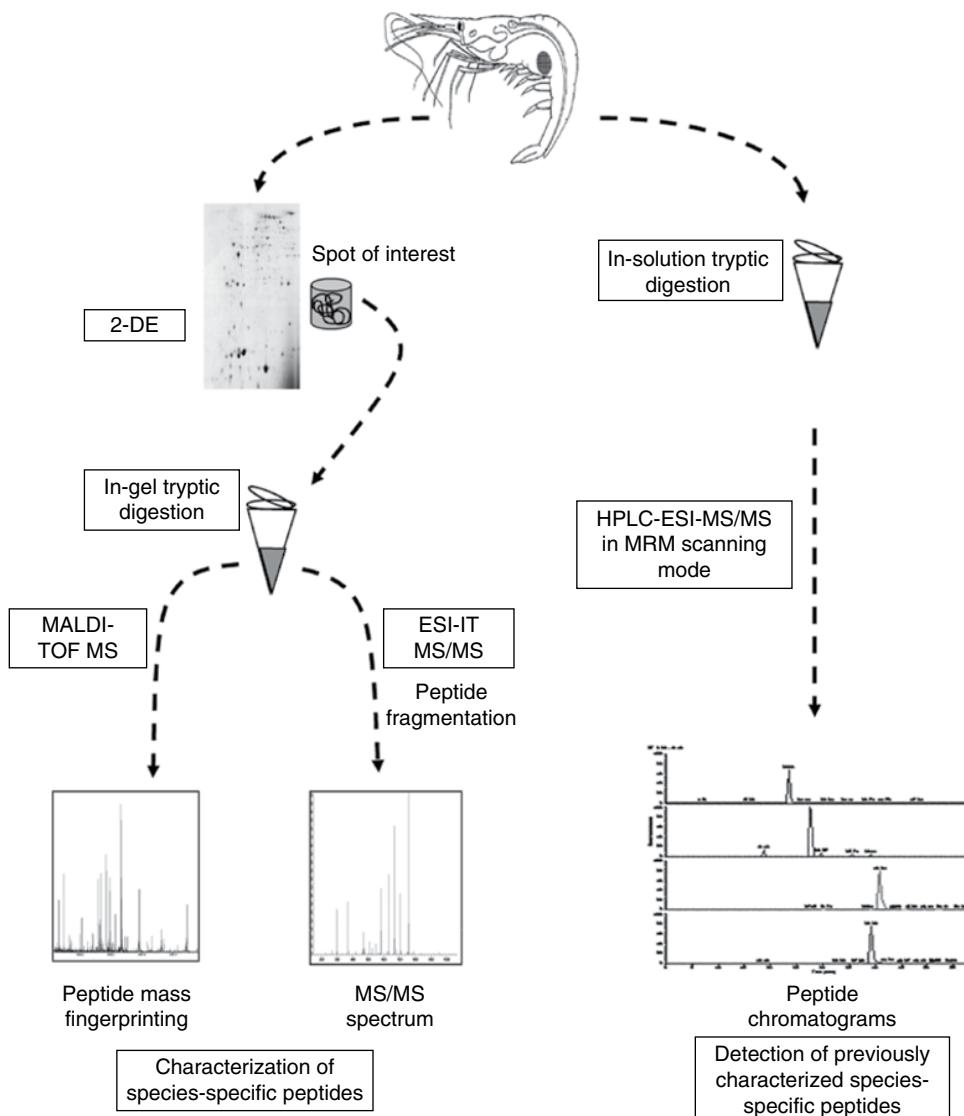


Figure 2.3 Proteomic approaches considered for the identification, characterization, and detection of species-specific diagnostic peptides from shrimp with food authentication purposes. Copyright © 2012 Elsevier. Used with permission from Ortea *et al.* (2012). Food authentication of commercially-relevant shrimp and prawn species: from classical methods to Foodomics. Electrophoresis 33, 2201–2211.

Regarding top-down like approaches, where the proteins are not subjected to tryptic digestion, Salla and Murray (2013) described a method using MS for the protein profiling of muscle extracts that was applied to the differentiation of species, geographic origin, and even fresh or frozen state in shrimp samples. Mazzeo *et al.* (2008) developed a similar protein profiling method able to obtain specific profiles for 25 different fish species, representing the highest number of species included in a fish authentication study.

2.3 Metabolomic Approaches

The objective of metabolomics is to identify and quantify as many metabolites as possible present in a cell, organism, or tissue (Fiehn, 2002). Two different approaches are used in metabolomics studies: metabolite profiling, where a specific group of metabolites are studied, and metabolic fingerprinting, which focuses on the comparison of patterns or fingerprints between the different sample groups. In recent years, due to advances in analytical technologies and in statistical and computing tools, metabolomics is increasingly being used in different scientific areas, including food quality.

Although these approaches can be applied simultaneously to a broad range of molecules, no single analytical platform is capable of analyzing all the different metabolites at once, due to the complexity and the wide dynamic range of the metabolome. Nuclear magnetic resonance (NMR) and mass spectrometry (MS) are the main analytical techniques in metabolomics, although some other methods, such as infrared (IR), Raman spectroscopy, and electronic sensors, have also been applied in authentication studies of different food commodities (Cubero-Leon *et al.*, 2014). Since metabolomics analysis usually produces data from a large number of variables, data analysis is critical in order to differentiate between the different conditions or sample groups that are being analyzed. Mathematical tools grouped under the term *chemometrics* – mainly multivariate data analysis tools such as principal component analysis (PCA), clustering methods, linear discriminant analysis (LDA), and regression analysis – are widely used for metabolomics data analysis. In Table 2.2, an overview of the metabolomics applications used to study the authenticity of seafood products is shown.

Metabolic profiling of water-soluble and liposoluble molecules extracted from skin and muscle, using ^1H NMR followed by PCA, has been studied for the discrimination of wild and farmed sea bass (Mannina *et al.*, 2008). Fatty acid composition, but also some other molecules such as cholesterol, phosphatidylethanolamine, choline, glutamine, and fumaric and malic acids, allows differentiation between the wild and farmed samples.

In a similar study using ^1H NMR, but in combination with advanced chemometric tools in addition to PCA, namely extended canonical variable analysis and discriminant analysis, Savorani *et al.* (2010) were able to differentiate sea bream samples coming from three different farming systems and determine whether the fish had been stored at -80°C or not.

The combination of near-infrared spectroscopy (NIRS) with different chemometric techniques was able to reliably discriminate between wild and farmed sea bass faster and more economically than using chemical methods (Ottavian *et al.*, 2012). NIRS, followed this time by PCA and a soft independent modeling class analogy (SIMCA) method, was also investigated for the classification of sea bass samples from two rearing systems (organic vs conventional feed) (Trocino *et al.*, 2012). The methodology was able to correctly classify 65% of the organic samples and 72.5% of the conventional samples.

Although not pure “omics” approaches, stable isotope ratio analysis (SIR) and multi-element analysis might be included within metabolic profiling methodologies, since they target selected classes of compounds. In this sense, SIR analysis by isotope ratio mass spectrometry (IRMS) and multi-element analysis by ICP-MS have become two of the most frequently used technique for assessing authenticity and traceability in food products, especially in geographical origin studies (Drivelos and Georgiou, 2012). The differences in elemental composition of soils are propagated through the trophic chain, and therefore the elemental composition profile of animals and plants may be used as a marker characterizing the geographical origin of food products (Kelly *et al.*, 2005). Likewise, the ratios of different element isotopes can also be used for determining geographical origin. $^{13}\text{C}/^{12}\text{C}$ ratio, due to differences in plants according to latitude (Kelly *et al.*, 2005) and $^{15}\text{N}/^{14}\text{N}$ ratio, which changes according to local agricultural practices (Oulhote *et al.*, 2011), are the most informative parameters for analyzing the geographical origin of animals and plants. In seafood species, most of these studies have applied SIR analysis to discriminate between wild and farmed specimens of commercially relevant fish species, such as gilthead sea bream (Moreno-Rojas *et al.*, 2007; Morrison *et al.*, 2007; Serrano *et al.*, 2007), sea bass (Gordon Bell *et al.*, 2007; Fasolato *et al.*, 2010), Atlantic salmon (Dempson and Power, 2004; Thomas *et al.*, 2008), and turbot (Busetto *et al.*, 2008). SIR analysis of C and N succeeded even in distinguishing among farmed cod originating from different farms (Turchini *et al.*, 2009) and differentiating farmed trout fed with plant- or fish-protein-based diets (Moreno-Rojas *et al.*, 2008). Nevertheless, it was able to differentiate organically farmed salmon from conventionally farmed salmon but only in combination with fatty acids analysis, although it differentiated farmed from wild specimens (Molkentin *et al.*, 2007).

Table 2.2 Metabolomics applications to assess authenticity of seafood products.

Main technique	Discrimination between/identify	Variables measured	Reference
¹ H NMR	wild and farmed sea bass sea bream samples from three different farming systems	29 chemical features Metabolic fingerprints	Mannina <i>et al.</i> (2008) Savorani <i>et al.</i> (2010)
NIRS	wild and farmed sea bass sea bass samples from organic and conventional farming systems	35 chemical features Chemical composition spectra	Ottavian <i>et al.</i> (2012) Trocino <i>et al.</i> (2012)
SIR analysis	wild and farmed gilthead sea bream wild and farmed sea bass wild and farmed Atlantic salmon wild and farmed turbot cod from different farms cod fed with plant or fish diets organically from conventionally farmed salmon gadoid fish species Atlantic cod and saithe wild and farmed Atlantic salmon	$\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$	Moreno-Rojas <i>et al.</i> (2007) Morrison <i>et al.</i> (2007) Serrano <i>et al.</i> (2007) Gordon Bell <i>et al.</i> (2007) Fasolato <i>et al.</i> (2010) Dempson and Power (2004) Thomas <i>et al.</i> (2008) Busetto <i>et al.</i> (2008) Turchini <i>et al.</i> (2009) Moreno-Rojas <i>et al.</i> (2008) Molkentin <i>et al.</i> (2007) Monteiro Oliveira <i>et al.</i> (2011)
Multi-element analysis	origin of farmed shrimp sea cucumbers from different origins caviar from different origins mussels from the European Protected Designation of Origin <i>Rias Baixas</i>	24 elements 19 elements Trace metal elements 15 elements 72 elements 40 elements	Adey <i>et al.</i> (2009) Anderson <i>et al.</i> (2010) Smith and Watts (2009) Liu <i>et al.</i> (2012) Rodushkin <i>et al.</i> (2007) Costas-Rodríguez <i>et al.</i> (2010)

Notes: NIRS, near-infrared spectroscopy; NMR, nuclear magnetic resonance; SIR, stable isotope ratio.

Regarding the use of multi-element analysis, Smith and Watts (2009) determined the origin of farmed shrimp by trace metal profiling and multivariate statistics using a database on the composition of shrimp from different countries. Wild and farmed salmon were differentiated using elemental analysis and different classification methods (Adey *et al.*, 2009; Anderson *et al.*, 2010), and sea cucumber samples from three water environments in China were identified using multi-element analysis and chemometric techniques (Liu *et al.*, 2012). The analysis of minor and trace elements by ICP-MS followed by LDA or SIMCA allowed the differentiation of cultivated mussels under the European Protected Designation of Origin *Rias Baixas* (Costas-Rodríguez *et al.*, 2010). Rodushkin *et al.* (2007) were able to differentiate between caviar from different origins measuring 17 elements also by ICP-MS. Regarding species identification, the SIRs of C and N were recently used to differentiate the gadoid fish species Atlantic cod and saithe (Monteiro Oliveira *et al.*, 2011).

2.4 Genomic Approaches

Molecular techniques based on the analysis of DNA have several advantages opposed to other molecular approaches, such as the ubiquitous presence in almost all cells and tissues of an individual and the high stability of DNA that allows analysis in highly processed food products. Furthermore, due to the high specificity of DNA, complex mixtures can be analyzed and closely related species differentiated. In this sense, authentication of seafood by DNA-based techniques is mainly focused on the detection of species substitution and mislabeling of food products of marine origin. In a number of studies, the discrimination of closely related species that have special interest in the seafood sector has been reported for groups of fish, such as codfish (Calo-Mata *et al.*, 2003; Teletchea *et al.*, 2006), eel (Itoi *et al.*, 2005; Rehbein *et al.*, 2002), flatfish (Comesaña *et al.*, 2003; Sotelo *et al.*, 2001), grouper (Asensio, 2008), hake (Machado-Schiaffino *et al.*, 2008; Pérez *et al.*, 2005), salmon (Carrera *et al.*, 2000; Russell *et al.*, 2000), sardine (Jérôme *et al.*, 2003), sea bream (Schiefenhövel and

Rehbein, 2013), shark (Blanco *et al.*, 2008), and thunnus (Lockley and Bardsley, 2000; Lowenstein *et al.*, 2009; Pedrosa-Gerasmio *et al.*, 2012), as well as for prawn and shrimp (Khamnamtong *et al.*, 2005; Pascoal *et al.*, 2008a), bivalves (Bendezu *et al.*, 2005; Rego *et al.*, 2002; Santaclarra *et al.*, 2006), and cephalopods (Chapela and Sotelo, 2003; Santaclarra *et al.*, 2007). In a further study, 50 commercial important fish species were studied and 30 out of the 50 species could be successfully distinguished and identified (Kochzius *et al.*, 2010).

Besides species identification, the determination of the geographical origin is of importance to register the location of capture, with the objective of controlling overfishing and monitoring the illegal trade of protected and endangered stocks. For example, geographical populations of the black tiger shrimp have been extensively studied (Khamnamtong *et al.*, 2009; Li *et al.*, 2007; Mandal *et al.*, 2012). Likewise, genetic stock identification of chum salmon has been carried out (Moriya *et al.*, 2007). The investigation of the genetic diversity and population studies is also of interest to aquaculture facilities in order to monitor inbreeding, assign parentage, and identify strains.

When talking about DNA-based approaches for food analysis purposes, the polymerase chain reaction (PCR) plays a crucial role. PCR, where a specific DNA fragment is amplified, is characterized by high sensitivity and specificity, as well as a short time required to perform the analysis. For the authentication of products of marine origin, both nuclear and mitochondrial DNA have been targeted, being the cytochrome B gene (cytb), 16s rRNA ribosomal gene, and cytochrome c oxidase I gene (COI), the most frequently used genes for species identification. Mitochondrial DNA is the preferred one when searching for inter-specific variability and conserved regions at intra-specific level to enable the amplification of regions that are common to a big group of specimens. Nuclear genes, in contrast, exhibit a higher inter- and intra-specific variability due to biological factors. Rasmussen and Morrissey (2009) extensively reviewed the application of DNA-based methods for seafood. In Table 2.3, an overview of the most applied PCR-based methods and related studies carried out in seafood authentication is given.

PCR-based methods can be divided into two groups, those that target many loci and those that amplify a single locus. In the first case, amplification results in a fingerprint-like amplification pattern on the base of which discrimination can be carried out by comparison to reference samples. Techniques such as Randomly Amplified Polymorphic DNA (RAPD) and simple sequence repeats (SSR) are included into this group. These methods have the advantage that they do not require a prior knowledge of the DNA sequences and false positive results are unusual, since a bad-quality fingerprint rarely matches a fingerprint of another species. The challenges arise when working with mixtures of different species and highly degraded DNA. In the second group, a specific biomarker DNA is selected and amplified by the application of specific or universal primers. Afterward, the amplification products are differentiated due to the variability in their DNA sequences and the presence of so called single nucleotide polymorphisms (SNPs) by methods such as Restriction Fragments Length Polymorphism (RFLP), Single Strand Conformation Polymorphism (SSCP), or DNA hybridization to specific probes. These techniques require the prior study of sequences of the target DNA that makes the development of the methodology more complex and expensive. However, the advantages are that analysis of mixtures can be carried out, as well as real-time PCR that allows a quantitative assessment of adulteration in a food product relative to the total amount of fish or seafood.

Forensically informative nucleotide sequencing (FINS) is by far the most reliable analysis technique; with that a precise and unequivocal identification can be obtained. Universal primers are selected for PCR amplification that may react with any species and afterwards sequencing of the obtained DNA-fragments is carried out. For species identification, the obtained DNA-sequences are compared to reference sequences deposited in public databases by bioinformatics tools such as the blast tool of the NCBI (National Center for Biotechnology Information) that determines the highest percentage of DNA-sequence similarity. Another approach represents the creation of a phylogenetic cluster with that an unknown target DNA-sequence is identified by grouping together with the corresponding reference species. Figure 2.4 shows the phylogenetic tree of the 16S rRNA gene sequences of a number of crustacean species. Pascoal *et al.* carried out various studies aiming the discrimination of prawn and shrimp species by the construction of phylogenetic relations of the DNA-sequences of the 16S rRNA/tRNA^{Val} (Pascoal *et al.*, 2008c) and cytb (Pascoal *et al.*, 2008b) genes. DNA-sequencing and FINS have frequently been applied to species discrimination in seafood as shown in Table 2.3; however, this approach can only be carried out in isolated specimens and not in mixtures of seafood products. Furthermore, it is also expensive and time consuming; therefore, its use in routine laboratories is not appropriate.

An important progress in DNA-sequencing has been done by DNA-barcoding. The Consortium for the Barcode of Life (CBOL) directs a global effort to assemble a sequence reference library for every species of fish on earth (Fish Barcode of Life; FISH-BOL; www.fishbol.org) (Hanner *et al.*, 2011). For that, the cytochrome c oxidase I (COI) gene acts as a “barcode” to identify and delineate all animal life (Changizi *et al.*, 2013). DNA-barcoding has been applied to seafood authentication aiming the detection of frauds and mislabeling of 37 commercial processed seafood products from markets

and groceries (Nicolè *et al.*, 2012), 68 samples of tuna sushi from restaurants (Lowenstein *et al.*, 2009), and 28 smoked fish products (Smith *et al.*, 2008).

Once the DNA sequences are known, the search for variability can be carried out and specific primers can be designed for the identification of certain species with importance in seafood authenticity (Pascoal *et al.*, 2011). Multiplex PCR has been carried out to detect various seafood species simultaneously (Asensio, 2008; Bendezu *et al.*, 2005; Infante *et al.*, 2006; Rasmussen *et al.*, 2010).

Table 2.3 *Genomics applications to assess authenticity of seafood products.*

Technique used and species studied	Target gene	Reference
PCR-RFLP		
Various fish species	Cyt b	Hold <i>et al.</i> (2001); Dooley <i>et al.</i> (2005a)
Gadoids	Cytb	Calo-Mata <i>et al.</i> (2003)
Salmon	Cytb, (P53)	Carrera <i>et al.</i> (2000); Russell <i>et al.</i> (2000); Dooley <i>et al.</i> (2005b)
Flatfish	12S rRNA, Cytb	Carrera <i>et al.</i> (2000); Comesáñ <i>et al.</i> (2003)
Thunnus	Cytb	Lin <i>et al.</i> (2005)
Hake	ITS1	Pérez <i>et al.</i> (2005)
Eel	Cytb	Rehbein <i>et al.</i> (2002)
Prawn and shrimp	16S rRNA, Cytb	Khamnamtong <i>et al.</i> (2005); Pascoal <i>et al.</i> (2008b, c, 2012)
Mussels	18S rDNA; ITS 1; Adhesive protein	Santaclara <i>et al.</i> (2006)
Cephalopods	Cytb	Chapela and Sotelo (2003); Santaclara <i>et al.</i> (2007)
SSCP		
Flatfish	Cytb	Céspedes <i>et al.</i> (1999)
Eel	Cytb	Rehbein <i>et al.</i> (2002)
Salmon	Cytb	Rehbein (2005)
Sparidae	Cytb	Schiefenhövel and Rehbein (2013)
Shrimp	16S rRNA	Khamnamtong <i>et al.</i> (2005)
Clams	α-actin gene	Fernández <i>et al.</i> (2002)
FINS		
Sardine	Cytb	Jérôme <i>et al.</i> (2003)
Thunnus	D-loop	Pedrosa-Gerasmio <i>et al.</i> (2012)
Hake	ITS1	Pérez <i>et al.</i> (2005)
Flatfish	Cytb	Sotelo <i>et al.</i> (2001)
Shark	Cytochrome b	Blanco <i>et al.</i> (2008)
Bivalves	18S rDNA	Santaclara <i>et al.</i> (2006)
Cephalopods	16S rRNA; Cytb	Chapela <i>et al.</i> (2002); Chapela and Sotelo (2003)
Species-specific primers		
Nile perch, grouper, wreck fish	5S rDNA	Asensio <i>et al.</i> (2001, 2008)
Atlantic mackerel	NADH dehydrogenase subunit 5	Infante <i>et al.</i> (2006)
Thunnus	Cytb	Lockley and Bardsley (2000)
Shrimp	16SrRNA	Pascoal <i>et al.</i> (2011)
Bivalves	16S rDNA, 18S rDNA	Bendezu <i>et al.</i> (2005)
Real time PCR		
Eel	16S rRNA	Itoi <i>et al.</i> (2005)
Haddock	transferrin gene	Hird <i>et al.</i> (2005)
Mackarel	Cytb	Velasco <i>et al.</i> (2013)
Salmon	COI	Rasmussen <i>et al.</i> (2010)
Thunnus	Cytb; d-loop; 16S rRNA	Lopez and Pardo (2005); Chuang <i>et al.</i> (2012)

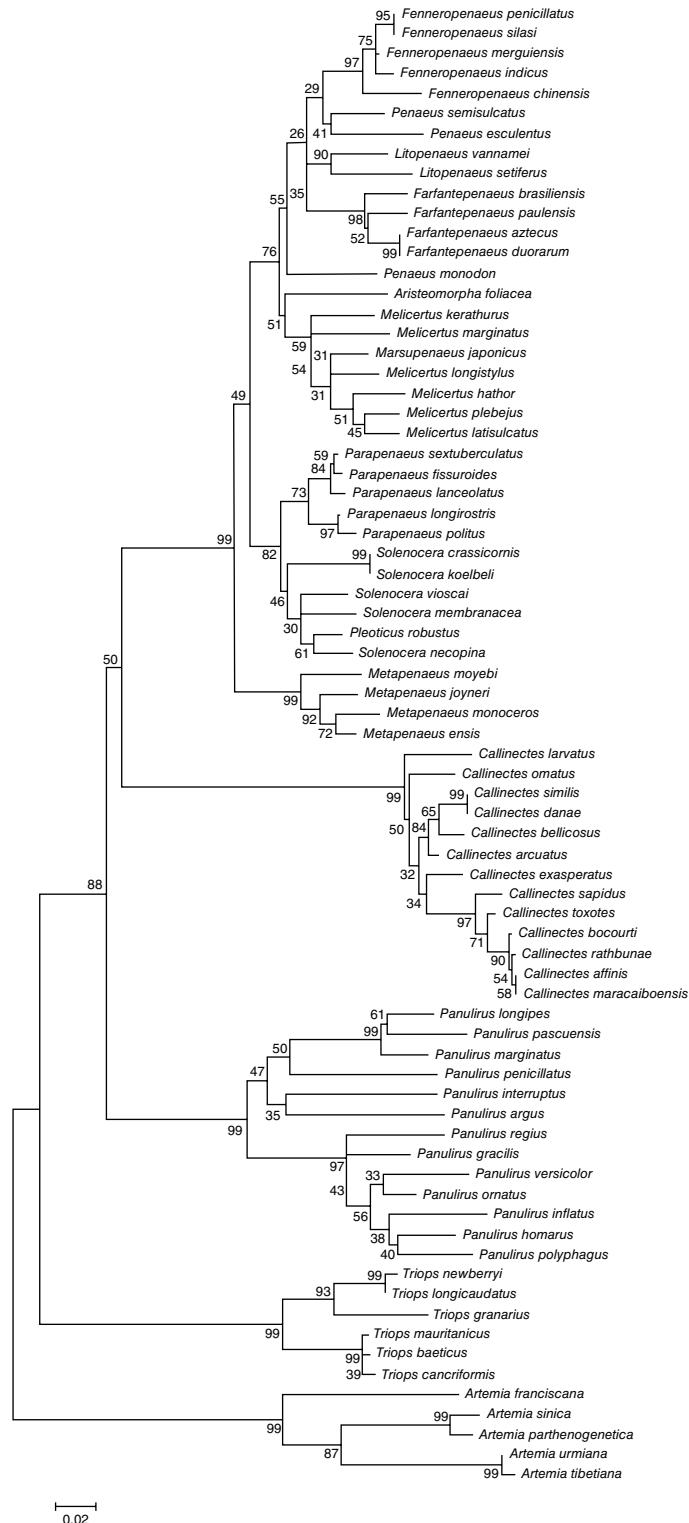


Figure 2.4 Phylogenetic tree of the 16S rRNA gene sequences of crustacean species.

The PCR amplification of a species-specific DNA region can be measured in real time by detection of a fluorescence signal that increases with the concentration of the amplified product, thus allowing the quantification of the initial concentration of the target DNA in the seafood product (Hird *et al.*, 2005; Lopez and Pardo, 2005). Real-time PCR is rapid and sensitive and has been successfully applied to the detection of thunus in canned products (Chuang *et al.*, 2012), the determination of mackerel (Velasco *et al.*, 2013), and the differentiation of two eel species (Itoi *et al.*, 2005). Although this approach is expensive, since it requires special thermal cycling machines that are able to detect specific probes labelled with a fluorophore and the generation of false-positive signals has frequently been reported, it is the only technique with which a quantitative assessment can be carried out.

Similar to the search of specific regions, the comparison of DNA-sequences has the objective to determine single-nucleotide-polymorphisms (SNPs). SNPs play an important role as molecular markers in genomics and are used to study the genetic diversity of organisms, as well as to differentiate species.

The simplest approach to detect SNPs is the single-strand conformation polymorphism (SSCP) that is based on the differences in the electrophoretic mobility of single strands of a PCR amplicon, which differs in their DNA-sequences. After realizing the PCR with primers universal to a certain group of interest, the fragments are denatured by heat or organic solvents and separated by conventional electrophoresis in polyacrylamide gels (PAGE), or by capillary electrophoresis (CE). This technique is inexpensive, convenient, and sensitive and has been successfully applied for seafood authentication (see Table 2.3).

A further, more advanced approach to detecting SNPs is the hybridization to specific probes. DNA microarrays are miniature high density arrays that can have from a few hundred to several hundred thousand oligonucleotides (probes) capable of taking part in hybridization reactions. The sample to be analyzed is usually labeled with a fluorophore and hybridized to the array. In this way, a high number of species can be analyzed simultaneously in a short time and subtle differences in the DNA sequences can be distinguished. The microarray technique is commonly applied to study the genetic diversity of populations of marine origin, such as for the genetic identification of salmon stocks (Moriya *et al.*, 2007). Less work has been done on the application of a DNA microarray to species identification in seafood authentication. Kochzius *et al.* (2010) developed a microarray platform that is able to identify 30 commercially important fish species. For a clear discrimination 64 species-specific probes were necessary. These authors also compared the different genes 16S rRNA, cytb, and COI for hybridization probe design and reported that a large number of probes designed on the COI gene were rejected after the hybridization experiment due to cross-hybridization. This challenges the utility of this gene as a DNA marker for barcoding. Likewise, the 16S rRNA gene was not able to distinguish closely related fish species (Kochzius *et al.*, 2010).

The most commonly used DNA-based technique in seafood authentication is PCR combined with restriction fragment length polymorphism (PCR-RFLP) that is based on the specific fragment patterns resulting after cutting PCR products with restriction endonucleases (Hold *et al.*, 2001; Sotelo *et al.*, 2001). If the sequences are known, the best restriction enzyme can be chosen by determining the cutting sites and calculating the fragment sizes theoretically. For an unequivocal identification of an elevated number of species, usually more than one restriction enzyme is required. Frequently applied enzymes are *Alu*I, *Dde*I, *Hae*III, *Hinc*II, *Nla*III, *Eco*RV, *Hinf*I, *Taq*I, *Msp*I, *Aci*I, *Mwo*I, and *Sau*3AI, which are applied to the amplification products obtained after PCR with universal primers commonly targeting the cytb and the 16S rRNA genes (Rasmussen and Morrissey, 2009). Figure 2.5 shows the theoretically determined fragment sizes when cutting an amplified 16SrRNA/tRNA^{Val} gene region of prawn and shrimp species with the restriction enzyme *Alu*I.

PCR-RFLP was successfully applied for species identification of cod (Calo-Mata *et al.*, 2003), flatfish (Sotelo *et al.*, 2001), salmon (Russell *et al.*, 2000), raw and processed eel (Rehbein *et al.*, 2002), and cephalopods (Chapela *et al.*, 2002; Chapela and Sotelo 2003). Calo-Mata *et al.* (2008) patented a methodology for the identification of 20 prawn and shrimp species based on the amplification of a 515–535 bp region of the 16S rRNA and part of the tRNA^{Val} gene. An improvement of the usually applied gel electrophoresis to visualize the specific fragment patterns has been the use of a lab-on-a-chip capillary electrophoresis (CE) (Dooley *et al.*, 2005b).

Finally, the multi-loci approaches have been frequently applied to genetic diversity and geographic population studies; for example, of shrimp species by RAPD (Rajakumaran *et al.*, 2013) and SSR (Li *et al.*, 2007). The use for species identification is less common; however, some studies have been carried out to obtain specific RAPD fingerprint patterns with the aim to differentiate commercial important species of marine origin. The technique of RAPD is based on the PCR amplification of random sequences in the corresponding specimen by using a set of arbitrary primers. As a result, every primer generates a large number of amplification fragments that are visualized by electrophoresis and exhibit a specific fingerprint. Species are then distinguished by the presence or absence of characteristic bands. The methodology has been

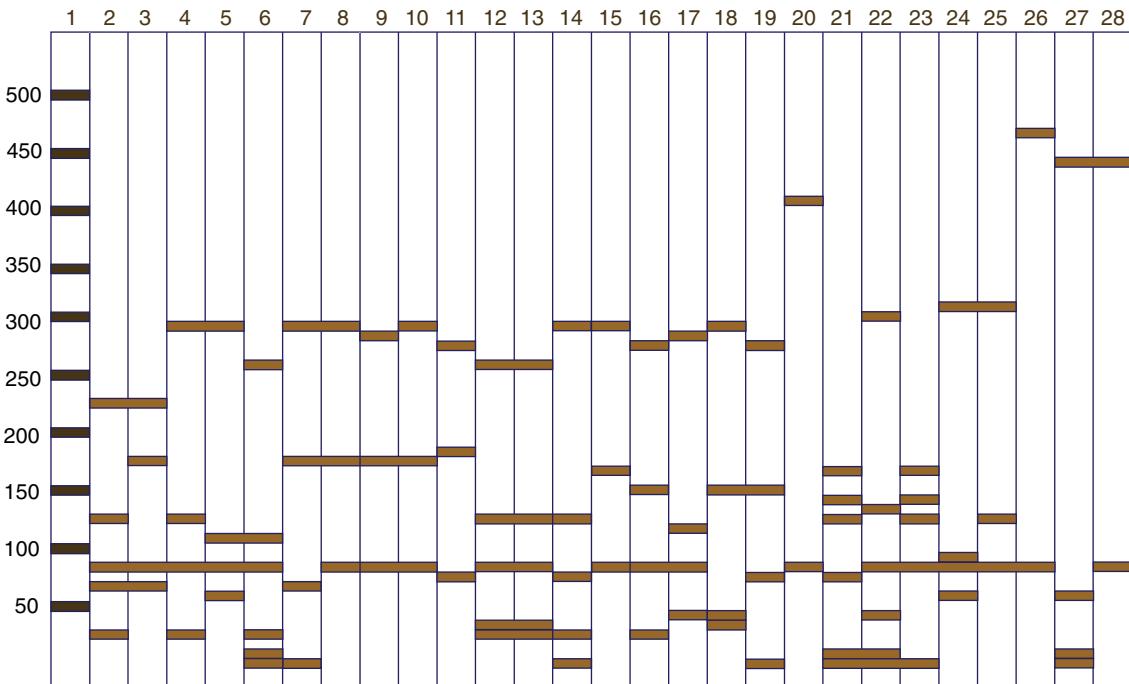


Figure 2.5 Theoretically determined fragment sizes obtained when cutting a 16S rRNA/tRNA^{val} gene region of prawn and shrimp species with the restriction enzyme Alul. Lanes: 1 Molecular weight marker; 2 *Litopenaeus vannamei* Genotype 1; 3 *Litopenaeus vannamei* Genotype 2; 4 *Litopenaeus vannamei* stylirostris; 5 *Farfantepenaeus notialis* Genotype 1; 6 *Farfantepenaeus notialis* Genotype 2; 7 *Farfantepenaeus brasiliensis*; 8 *Farfantepenaeus brevirostris*; 9 *Farfantepenaeus aztecus*; 10 *Farfantepenaeus californiensis*; 11 *Farfantepenaeus indicus*; 12 *Farfantepenaeus merguensis*; 13 *Farfantepenaeus* sp. 29; 14 *Farfantepenaeus monodon*; 15 *Penaeus semisulcatus*; 16 *Penaeus setiferus*; 17 *Marsupenaeus japonicus*; 18 *Melicertus latisulcatus*; 19 *Melicertus* sp. 30; 20 *Aristaeomorpha foliacea*; 21 *Solenocera* sp. 15; 22 *Solenocera agassizii*; 23 *Solenocera* sp. 18; 24 *Pleoticus muelleri* Genotype 1; 25 *Pleoticus muelleri* Genotype 2; 26 *Metapenaeus* sp. 9; 27 *Metapenaeus* sp. 21; 28 *Parapenaeus longirostris*.

used to differentiate groupers and wreck fishes from fraudulent Nile perch filets (Asensio *et al.*, 2002). In a similar study, RAPD has been successfully applied to differentiate four mussel species (Rego *et al.*, 2002).

2.5 Conclusions

Implementation of food regulations requires a thorough control of food quality and labeling in order to protect consumer interests and make informed choices about the food they consume. In recent years, molecular techniques, such as genomics, metabolomics, and proteomics, have increased the range of available tools for the definition and monitoring of food authenticity markers. Molecular markers or profiles for seafood authenticity, including aspects such as the biological species, type of raw material used, geographical origin, production method, and technological process used, have been discovered using these advanced omics methodologies. Many new developments already used in biomedical research, such as high-resolution mass spectrometers, protein and DNA arrays, digital PCR, high resolution melting analysis, biosensors, and nanomaterials, are promising alternatives for the development of new fast methods to monitor and quantify authenticity markers in seafood products.

The demonstrated potential of foodomics-related methodologies, namely genomics, metabolomics, and proteomics, indicates that they will play an important role in seafood authenticity assessment, from the discovery of authenticity markers or profiles to the development of reference methods designed to check if a specific product meets the characteristics stated by the seller, therefore helping to comply with food labeling regulations and fight against food adulteration, and improving consumers' well-being, health, and confidence.

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3

A Foodomics Approach Reveals Hypocholesterolemic Activity of Red Microalgae

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3.1 Introduction

The American Society for Nutrition has identified “omics” as a critical tool for advancing nutrition research, with the ultimate goal of improving the health of the global population (Ohlhorst *et al.*, 2013). Thus, future investigations of the impact of diet on health will most likely focus on “foodomics”: the comprehensive, high-throughput approach for food science that focuses on improving human nutrition (Capozzi and Bordon, 2013). While the more traditional genomics and nutrigenomics approaches emphasize gene expression and the interaction between genes and diet, the foodomics approach employs a wider perspective. Thus, it allows a more holistic evaluation of the health benefits of different food ingredients as it integrates findings from more traditional “omics” methodologies (e.g., proteomics, genomics, transcriptomics, and metabolomics). Foodomics is, therefore, a valuable tool for discovering the mechanisms of action of food ingredients, as it incorporates data obtained by several scientific approaches. Such comprehensive data will enable health and nutrition professionals to provide better scientific evidence for the therapeutic effects of functional foods and food supplements (Ibanez *et al.*, 2012).

The oceans and seas provide a wide variety of food products that can potentially be used as unique sources of functional foods. Seafood – and, in particular, fish and fish oils – have been shown repeatedly to improve metabolic and biochemical processes, and their therapeutic effects, especially with respect to heart disease, have been widely investigated (Gerber *et al.*, 2013). However, other marine products, such as macro- and microalgae, have not been extensively studied and much less is known of their potential use as functional foods. The current chapter focuses on research performed in our laboratory, wherein we employ a foodomic approach to investigate how consumption of red microalgae affects cholesterol metabolism.

3.2 Marine Functional Foods and Supplements

The varied environmental conditions of the oceans and seas, including the wide ranges of temperatures, light exposure conditions, salinities, tides, depths, and nutrient availability, have promoted different survival adaptations in marine organisms; as a result, marine fauna and flora are extremely diverse (Markou and Nerantzis, 2013). Many marine organisms have been identified as sources of high-quality protein, omega-3 fatty acids, antioxidants, essential vitamins, and minerals, and, in the case of seaweed and microalgae, dietary fiber and carotenoids (Kadam and Prabhasankar, 2010). Hence, marine organisms can provide important ingredients to be used as food or food supplements; for instance, the supplement glucosamine, which is produced from hydrolyzed chitosan obtained from shrimp and crab shells, is a popular marine functional ingredient that is thought to assist the treatment of arthritis (Henrotin and Lambert, 2013). Other marine products are rich sources of minerals; for example, whole sardines are often consumed to provide dietary calcium, while supplemental calcium can be isolated from corals and from shark cartilage (Kim *et al.*, 2012).

3.2.1 Algae as a Functional Food

Algae are a diverse group of macroscopic and microscopic eukaryotic organisms that can be found in both unicellular and multicellular forms. Macroalgae (“seaweed”) is the larger and more complex form of algae, whereas microalgae are organisms that live as individual cells or as cell aggregates. Most algae grow in aquatic environments, including freshwater lakes and rivers, as well as oceans, seas, and hot springs. Throughout the world, algae thrive under a wide range of conditions. Because algae adapt to the local environment, their chemical composition varies greatly (Markou *et al.*, 2012; Marsham *et al.*, 2007).

Throughout Asia and Africa, and in many coastal areas around the world, algae have served, for centuries, as an important food source; indeed, in those regions, algae are considered an excellent source of macronutrients, vitamins, minerals, dietary fiber, and phytochemicals. In Western countries, by contrast, algae are predominately used as food additives or, more recently, as innovative ingredients in the cosmetic and pharmaceutical industries, and even as a possible biofuel (Markou and Nerantzis, 2013; Thomas and Kim, 2013). Nonetheless, algae have recently gained interest with regard to their possible role as a functional food or a dietary supplement. This is because algae have a distinctive nutritional make-up and produce a variety of unique secondary metabolites that have important biological activities (Ibanez and Cifuentes, 2013).

Algae can be harvested directly from their natural environments or cultivated commercially, and the production of secondary metabolites can be increased by controlling cultivation conditions or via genetic engineering. Some of the abundant bioactive compounds that algae produce have been identified as antihypertensive, antioxidant, antiinflammatory, antimicrobial, antiviral, antitumorogenic, anticoagulant, and hypocholesterolemic agents (Raposo *et al.*, 2013). Thus, it is becoming clearer that algae could be used as an important food source or supplement in the Western world.

3.2.2 The Nutritional Value of Algae

Algae contain several important ingredients that have the potential to positively influence human health. These include macronutrients, phytochemicals, vitamins, and minerals. Algae also contain unique dietary fiber, which will be described in detail next.

3.2.2.1 *Macronutrients*

Protein content in algae ranges from 5% to more than 40% of the dry weight, and includes bioactive peptides that are thought to reduce hypertension, act as antioxidants, and lower cholesterol levels (Kim and Kang, 2011). The lipid content in algae is greatly influenced by environmental conditions, which determine both the quantity and quality. In general, algae contain glycolipids, neutral lipids, and phospholipids. However, probably the best-known lipid compounds in algae are members of the omega-3 family of fatty acids, including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which reduce the levels of blood triglycerides (Bernstein *et al.*, 2012), reduce risk of heart disease, and have anti-inflammatory effects (Calder, 2012).

Algae also contain phytosterols, which have been shown to lower total and LDL cholesterol (Micallef and Garg, 2009). A relatively large portion (33–75%) of the dry weight of algae is complex carbohydrates; the structure of these carbohydrates is often significantly different from those found in terrestrial plants and they are characterized by low digestibility (Tosh and Yada, 2010). Polysaccharides are found in cell walls, as intercellular mucilages, and as storage

molecules. The structural polysaccharides include carrageenans, agar, and alginates, which are widely used in the hydrocolloid industry as gelling, stabilizing, and thickening agents (Kilinç *et al.*, 2013).

3.2.2.2 Phytochemicals

Alongside macronutrients, algae contain vital phytochemicals, including carotenoids and polyphenols. Carotenoids are abundant in algae; they are potent antioxidants and are considered to have different health benefits (e.g., reduced risk of coronary heart disease), and several carotenoids are precursors of Vitamin A (Ibanez and Cifuentes, 2013; Kumar *et al.*, 2008). The major carotenoids in algae are β -carotene, lutein, violaxanthin, neoxanthin, zeaxanthin, astaxanthin, and fucoxanthin. However, each variety of algae has a unique combination of carotenoids that contribute to its distinct color and act as antioxidants (Ibanez and Cifuentes, 2013). Algal polyphenols, also known as phlorotannins, can also act as antioxidants; they are considered protective against oxidative degradation and may play a role in preventing diseases caused by free-radical damage (Kumar *et al.*, 2008).

3.2.2.3 Vitamins and Minerals

Algae contain many vitamins, most prominently the water-soluble Vitamin C and B complex, and the fat-soluble Vitamins A and E. In addition, algae are one of the only vegan sources of vitamin B₁₂ (Bocanegra *et al.*, 2009). Various minerals are also found in algae and, in many cases, their concentrations are higher than in other traditional food sources. The list includes magnesium, phosphorus, calcium, potassium, iodine, iron, copper, manganese, selenium, and zinc (MacArtain *et al.*, 2007; Maehre *et al.*, 2014).

3.2.2.4 Dietary Fiber

Most algal carbohydrates can be categorized as dietary fiber as they are resistant to digestion by enzymes in the human gastrointestinal tract and can be partially or totally fermented by colonic bacteria (Lattimer and Haub, 2010; Mišurcová *et al.*, 2012). Algal fiber content ranges from 33 to 50% of the dry weight, but the specific composition of these fibers (i.e., the total water-soluble and water-insoluble dietary fiber values) vary greatly with the species, time of harvesting, geographical origin, growing conditions, and post-harvest processing of the algae (Bocanegra *et al.*, 2009). Overall, algae are a rich source of total dietary fiber, which are found in significantly greater amounts in algae than in most vegetables, whole grains or legumes.

The physicochemical properties of algal polysaccharides are often significantly different from those in plant food sources (Mišurcová *et al.*, 2012). In addition to cellulose, which can be found in some varieties of algae, many other prevalent polysaccharides are unique to algae. One example is alginic compounds: a family of linear polysaccharides that have an irregular arrangement of mannuronic and guluronic acid residues, which are capable of absorbing water at 200–300 times their own weight. A second example is agar, a highly soluble fiber composed of a mixture of sulfated galactans, D-galactose, and L-lactose. Yet another example is carrageenan, a group of linear sulfated galactans that is found in many species of marine red algae (Bocanegra *et al.*, 2009).

Green algae provide a notable collection of dietary fiber; they contain sulfated heteropolysaccharides in their mucilaginous matrix (Jiménez-Escríg and Sánchez-Muniz, 2000), water-soluble ulvans, and water-insoluble glucoxylans, glucuronomannans, and other ionic polysaccharides with sulfate groups and uronic acids (Lahayen, 1991).

Similar to dietary fiber from terrestrial plants, the unique physicochemical characteristics of each variety of algae will determine its physiological and metabolic impact on the human body. Thus, the specific chemical make-up of each algal polysaccharide will determine its viscosity, fermentability, water-holding capacity, bile-acid-binding ability, cation-exchange capacity, and fecal-bulking properties (Bocanegra *et al.*, 2009).

The distinctive combination of macronutrients, vitamins, minerals, dietary fiber, and phytochemicals found in algae provide a strong nutritional foundation for incorporating algae and algal supplements into the daily diet. New evidence demonstrating the therapeutic properties of algae add to its popular appeal and increase its potential to gain a more prominent place in Western nutrition.

3.3 Microalgae

Microalgae are a diverse group of more than 35,000 species of microorganisms, which can be found as individual cells or in chains or groups (Ebenezer *et al.*, 2012). Being photosynthetic autotrophs, microalgae have a crucial ecological role as primary producers of essential biomass compounds and nutrients, including omega-3 polyunsaturated fatty acids, proteins,

sterols, pigments, carbohydrates, and dietary fiber (de Jesus Raposo *et al.*, 2013). Microalgae also produce numerous secondary metabolites that can be isolated as nutraceuticals and supplements, which are considered to have health benefits because they are antioxidants, anti-inflammatories, or analgesic agents (Markou and Nerantzis, 2013).

The specific chemical composition of microalgae varies greatly with species and environmental conditions; hence, the biomass composition of microalgae can be altered dramatically by modifying the cultivation approach. Microalgae are cultivated in open culture systems or in closed (photobioreactor) systems (Ugwu *et al.*, 2008). For optimal growth, several parameters should be considered when cultivating microalgae, including the characteristics of the species being cultivated (e.g., its pigments, size, etc.), the environmental conditions that it requires (e.g., light, temperature), its physiological characteristics (e.g., response to stress or starvation), its optimal mode of operation (e.g., maximal production at the logarithmic or stationary phase), the location of the desired product within the organism (intra/extracellular), and the nature of the desired product (polysaccharide, protein, or pigment) (Arad and van-Moppes, 2013). Choosing an optimal cultivation methodology allows increased production of specific compounds for commercial purposes.

3.3.1 Red Microalgae

The phylum *Rhodophyta* (red algae) comprises both multicellular macroalgae, which are commonly found as ocean seaweed, and unicellular microalgae, which are found predominately in marine environments, brackish or fresh water, and soil (Arad and van-Moppes, 2013). In the study of red microalgae, four species, each with its own distinctive habitat, have been investigated systematically: *Porphyridium* sp. and *P. cruentum* (both seawater microalgae), *P. aerugineum* (fresh water microalgae), and *Dixoniella grisea* (brackish water microalgae).

Red microalgae have efficient photosynthetic machinery made possible by a globular chloroplast that fills most of the cell with a lamellar composition (Gantt, 1981). The main products of the photosynthetic process are sulfated algal polysaccharides (SAPs), which encapsulate the cells (Arad and van-Moppes, 2013). The colors of red microalgae are determined by the accessory pigments phycoerythrin and/or phycocyanin, which can be present in addition to chlorophyll a. The reproduction of red microalgae is asexual with a relatively fast growth rate (Adda *et al.*, 1985), making them very suitable for cultivation. For instance, the Arad group has developed an advanced system for cultivating red microalgae for large-scale production of extracellular polysaccharides. The system is comprised of closed vertical bio-reactors made of disposable polyethylene sleeves hung from iron frames. In the sleeves, cultures of red microalgae are mixed by an air stream with 2–3% CO₂, and the temperature is kept below 25 + 3°C by using an automatic water-spraying mechanism. Overall, this red algae cultivation system is efficient, inexpensive, and not easily contaminated (Arad and Richmond, 2004; Cohen and Arad, 1989).

Our ability to successfully cultivate large quantities of red microalgae *Porphyridium* sp. and to isolate SAPs led our research group to focus on this particular algal source. Our research uses a foodomic approach that integrates chemical characterization, rheological studies, biochemical investigations of polysaccharide formation, and metabolic studies that evaluate the physiological impact of consumption. Taken together, this information, along with additional molecular and genetic data, provides a rigorous scientific evaluation of the ability of *Porphyridium* sp. to act as a therapeutic agent to improve health.

3.3.2 Sulfated Polysaccharides from Red Microalgae

As mentioned earlier, the cells of red microalgae are encapsulated within SAPs that dissolve from the cell surface into the containing liquid medium (“soluble polysaccharides”; Ramus, 1972, 1986) and increase its viscosity. The biological function of those cell-wall SAPs is not clear, but it has been proposed that they serve as a “buffer layer” that surrounds the cells and protects them against unfavorable environmental conditions (e.g., drought or extreme pH values or temperatures) (Arad, 1988). In *Porphyridium* sp., on which our research has largely focused, this explanation seems very plausible. This red microalgae species was isolated from sea sand, a natural environment wherein conditions are harsh and fluctuate greatly throughout the day. At ebb tide, for instance, the microalgae are exposed to high solar irradiation and drought, from which the SAP gel layer may provide protection by maintaining the humidity that the cells require. Indeed, it has been reported that SAPs isolated from *Porphyridium* sp. are relatively stable over a wide range of temperatures (30–160°C), pH values (2–9), and salinity levels (Geresh and Arad, 1991). In addition, these SAPs may also act as free-radical scavengers and as antioxidants, functions that are critical for the survival of unicellular organisms (Arad and Richmond, 2004; Tannin-Spitz *et al.*, 2005).

The cell-wall SAPs of red microalgae are anionic heteropolymers with a high molecular mass (Dubinsky *et al.*, 1992; Geresh and Arad, 1991; Heaney-Kieras and Chapman, 1976). They contain about 10 different sugars, most prominently xylose,

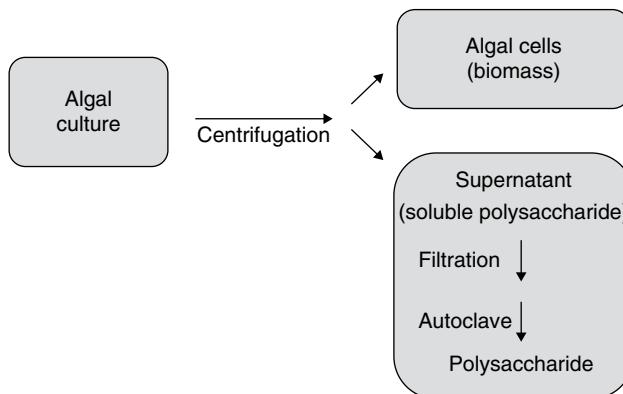


Figure 3.1 Production of red microalgae food supplements. The *Porphyridium* sp. algal culture is centrifuged to produce the two main dietary ingredients: a pellet containing algal cells (biomass) and a supernatant containing the soluble sulfated algal polysaccharides (SAPs). The SAPs are then filtrated and autoclaved to produce the isolated SAPs.

glucose, and galactose, in various ratios (Geresh and Arad, 1991; Geresh *et al.*, 1992). Their negative charge is due to the presence of glucuronic acid and half-ester groups (Heaney-Kieras and Chapman, 1976; Percival and Foyle, 1979), and their sulfate ratio varies among the different species, ranging from about 0.5 to 10% (w/w) (Capek *et al.*, 2008; Evans *et al.*, 1974; Geresh and Arad, 1991; Percival and Foyle, 1979; Ramus, 1972). The functional properties of the polysaccharides depend upon their molecular structures; however, their precise structures have not been fully elucidated due to their complex nature and the lack of known carboxyhydrolases that can degrade them (Arad *et al.*, 1993; Arad and Levy-Ontman, 2010; Ucko *et al.*, 1989).

In our laboratory, SAPs are produced from red microalgae following large-scale production and separation from the biomass (pelleted cells) in a down-stream process. First, the culture medium with the microalgae is continuously centrifuged to separate the cells from the growth medium (Figure 3.1). The pellet containing the algal cells (biomass) is then washed with distilled water, centrifuged again, frozen and lyophilized. The supernatant fraction, which contains the soluble SAPs excreted by the algae during growth, is then dialyzed to remove salts, and the polysaccharides are concentrated via ultrafiltration. Similar to the biomass, the polysaccharide fraction is frozen, lyophilized, and powdered.

Several studies have documented the therapeutic effects of SAPs isolated from red microalgae. For instance, a hypoglycemic effect was reported for SAPs from *P. cruentum* in a rodent model of diabetes (Liu *et al.*, 2005); (anti-inflammatory properties have been attributed to the SAPs of *Porphyridium* sp. (Matsui *et al.*, 2003; Tannin-Spitz *et al.*, 2005); and SAPs from various red algae have been reported to exert an antiviral activity (Arad *et al.*, 2006; Huleihel *et al.*, 2001, 2002; Huleihel and Arad, 2001). Next, we report studies that suggest another role for SAPs obtained from red microalgae, namely, their activity as hypocholesterolemic agents. These studies employ the foodomics approach, with an emphasis on metabolomics. Analyses of the products of metabolic pathways that are affected by the consumption of algal products allow us to better understand the physiological and biochemical impact of these algal products, with the ultimate goal of improving health.

3.3.3 Red Microalgae as a Hypocholesterolemic Agent

Extensive research has been conducted in our laboratory to elucidate the heart-protective properties of red microalgae. This work has employed animal models to illuminate the mechanisms by which red microalgae can lower risk factors for coronary vascular diseases (Dvir *et al.*, 2000, 2009). To achieve this goal, male Sprague-Dawley rats were fed experimental diets that were supplemented with cellulose (control), pectin (a known soluble hypocholesterolemic dietary fiber), *Porphyridium* sp. cells (“biomass diet”, comprising 27% insoluble and 8.5% soluble dietary fiber), or isolated SAPs (comprising 37% soluble and 8% insoluble dietary fiber). The rats were weighed regularly and their feces were collected throughout the experimental periods (15–30 days). Gastrointestinal transit times (GTT) were measured and fasting blood samples were collected at the end of all experiments. Intestinal length was measured and tissue samples and intestinal content were stored for further analyses.

Although rats in all groups ate similar amounts of food, rats fed on the SAP-supplemented diet demonstrated significantly lower weight gain than rats fed on the other diets. In addition, rats fed on the SAP diet or on the algal biomass diet showed a significantly higher fecal dry weight, and rats fed on the biomass diet had a significantly reduced GTT. As compared with

the control diet, all three experimental diets lowered plasma cholesterol levels, but the SAP-supplemented diet demonstrated the greatest hypocholesterolemic effect.

How did the algal components modify cholesterol metabolism? Our results indicate a twofold enhancement of fecal bile acid excretion and an increased neutral sterol excretion in the SAP-fed group, while a four-fold increase in fecal bile acid excretion was found in the biomass-fed group. Loss of bile acids through the feces is thought to decrease cholesterol levels due to interference with enterohepatic circulation (Ebihara and Schneeman, 1989), and decreased availability of bile acids reduces micelle formation and inhibits lipid absorption. In addition, bile acids are synthesized from cholesterol in the liver and are continuously recycled in the body; their loss in the feces thus contributes to the depletion of cholesterol stores and potentially depresses overall cholesterol metabolism (Jones, 2008). A direct loss of neutral sterols in the feces or an indirect loss through bile acids are both thought to be important pathways for lowering the overall levels of cholesterol. In addition, our data indicate that plasma cholecystokinin (CCK) levels were significantly higher in the biomass-fed group than in the SAP-fed or the pectin-fed groups. Because CCK is responsible for gut smooth muscle contractility, it is likely that the reduced GTT observed in biomass-fed rats was the result of increased CCK secretion. The reduced GTT may have contributed to the disrupted enterohepatic circulation of bile acids and decreased lipid digestion efficiency.

One of the most dramatic findings in our studies was the increase in cecal content viscosity. The SAP supplement produced an extremely viscous chyme, which was more than 10 times more viscous than that of the biomass-fed rats and more than a 100 times more viscous than that of the cellulose- or pectin-fed rats. A highly viscous chyme is thought to impede nutrient absorption and hamper micelle formation, which can decrease overall nutrient and lipid absorption. Thus, changes in intestinal content viscosity following consumption of red microalgae products provides a possible explanation for the weight differences observed in our studies and possibly also in other studies that have shown inhibited weight gain in rats fed on fiber-rich diets (e.g., Schneeman and Richter, 1993).

Intestinal morphology was also affected by the consumption of algal compounds. As compared with rats fed on cellulose, rats fed on SAP or on algal biomass demonstrated a significant elongation of the small intestine and colon, and rats fed on SAP showed the greatest increase in the surface area of the mucosa and tunica muscularis layers of the jejunum (Dvir *et al.*, 2000). Similar to changes in intestinal content viscosity, changes in intestinal morphology are also likely to affect nutrient absorption and thereby facilitate an overall decrease in cholesterol levels. Additional experiments with cholesterol-rich diets (Dvir *et al.*, 2009) similarly showed that the SAP and algal biomass diets are effective in lowering total plasma cholesterol levels, in preventing cholesterol accumulation in the liver, in significantly improving HDL/LDL ratios, and in decreasing plasma triglyceride concentrations.

Our data indicate that the mechanisms by which the algal biomass and the SAP diets affected lipid metabolism may be very different (Figure 3.2). The algal biomass appears to act as a hypocholesterolemic agent by enhancing bile acid excretion.

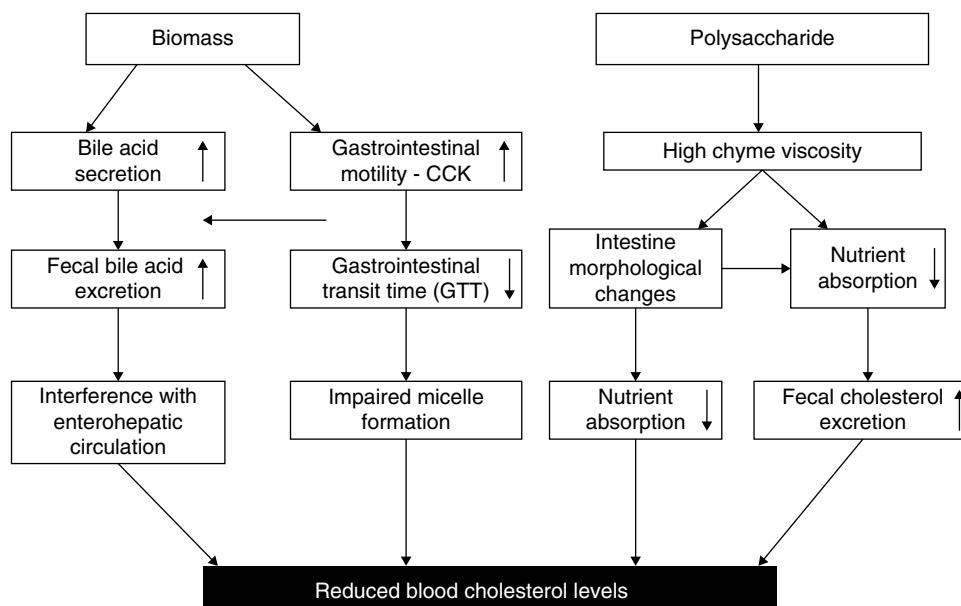


Figure 3.2 Suggested mechanisms for the hypocholesterolemic effects of biomass and SAPs from *Porphyridium* sp. See text for details.

Increased gastrointestinal motility, stimulated by greater CCK secretion and shortened GTT, may impair micelle formation, limit lipid absorption and, overall, impair intestinal nutrient absorption. The consumption of isolated SAPs, on the other hand, leads to a prominent increase in chyme viscosity, which also has the potential to interfere with nutrient absorption and increase cholesterol (neutral sterol) loss in fecal matter. The high viscosity of the intestinal contents due to SAP consumption affects the intestinal morphology, as has been also shown for other soluble dietary fiber sources (Stark *et al.*, 1996). Such changes in morphology are considered a compensatory mechanism that allows a greater surface area for nutrient absorption in response to a highly viscous chyme. In addition, increased intestinal muscle (muscularis) volume helps providing mechanical support to move the intestinal contents forward along the digestive tract.

Disparities in the physiological and metabolic effects between the red microalgal fractions are attributed to differences in the chemistry and composition of the dietary fiber and possibly of other biologically active compounds. The algal biomass, containing whole cells, is a predominately-insoluble dietary fiber. As such, its effect on viscosity is considerably lower than that of the isolated soluble SAPs. The algal biomass also contains other compounds, such as omega-3 fatty acids, which may contribute to the triglyceride lowering effects observed in our studies.

3.4 Summary

Marine sources of functional foods provide a promising area for development in the food industry. Among the available marine sources, algae – and, in particular, red microalgae – appear to be an especially attractive novel food source. Mainly, red microalgae could be used to produce food supplements that can decrease risk factors for heart disease, for instance by lowering cholesterol levels. The physicochemical properties of the dietary fiber in the algal biomass and in the isolated SAPs are unique among the more commonly eaten fiber sources obtained from terrestrial plants. It is therefore important to further investigate the potential health benefits of these products. A foodomics approach to identifying the molecular and metabolic mechanisms underlying the physiological effect of red algal products provides scientific data for developing evidence-based recommendations for the use of therapeutic supplements.

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Part II

Genomics

4

Gene-Diet Interaction and Weight Management

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4.1 Introduction

Obesity has been increasing rapidly, doubling all over the world since 1980 and now has become a global epidemic. Two thirds of adults in the US are currently affected by obesity and being overweight, and the prevalence of these conditions is predicted to be greater than 80% by 2030 (Malik *et al.*, 2012; Nguyen and El-Serag, 2010). The alarming escalation of obesity is believed to be largely due to considerable transition from a “traditional” to “obesogenic” environment, featured by increased access to highly palatable, calorie-dense foods and beverages, as well as sedentary lifestyles (Chaput *et al.*, 2012; Wells, 2012). Obesity is the major risk factor for various disorders such as Type 2 diabetes, coronary heart disease, stroke, hypertension, and cancers. In the USA, approximately 300 million adults die each year as a result of being overweight or obese, which has become the leading cause of preventable death (Nguyen and El-Serag, 2010).

There is an array of approaches to obesity prevention and management. These include modifications of dietary habits and lifestyle, pharmacological therapy, and bariatric surgery (Acosta *et al.*, 2014; Bray and Ryan, 2014; Makris and Foster, 2011; Wadden *et al.*, 2012). Even though medications and bariatric surgery have been demonstrated to be effective for obese patients, these approaches are usually employed for severely obese patients and are limited by side effects, not suitable for application to the free-living population, and current evidence is limited by the lack of long-term data demonstrating net benefit. Indeed, current guidelines emphasize diet and lifestyle modifications are best to achieve slow weight loss.

As one of the mainstream efforts on weight management in overweight and obese patients, various diet interventions have been proposed to improve weight loss and long-term weight maintenance (Makris and Foster, 2011; Malik and Hu, 2007). In previous diet intervention trials, considerable inter-individual heterogeneity has been noted in participants' responses, and accumulating evidence suggests that genetic variations likely underpin the heterogeneous response (Qi, 2012; Qi and Cho, 2008; Sacks *et al.*, 2009). Body weight is one of the strongest genetically influenced traits. In the past few years, genetic investigations have identified several dozens of variants associated with body weight and obesity (Ramachandrappa and Farooqi, 2011; Temelkova-Kurtktschiev and Stefanov, 2012). The studies have also suggested that many of the obesity-associated genes are involved in neurological regulation of appetite and food intakes, providing biological basis for the potential gene-diet interactions (Spelioites *et al.*, 2010). In addition, several recent studies in

prospective cohorts and randomized controlled trials (RCTs) provide promising evidence to support the potential gene-diet interactions on obesity and weight loss (Qi and Cho, 2008; Qi *et al.*, 2012a, b).

This chapter will summarize the recent advances in the areas of diet and lifestyle interventions on weight management, genetic discovery, and gene-diet interactions in relation to obesity, weight loss, and maintenance. The article will also introduce the concept of personalized weight management and emphasize the potential challenges lie in the area, the gap between the translational potential and practices, and future directions.

4.2 Diet and Lifestyle Modifications in Weight Management

Overweight and obese individuals are encouraged to lose weight and it is recommended that patients with obesity lose at least 10% of their body weight. Compelling evidence has shown that even modest reduction in body weight has quantifiable benefits on improvement of obesity-associated comorbidities (Sacks, *et al.*, 2009; Shai *et al.*, 2008). Diet and lifestyle modifications, such as decreasing energy intake and increasing physical activity, have been the preferred first line for weight management.

Energy-restricted diets are traditionally prescribed for weight loss (Finer, 2001). In recent years, there has been substantial focus on the macronutrient profile of a diet in potentiating weight loss and maintenance (Ebbeling *et al.*, 2012a; Goss, *et al.*, 2013; Te Morenga and Mann, 2012). Four types of dietary regimens are commonly used in weight loss interventions: low-calorie diet (LCD), very low-calorie diet (VLCD), low-fat diet, and low-carbohydrate diet (Fock and Khoo, 2013). While LCD and VLCD have consistently shown effects on reduction of body weight, low-fat diet, and low-carbohydrate diet generally show no significant difference in effectiveness on weight-loss. In a meta-analysis, Hooper *et al.* reported that lower total fat intake led to moderate but significant weight loss in adults with baseline fat intakes of 28–43% of energy intake and durations from 6 months to over 8 years (Hooper *et al.*, 2012). In another study, it was found that the low carbohydrate diet was associated with ~7 kg reduction in body weight (Santos *et al.*, 2012). Sacks *et al.* (2009) compared four popular weight-loss diets varying in fat, protein, or carbohydrates, and found there was no significant difference in promoting weight loss in a 2-year intervention trial. Wycherley *et al.* conducted a systemic review on the carbohydrate-to-protein ratio of low-fat diets. Compared with an energy-restricted standard protein diet, an isocalorically prescribed high-protein diet provided modest benefits for reductions in body weight and fat mass (Wycherley *et al.*, 2012).

Several specific dietary components have been also related to weight loss. For example, high intakes of whole grains have been consistently associated with reduced risk of obesity (Karl and Saltzman, 2012). Dairy products have also been found to facilitate moderate weight loss in several short-term RCTs (Chen *et al.*, 2012). Recently, it was found that reduced consumption of sugar sweetened beverages (SSBs) promoted weight loss in children (de Ruyter *et al.*, 2012; Ebbeling *et al.*, 2012b).

Although the value of diet interventions in weight management has been established, adherence to dietary modifications has been problematic in the long term. It has been estimated that significant weight regain occurs in 80–90% of individuals who lost body weight through diet interventions (Ramachandrappa and Farooqi, 2011). The DIOGENES is a randomized, controlled dietary intervention study that examines the effects of dietary protein and glycemic index (GI) on weight regain and metabolic risk factors in overweight and obese families, after an 8-week weight loss period on a low-calorie diet (Larsen *et al.*, 2010). In a recent follow-up analysis, it was found that average weight regain over the 12-month intervention period was 3.9 kg. Subjects on the high-protein diets regained less weight than subjects on the low-protein diets. Diets with different GI did not exhibit significant difference on weight regain. The data suggest that a higher protein content of an *ad libitum* diet may improve weight loss maintenance in overweight and obese adults (Aller *et al.*, 2014).

4.3 The Role of Genetic Factors in Determining Body Weight and Weight Loss

It has long been acknowledged that body weight is not only determined by the environment alone; in fact, body weight and obesity are among the most strongly genetically influenced traits. Scores of genes have been implicated through screening rare mutations in extreme obesity. The success in discovering rare genetic mutations in the leptin gene for the first time demonstrated the role of genetic variants in causing monogenic form of obesity in humans; and the identification of genes elsewhere in the genome such as LEPR (leptin receptor), MC4R (melanocortin 4 receptor), and POMC (pro-opiomelanocortin) for extreme obesity further proves the genetic contribution (Farooqi and O’Rahilly, 2006). Classic genetic research, such as twin studies and family studies in the 1980s and 1990s, has suggested that 40–70% of variation in body size is due to genetic factors (Farooqi and O’Rahilly, 2006).

Over the past two decades, researchers have made efforts to zero in on the genes affecting common form of obesity, the condition influencing a high proportion of the population. However, studies employing traditional linkage analysis within affected family pedigree and candidate gene approach focusing on biological or positional candidate regions were not fruitful in identification of novel genes harboring variants that affect body weight or obesity risk (Qi and Cho, 2008). This is largely due to that these studies are usually underpowered to detect moderate genetic effects which have been clearly demonstrated in later genome-wide association studies (GWAS). In addition, lack of adjustment for multiple testing and lack of replication are also among the major reasons for the little success of these approaches.

The vast majority of common genetic variants related to BMI or/and obesity risk, with minor allele frequency greater than 5%, were identified through GWAS (El-Sayed Moustafa and Froguel, 2013). The first GWAS of BMI in 2007 found that individuals who carry two copies of a common variant of the *FTO* gene had on average 3 kg more than those who do not carry any risk allele. Since then, the application of GWAS in large-scale populations has led to discovery of more than 30 genomic loci containing variants affecting BMI (Figure 4.1). It is notable that each variant on its own only had a very

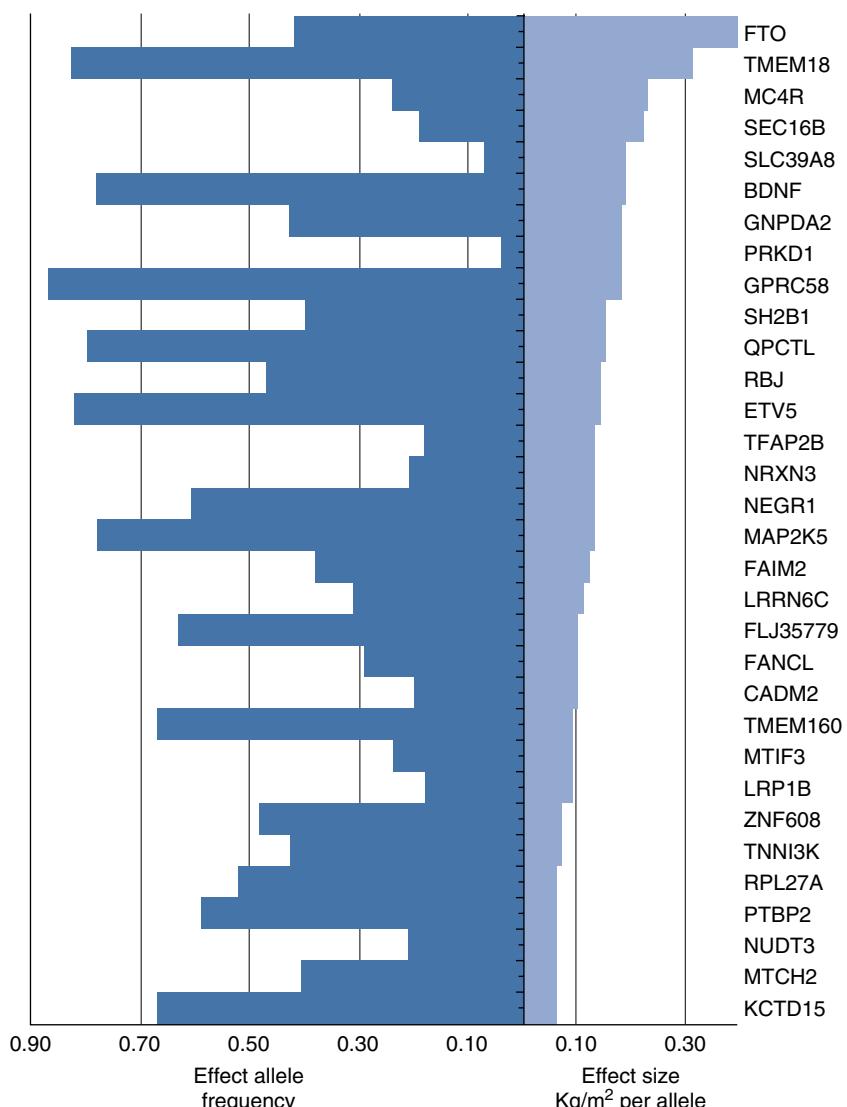


Figure 4.1 The effect sizes and frequencies of the risk alleles of the established SNPs associated with body mass index and obesity.

modest effect on adiposity phenotypes, and each risk allele is on average associated with 0.17 (0.06 to 0.39) kg/m² higher BMI; and all the variants taken together only account for 2–4% of variability of the phenotype (El-Sayed Moustafa and Froguel, 2013). Interestingly, many obesity genes are highly expressed in brain especially regions involved in regulation of appetite and food intakes, highlighting a neuronal influence on body weight regulation. In addition, genetic analyses indicate that different variants are involved in determining distribution of body fat; and GWAS of measures of fat distribution such as waist circumference and waist to hip ratio have detected common variants that are specifically associated with these markers, independent of BMI (Heid *et al.*, 2010).

Similar to body weight itself, weight loss is a complex phenomenon dependent on both genetic and environmental influences. However, very few GWASs have been performed on weight changes in response to weight-loss interventions. This is largely due to the fact that most of the existing intervention studies on weight-loss are small in size and it is extremely difficult to find samples for replications. By the year 2014, there were only two genome-wide studies performed on weight loss after gastric bypass surgery. In one study, Hatoum *et al.* conducted GWAS of 693 individuals undergoing Roux-en-Y gastric bypass (RYGB) surgery with replication in an independent population of 327 individuals undergoing RYGB. A locus on chromosome 15 locus near ST8SIA2 and SLCO3A1 was found to be associated with weight loss after RYGB (Hatoum *et al.*, 2013). In addition, it was found that expression of ST8SIA2 in omental fat of the participants was significantly associated with weight loss after RYGB. Rinella *et al.* carried out a similar but smaller GWAS (n = 89 and 169 in the discovery and replication stages, respectively). Seventeen SNPs near genes including PKHD1, HTR1A, NMBR, and IGF1R were identified to be nominally related to percent excess body weight loss at 2 years after surgery. However, the associations did not reach genome-wide significant level, raising concerns about potentially false positive findings (Rinella *et al.*, 2013).

The Look AHEAD is a randomized trial to determine the effects of diabetes support and education (DSE) and intensive lifestyle intervention (ILI), which combined diet modification and increased physical activity designed to produce an average of 7% weight loss and maintenance on cardiovascular morbidity and mortality in overweight and obese subjects with Type 2 diabetes. In a recent study of 3899 individuals from The Look AHEAD (2013b) McCaffery *et al.* analyzed 31,959 common SNPs on the Illumina CARE iSelect (IBC) chip with weight change at year 1 and year 4, and weight regain at year 4 among those who lost $\geq 3\%$ at year 1. Even though two novel regions were found to show chip-wide significant association with year-1 weight loss in ILI arm ($p < 2.96E-06$), these findings have not been further validated in other independent studies. No GWAS has been reported on weight loss in settings involving diet or/and lifestyle interventions. Apparently, large-scale collaborations of multiple weight-loss trials are essential to improve study power to identify new genetic loci.

4.4 Gene-Diet Interactions on Body Weight and Risk of Obesity

Mounting evidence has shown that dietary factors and genetic factors may interplay with each other in affecting body weight and obesity risk. A group of studies have examined interactions of individual nutrients such as total fat, P:S ratio, carbohydrate, and vitamins with the variants in candidate genes in relation to obesity traits (Qi and Cho, 2008). For example, in an early study, Memisoglu *et al.* assessed interactions between dietary fat intakes and PPAR gamma proline to alanine substitution polymorphism (Pro12Ala) in 2141 women from the Nurses' Health Study (NHS), a large ongoing prospective cohort study. It was found that intakes of total and monounsaturated fat significantly interacted with the polymorphism in relation to BMI (Memisoglu *et al.*, 2003). In a recent study, Larsen *et al.* examined genetic markers for BMI, WC, and WHR interacted with dietary calcium in relation to subsequent annual change in of the adiposity measures among 7569 individuals from the from the Danish Diet, Cancer and Health Study and the INTER99 study. Significant interaction between a WC genetic score calculated from six SNPs and calcium was found to be in relation to change in WC. Each risk allele was associated with -0.043 cm of WC ($P = 0.038$; 95% CI: -0.083 , -0.002) per 1000 mg Ca. However, no replication evidence was provided (Larsen *et al.*, 2014).

In fact, few findings from the previous studies are reproducible, and detection of gene-diet interactions remains a challenge in population studies (Qi and Cho, 2008; Qi, 2014). The majority of the previous studies is cross-sectional and is subject to confounding bias and reverse causation. Prospective design is advantageous in studying gene-environment interactions. In addition, measurement errors are usually considerable in assessing diet and lifestyle factors in large population studies, and therefore add substantial noise in analysis and lower the study power. Moreover, methods used in assessment of diet and lifestyle factors vary significantly across studies, and make the efforts to combine multiple datasets difficult. Statistical modeling of gene-environment interaction usually simplifies complex biological events and therefore performs poorly in capturing the diverse patterns of interactions. In addition, large sample size is essential to detect

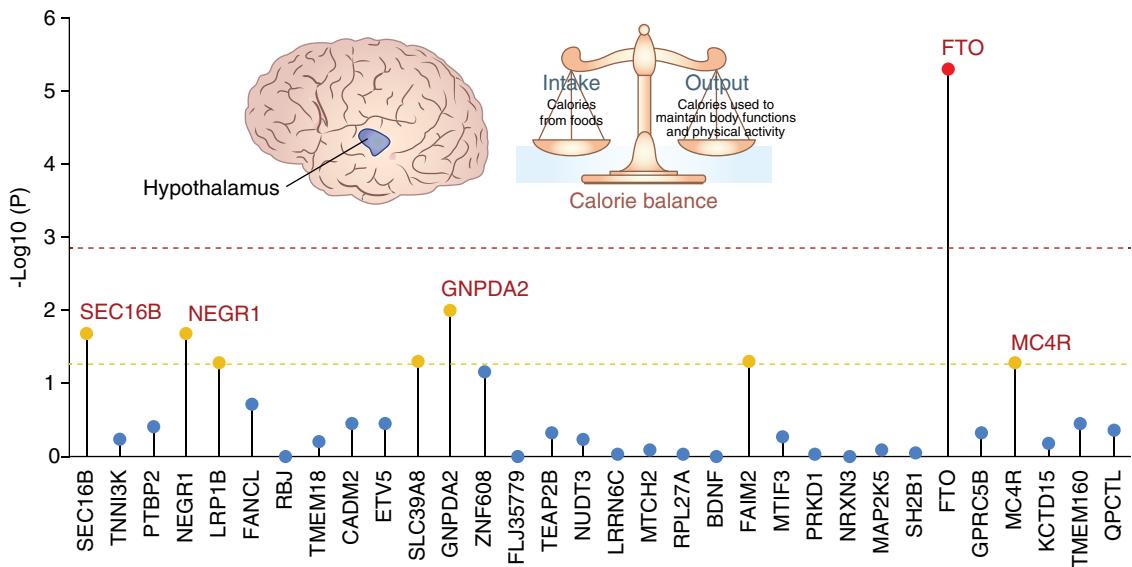


Figure 4.2 Interactions of the obesity-associated genes with fried food consumption in relation to body mass index. The genes showing significant interactions ($p \leq 0.05$) are highlighted in lighter shading. The majority of the significant genes are highly expressed in the brain, especially the regions involved in regulation of energy balance.

moderate interactions. The experience in GWAS has emphasized importance of replication in genetic discovery. Similarly, replication would be critical to validate any findings of gene-environment interactions.

Recently, we performed several analyses to address the interactions between genetic factors and dietary factors in relation to body weight and obesity risk in large, prospective cohorts. In a study (Qi *et al.*, 2012a), we examined interactions between intake of sugar sweetened beverage (SSB) and genetic susceptibility to obesity (evaluated on 32 BMI-associated loci) in relation to BMI and obesity among three cohorts – the NHS, the Health Professional Follow-up Study (HPFS), and the Women Genome Health Study (WGHS). We observed directionally consistent interactions between genetic susceptibility and SSB. In the three cohorts combined, the pooled relative risks (95% CI) for incident obesity per increment of 10 risk alleles were 1.34 (1.16–1.52), 1.58 (1.30–1.87), 1.52 (1.21–1.83), and 3.24 (1.90–4.58) across the four categories of SSB intake (P for interaction <0.001). In a similar analysis among the three cohorts NHS, HPFS, and WGHS, consistent interactions were found between the obesity GRS and fried food consumption in relation to BMI (Qi *et al.*, 2014). In the NHS and HPFS (P for interaction ≤ 0.001), among participants in the highest tertile of the GRS, the differences in BMI between individuals who consumed fried foods ≥ 4 times/week and those consumed <1 time/week amounted to 1.0 (SE 0.2) in women and 0.7 (0.2) kg/m^2 in men, whereas the corresponding differences were 0.5 (0.2) and 0.4 (0.2) kg/m^2 in the lowest tertile of the GRS. The gene-diet interaction was replicated in the WGHS (P for interaction < 0.001). It was also found that the individual variants in genes such as FTO, MC4R, and NEGR1 showed significant interactions with fried food intakes. Interestingly, these genes are all highly expressed in brain and play key roles in regulating energy intake and expenditure, suggesting gene-diet interactions likely occur in pathways of the central nervous system (Figure 4.2).

4.5 Gene-Diet Interactions on Weight Loss in Randomized Clinical Trials

The Preventing Overweight Using Novel Dietary Strategies (Pounds Lost) is a clinical trial including in total of 811 overweight or obese adults assigned to weight-loss diets varying in macronutrient contents for 2 years (Sacks *et al.*, 2009). The participants had lost an average of 6 kg at 6 months; but began to regain weight after 12 months. By the end of intervention at 2 years, weight loss was found to be similar in those who were assigned to various diets. In the Pounds Lost trial, significant interactions was reported between the *IRS1* SNP rs2943641 and carbohydrate intake in relation to changes in weight loss and insulin resistance (Qi *et al.*, 2011). At 6 months, participants with the risk-conferring CC genotype were

found to have greater decreases in weight loss ($P = 0.018$) than those without this genotype in the highest-carbohydrate diet group; however, the IRS1 genotype was not related to weight loss in those with the lowest-carbohydrate intake (P for interaction = 0.03). The gene-diet interaction was attenuated at 2 years due to weight regain. In another study, it was found that SNP rs1558902 in obesity gene FTO interacted with dietary protein on 2-year changes in measures of body compositions and fat distribution including fat-free mass, total percentage of fat mass, and total-, visceral-, and superficial adipose tissue mass (Zhang *et al.*, 2012). It appeared that a high-protein diet were more beneficial in individuals with the risk allele A. These data indicate considerable genetic heterogeneity in weight loss in response to diet interventions. Interestingly, in the same study population, it was found that the FTO genotype might interact with diet protein in relation to changes in appetite-related traits such as food cravings and appetite scores. The A allele of FTO SNP rs9939609 was associated with a greater decrease in food cravings and appetite scores in participants with high-protein-diet intake ($P = 0.027$ and 0.047, respectively) but not in subjects in the low-protein-diet group ($P = 0.384$ and 0.078, respectively) (Huang *et al.*, 2014).

In a subset of 776 high cardiovascular risk subjects from the PREDIMED, a randomized trial aimed at assessing the effect of the Mediterranean diet (MD) for primary cardiovascular disease prevention, Razquin *et al.* found that subjects carrying the A allele if FTO SNP rs9939609 had the lowest body weight gain ($B = -0.685$; $P = 0.022$) after 3 years of nutritional intervention compared those without the allele. Moreover, the effect was significant only in carriers of the A allele allocated to the MD groups but not in the control group (P for interaction = 0.649) (Razquin *et al.*, 2010). In the Diabetes Prevention Program (DPP), 3819 participants were randomized into intensive lifestyle modification (eating less fat and calories and exercising for a total of 150 min a week), metformin or placebo control. Pan *et al.* genotyped 20 tagging SNPs for MC4R, a gene involved in the melanocortin system and energy homeostasis. The minor allele of rs17066866 was associated with less short-term (baseline to 6 months; $P = 0.006$) and long-term (baseline to 2 years, $P = 0.004$) weight loss in the lifestyle intervention group, but not in placebo group (Pan *et al.*, 2013). However, it is difficult to tease out which lifestyle components (diet or exercise) interacted with the genotype. In the TULIP, a trial consisted of exercise and diet intervention with decreased intake of fat and increased intake of fibers (>15 g fiber per 1000 kcal). SNP rs7903146 in diabetes-associated TCF7L2 gene was found to be related to weight loss and the CC genotype of was associated with significantly greater weight loss in participants with high fiber intake, but not in those with low fiber intake (Heni *et al.*, 2012). Gene-diet interactions on weight loss were also observed in many other studies (Qi, 2014). (Table 4.1) It is notable in all these studies, no replication was performed.

In addition to nutrient components, several recent studies have also found that eating behaviors such as dietary restraint and disinhibition might also influence inter-individual differences in response to weight loss, and interact with genetic factors (Lopez-Guimera *et al.*, 2014; Verhoef *et al.*, 2014). However, the evidence is far from robust.

4.6 Gene-Diet Interactions on Weight Maintenance

Weight regain after intentional weight loss remains a major challenge in long-term weight maintenance. Although diet and lifestyle modification may usually lead to weight loss, a proportion of the participants regain body weight later during the course of intervention and the majority of them regain weight after intervention. The mechanisms underlying weight regain after weight loss has been extensively studies. It is believed that physiological adaptation to weight loss encouraging restore of body weight, including alterations in energy expenditure, appetite regulation, and substrate metabolism plays a critical role in driving regain of body weight (Sumithran and Proietto, 2013).

The gene-diet interactions on weight maintenance after weight loss have been specifically assessed in several clinical trials. In the DIOGENES trial, Larsen *et al.* recently examined 768 tagging SNPs for nutrient-sensitive candidate genes among 742 participants who had significantly lost body weight by low-calorie diet in the DIOGENES. It was found that the genetic variants in *CCK*, *MLXIPL*, *GHRL*, and *LEPR* significantly interacted with dietary protein on weight regain in the participants. In addition, *PPARD*, *LPIN1*, *PLAUR*, and *FABP1* variants significantly interacted with dietary protein on fat mass regain (Larsen *et al.*, 2012).

In The Look AHEAD, McCaffery *et al.* recently examined 13 obesity-predisposing polymorphisms in predicting weight change at year 1, and weight regain at year 4 among individuals who lost 3% or more of their baseline weight by year 1. It was found that SNP rs3751812 near FTO gene showed significant prediction on weight regain in the DSE group ($P = 0.005$), but not within the ILI group, suggesting potential gene-lifestyle intervention interactions (McCaffery *et al.*, 2013a). In another study, Erez *et al.* examined potential predictors for weight changes during the “weight loss phase” (0–6 months) and the “weight maintenance/regain phase” (7–24 months) among the participants from a 2-year weight-loss diet intervention trial. It was found that SNPs rs4731426 and rs2071045 in LEP gene were associated with weight regain (Erez *et al.*, 2011).

Table 4.1 Selected studies on gene-diet interactions on weight loss and weight regain.

Studies	Participants and interventions	Major findings
Weight loss		
Qi <i>et al.</i> , 2011	2-y diet intervention; 738 overweight or obese adults	<i>IRS1</i> genetic variants modified effects of dietary carbohydrate on weight loss and insulin resistance
Mattei <i>et al.</i> , 2012	2-y diet intervention; 591 overweight or obese adults	Dietary fat intake interacted with <i>TCF7L2</i> rs7903146 genotype in relation to changes in BMI, total fat mass, and trunk fat mass
Zhang <i>et al.</i> , 2012	2-y diet intervention; 742 overweight or obese adults	High-protein diet interacted with <i>FTO</i> rs1558902 genotype in relation to weight loss and improvement of body composition and fat distribution
Heni <i>et al.</i> , 2012	N = 304; 9-m diet intervention	<i>TCF7L2</i> SNP rs7903146 CC genotype was associated with greater weight loss in participants with high fiber intake, but not those with low fiber intake
Qi <i>et al.</i> , 2012	2-y diet intervention; 737 overweight or obese adults	Dietary carbohydrate modified <i>CIPR</i> SNP rs2287019 genotype effects on changes in body weight, fasting glucose, and insulin resistance
Pan <i>et al.</i> , 2013	N = 3,819; 2-y intervention; lifestyle modification and metformin	<i>MC4R</i> SNP rs17066866 was associated with less short-term (baseline to 6 months) and less long-term (baseline to 2 years) weight loss in the lifestyle intervention group, but not in placebo group
Xu, <i>et al.</i> , 2013	2-y diet intervention; 734 overweight or obese adults	Dietary fat significantly modified genetic effects of BCAA associated <i>PPM1K</i> SNP rs1440581 on changes in weight, fasting insulin
Weight regain		
Erez <i>et al.</i> , 2011	2-y diet intervention; 322 overweight or obese adults	<i>LEP</i> genotype was related to weight regain from 7 to 24 months
Larsen <i>et al.</i> , 2012	N = 742; 6-m diet intervention on weight loss maintenance	Diet glycemic index and protein intakes interacted with multiple variants in nutrient-sensitive genes on regain of waist and fat mass
McCaffery <i>et al.</i> , 2013a	N = 3899; 4-y lifestyle intervention in diabetic patients	Variations in the <i>FTO</i> and <i>BDNF</i> loci were related to weight regain after weight loss

Abbreviations: BCAA, branched chain amino acid; SNP, single nucleotide polymorphism.

4.7 Personalized Weight Management through Diet and Lifestyle Modifications

Modifications of diet and lifestyle have been widely accepted as mainstream strategy to prevent obesity and lower its complications; and a one-size-fits-all strategy is currently adopted in healthy diet and lifestyle recommendation. However, the conventional approach requires substantial simplification and strong assumption that there is no inter-individual variance in responses to diet interventions. Emerging evidence has shown that human genotypes may modify dietary effects on weight loss and maintenance, suggesting that to consider genomic variations seems to become the leading edge of personalized interventions, and some observers enthused that the new technology would revolutionize the strategy to prevent and treatment human diseases. Empowering genotyping and sequencing technologies enables assessment of individual's genomic feature in unprecedented efficiency and detail. This leads to an expectation of the switch from the traditional, one-size-fits-all diet intervention toward a more personalized manner, by referring to the "individuality" of the human genome. Commercial companies have sprung up and begun to market direct-to-consumer DNA testing. However, it remains questionable whether the data collected are appropriately interpreted and there is little practical guidance for health professionals and consumers concerning their use.

A potentially important role of genetic findings is to stratify risk for personalized screening. Pashayan *et al.* have shown that polygenic risk stratification can potentially improve the effectiveness and cost-effectiveness of screening programs. However, compared with "one-size-fits-all" screening programs, personalized screening adds further layers of complexity to the organization of screening services and raises ethical, legal and social challenges (Pashayan *et al.*, 2013). Currently, little is known whether personalized genetic counseling may improve modifications of diet habits or lifestyle. Grant *et al.*

recently examined whether diabetes genetic risk testing and counseling could improve diabetes prevention behaviors among 108 overweight patients (Grant *et al.*, 2013). Participants in the higher- and lower-genetic risk received individual genetic counseling before being enrolled with untested control participants in a 12-week, validated, diabetes prevention program. It was found that there were few statistically significant differences in self-reported motivation, program attendance, or mean weight loss when higher-risk recipients and lower-risk recipients were compared with control subjects. The data from this study suggest genetic counseling might not alter self-reported motivation or prevention program adherence. However, the study is small in size and likely to be underpowered to detect the differences as proposed. In addition, it remains unclear how genetic information would be translated to general patients regarding its interactive relation with diet/lifestyle modifications and implication on improvement of health. To date, no study has assessed how genetic counseling may affect diet and lifestyle modifications on weight loss.

4.8 Summary and Concluding Remarks

For obese and overweight patients, the best weight management strategy is to eat less and exercise more. Even with a tremendous body of research conducted, controversy still abounds regarding the relative effectiveness of various weight-loss and weight-maintenance diets in weight management. In addition, weight management prescriptions are usually ineffective over the long term and weight regain occurs due to low compliance. Moreover, considerable heterogeneities in response to diet interventions have been noted for long time and genetic variations are considered to play a critical role. In the past few years, growing studies have shown evidence for interactions between genetic factors and dietary factors on obesity, weight loss, and maintenance, suggesting genetic variations may modulate the effects of diet interventions on management of obesity. GWAS have been a huge success for identification of genetic loci affecting obesity; however, large-scale GWAS on weight management is still lacking. To have the knowledge about genetic variants involved in affecting response to diet and lifestyle interventions may be useful in a number of ways, including improved biological understanding and discovery of more efficient intervention strategies.

Study of gene-diet interaction may inform development of personalized diet and lifestyle interventions based on genetic profile that are better tailored to meet the individuals' needs. In 2005, public health genomics was proposed as "the responsible and effective translation of genome-based knowledge and technologies for the benefit of population health" (Pashayan *et al.*, 2013). Currently, genomic risk profiles based on the known common susceptibility variants have limited utility in weight management; however, the increasing availability of genotyping and next-generation sequencing (NGS) provides a potential that genomic testing may have an active role in promoting diet and lifestyle interventions. A more provocative question is whether a "personalized" strategy should replace the current practice of weight management. Even putting aside the lack of long term safety data, there are clearly significant concerns regarding ethical and cost-efficient issues. In addition, the gene-diet interactions are complex. Exploration into the function and mechanisms, and thoroughly understanding the interactions will help define strategies as we move towards personalized weight management.

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5

NutrimiRomics: The Promise of a New Discipline in Nutrigenomics

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5.1 Introduction

The field of nutrient–gene interactions has gradually unfolded over the last decade and established itself as a key cornerstone in nutritional research. Studies have focused on genes that encode proteins whose functions are of interest in health and disease. Noncoding genes, which have been referred to as “junk DNA” (Downs *et al.*, 2005; Flam, 1994; Nowak, 1994), have been of no interest. Times have changed. What used to be “junk” is now under the spotlight center stage (Khajavina and Makalowski, 2007; *Nature* Technology Feature, 2009). The central dogma in molecular biology ignored a significant part of the genetic code, which remained under veils for decades. Noncoding RNA (ncRNA) genes produce functional RNA molecules rather than encoding proteins. Several different systematic screens have identified a surprisingly large number of ncRNA genes. ncRNAs seem to be particularly abundant in roles that require highly specific nucleic acid recognition without complex catalysis, such as in directing post-transcriptional regulation of gene expression or in guiding RNA modifications. Although it has been generally assumed that most genetic information is transacted by proteins, recent evidence suggests that the majority of the genomes of mammals and other complex organisms are in fact transcribed into ncRNAs, many of which are alternatively spliced and/or processed into smaller products (Mattick and Makunin, 2006). These RNAs (including those derived from introns) appear to comprise a heretofore hidden layer of internal signals that control various levels of gene expression in physiology and development, including chromatin architecture/epigenetic memory, transcription, RNA splicing, editing, translation, and turnover. This hidden layer of internal signals is now emerging to be of such critical significance that a lack of consideration for that layer poses the serious risk of clouding our ability to understand the molecular basis of health and disease (Goodrich and Kugel, 2006; Mattick and Makunin, 2006; Racz and Hamar, 2006; Tomaru and Hayashizaki, 2006). Post-transcriptional gene silencing (PTGS), which was initially viewed upon as an isolated regulatory mechanism in some plant species, now represents a major frontier in molecular medicine (Filipowicz *et al.*, 2006; Racz and Hamar, 2006). RNAi was first observed inadvertently in an experiment to increase the purple pigment in petunias. However, the experiment backfired when the gene introduced caused PTGS of the pigment production gene. Subsequent studies on *C. elegans* and the fruit fly *Drosophila* revealed that PTGS could be triggered by dsDNA. A similar phenomenon in fungus was termed “quelling”

in 1992. Andrew Fire and Craig Mello (Nobel Prize winners in Physiology/Medicine, 2006) are credited with the 1998 discovery of RNAi (Fire *et al.*, 1998). Earlier works had identified that both antisense (Izant and Weintraub, 1984) as well as sense (Guo and Kemphues, 1995) RNA could silence genes, although the results were inconsistent and the effects usually modest. In light of the observation that both sense and antisense RNA could cause silencing, Mello argued that the mechanism could not just be a pairing of antisense RNA to mRNA, and he coined the term RNAi for the unknown mechanism (Rocheleau *et al.*, 1997). The discovery that short RNA is the effector of RNAi was rapidly followed by the identification of a class of endogenous RNA molecules of the same size in worms, flies, mice, and humans. This small RNA was called miRNA (Lagos-Quintana *et al.*, 2001; Lee and Ambros, 2001; Reinhart *et al.*, 2000). In all forms of life, ncRNA includes ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), interference RNA (RNAi), short interfering RNA (siRNA), and micro RNA (miRNA or miR).

miRNAs are powerful regulators of gene expression (Sen and Roy, 2007). It is estimated that about 3% of human genes encode for miRNAs (Sassen *et al.*, 2008). A miRNA is approximately 22 ribonucleotides long, noncoding RNAs, with a potential to recognize multiple mRNA targets guided by sequence complementarity and RNA-binding proteins. Recent evidence suggests that the number of unique miRNA genes in humans exceeds 1000 (Perera and Ray, 2007). The numbers of miRNAs and their targets turn out to be much greater than what we previously thought (Ro *et al.*, 2007). miRNAs are functionally versatile, with the capacity to specifically inhibit translation initiation or elongation, as well as induce mRNA destabilization, through predominantly targeting the 3'-untranslated regions of mRNA. Briefly, miRNAs are transcribed in the nucleus by conventional mechanisms and are exported to the cytoplasm (Yi *et al.*, 2003), where after biological processing they form the mature miRNA that can interact with matching mRNAs by RNA–RNA binding. This binding, with the assistance of the RNA-induced silencing complex (RISC), leads to modes of action, resulting in mRNA degradation or translational inhibition (see Figure 5.1 later) (Tang, 2005). This mechanism of action is termed *post-transcriptional gene regulation* (PTGS). In animals, in contrast to plants, there is not a 100%-nt match between miRNA and its target mRNA, leading to a mode of action causing mRNA translational inhibition and not mRNA degradation (Carrington and Ambros, 2003). The interaction between the miRNA and its matching mRNA occurs between the 5' UTR of the miRNA to the 3' UTR region of the mRNA by a matching seed element in the miRNA. Utilizing this data, computational prediction approaches estimate that miRNAs can target 30% of the human genome (Kruger and Rehmsmeier, 2006; Lewis *et al.*, 2005; Sassen *et al.*, 2008; Smalheiser and Torvik, 2006). Other estimates claim that more than 50% of human protein-coding genes might be regulated by miRNAs (Wu *et al.*, 2007). Furthermore, one miRNA can regulate hundreds of genes (Wu *et al.*, 2007) and that one gene can be regulated by a number of miRNAs.

The first step in any gene expression is its transcription. Initially, it was believed that miRNA transcription is mediated by RNA polymerase III, because it transcribes most of the small RNAs. However, pri-miRNAs are sometimes several kilobases long and contain stretches of more than four uracils, which would have terminated transcription by pol III. Lee *et al.* (2004) have concluded that miRNA transcription is accomplished by RNA polymerase II. The miRNA is first transcribed as a hundreds to thousands of nucleotide long miRNA precursor named a primary miRNA (pri-miRNA). Analysis of several, pri-miRNA precursors has shown that they all contain a 5'7-methyl guanosine cap and a 3' poly-A tail. Therefore, this data indicates that pri-miRNAs are structurally analogous to mRNAs (Cullen, 2004). Following transcription, the miRNA goes through the first step of cleavage. It is initiated by the nuclear RNA's III Drosha, a double-stranded RNA (dsRNA)-specific endonuclease that introduces staggered cuts on each strand of the RNA helix (Lee and Kim, 2005). It is responsible for nuclear processing of the pri-miRNAs into stem-loop (hairpin-shaped) precursors of ~70 nucleotides named precursor miRNA (pre-miRNAs). It has been shown that RNA interference of Drosha, results in the strong accumulation of pri-miRNA and the reduction of pre-miRNA and mature miRNA *in vivo* (Lee *et al.*, 2003). RNA stem-loops with a large, unstructured terminal loop (above 10 nt) are the preferred substrates for Drosha cleavage (Zeng *et al.*, 2005). In the nucleus, Drosha functions as a large complex where it interacts with DGCR8, an essential cofactor for Drosha, which contains two dsRNA-binding domains (Han *et al.*, 2004; Yeom *et al.*, 2006). Recombinant human Drosha alone shows nonspecific RNase activity, but the addition of DGCR8 renders it specific for pri-miRNA processing (Tomari and Zamore, 2005). The primary and secondary structure of miRNA precursors is conserved as internal loops and bulges commonly appear in specific positions in the miRNA stem. This enables correct future processing by the following enzymes in the maturation of the miRNA (Saetrom *et al.*, 2006).

Export of the pre-miRNA from the nucleus to the cytoplasm is mediated by Exportin 5 (Yi *et al.*, 2003). It is a nuclear export receptor for certain classes of dsRNA, including pre-miRNAs, viral hairpin RNAs, and some tRNAs (Chen *et al.*, 2004). It was demonstrated that the export of pre-miRNAs is sensitive to depletion of nuclear RanGTP, therefore mediated by it (Bohnsack *et al.*, 2004). Once in the cytoplasm, there is a disassembly of the exported complex by GTP hydrolysis (Matsuura and Stewart, 2004). Besides the role of Exportin 5 in the nuclear export of pre-miRNA, there is evidence that it

also has a role in preventing nuclear pre-microRNA degradation (Zeng and Cullen, 2004). The second step of miRNA processing is confined to the cytoplasm (Lee *et al.*, 2002). The pre-miRNA goes through another cleavage step, conducted by Dicer. Dicer is a multidomain ribonuclease that processes the hairpin precursor to a ~22-nt small dsRNA mature miRNA (Kolb *et al.*, 2005). Dicer functions through intramolecular dimerization of its two RNase III domains, assisted by the flanking RNA-binding domains, PAZ and ds RNA-binding domains (dsRBD) that generate products with 2 nt 3' overhangs (Zhang *et al.*, 2004). PAZ domains are highly conserved domains of 130 amino acids that bind to RNA found only in Dicer and Argonaut proteins (discussed later) (Carmell and Hannon, 2004). Following Dicer cleavage of the pre-miRNA, the mature miRNA is incorporated into a RISC complex whose diverse functions can include mRNA cleavage, suppression of translation, transcriptional silencing, and heterochromatin formation (Andl *et al.*, 2006). This complex functions as well in RNA interference. RISC is a multiple-turnover enzyme complex, meaning that miRNA can direct multiple rounds of target cleavage once incorporated. One strand of the ds miRNA is preferentially incorporated into RISC depending upon the thermodynamics of the duplex. Gregory *et al.* (2005) have isolated a trimeric protein complex of ~500 kDa that contains Dicer, human immunodeficiency virus transactivating response RNA-binding protein (TRBP), and Argonaute2 (Ago2), and have demonstrated that this complex is required for miRNA biogenesis. There is evidence that the complex forms prior to miRNA loading (Maniataki and Mourelatos, 2005). TRBP is a protein with three dsRBDs that has been shown to be essential for the miRNA processing (Haase *et al.*, 2005). Ago2 is a member of the Argonaute protein family and the only member in humans associated with both siRNA and miRNA silencing (Sontheimer and Carthew, 2004). It serves as the catalytic engine of RISC by a PIWI domain that contains a RNaseH-like structure for its endonucleolytic-slicer activity (Miyoshi *et al.*, 2005; Roy *et al.*, 2009). It was found that Ago2 is essential for mouse development, and cells lacking it are unable to respond to siRNAs experiments. Moreover, mutations within its RNaseH domain inactivate RISC, proving its fundamental role in miRNA induced mRNA silencing.

Match between miRNAs and their target mRNAs inhibits translation. Therefore, RISC containing miRNA may directly interfere with translation initiation or elongation and perhaps target the mRNA to centers of degradation. These centers,

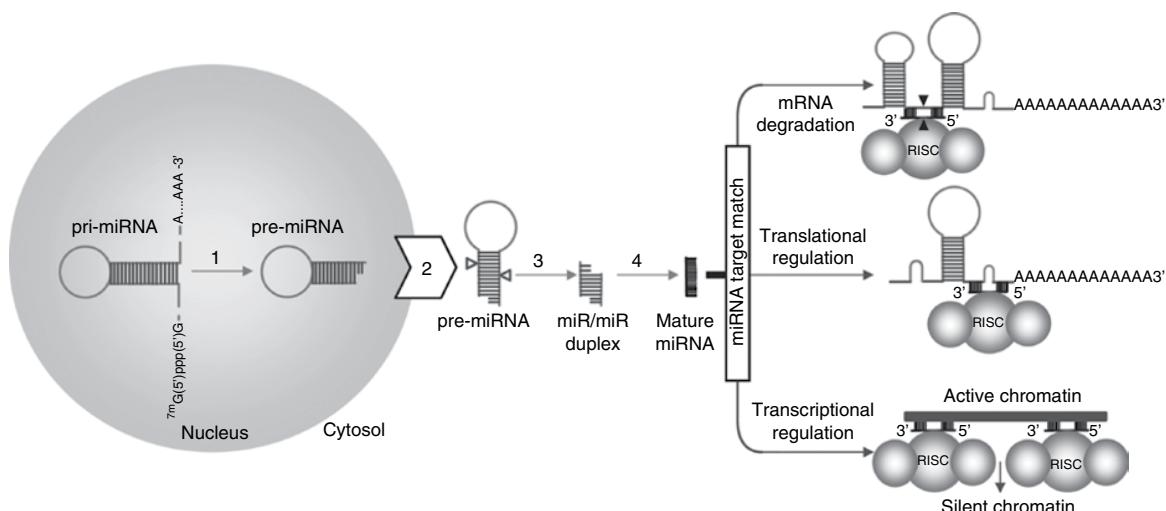


Figure 5.1 Overview of the major mechanisms involved in the generation and function of miRNA. Primary transcripts of miRNA (pri-miRNA) are generated by polymerase II and possess a 5' 7-methyl guanosine cap and are polyadenylated. Processing of pri-miRNA in the nucleus is mediated by a microprocessor complex [01] including Pasha and Drosha. Drosha is an RNase III endonuclease that asymmetrically cleaves both strands of the hairpin stem at sites near the base of the primary stem loop thus releasing a 60-to 70-nucleotide pre-miRNA that has a 5' phosphate and a 2-nucleotide 3' overhang. Specific RNA cleavage by Drosha predetermines the mature miRNA sequence and provides the substrate for subsequent processing events. The pre-miRNAs are transported to the cytoplasm by Exportin-5 [02]. Once in the cytosol, a second RNase III endonuclease, Dicer [03] cleaves the pre-miRNA. Dicer releases a 22-nucleotide mature double-stranded miRNA with 5' phosphates and a 2-nucleotide 3' overhang. One strand of the miRNA duplex is subsequently incorporated into an effector complex termed RNA-induced silencing complex [04] or RISC that mediates target gene expression. Ioshikhes, *et al.*, (2007). Reproduced with permission of Mary Ann Liebert, Inc.

which contain untranslated mRNAs, are sites of mRNA degradation previously observed in yeast and animal cells and are called processing (P) bodies (Jabri, 2005). Supporting this notion is the evidence of the presence of Argonaute family proteins in these P-bodies, though it is not clear whether as a cause or as a consequence of inhibiting protein synthesis. Chu *et al.* (2006) showed that RCK/p54 is the effector molecule in miRNA-RISC that represses translation. RCK/p54, the human homolog of yeast Dhh1p, is a P-body protein and a member of the ATP-dependent DEAD box helicase family. In human cells, RCK/p54 interacts in P-bodies with the translation initiation factor eIF4E. The overall result of the binding of mRNAs in the RISC complex by matching miRNAs will be inhibition of translation of the mRNAs, and therefore decreased protein levels of miRNAs target mRNAs. Thus, miRNAs play a key role in regulating functionality of coding genes. The mechanism of miRNA synthesis is summarized in Figure 5.1.

5.2 miRomics: A New Cornerstone

A single miRNA has the ability to regulate hundreds of mRNAs, which may have a significant effect on gene expression networks (Cheng *et al.*, 2012). Since a variation in expression of hundreds of mRNAs may be due to the expression patterns of one or a few miRNAs that regulate them, miRNA expression patterns can be especially rich in biological information (Cheng *et al.*, 2012). Genome-wide analysis of miRNA expression provides powerful insight into the functional status of the coding genome. miRNA expression profiling has been helpful in identifying miRNAs that regulate a wide range of processes, including organismal development and establishment and maintenance of tissue differentiation (Alvarez-Garcia and Miska, 2005; Wienholds *et al.*, 2005). Hence, miRNAs are used not only as investigative reagents for the reprogramming of cell fate in stem cell applications, but also as biomarkers for identifying the tissue differentiation state of cancers of unknown tissue origin (Lu *et al.*, 2008; Rosenfeld *et al.*, 2008). During the last 5 years, there has been a sharp improvement in the profiling technologies for miRome screening. What started in 2004 with laborious Northern blot analyses to screen 119 miRNA (Sempere *et al.*, 2004) has today matured into multiple technology platforms that can robustly screen more than 1000 miRNA (Li and Ruan, 2009; Pene *et al.*, 2009; Wei and Soteropoulos, 2008). One of the earliest versions of microchip contained oligonucleotides corresponding to 245 miRNAs from human and mouse genomes (Liu *et al.*, 2004). Contemporarily, 18–26 nucleotide RNAs were isolated from developing rat and monkey brains. From the sequences of these RNAs and the sequences of the rat and human genomes, the small RNAs likely to have derived from stem-loop precursors typical of miRNAs were selected. Using this approach, a microarray technology suitable for detecting 138 mammalian miRNAs was developed (Miska *et al.*, 2004). The short nature of the target sequences makes it difficult to achieve sufficient specificity with standard DNA oligonucleotide technologies. Direct random-primed cDNA synthesis on either chemically synthesized small RNAs (21–22 nucleotides) or gel-purified mature miRNAs from human cells can produce specific and sensitive full-length cDNA probes. Internally labeled cDNA probes are sensitive for detecting differential miRNA expression between control and test groups (Sioud and Rosok, 2004). Although the goal of miRNA profiling remained the same, the technology has markedly improved over time, enabling more robust analyses. Some miRNAs differ from each other by as little as a single nucleotide, emphasizing the importance of good mismatch discrimination. To address this issue, in our laboratory, we utilize a locked nucleic acids- (LNAs) based approach (Roy *et al.*, 2009). LNAs are a class of conformationally restricted nucleotide analogs with a 2'-O, 4'-C-methylene bridge (Nielsen *et al.*, 1999). The incorporation of LNA in an oligonucleotide increases the affinity of that oligonucleotide for its complementary RNA or DNA target by increasing the melting temperature of the duplex. Additionally, the Tm difference between a perfectly matched target and a mismatched target is substantially higher than that observed when a DNA-based oligonucleotide is used. These properties, high Tm and excellent mismatch discrimination, make LNA-modified probes ideal for analysis of short and similar targets like miRNAs. Furthermore, by adjusting the LNA content and probe length, it is possible to design Tm-normalized probes, allowing hybridization conditions that are optimal for all probes used on, for example, an array (Busch *et al.*, 2007). Recently, deep sequencing has been applied for miRNA profiling and has emerged as an attractive tool for performing global miRNA analysis, and has advantages that include pooling of samples for high-throughput purposes, having a wide detectable expression range, the potential to analyze expression of all annotated miRNAs, and the possibility of detecting novel miRNAs (Schee *et al.*, 2013). Deep sequencing uses massively parallel sequencing, generating millions of small RNA sequence reads from a given sample. Profiling of miRNAs by deep sequencing measures absolute abundance and allows for the discovery of novel microRNAs that have eluded previous cloning and standard sequencing efforts. Public databases provide *in silico* predictions of miRNA gene targets by various algorithms. To better determine which of these predictions represent true positives, microRNA expression data can be integrated with gene expression data to identify putative microRNA:mRNA functional pairs (Creighton *et al.*, 2009).

miRNA expression profiling analysis can also be performed using a low-cost PCR-based assay platform. Primers associated with these miRNA assays were designed using a novel bioinformatics algorithm that has incorporated many primer selection features for assay specificity, sensitivity, and homogeneity (Wang, 2009).

5.3 Nutrigenomics and miR

Nutrient–gene interaction represents a major cornerstone that positions nutritional research for the future. Until recently, the influence of nutrient signaling on the global effects of translational control was not very clear (Liu and Qian, 2011). An emerging notion of translational reprogramming explains how to sustain the expression of specific proteins during pathophysiological conditions by the translation of selective mRNAs (Liu and Qian, 2011).

Successful conclusion of the Human Genome Project and emergence of the powerful “omics” tools have ushered in a new era of medicine and nutrition. Application of omics technologies to enhance the understanding of nutritional sciences has led to the development of nutrigenomics. The term “nutrigenomics” was coined less than a decade ago to develop a subdiscipline that would address the application of high-throughput genomics tools in nutrition research and is aimed at unbiased system wide screening of genes with the goal to uncover specific candidate genes relevant to a pathology or simply responsive to a dietary phytochemical. Broadly, nutrigenomics refers to the study of the effects of foods and food constituents on gene expression in host tissue. The overall goal was to understand how nutrition influences metabolic pathways and homeostatic control, how this regulation is disturbed in the early phase of a diet-related disease, and to what extent individual sensitizing genotypes contribute to such diseases (Muller and Kersten, 2003). Nutrition–health relationship is largely influenced by nutrient–gene interaction. Increasing data has started to connect signaling cascades and miRNA regulation by nutrients. With the advancements in our understanding about how nutrients regulate epigenome, genome, proteome and miRome, there is an additional layer of sophistication that has been added which emphasizes the relationship of genetics and nutrients (Farooqi, 2013). The functional responsiveness of genes to nutritional elements consumed provides the fundamental basis of nutrigenomics. In humans, early-life metabolic imprinting has been evident in several epidemiological studies. Both in the uterus and during the first years of life, under and overfed mother–child units imprint gene changes that lead to chronic metabolic problems in later life (Chavez and Munoz de Chavez, 2003). During the last decade, nutrigenomics has emerged as a key driver of food commercialization. Range of products covered has been wide, spanning from functional food to individual nutrients. Development of miRNA biology has exposed a major crack in the armor of nutrigenomics. As of now, the field is limited to addressing coding genes only. In its current form, nutrigenomics fails to appreciate the key significance of noncoding genes in human health and disease. Also the primary challenge in this young discipline is to develop the fundamental knowledge base required to start for addressing this complex system (Zeisel, 2010). Current developments in the biology of noncoding genes, especially miRNA, provide an extraordinary opportunity to invigorate nutrigenomics by incorporating nutrigenomics as a key subcomponent. Nutrigenomics will help understand the relationship between food elements and the response of miRNA in specific body compartments. Such knowledge, taken together with information of nutrient-responsive coding genes, will help understand nutrigenomics as a whole. For example, let us assume that a nutrient induces a set of coding genes named CG1–100. Say the same nutrient also induces a set of noncoding genes NG50–150 such that NG50–100 are miRs that specifically target CG50–100. In that case, although expression profiling of coding genes would identify CG1–100 as candidate genes that are sensitive to the given nutrient, CG50–100 could be functionally inert because NG50–100 would silence them. Thus, when expression profiling of both coding as well as noncoding genes are taken into account, CG1–50 would emerge as the actual subset of coding genes sensitive to the given nutrients that are functionally active. This is an oversimplified example to communicate the broad point. A more specific example is provided next where obesity is discussed as a use case.

According to the Centers for Disease Control (CDC) and World Health Organization (WHO), in adults, a body mass index (BMI) of 25 or more is considered “overweight” and a BMI of 30 or more is considered “obese”. In 2007, more than 1.1 billion adults worldwide were overweight and 312 million of them were obese (Hossain *et al.*, 2007). The WHO estimates that by 2015, the number of overweight people worldwide will increase to 2.3 billion, and more than 700 million will be obese (American Heart Association, 2008). According to the World Health Organization, the United States ranks fifth among all countries for obesity-related deaths. Two-thirds of Americans are overweight, defined as having a BMI greater than 25. Americans spend close to \$117 billion on obesity-related complications, with another \$33 billion spent annually in attempts to control or lose weight. Nutritional supplements are considered a potentially valuable countermeasure to fight obesity (Downs, *et al.*, 2005; Lau *et al.*, 2008; Li *et al.*, 2008). Studies identifying nutritional supplement sensitive coding genes utilizing full-genome screening approaches are powerful in formulating hypotheses that would explain the

mechanism of action of the supplement in question (Roy *et al.*, 2004, 2007). Obesity is the result of an imbalance between food intake and energy expenditure resulting in the storing of energy as fat. Microarray-based expression profiling studies have provided scientists with a number of new candidate genes whose expression in adipose tissue is regulated by obesity. Integrating expression profiles with genome-wide linkage and/or association analyses is a promising strategy to identify new genes underlying susceptibility to obesity (Dahlman and Arner, 2007). This promise can only be realized when the significance of miRNA in adipose tissue is factored in. Because miRNAs have a major say in the functional status of coding genes, upregulation of miRNA that target adipogenic coding genes is likely to provide productive solutions aimed at managing obesity. Support for this notion is provided by the recent observation that miRNAs induced during adipogenesis that accelerate fat cell development are downregulated in obesity. A number of differentially expressed miRNAs have been identified by several high-throughput studies in adipose tissue pathology and during adipogenesis and a number of which have now been characterized functionally as per their actions and targets (Hilton *et al.*, 2013). Ectopic expression of miR-103 or miR-143 in preadipocytes accelerated adipogenesis demonstrating that miRNA play a key role in obesity (Xie *et al.*, 2009). Human adipose microRNA-221 is found to be upregulated in obesity and contributes to the development of the insulin resistance (Meerson *et al.*, 2013). Angiogenesis is another factor that feeds adipose tissue growth. During obesity, expansion of adipose tissue is accompanied by its vascularization, through the processes of angiogenesis (Lemoine *et al.*, 2013; Bruemmer, 2012). Recent studies demonstrate that angiogenesis is under the tight control of miRNAs (Sen *et al.*, 2009). Nutritional supplements aimed at managing obesity should be investigated for their ability to favorably regulate adipose tissue miRNA response minimizing storage and accelerating catabolism of cellular and tissue fat. NutrimiRomics represents a powerful tool in that regard and is likely to emerge as a major driver of the nutritional supplement industry in the near future.

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6

Genomics as a Tool to Characterize Anti-inflammatory Nutraceuticals

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A rapid physiological response to tissue injury is to set the site on fire (the Latin, *inflammatio*, means “to set on fire”) by recruiting leukocytes from the blood into the injured tissues. Inflammation, an important component in the wound healing cascade, is a protective response of the body to infection or injury, which is designed for removal of the causative agent and restoration of tissue structure and function (Rodriguez-Vita and Lawrence, 2010). A cascade of biochemical events propagates and matures the inflammatory response, involving the local vascular system, the immune system, and various cells within the injured tissue. Inflammation is viewed as an integral component of tissue repair or wound healing. The benefits of inflammation are only realized when inflammation is transient and is resolved in a timely manner. Impairments in the resolution of inflammation lead to chronic inflammation, which is now known to be implicated in a wide range of disease processes. Prolonged inflammation, often associated with oxidative stress, may cause epigenetic changes and lead to a progressive shift in the type of cells present at the site of inflammation, and is implicated in a variety of disease conditions. For example, chronic inflammation has been linked with an increased risk of Type 2 diabetes and cardiovascular diseases. In this chapter, the broad-based implications of chronic inflammation in human health and disease are discussed. Next, the significance of nutraceuticals in managing chronic inflammation is addressed. Finally, a case of genomics as a tool in the characterization of the anti-inflammatory properties of nutraceuticals is discussed.

6.1 Chronic Inflammation in Disease

6.1.1 Vascular Disorders

Chronic inflammation is known to support angiogenesis, which in turn is expected to support tumor growth. Angiogenesis and unresolved inflammation have long been coupled together in many chronic inflammatory disorders with distinct etiopathogenic origin, including psoriasis, rheumatoid arthritis, Crohn’s disease, diabetes, and cancer. Lately, this concept has been substantiated by the finding that several previously established noninflammatory disorders, such as osteoarthritis and obesity, display both inflammation and angiogenesis in an exacerbated manner. In addition, the interplay between inflammatory cells, endothelial cells, and fibroblasts in chronic inflammation sites, together with the fact that inflammation and angiogenesis can actually be triggered by the same molecular events,

further strengthen this association (Costa *et al.*, 2007). There is substantial evidence suggesting that chronic inflammation and angiogenesis are mutually dependent; recent studies reveal that the nature of this link involves both escalation of cellular infiltration and proliferation as well as overlapping roles of regulatory growth factors and cytokines (Jackson *et al.*, 1997). Atherosclerosis is a chronic inflammatory disorder supported by inflammation that may be persistent for years and even decades (Rottenstrich and Rohana, 1999). Polyphenols have a potentially profound impact on chronic inflammatory mechanisms, as recent study has shown polyphenols have several regulatory implications. Examples of polyphenols' regulatory characteristics include reducing endothelial inflammatory markers and cytokine expression (Tangney and Rasmussen, 2013).

6.1.2 Respiratory Disorders

The immune system of the respiratory tract encounters unequal demands, and is a special environment where both inflammatory and anti-inflammatory responses must take place. In order to process foreign antigens without interfering with its primary biologic functions, the lung has adopted novel pathways of immune control. Elucidation and a thorough understanding of the mechanisms that not only governs the pulmonary immune system and the associated inflammatory response but also provokes a quick response to potentially detrimental or disease-causing organisms or materials is important in developing knowledge-based approaches to intervention in many pulmonary diseases (Crapo *et al.*, 2000). Inhaled environmental stressors damage the airways and lung parenchyma, producing irritation, recruitment of inflammatory cells, and oxidative modification of biomolecules. Oxidatively modified biomolecules, their degradation products, and adducts with other biomolecules can reach the systemic circulation and when found in higher concentrations than normal they are considered to be biomarkers of systemic oxidative stress and inflammation. Metabolic stressors produced in the lung have a number of effects in tissues other than the lung, such as the brain, and they can also abrogate the mechanisms of immunotolerance (Gomez-Mejiba *et al.*, 2008). Endogenous anti-inflammatory mediators and immune regulating mechanisms are important for the resolution of inflammatory processes. A disruption of these mechanisms can be causally related not only to the initiation of unnecessary inflammation but also to the persistence of several chronic inflammatory diseases. The recruitment of neutrophils into the airway due to the presence of pro-inflammatory mediators causes a persistent decline in overall lung function (Elizur *et al.*, 2008). In asthma, chronic Th-2-driven eosinophilic inflammation of the airways represents one of the central abnormalities (van Hove *et al.*, 2008). Oxidative and nitritative stress and severe inflammation account for the amplified inflammation in chronic obstructive pulmonary disease (COPD) (Mroz *et al.*, 2007). A vicious cycle of airway obstruction, infection, and inflammation continues to cause most of the morbidity and mortality in cystic fibrosis (CF). The chronic inflammatory process damages and obstructs the airways and eventually claims the life of the patient (Nichols *et al.*, 2008).

6.1.3 Gastrointestinal Tract

Inflammatory disorders in the gastrointestinal (GI) tract may range from esophagitis and gastritis in the upper GI tract to Crohn's disease, hemorrhoids, and ulcerative colitis in the lower GI tract (Gastroenterology Consultants, 2014). Intestinal inflammation is implicated in inflammatory bowel disease (IBD) including Crohn's disease and ulcerative colitis. Enteric bacteria are a critical component in the development and prevention/treatment of chronic intestinal inflammation (Werner and Haller, 2007). At the interface between the luminal content and host tissues, the intestinal epithelium must integrate pro- and anti-inflammatory signals to regulate innate and adaptive immune responses; that is, to control inflammation. However, under the influence of environmental factors, disturbance of the dialog between enteric bacteria and epithelial cells contributes to the development of chronic inflammation (Clavel and Haller, 2007b). The genetic predisposition to deregulated mucosal immune responses and the concurrent prevalence of certain environmental triggers in developed countries, are strong etiologic factors for the development of inflammatory bowel diseases in human subjects, including Crohn's disease and ulcerative colitis (Clavel and Haller, 2007a). Genome-wide association studies (GWAS) have clearly outlined specific loci that present potential genetic risk associated with inflammatory bowel disorders. Well established environmental triggers of IBD include smoking, vitamin D deficiency, use of antibiotics, and stress. While trying to further understand the triggers of IBD, it is important to note both environmental factors as well as any genetic predisposition to the disease (Ko and Urban, 2013).

6.1.4 Neurodegenerative Diseases

Neurodegeneration is the most significant pathological characteristic of several neurodegenerative conditions, such as Alzheimer's disease (AD) and Parkinson's disease (PD). Irrespective of the different triggering events in these diseases, chronic immune activation, in particular of microglia and the resident macrophages of the central nervous system, is a common feature (Amor *et al.*, 2010). Recent studies demonstrate a strong link between neurodegeneration and chronic inflammation. The central nervous system (CNS) has limited regenerative capacity. Neural cell death occurs by apoptosis and necrosis. Necrosis in the CNS usually follows ischemic or traumatic brain injury (DeLegge and Smoke, 2008). Infection-triggered chronic inflammation may initiate a cascade of events leading to chronic inflammation and amyloid deposition in AD. Thus, anti-inflammatory therapy is considered to prevent dementia (Miklossy, 2008). Recent data suggests that non-steroidal anti-inflammatory drugs may be effective against AD in the early stages, yet may be harmful in the later stages (Breitner *et al.*, 2012; Etminan *et al.*, 2003). The immunohistochemical demonstration of reactive microglia and activated complement components suggests that chronic inflammation occurs in affected brain regions in PD. Evidence from humans and monkeys exposed to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) indicates this inflammation may persist many years after the initial stimulus has disappeared. Chronic inflammation can damage host cells and contributes to the pathogenesis of PD (McGeer and McGeer, 2004). Chronic inflammation in the brain that is present in PD is often a result of MPTP-induced production of excess astrocytes (Niranjan *et al.*, 2010). A process known as *reactive gliosis* characterizes a change in expression and levels of astrocytes in the presence of brain injury or neuroinflammation (Kanemaru *et al.*, 2013).

6.1.5 Cancer

The word "inflammation" in Latin means "I ignite, set alight" and, like a fuel, that's exactly what it does to cancer (Envita, n.d.). Chronic inflammation, carcinogenesis, and cancer prognosis are tightly related. Inflammatory cells and cancer cells themselves produce free radicals and soluble mediators such as metabolites of arachidonic acid, cytokines, and chemokines, which act by further producing reactive species. These, in turn, strongly recruit inflammatory cells in a vicious circle. Reactive intermediates of oxygen and nitrogen may directly oxidize DNA or may interfere with mechanisms of DNA repair. These reactive substances may also rapidly react with proteins, carbohydrates, and lipids, and the derivative products may induce a high perturbation in the intracellular and intercellular homeostasis until DNA mutation. The main substances that link inflammation to cancer via oxidative/nitrosative stress are prostaglandins and cytokines (Federico *et al.*, 2007). Several recent studies have identified nuclear factor- κ B as a key modulator in promoting inflammation to cancers. (Lu *et al.*, 2006). IL-6 and TNF are two cytokines that have been linked to various inflammation related cancers. These cytokines affect different cell types and create a suitable environment for tumor growth (Grivennikov and Karin, 2011).

The proportion of total cancer deaths attributable to infectious agents is estimated to be about 20–25% in developing countries and 7–10% in more industrialized countries. Recurrent or persistent inflammation may induce, promote, or influence susceptibility to carcinogenesis by causing DNA damage, inciting tissue reparative proliferation, and/or creating a stromal "soil" enriched with cytokines and growth factors (Schottenfield and Beebe-Dimmer, 2006). There is a proven association between carcinoma of the pancreas and both the sporadic and hereditary forms of chronic pancreatitis (McKay *et al.*, 2008). Kaposi's sarcoma (KS) is a complex cancer characterized by angioproliferative multifocal tumors of the skin, mucosa, and viscera. KS lesions comprised both distinctive spindle cells of endothelial origin and a variable inflammatory infiltrate. KS may result from reactive hyperproliferation induced by chronic inflammation (Douglas *et al.*, 2007). Chronic inflammation is likely to have an important role in bladder carcinogenesis in developed countries (Michaud, 2007).

6.1.6 Rheumatic Diseases

Management of chronic inflammation represents the central mechanism targeted by most therapies addressing rheumatic diseases (Sacre *et al.*, 2007). Rheumatoid arthritis (RA) is by definition a chronic disease with an autoimmune inflammatory attack on diarthrodial cartilaginous joints (Holmdahl, 2006).

6.2 Nutraceuticals in the Management of Chronic Inflammation

At present, monoclonal antibody-based therapeutics show clear promise in treating inflammatory disorders (Feldman and Maini, 2001; Kaplan, 2002). The central role of $\text{TNF}\alpha$ in causing inflammation was initially provided by the demonstration that anti- $\text{TNF}\alpha$ antibodies added to *in vitro* cultures of a representative population of cells derived from diseased joints inhibited the spontaneous production of IL-1 and other proinflammatory cytokines. Systemic administration of anti- $\text{TNF}\alpha$ antibody or sTNFR fusion protein to mouse models of rheumatoid arthritis was shown to be anti-inflammatory and protective for joints. Clinical investigations, in which the activity of $\text{TNF}\alpha$ in rheumatoid arthritis patients was blocked with intravenously administered infliximab, a chimeric anti- $\text{TNF}\alpha$ monoclonal antibody (mAB), have provided evidence that TNF regulates IL-6, IL-8, MCP-1, and VEGF production, recruitment of immune and inflammatory cells into joints, angiogenesis, and reduction of blood levels of matrix metalloproteinases-1 and -3 (Feldmann *et al.*, 2004; Feldmann and Maini, 2001). The development of anti-TNF therapy is a key step forward in rheumatology as it is the first new therapy based on investigating the molecular mechanisms of this disease. Recent studies show the body develops antibodies against anti-TNF therapies, showing the necessity to use variations of the therapy simultaneously for a synergistic effect (Scott and Lichtenstein, 2014). Despite such major breakthroughs in investigative medicine, the fact remains that a vast population of individuals in developing countries suffering from inflammatory disorders do not benefit from monoclonal antibody-based therapy, primarily because of excessive cost of acquisition and limited availability. For example, the cost associated with a single dose of infliximab is several thousand US dollars (Valle *et al.*, 2001).

The medical cost of rheumatoid arthritis averages \$US 5919 per case per year in the USA and approximately £2600 per case per year in the UK. Current slow-acting antirheumatic drugs have limited efficacy and many side-effects. Moreover, they do not improve the long-term prognosis of rheumatoid arthritis (Choy and Panayi, 2001). The use of medicinal plants to treat inflammatory disorders continues to be in practice worldwide (Borchers *et al.*, 2000; Chainani-Wu, 2003; Cohen *et al.*, 2000; Ernst and Chrubasik, 2000; Long *et al.*, 2001; Phillipson, 2003). Medicinal plants often contain complex mixtures of phytochemicals that have additive or synergistic interactions. Further research regarding the potential anti-inflammatory properties of phytochemicals is important because of the availability and relatively low-risk of the phytochemicals (Kim *et al.*, 2011). Also, further research is needed to identify biomarkers of inflammation, oxidative stress, and antioxidant status, necessary for identifying new anti-inflammatory nutraceuticals (Al-Okbi, 2012).

The anti-inflammatory properties of herbal preparations have been recognized in ancient Indian and Chinese medical literature. Inflammatory pathways in obesity and metabolic diseases have been targeted by curcumin. Other spice-derived nutraceuticals effective in obesity include capsaicin, gingerol, piperine, cinnamaldehyde, and fenugreek (Aggarwal, 2010). The gum resin of *Boswellia serrata*, known in the Indian Ayurvedic system of medicine as *Salai guggal*, contains Boswellic acids (BA), which have been shown to inhibit leukotriene biosynthesis. Compounds from the gum with proven anti-inflammatory effects are pentacyclic triterpenes of the BA type. Recently, the tetracyclic triterpene 3-oxo-tirucalllic acid has been identified as a key active principle in *Boswellia* resin (Boden *et al.*, 2001). BA function as specific, nonredox inhibitors of leukotriene synthesis either interacting directly with 5-lipoxygenase or blocking its translocation (Ammon, 1991, 1996; Ammon *et al.*, 1993). Among the BA, acetyl-11-keto-beta-boswellic acid potently inhibits 5-lipoxygenase product formation with an IC_{50} of 1.5 μm . In contrast to the redox type 5-lipoxygenase inhibitor nordihydroguaiaretic acid, BA in concentrations up to 400 μm did not impair the cyclooxygenase and 12-lipoxygenase in isolated human platelets and the peroxidation of arachidonic acid by Fe-ascorbate. These data support that BA are specific, nonreducing-type inhibitors of 5-lipoxygenase (Safayhi *et al.*, 1992). In addition to their effects on the lipoxygenase system, certain BA inhibit elastase in leukocytes, inhibit proliferation, induce apoptosis, and inhibit topoisomerases of leukemia and glioma cell lines. A series of chronic inflammatory diseases are plausibly perpetuated by leukotrienes. In clinical trials promising results supporting the anti-inflammatory effects of *Boswellia* extract (BE) were observed in patients with rheumatoid arthritis, chronic colitis, ulcerative colitis, Crohn's disease, bronchial asthma, and peritumoral brain edema (Ammon, 2002; Gupta *et al.*, 1998, 2001). The enzymatic oxidation of arachidonic acid yields potent pathological agents by two major pathways, prostaglandin and lipoxygenase. The lipoxygenase pathway generates a new class of arachidonic acid oxygenation products, called the leukotrienes, which mediates inflammation. Recent studies have shown therapeutic advances have been studied via leukotriene antagonists or implementing leukotriene inhibitors (Sharma and Mohammed, 2006). Unlike the prostaglandins, some of which play important roles as biological regulators, the action of the lipoxygenase products

appear to be exclusively of a pathological nature (Kuehl and Egan, 1980). Thus, the antilipoxygenase effects of BA are likely to have therapeutic implications.

Herbal medicines are widely used in the USA, with approximately one-quarter of adults reporting use of an herb to treat a medical illness within the past year. The relatively low risk of herbal medicines are oftentimes preferred over traditional prescriptions (Girard and Vohra, 2011). Of the 10 most commonly used herbs in the USA, systematic reviews have concluded that only four are likely to be effective, and there is limited evidence to evaluate the efficacy of the approximately 20,000 other available products (Bent and Ko, 2004). The emergent “omics” technology platform represents a powerful tool to examine the efficacy of herbals and nutraceuticals. Medicinal plants belonging to the *Burseraceae* family, including *Boswellia*, are especially known for their anti-inflammatory properties (Duhieua *et al.*, 1993). *Boswellia serrata* (frankincense) has been used in traditional medicine for treatment of inflammatory diseases since antiquity. BAs have been studied for more than 30 years (el-Khadem *et al.*, 1972). Acetyl-11-keto-beta-boswellic acid (AKBA) is a naturally occurring pentacyclic triterpene isolated from the gum resin exudate from the stem of the tree *Boswellia serrata*. AKBA has been recently identified as a novel, orally active, nonredox, and noncompetitive 5-lipoxygenase inhibitor that also inhibits topoisomerase I and II *in vitro* (Park *et al.*, 2002a; Park *et al.*, 2002b). In humans, orally taken BE manifests as plasma KBA. The peak plasma levels of BE were reached at 4.5 h. The plasma concentration attained a steady state after approximately 30 h. BE has been proven to be safe and well tolerated on oral administration in humans. No adverse effects were seen with this drug when administered as a single dose in 333 mg (Sharma *et al.*, 2004).

6.3 GeneChip™ as a Tool to Characterize the Anti-Inflammatory Properties of Nutraceuticals

Almost two decades ago, TNF was identified as a protein produced by the immune system that suppressed tumor cell proliferation. Extensive research since then has revealed that TNF α is a major mediator of inflammation (Streiter *et al.*, 1993; Sullivan, 2003; Warren *et al.*, 1988). Endothelial cells are critical elements in the pathophysiology of inflammation. They participate through the synthesis and secretion of proinflammatory cytokines, including interleukin 1 (IL-1), IL-6, and IL-8, as well as M-CSF, G-CSF, GM-CSF, GRO alpha, and MCP. They also express a series of cell-surface proteins and glycoproteins known as cell adhesion molecules that allow circulating leukocytes to bind to endothelial cells and allow endothelial cells to bind to matrix proteins (Swerlick and Lawley, 1993). TNF α potently induces inflammatory responses in endothelial cells (Pober, 2002). Thus, the molecular basis of the anti-inflammatory effects of nutraceuticals may be tested in a system of TNF α -induced gene expression in human microvascular endothelial cells (HMEC) (Roy *et al.*, 2005).

With the objective to identify sets of TNF α sensitive genes in HMEC, GeneChip™ analysis was performed in our own laboratories (Roy *et al.*, 2002a, b, 2003). After 24 h of seeding, HMEC cells were either treated with BE (50 μ g/ml) or matching volume of dimethyl sulfoxide (DMSO) for 48 h. As required, this was followed by treatment with recombinant human TNF α (50 ng/ml) for 6 h. Cells were harvested after 6 h of TNF α treatment and the total RNA was extracted using the RNeasy kit (Qiagen). Extracted RNA was treated with DNA-free (Ambion) to remove any possible DNA contamination present in the extracted RNA. The quality of RNA was assessed using Bioanalyzer 2100 (Agilent). To assess the quality of the labeled targets, the samples were hybridized for 16 h at 45°C to GeneChip™ test arrays. Satisfactory samples were hybridized to the human genome arrays (U133 Plus 2.0) for the screening of more than 47,000 transcripts including the entire human genome. The arrays were washed, stained with streptavidin-phycoerythrin, and were then scanned with the high-resolution GeneChip scanner 3000 (Affymetrix) in our own facilities. To allow for statistical treatment, data were collected from three experiments. Raw data were analyzed using Affymetrix Microarray Suite 5.0 (MAS) and Data Mining Tool 2.0 (DMT) software. Additional processing of data was performed using dChip software (Li and Wong, 2001). We employed a stringent approach by taking a cutoff of 100%; that is, in a 3 \times 3 comparison only those genes called increased in all nine of nine pair-wise comparisons were considered to increase in their expression. The magnitude of the change in gene expression (fold change) was reported for each comparison. These values were averaged to obtain an average fold change for each gene. Such conservative analytical approach limits the number of false-positive gene identifications. For data visualization and identification of BE-sensitive TNF α -inducible genes, genes filtered using the comparison analysis approach were subjected to hierarchical clustering using dChip

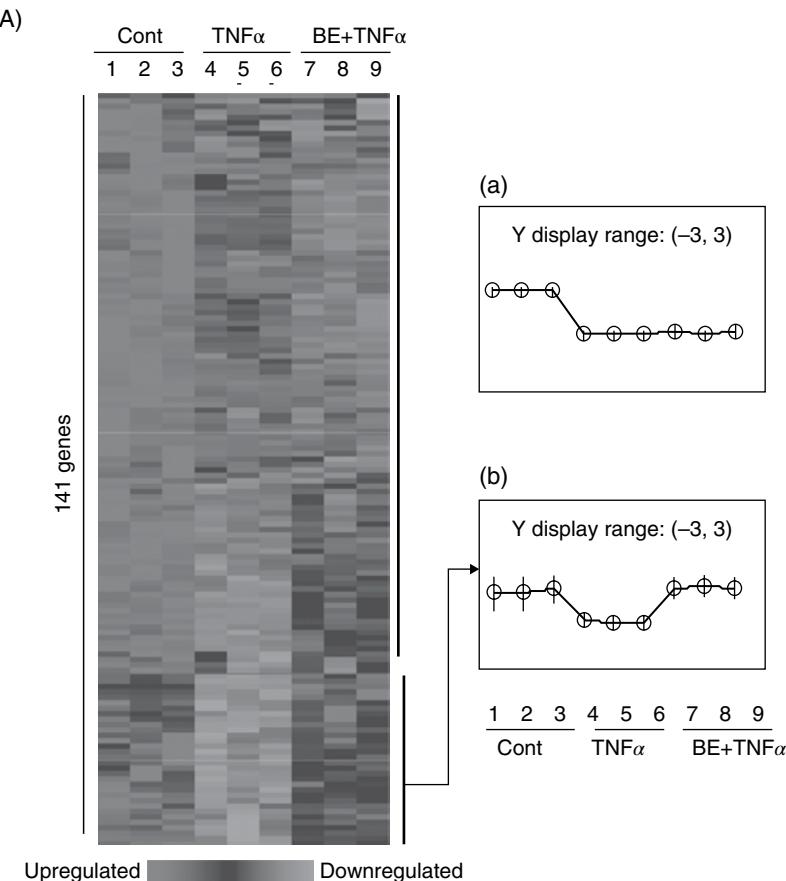


Figure 6.1 Hierarchical cluster images illustrating *Boswellia*-sensitive genes induced or downregulated by $TNF\alpha$ in human microvascular endothelial cells. For clear graphic display of the specific clusters of genes showing an increase (A) or decrease (B) in expression following $TNF\alpha$ treatment to HMEC cells, a count percentage analysis was performed. Genes that were found up- or downregulated in 100% of replicates and all comparisons (nine out of nine) following $TNF\alpha$ treatment were selected. These select candidate genes were subjected to hierarchical clustering to identify clusters of genes induced/downregulated by $TNF\alpha$ and are sensitive to *Boswellia*. The gradation in shading represents a higher to lower expression signal. Roy et al. (2005). Reproduced with permission of Mary Ann Liebert, Inc.

software. This approach recognizes distinct clusters of transcriptome (Figure 6.1). The genes that were significantly changed in BE and TNF α cotreated group compared to the group treated with TNF α alone were selected. Functional categorization and pathway construction were performed using the following software and web resources: Gene Ontology Data Mining Tool (Affymetrix), *KEGG* (*Kyoto Encyclopedia of Genes and Genomes*), GenMAPP, DAVID (Database for Annotation, Visualization, and Integrated Discovery Verification), and LocusLink (Swiss-Prot). Acutely, TNF α induced 522 genes and downregulated 141 genes in nine out of nine pair-wise comparisons. Of the 522 genes induced by TNF α in HMEC, 113 genes were clearly sensitive to BE treatment. Such genes directly related to inflammation, cell adhesion, and proteolysis. The robust BE-sensitive candidate genes were then subjected to further processing for the identification of BE-sensitive signaling pathways. The use of resources such as GenMAPP, *KEGG*, and Gene Ontology (GO) led to recognition of the primary BE-sensitive TNF α -inducible pathways (Figure 6.2). BE prevented the TNF α -induced expression of matrix metalloproteinases. BE also prevented the inducible expression of mediators of apoptosis. Most strikingly, however, TNF α -inducible expression of VCAM-1 and ICAM-1 were observed to be sensitive to BE. Real-time PCR studies showed that although TNF α potently induced

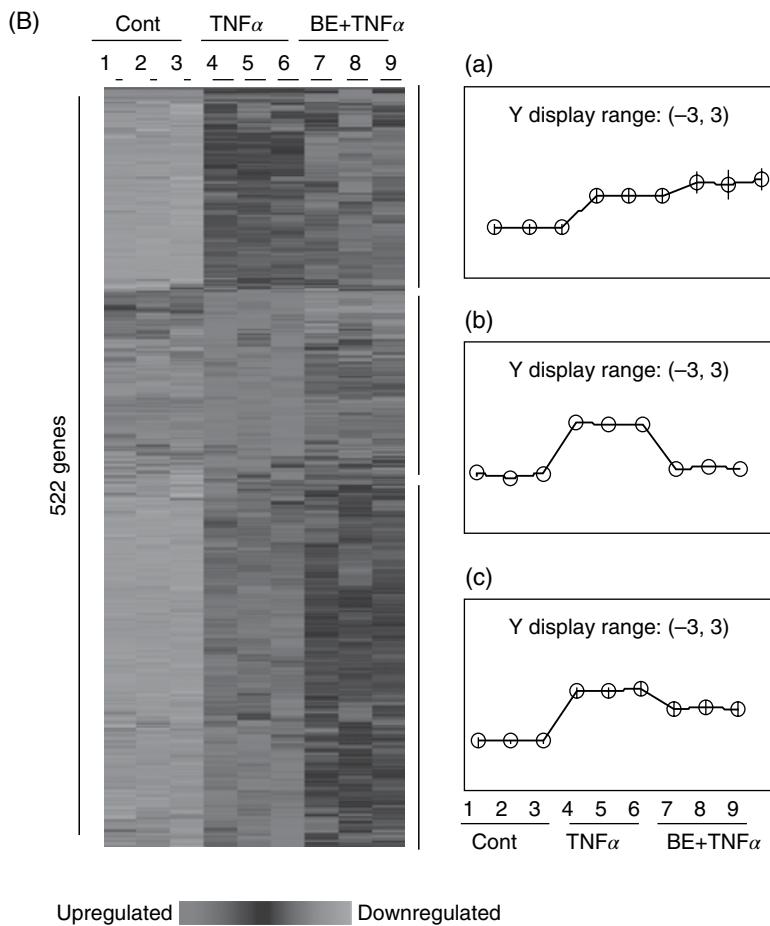


Figure 6.1 (Continued)

VCAM-1 gene expression, BE completely prevented it. This result confirmed our microarray findings and built a compelling case for the anti-inflammatory property of BE. In an *in vivo* model of carrageenan-induced rat paw inflammation, a significant anti-inflammatory property of BE was noted (Roy *et al.*, 2005).

As a follow-up to the previously mentioned GeneChip study, which recognized matrix metalloproteinases (MMP) as a key TNF α -inducible pathway that is BE-sensitive, the effects of BE on TNF α -inducible MMP expression in human microvascular endothelial cells was tested. MMPs are a family of zinc-containing enzymes involved in the degradation and remodeling of extracellular matrix proteins. Under normal physiological conditions, the activities of these enzymes are well regulated by endogenous tissue inhibitors of metalloproteinases (TIMPs). Chronic stimulation of MMP activities, due to an imbalance in the levels of MMPs and TIMPs, has been implicated in the pathogenesis of a variety of diseases such as cancer, osteoarthritis, and rheumatoid arthritis. Thus, MMP inhibitors are expected to be useful for the treatment of these disorders. Because of their importance in a variety of pathological conditions, a number of small molecular weight MMP inhibitors have entered clinical trials in humans. However, the results of these trials have been disappointing (Skiles and Gonnella, 2004). In our study, to evaluate the significance of AKBA in the anti-inflammatory properties of BE, effects of BE containing either 3% (BE3%) or 30% (BE30%) were compared. Pretreatment of HMEC for 2 days with BE potently prevented TNF α -induced expression and activity of MMP3, MMP10, as well as MMP12. The Freund's adjuvant-induced rat paw edema experimental system was utilized to test the significance of the anti-inflammatory properties of BE *in vivo*. *In vivo*, BE protected against experimental arthritis. In all experiments, both *in vitro* and *in vivo*, BE30% was more effective

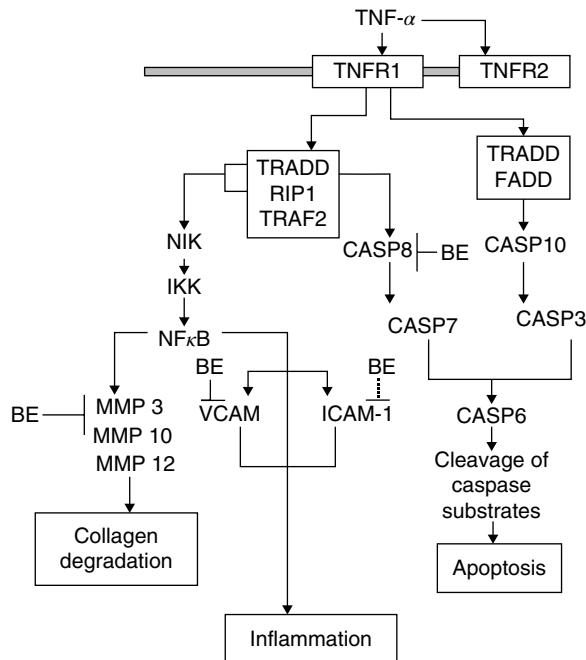


Figure 6.2 Boswellia-sensitive TNF- α induced signaling pathways in human microvascular endothelial cells. Pathway construction is based on GeneChip™ expression data and appropriate software resources (see Materials & Methods). To obtain insights on the effects of Boswellia on specific pathways induced by TNF α in endothelial cells, the results of GeneChip analysis were mapped onto known pathways associated with inflammation, apoptosis, and collagen degradation. GenMAPP, KEGG, and Gene Ontology (GO) were used to develop the pathways. Genes shown in red are candidates identified using GeneChip assay that were upregulated following TNF α . Blunt arrow marked with BE marks the genes whose expression levels are fully (solid line) or partly (broken lines) normalized by BE pretreatment. TNFR, TNF α receptor; CASP, caspase; BE, Boswellia extract; TRADD, TNFR1-associated protein with death domain; NIK, NF κ B-inducing kinase; IKK, I κ b kinase; MMP, matrix metalloproteinase; VCAM, vascular cell adhesion molecule; ICAM, intercellular adhesion molecule. Roy *et al.* (2005). Reproduced with permission of Mary Ann Liebert, Inc.

than BE3%. These observations lend support to the GeneChip studies demonstrating that BE has potent anti-inflammatory properties both *in vitro* as well as *in vivo* (Roy *et al.*, 2006). Thus, the GeneChip screening and data mining approach adopted represents a powerful tool for hypothesis discovery.

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7

Nutrigenomics, Inflammaging, and Osteoarthritis: A Review

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7.1 Introduction

The global incidence of age-related diseases of the bone, joint, and muscle is steadily rising, seriously affecting the health of millions of people across the world. According to the United Nations (UN) (www.un.org) and the World Health Organization (WHO) (www.who.int) musculoskeletal and arthritic conditions are leading causes of morbidity and disability throughout the world, giving rise to enormous healthcare expenditures and loss of work (sources: The Arthritis Foundation (AF) and WHO: Ehrlich, 2003; Salminen *et al.*, 2012a; Symmons *et al.*, 2000; Woolf *et al.*, 2003;^{1, 2, 3}). Many types of rheumatic diseases and arthritic conditions are essentially “inflammatory” disorders wherein that inflammation promotes disease progression. The term “arthritis” characterizes a group of conditions involving inflammatory damage to synovial joints (Di Paola *et al.*, 2008). Arthritis literally means inflammation (*itis*) of the joints (*arthr*). It involves pain, redness, heat, swelling, and other harmful effects of inflammation within the joint. There are over 200 different forms of arthritis.

¹ www.arthritis.org

² www.who.int/healthinfo/statistics/bod_osteoarthritis.pdf

³ <http://www.who.int/bulletin/volumes/81/9/Ehrlich.pdf>

However, the most common and economically important form of arthritis is osteoarthritis (OA), also known as osteoarthritis or degenerative joint disease (DJD) although it must be noted that osteoarthritis and DJD are now considered to be inappropriate and incorrect terms for describing OA. OA is the major cause of pain and disability affecting the elderly (Aigner *et al.*, 2004). Other forms of arthritis include psoriatic arthritis and rheumatoid arthritis (RA), an autoimmune disease in which the body's own immune system attacks synovial joints. The risk factors for arthritis are gender, age, family history, ethnic background, and smoking tobacco (Oliver *et al.*, 2009). It is important to note that OA and RA are both now considered to be systemic disturbances. Although synovial joints are primarily affected in both arthritides, these diseases can also wreak havoc in other organs. For example, in RA the pancreas and the heart also undergo significant changes. Ultimately, the major consequences of all forms of arthritis include disability, chronic pain and significant morbidity. Pain is a constant and daily feature in well-established forms of the disease. A large component of arthritic pain arises from the inflammation that occurs around and within the joint. Disability in patients with arthritis is a consequence of degeneration in the joint and surrounding tissues, and is further enhanced by this inflammation-induced ("inflammatory") pain. Aside from analgesics, there are currently no effective pharmacotherapies capable of restoring the structure and function of the damaged synovial tissues in any form of arthritis. Consequently, there is significant interest from patient groups, rheumatologists, and commercial companies in any nutrients, nutraceuticals, and natural products that may provide complementary therapeutic support for patients with inflammatory diseases of joints. In this chapter we introduce readers to clinical aspects of OA and discuss factors that lead to its development and progression. We then explore the concept of inflammaging and how the emerging field of nutrigenomics may be applied to understanding the effects of nutraceuticals and functional foods on synovial joint tissues, particularly muscle.

7.2 Osteoarthritis (OA)

OA is the most common form of arthritis. A study carried out in the USA estimated that OA is one of the top five causes of disability amongst non-hospitalized adults (source: Centers for Disease Control and Prevention, USA (CDC): Salminen *et al.*, 2012b). According to estimates from the National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS) more than 20 million Americans currently suffer from OA. In 2006, it was estimated that around 35 million to 40 million Europeans had OA. It is anticipated that by the year 2030, 20% of adults will have developed OA in Western Europe and North America. Furthermore, since our population appears to have ever increasing longevity, OA is expected to place a growing economic burden on health and social care systems, and community services in Europe and the rest of the world.

OA is rare in people under 40 and advancing age is a major risk factor. Perhaps not surprisingly, there is radiographic evidence of OA in at least one joint in the majority of people aged 65 or over. The end stage treatment for OA is surgery, either to modify or replace the joint. With increasing life expectancy, growth in the elderly population and an alarming escalation of chronic, inflammatory and age-related conditions (such as OA), there is increased demand for new treatments and preventative approaches. Although OA is primarily associated with aging, there are other important contributing factors (Figure 7.1) (Lotz *et al.*, 2010). These include genetics, underlying anatomical and orthopedic disorders (i.e., congenital hip dislocation), obesity, underlying inherited or acquired metabolic disease, endocrine disease, various disorders of bone turnover and blood clotting, joint infection, crystal deposition, previous RA or a history of joint trauma, repetitive use, muscle weakness, or joint instability. The mechanical and metabolic alterations that occur in obesity, along with the pro-inflammatory factors produced by white adipose tissue in the chronically overweight, are thought to be major factors in the progression of the disease (Yusuf *et al.*, 2010). The factors that contribute to OA progression are summarized in Figure 7.2.

Symptoms of OA in the most frequently affected joints include pain, stiffness (crepitus), and limited mobility, as well as swelling, and, occasionally, warmth. These manifestations are highly variable, depending on joint location and disease severity. OA can affect any synovial joint but it commonly affects large load-bearing joints such as the hip and knee. The disease was traditionally thought of as being due to daily wear and tear of the joint and, indeed, the accumulation of microtrauma to cartilage and bone contribute to pathogenesis. The most prominent anatomical feature is the progressive destruction of articular cartilage (Buckwalter *et al.*, 2005). However, OA is an inflammatory disease involving not only articular cartilage but also the synovial membrane, subchondral bone and peri-articular soft tissues (Goldring and Goldring, 2007). Inflammation of the synovium occurs in both the early and late phases of OA and is associated with alterations in the adjacent cartilage. This inflammatory synovitis is qualitatively highly similar to that seen in RA. Catabolic and pro-inflammatory mediators such as cytokines, nitric oxide (NO), prostaglandin E₂ (PGE₂), and neuropeptides are produced by the inflamed synovium, which alter the balance of cartilage matrix degradation and repair (Sutton *et al.*, 2009). These events lead to excess production of the proteolytic enzymes

Systemic and OA Risk Factors:
Age
Obesity, overweight and sedentary lifestyle
Mechanical factors
Joint trauma, joint instability, joint overuse, increased joint laxity - increased stiffness of periarticular ligaments and tendons
Anatomic factors, joint shape and conformation
Genetics, epigenetics
Metabolic disease
Diabetes mellitus – formation of advanced glycation end-products (AGEs), oxidative stress
Endocrine disease (inherited or acquired)
Disorders of bone turnover
Disorders of blood clotting (haemophilia)
Previous septic arthritis (joint infection)
Previous rheumatoid arthritis (RA)
Gout or other forms of inflammatory crystal deposition in joints

Figure 7.1 Systemic and OA risk factors.

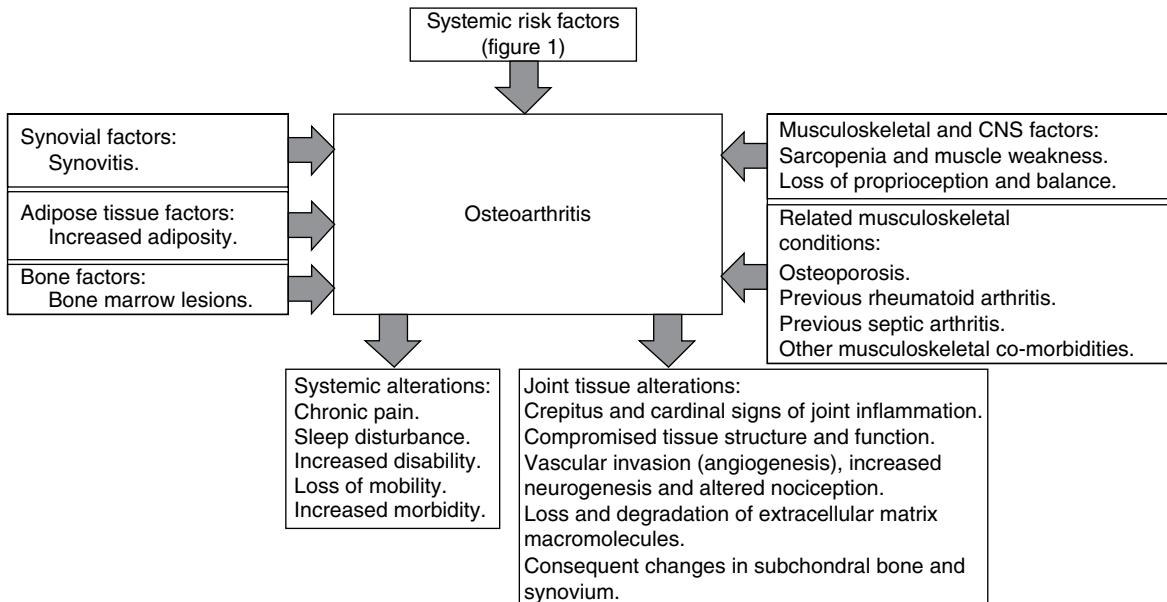


Figure 7.2 Factors that contribute to the development and progression of OA.

responsible for cartilage breakdown (Sellam *et al.*, 2010). Cartilage degeneration induces further synovial inflammation, creating a vicious circle. The progressing synovitis will then exacerbate clinical symptoms and joint degradation in OA (Sellam *et al.*, 2010).

7.3 Antioxidants and the Inflammatory Microenvironment

Antioxidants are naturally occurring reducing agents capable of inhibiting the oxidation of biological molecules. Oxidation reactions in living cells produce free radicals; reactive oxygen species (ROS) and their derivatives. These dangerous and harmful chemical products can accumulate over time, causing extensive structural damage or even cell death. The cytotoxic effects of

ROS can cause a variety of health problems including inflammatory disease, tissue necrosis, organ failure, atherosclerosis, infertility, birth defects, premature aging, mutations, and malignancy (Parke and Sapota, 1996). ROS production initiates an “inflammatory state” which unless quenched may result in chronic inflammatory disease states, for example, arthritis, hepatitis, nephritis, myositis, scleroderma, lupus erythematosus, or multiple system organ failure (Parke and Sapota, 1996). However, ROS can also be involved in the *initiation* of inflammatory responses (Gloire *et al.*, 2006). For example, ROS such as H_2O_2 can stimulate the transcription factor NF- κ B, which is crucial for cellular processes such as inflammation, immunity, cell proliferation, and apoptosis (Schreck *et al.*, 1991). Therefore, ROS mediated upregulation of NF- κ B can cause dysregulation of many inflammatory responses. NF- κ B. ROS are also linked to mitochondria and the inflammasome (Zhou *et al.*, 2011). The inflammasome is a protein complex that stimulates caspase-1 activation to promote the processing and secretion of proinflammatory cytokines (Ogura *et al.*, 2006). This multiprotein oligomer consists of caspase 1, PYCARD, NALP, and sometimes caspase 5 (also known as caspase 11 or ICH-3). Inflammasome-dependent inflammatory responses can be triggered by a variety of stimuli including infection, tissue damage, and metabolic dysregulation (Tschoopp, 2011). Recent work suggests that mitochondria are involved in integrating distinct signals and relaying information to the inflammasome. Dysfunctional mitochondria generate ROS, which is required for inflammasome activation. Interestingly, mitochondrial dysfunction has been linked to OA (Blanco *et al.*, 2004; Terkeltaub *et al.*, 2002). Analyses of mitochondrial electron transport chain activity in chondrocytes from OA affected cartilage show decreased activity of complexes I, II, and III compared to normal chondrocytes (Blanco *et al.*, 2011). Therefore, it is possible that mitochondrial dysfunction in arthritis is exacerbated by ROS and catabolic processes that alter cellular metabolism. The inflammasome is negatively regulated by autophagy, which is a catabolic process that removes damaged or otherwise dysfunctional organelles, including mitochondria (Tschoopp, 2011). Autophagy has been shown to be a protective mechanism in normal cartilage, and its aging-related loss is linked with cell death and OA (Carames *et al.*, 2010). These studies suggest that the connections between mitochondria, metabolism, and inflammation are important for cell function and that malfunctioning of this network is associated with many chronic inflammatory diseases. ROS generation and inflammasome activation are linked with mitochondrial dysfunction and may explain the frequent association of mitochondrial damage with inflammatory diseases.

Antioxidants in foods and natural products are thought to interfere with inflammatory reactions by inhibiting ROS formation, scavenging free radicals, or removing ROS derivatives. They do this by being oxidized themselves, so antioxidants are often reducing agents such as vitamin C (ascorbic acid), vitamin E, thiols (glutathione), or a variety of plant polyphenols. Living cells maintain a complex and interrelated protective system of antioxidant vitamins, minerals such as selenium and manganese as cofactors, and glutathione to protect themselves from the harmful effects of ROS (Meister, 1994a, b). Cells also use a variety of antioxidant enzymes such as catalase, superoxide dismutase, and various peroxidases to quench and control cellular levels of ROS. Deficiency in antioxidants, or inhibition of the antioxidant enzyme systems, may cause oxidative stress and may damage or kill cells. Oxidative stress is an important component of many diseases. Therefore, the biology of ROS and antioxidants is widely investigated in the context of understanding the role of these chemicals in chronic diseases characterized by oxidative stress.

The redox state of chondrocytes is relevant in the context of OA because they exist in an avascular microenvironment, with low nutrient and oxygen levels (Mobasher *et al.*, 2002, 2008). Although chondrocytes rely on glycolysis (Archer *et al.*, 2003), some of the metabolic functions of these cells are oxygen dependent (Henrotin *et al.*, 2003; Henrotin and Kurz, 2007). Oxygen is mainly supplied by diffusion from the synovial fluid (Mobasher *et al.*, 2002; Pfander and Gelse, 2007). Consequently, the lack of oxygen means that chondrocytes display a metabolism adapted to anaerobic conditions (Henrotin *et al.*, 2003; Henrotin and Kurz, 2007; Lafont, 2010). There is little published information about the regulation of antioxidant enzymes within cartilage. Equally, little is known about the transport of antioxidants from the circulation to chondrocytes. However, some transport of nutrients, oxygen, and antioxidants to chondrocytes is thought to occur by diffusion from subchondral bone (Imhof *et al.*, 2000) and the synovial microcirculation (Levick, 1995). The role of subchondral bone in the pathogenesis of cartilage damage has been underestimated (Imhof *et al.*, 2000). There is increasing evidence that vascular pathology plays a role in the initiation and/or progression of OA (Findlay, 2007). In pathological conditions, oxygen tension in synovial fluid is subject to fluctuation as blood flow may be reduced by venous occlusion and stasis, vascular shunt, and fibrosis in synovium and/or by the development of microemboli in the subchondral vessels (Findlay, 2007). In response to oxygen variations induced through ischemia/reperfusion injury, mechanical stress, immunomodulatory, and inflammatory mediators, chondrocytes produce abnormal levels of ROS that are more usually produced by immune cells (Henrotin *et al.*, 1992, 2003; Henrotin and Kurz, 2007). The main ROS produced by chondrocytes are NO and superoxide anion that generate derivative radicals, including peroxynitrite and hydrogen peroxide (H_2O_2) (Hiran *et al.*, 1997, 1998). NO and its redox derivatives appear to have a number of different functions in both normal and pathophysiological joint conditions (Abramson, 2008b). Low NO concentrations have protective effects on other cell types and the literature

that deals with this area is beyond the scope of this chapter. Chondrocytes stimulated with pro-inflammatory cytokines produce large amounts of NO, which have been implicated in OA and has the capacity to inhibit extracellular matrix production by interfering with important autocrine and paracrine factors (Studer *et al.*, 1999). The published literature suggests important roles for NO in inflammation and pain associated with OA but this area is highly controversial and more work needs to be done to clarify the role of NO in joint health and disease (Abramson, 2008a). NO is synthesized by nitric oxide synthase (NOS) enzymes. Chondrocytes express both endothelial (eNOS) and inducible (iNOS) forms of the enzyme. NO production is stimulated by cytokines (i.e., IL-1 β , TNF- α), interferons (i.e., interferon γ : IFN- γ), and lipopolysaccharides (LPS). In fact, the increased expression of iNOS and cyclo-oxygenase-2 (COX-2) in OA is largely due to the increased expression of pro-inflammatory cytokines, particularly IL-1 β , which act in an autocrine/paracrine fashion to perpetuate a catabolic state that leads to progressive destruction of articular cartilage (Abramson *et al.*, 2001). In contrast, NO production is inhibited by growth factors such as transforming growth factor β (TGF- β).

In healthy cartilage, chondrocytes are thought to maintain robust defense mechanisms against attack by NO, free radicals, and ROS. However, as discussed earlier, responses to ROS generation will be dependent on redox status at the cellular level and influenced by systemic levels of inflammatory mediators, if present. When the oxidant level does not exceed the reducing capacities of cells, ROS are strongly involved in the normal physiological control of cellular functions including signal transduction. In contrast, in some pathological situations, when the cellular antioxidant capacity is insufficient to detoxify ROS, oxidative stress may occur degrading not only cellular membranes and nucleic acids, but also extracellular components including proteoglycans and collagens. This is likely to happen in certain OA phenotypes. Furthermore, ROS can modify proteins by oxidation, nitrosylation, nitration, or chlorination of specific amino acids, leading to impaired biological activity, changes in protein structure, and accumulation of damaged proteins in the tissue.

A further point that needs to be made in connection with oxidative stress is the fact that redox sensitive transcription factors (e.g., NF- κ B) are upregulated, which might result in an uncontrolled inflammatory response. Oxidative stress may also cause cell death and release of cellular content into extracellular environment, activating clearance mechanisms in the microenvironment. Altogether, degradation products and cellular material containing oxidized molecules may contribute to the exacerbation of synovial inflammation and form a vicious circle, constituted by newly formed ROS and further degradation products.

7.4 Inflammaging

“Inflammaging” is defined as “low-grade chronic systemic inflammation established during physiological aging” (Franceschi and Bonafe, 2003). The aging phenotype is characterized by immunosenescence and is explained by an imbalance between inflammatory and anti-inflammatory pathways, which results in a “low grade chronic pro-inflammatory status” (Franceschi *et al.*, 2007). Inflammaging is thought to be a driving force behind many forms of age-related pathologies, such as neurodegeneration, atherosclerosis, metabolic syndrome, diabetes mellitus, and sarcopenia (Franceschi and Bonafe, 2003). There is increasing evidence to suggest that inflammaging is associated with inflammatory diseases of the musculoskeletal system (i.e., osteoporosis, OA, and RA) (Berenbaum, 2013; Lencel and Magne 2011; Sellam *et al.*, 2013). In this context, humans and other animals must maintain homeostasis as they age, despite incessant attack from both intrinsic and extrinsic stimuli (Goto, 2008). Increased longevity results in a reduced capacity to mount inflammatory responses to infections and coordinate efficient anti-inflammatory responses to antigens and other noxious agents in our food and environment. Molecular evidence points to a disturbed interplay between autophagy and inflammasomes (Salminen *et al.*, 2012a). Declined autophagic capacity in aging cells impairs the process of cellular housekeeping. This leads to protein aggregation, accumulation of misfolded proteins, and the formation of dysfunctional mitochondria, which increases the generation of ROS thus promoting oxidative stress. In turn, oxidative stress can induce the assembly of inflammasomes (Salminen *et al.*, 2012b). Nod-like receptor protein 3 (NLRP3) is the major immune sensor for cellular stress signals. NLRP3 inflammasome-dependent inflammatory responses are triggered by a variety of signals of host danger, including infection, tissue damage, and metabolic dysregulation (Tschoopp, 2011; Zhou *et al.*, 2011). Inflammatory signals activate inflammasome-dependent responses and caspases, predominantly caspase-1, which cleaves the inactive precursors of interleukins, thus stimulating their elevated secretion and activity (Salminen *et al.*, 2012a). Consequently, these cytokines promote inflammatory responses and accelerate the aging process by inhibiting autophagy, which is believed to be a protective mechanism in cartilage. Autophagy may be a protective or homeostatic mechanism in normal cartilage (Lotz *et al.*, 2011). However, in OA it is associated with a reduction and loss of Unc-51-like kinase 1 (ULK1), an inducer of autophagy, Beclin1, a regulator

of autophagy, and microtubule-associated protein 1 light chain 3 (LC3), which initiates autophagy and increases chondrocyte apoptosis (Carames *et al.*, 2010).

7.5 Nutrigenomics

Nutrigenomics is the study of the effects of foods and food constituents on gene expression (Figure 7.3) (van Ommen and Stierum, 2002). This field of study has emerged because of the modern realization that the health effects of food-derived substances start at the molecular level (van Ommen, 2004; van Ommen and Stierum, 2002). Therefore, nutrigenomics is a form of personalized nutrition that involves tailoring diets to an individual's genetic makeup, considering genetic variation, allergies, and intolerances (van Ommen, 2007). The changes in gene expression translate to changes in the proteome and metabolome and consequently result in an altered metabolic state, which may have beneficial health effects. An important aim of nutrigenomic research is defining the relationship between genes and nutrients from basic biology to clinical states. We often overlook the fact that nutrigenomics and systems biology apply the same set of tools and technologies. The nutrigenomics approach extracts relevant differences, which become leads for further hypothesis driven and mechanistic research. The application of systems biology approaches in nutritional research aim to describe the physiological responses of culture models, experimental animals, and human subjects by exploiting the datasets, focusing on biochemical pathways, molecular targets for therapy, and potential biomarkers. Within this nutrigenomic framework, the term "nutritargeting" can be applied. Nutritargeting is defined as the targeting of a nutrient or nutrients to specific "target" tissues and is analogous to the term "drug targeting" (Biesalski and Tinz, 2008). There is a good scientific rationale for this intuitive idea. Some tissues are able to accumulate and utilize micronutrients selectively. For example, the antioxidant vitamin C is selectively accumulated in astroglial cells in the brain and in the lens of the eye (where it fulfills antioxidative and metabolic functions, facilitating the formation of collagen structures) (Biesalski and Tinz, 2008). Dehydroascorbic acid, the oxidized form of vitamin C, can enter the cell via the glucose transporter GLUT1 (Troadec and Kaplan, 2008). GLUT1 is expressed in tissues as a consequence of low oxygen pressure leading to upregulation of HIF1- α (a finding that is well documented in cancer cells; Airley *et al.*, 2007). These observations highlight the importance of vitamin C and its oxidized form dehydroascorbate in clinical nutrition, particularly in critically ill patients (Biesalski, 2008; McGregor and Biesalski, 2006).

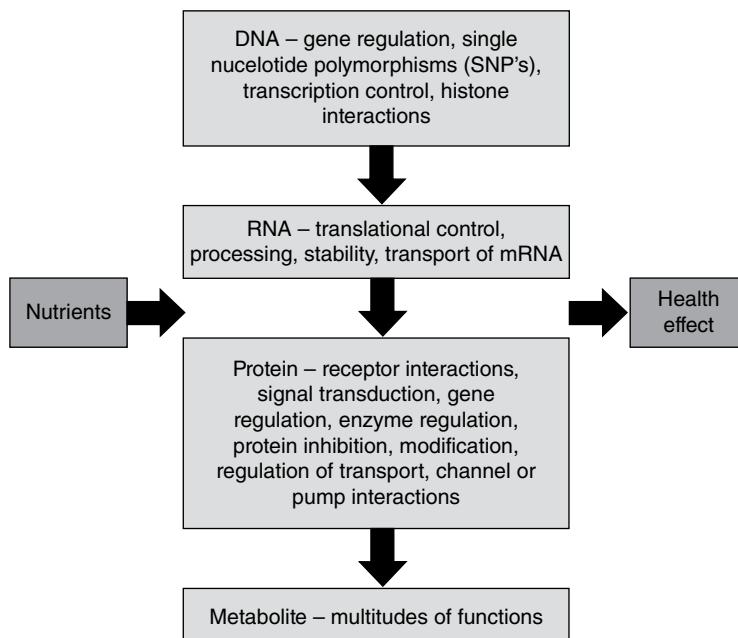


Figure 7.3 Nutrigenomics. The study of the effects of nutrients on gene expression.

Approximately 40% of the body's ascorbate is stored in skeletal muscle because this tissue is relatively abundant and its cellular concentration is ten-fold higher than the plasma level. The concept of nutritargeting relates to the gradual accumulation of micronutrients in target tissues using specific "carriers" or removal of the main barriers to absorption and tissue accumulation. For example, naturally occurring curcumin, which is poorly absorbed, exhibits increased bioavailability when complexed with polysorbate. "Nutritargeting" may play an important role in diseases where either systemic absorption is not possible (e.g., malabsorption/ maldigestion) or where significant local deficits occur, which may not adequately be supplied by the systemic application of that nutrient (Biesalski and Tinz, 2008). One of the goals of nutrition research is to optimize health and prevent or delay disease (van Ommen *et al.*, 2009). Research that targets certain aspects of the overarching drivers of health (metabolism, oxidation, inflammation, and stress responses) may be instrumental in creating knowledge for maintaining health and preventing disease through nutrition (van Ommen *et al.*, 2009).

We have discussed the importance of oxidative stress and inflammaging in cartilage biology in two recently published book chapters (Mobasheri *et al.*, 2013, 2014). The remaining part of this chapter will deal with inflammation in muscle and its relevance to OA. We also review some data of the literature that focuses on the effects of nutrients and polyphenols on muscle.

7.6 Muscle Inflammation in OA

An often-overlooked contributor to the progression of OA is sarcopenia and muscle weakness. Muscular support for the joint is important for the health of the whole joint organ. A considerable body of data shows that there is a strong correlation between muscle weakness and the presence of OA in patients (Palmieri-Smith *et al.*, 2010; Segal *et al.*, 2010), and indeed, muscle weakness is a better predictor of knee OA than joint space narrowing itself (McAlindon *et al.*, 1993). Although correlation does not imply causation, animal models confirm that experimentally induced muscle weakness does precipitate joint degeneration (Egloff *et al.*, 2014; Rehan Youssef *et al.*, 2009). It also seems logical that some of the amelioration of OA claimed to occur with moderate exercise could be because the joint receives more support from the strengthened (trained) musculature (Roos *et al.*, 2011). Skeletal muscle itself is well known to be vulnerable to the effects of cytokines (Roubenoff *et al.*, 2003) and these are, in turn, well known to increase with aging (Table 7.1). It is of note that changes of cytokines can include both those typically thought of as pro and anti-inflammatory, and that "successful aging" (i.e., aging without co-morbidity, is still associated with increased inflammatory activity; Krabbe *et al.*, 2004).

There is substantial evidence from studies of rodent models that skeletal muscle is highly sensitive to regulation by factors in the circulation. Experiments have shown that age-related deficits in muscle (loss of mass and contractile force generation, and impaired regeneration) can be restored by exposure of the tissue to young blood (Conboy *et al.*, 2005). Furthermore, recent work has shown that the protein growth differentiation factor (GDF)-11, as well as the hormone testosterone, which are both systemically depleted with aging, are key contributors to the rejuvenation of muscle function by young blood (Sinha *et al.*, 2014a, b). Other circulating factors are also likely to influence muscle function with aging. Consistent with the inflammaging hypothesis, injection of the pro-inflammatory cytokines TNF- α or IL-6 into rodents induces muscle atrophy (Fujita *et al.*, 1996; Haddad *et al.*, 2005; Janssen *et al.*, 2005; Li *et al.*, 2005). Transgenic mice over expressing IL-6 exhibit muscle atrophy resembling early-onset sarcopenia (muscle wastage or loss), which was prevented by regular subcutaneous injection of an IL-6 receptor-blocking antibody (Tsujinaka *et al.*, 1996). Both TNF- α and IL-6 have been reported to be increased in the human circulation with aging (Bartlett *et al.*, 2012; Forsey *et al.*, 2003), and in older people, circulating IL-6 and C-reactive protein (CRP) concentrations were inversely correlated with muscle strength, while the anti-inflammatory protein α 1-antichymotrypsin was found to protect against losses of muscle strength and mass (Schaap *et al.*, 2006).

An area garnering rising interest connected with inflammaging is the nutraceutical intervention concept, that is, supplementation of dietary non-medicinal extracts from herbs, spices, fruits, vegetables, teas, and other sources to deliver bioactive compounds that ameliorate age-related chronic inflammation. Certain dietary interventions such as these may modify the circulating milieu of cytokines and growth hormones, and thus act to maintain or restore muscle mass and function in older people. It is hoped that interdicting the age-related increase in muscle cytokines and downstream weakness will improve joint mobility in the elderly through reductions of both sarcopenia itself and of joint degeneration. Polyphenols, a class of compounds found in some edible plants, are under active investigation for their potential to reduce chronic inflammation. In plants, polyphenols function as antimicrobials, stress response factors, and pigments. However, they also induce structure-specific responses in mammalian cells, either directly or via secondary metabolites. At physiological doses, polyphenols have been shown to modulate ion channels (Wallace *et al.*, 2006), inhibit pro-inflammatory NF- κ B signaling (Kundu *et al.*, 2006) and cyclooxygenase and lipoxygenase activities (Kimura *et al.*, 1985; Kundu *et al.*, 2006), suppress intracellular reactive oxygen species (Lombardo *et al.*, 2013), interact with enzymes involved in redox signaling

Table 7.1 Cytokines linked to aging. A number of cytokines have been shown to change abundance as humans and non-human animals age. The arrows indicate whether levels tend to increase or decrease during aging. Asterisks indicate those cytokines typically thought of as “anti-inflammatory” (Marietta et al., 1996; Yugesha et al., 2009) (IL3), (Hart et al., 1989; Nolan et al., 2005) (IL4), (Marie et al., 1996) (IL-4, IL-10, and IL-13).

Significantly associated with aging	Not significantly associated with aging
↑ TNF- α (Diniz et al., 2010; Fagiolo et al., 1993; Morrisette-Thomas et al., 2014; Mooradian et al., 1991; Paganelli et al., 1994)	MCP1 (Morrisette-Thomas et al., 2014)
↓ IFN- γ (Paganelli et al., 1994; Rink et al., 1998)	IL-7 (Nikolich-Zugich, 2008)
↑ IL-18 (Morrisette-Thomas et al., 2014)	IL-8 (Morrisette-Thomas et al., 2014)
↑ hsCRP (Krabbe et al., 2004; Morrisette-Thomas et al., 2014)	IL-12 (Morrisette-Thomas et al., 2014)
↑ IL-1 (Paganelli et al., 1994; Rink et al., 1998)	
↓ IL-2 (Gillis et al., 1981; Rink et al., 1998)	
↑ IL-3* (Paganelli et al., 1994; Rink et al., 1998)	
↑ IL-4* (Paganelli et al., 1994)	
↑ IL-6 (Ershler et al., 1993; Fagiolo et al., 1993; Fernandez-Real et al., 2001; Morrisette-Thomas et al., 2014; Paganelli et al., 1994)	
↑ IL-10* (Cakman et al., 1996; Miles et al., 2008; Morrisette-Thomas et al., 2014; Rink et al., 1998)	
↓ IL-15 (Morrisette-Thomas et al., 2014)	
↑ IL-18 (Morrisette-Thomas et al., 2014)	
↑ MCP-1 (Miles et al., 2008)	
↑ 6Ckine (Miles et al., 2008)	
↑ Eotaxin-1 (Shurin et al., 2007)	
↑ MIG (Shurin et al., 2007)	

(Buryanovskyy et al., 2004; Lu et al., 2006; Takahashi et al., 2012), influence cellular metabolism, autophagy, and protein acetylation (El-Mowafy and Alkhalaif, 2003; Gu et al., 2014; Pietrocola et al., 2012), and bind directly to microRNAs (Baselga-Escudero et al., 2014). As little as 200 ml of red wine is reported to potentially contain sufficient polyphenols to modulate ATP dependent potassium channels (Mosca et al., 2002). Potassium channels are well known to be expressed by both joint chondrocytes (Mobasher et al., 2007) and a variety of mammalian muscle cell types (Wellman et al., 1999).

A wide range of polyphenols are potentially of interest to the study of muscle inflammaging and nutrigenomics, including quercetin and its glycosylated/methylated derivatives, which are found in many different common fruits and vegetables, including onions and apples; curcumin, from the Indian spice turmeric, which is a core component of the Ayurvedic and Unani traditional medical systems; gingerol, from ginger, which is closely related to turmeric; resveratrol, from red grapes, and epigallocatechin gallate (EGCG), from green tea. These structures have been reported to inhibit pro-inflammatory signaling *in vitro* and *in vivo*, and while the existing literature is somewhat limited in respect to their actions on skeletal muscle, those studies that have been performed have shown some interesting results. Long-term dietary supplementation of mdx mice (a widely used genetic model of muscular dystrophy) with quercetin significantly reduced the progression of the dystrophy phenotype (Hollinger et al., 2014). The limb immobilization/suspension rodent model is frequently used for the study of skeletal muscle atrophy; muscles thus unloaded show increased proteasomal/apoptosomal activity, indicating atrophy. Daily injection of hindlimb-immobilized rats with curcumin was found to abrogate the muscle atrophy response (Vazeille et al., 2012). Oral supplementation with resveratrol also maintained muscle mass and protected against metabolic dysfunction in a rat muscle unloading model (Momken et al., 2011). There is accumulating evidence that polyphenols may inhibit the induction of atrophy by factors involved in inflammaging. An *in vitro* model of muscle showed significantly less atrophy when treated with resveratrol and TNF- α together, compared with TNF- α treatment only (Wang et al., 2014). This study highlighted the Akt/mTOR/FoxO1 signaling pathway as a regulatory target of resveratrol in alleviating TNF- α -induced atrophy. However, long-term dietary supplementation of mice with resveratrol did not prevent age-related sarcopenia, indicating that other polyphenols may be better candidates for use as interventions in aging (Jackson et al., 2011). The green tea polyphenol, EGCG, has come to the fore as an anti-atrophic polyphenol following several studies over

the last decade. Although EGCG supplementation in the diet did not protect against primary muscle atrophy in an aged rat hindlimb-immobilization model, EGCG-supplemented rats showed significantly increased muscle recovery after remobilization (Alway *et al.*, 2014). Loss of force in a mouse model of muscle unloading was also reduced by dietary EGCG supplementation (Ota *et al.*, 2011), and EGCG delivered either through the diet or by subcutaneous injection protected against muscular dystrophy in *mdx* mice (Dorchies *et al.*, 2006; Nakae *et al.*, 2008). In mice with tumor-driven cachexia, EGCG attenuated muscle atrophy and suppressed NF- κ B signaling in muscle (Wang *et al.*, 2011). The induction of myotube atrophy markers in a physical *in vitro* model of muscle unloading (three-dimensional clinorotation) was significantly inhibited by treatment by EGCG (Hemdan *et al.*, 2009) and EGCG treatment also significantly reduced serum starvation-induced myotube atrophy (Mirza *et al.*, 2014). The evidence that is presently available therefore points towards EGCG (or green tea) as a promising dietary intervention to potentially reduce muscle dysfunction and atrophy in aging. A trial was conducted in sarcopenic Japanese women; the subjects either underwent a program of exercise, were supplemented with green tea catechins, or undertook both interventions together. Interestingly, the only group to show a significant increase in leg muscle mass (2% increase after 3 months) was the combined catechins and exercise group, neither catechins alone nor exercise alone were effective (Kim *et al.*, 2013). The structurally similar cocoa polyphenol, (-)-epicatechin, has also been reported to protect against sarcopenia in mice (Gutierrez-Salmean *et al.*, 2014), and normalize aberrant metabolic processes in skeletal muscle of patients with systemic age-related conditions (Ramirez-Sanchez *et al.*, 2013). In addition to these promising reports on EGCG and (-)-epicatechin, there are hundreds of polyphenols relevant to the human diet that have not been studied in the context of skeletal muscle atrophy, and other anti-sarcopenic compounds may remain among this unexplored pool of potential bioactives. Also of interest is the non-polyphenolic organosulfur compound diallyl sulfide, found predominantly in garlic, which was found to inhibit muscle atrophy in mice with cancer cachexia, and also led to increased muscle weights in healthy control subjects (Olivan *et al.*, 2011).

In summary, there is clear evidence that inflammaging exerts effects on muscle that contribute to progression of OA, and several promising candidate plant-derived bioactive molecules may beneficially modulate one or more aspects of the inflammaging/muscle weakness/joint degradation trinity in the development of OA (Figure 7.4). Of these compounds, EGCG from green tea is arguably best evidenced to improve muscle function, and further study is warranted for diallyl sulfide from garlic, curcumin from turmeric, (-)-epicatechin from cocoa, and quercetin from onions and apples.

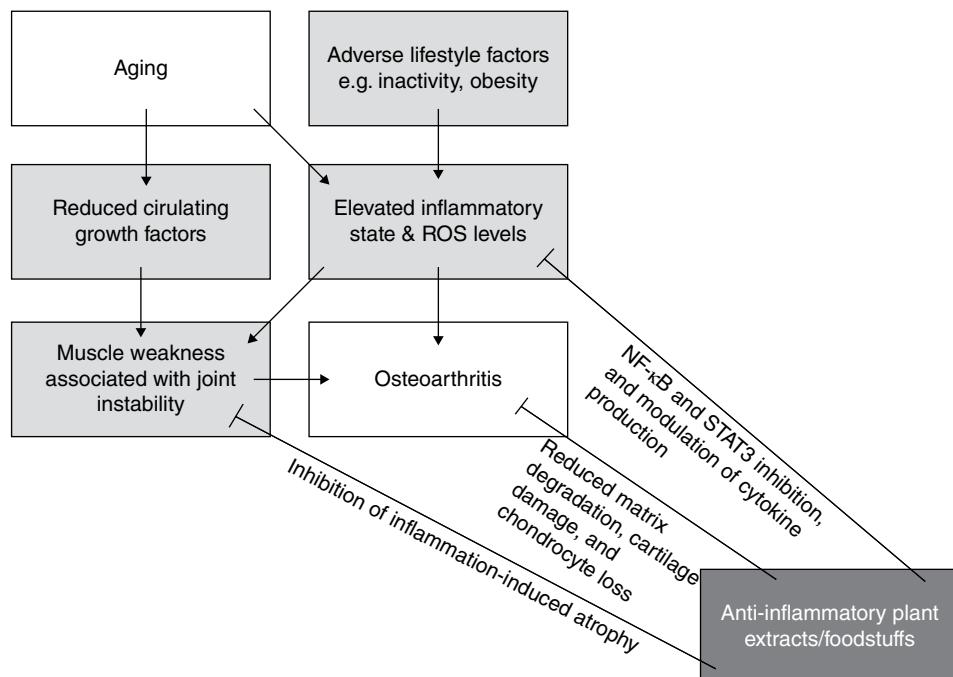


Figure 7.4 The relationship between aging, OA and anti-inflammatory nutraceuticals, functional foods, and natural plant products/extracts.

7.7 Conclusions

Nutraceuticals, functional foods and dietary supplements have become important areas of research and clinical practice in orthopedics and rheumatology. The elderly population is rapidly growing and expanding throughout the developed and developing worlds. Therefore, the use of herbal and complementary medicines for the treatment of persistent musculoskeletal pain will continue to increase. Therefore, it is important that patients and health-care providers are aware of the evidence for or against these approaches. Some of the published evidence suggests that several herbal medicines and dietary supplements have the capacity to alleviate the pain of OA and RA. For several treatments, the risk-benefit profile is encouraging. Some herbal remedies are inhibitors of NF- κ B and may be able to reduce the consumption of NSAIDs. Nutraceuticals, functional foods and dietary supplements may be used to supplement some of the benefits from existing pharmaceutical treatment modalities for OA. In such situations the aim is to reduce the frequency of consumption and dosages of conventional drugs such as NSAIDs, which have significant side-effects. However, the objective is not to replace NSAIDs altogether because they not only provide pain relief, but also possess valuable anti-inflammatory activity. However, patients with OA routinely use prescribed and alternative products at the same time. There is potential for adverse drug interactions and patients should be made aware of the risks associated with taking multiple products. Also, many nutraceuticals and functional foods have not been tested properly in rigorous clinical trials examining the efficacy of herbal remedies are needed before definitive recommendations regarding the application of these modalities can be made.

The European Food Safety Authority (EFSA) based in Parma, Italy has recently issued new guidelines and proposed new scientific requirements for health claims related to the maintenance of joints and to the reduction of the risk of developing OA. EFSA has proposed that clinical trials of functional foods and nutraceuticals should be designed in new and innovative ways to demonstrate a “beneficial physiological effect” on healthy joints. According to these new guidelines, only clinical trials designed to demonstrate a beneficial physiological effect on joints or a reduction in joint degradation in people without OA should be accepted as indicative. These guidelines present some major new challenges to the scientific and clinical communities. Furthermore, they create a number of opportunities for new types of clinical trials. Studies performed in non-diseased (but including high risk) population subgroups in which the incidence of OA is the outcome measure could be used for substantiation of health claims relating to the normal maintenance of the joint. Whilst attempting to address these requirements, we need to discriminate between food and non-food supplements. Studies dealing with “non-foods” will require a much more traditional pharmacological design compared to studies on “foods”. Clearly, addressing these issues requires new strategies and large scale clinical studies lasting several decades. Such new trials will require radical rethinking of the concept of clinical trials in the OA research community. Human studies will be necessary for substantiation of clinical data and study groups should be representative of the entire population. The hierarchy of evidence should also be considered; for example, double blind interventional studies are of greater significance compared to observational studies and reproducibility of the effect much be demonstrated. In addition, demonstrating efficacy of food supplements to organizations such as EFSA will require data on tolerance and safety, specifically gastric tolerance, hepatotoxicity, renal toxicity, and allergenicity.

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8

Genetic Basis of Anti-Inflammatory Properties of *Boswellia* Extracts

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8.1 Introduction

Inflammation is typically a protective mechanism that is triggered in response to noxious stimuli, trauma, or infection, in order to guard the body and to hasten the recovery process. As such the complex inflammatory process per se may not constitute a serious disease condition. However, inflammation that is unchecked or fails to respond poses health risks to millions of people worldwide. Inflammation is also a common factor underlying many disease indications including wounds, burns, heart attacks, arthritis, asthma, and cancer.

The study of genetic basis and molecular mechanisms of inflammatory diseases has thus become one of the hottest areas in biomedical research these days with a particular emphasis on gene regulation. Nutrigenomics constitutes the study of the effects of bioactive compounds from food on gene expression. An increasing body of scientific evidence hoarded during the last few years has demonstrated that phytochemicals derived from food ingredients and plants alter the expression of genes in the human body. Detailed mechanisms of action for their active role in the prevention of inflammation have been elucidated for many natural ingredients. Employing novel genomic approaches to identify key elements of acute and chronic inflammation is thus a key aspect of nutrigenomics to identify individual compounds, as well as extracts, derived from food ingredients to alter the expression of inflammation related genes in the human body.

Inflammatory mediators and inflammatory cells modulate the inflammatory response. A number of inflammatory mediators, such as kinins, cytokines, eicosanoids, enzymes, and adhesion molecules act on specific targets leading to the local release of other mediators from leukocytes, and also attract leukocytes to the site of inflammation. Inflammation can be controlled effectively by inhibiting the formation of inflammatory mediators, such as eicosanoids. Eicosanoids, prostaglandins, and leukotrienes are produced primarily from arachidonic acid (Figure 8.1) that has been released from the cell membranes. The formation of prostaglandins and leukotrienes from arachidonic acid can be suppressed by inhibiting cyclooxygenase and lipoxygenase, respectively. The two known isoforms of cyclooxygenase (COX) are COX-1 and -2. Although these two isoforms share similar structure, they differ markedly in their pattern of regulation and physiological functions. COX-1 is

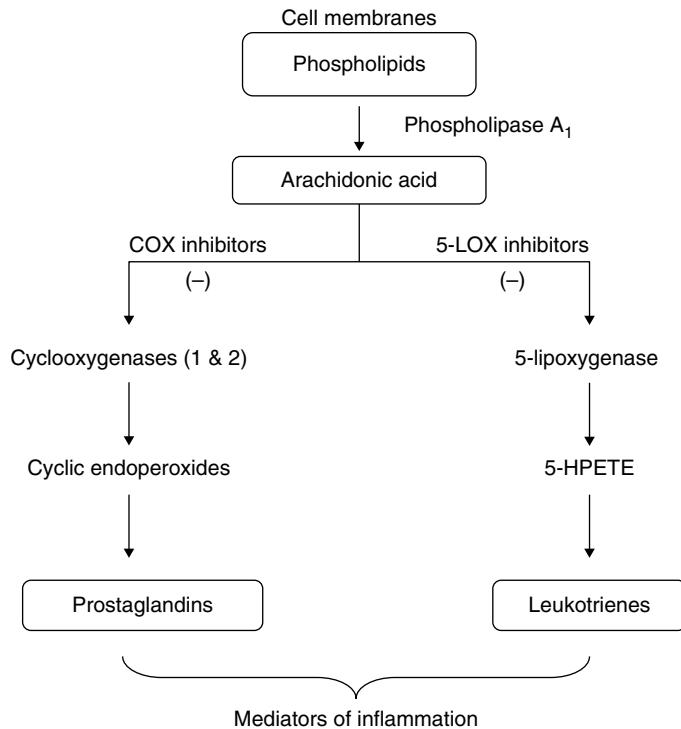


Figure 8.1 Eicosanoid biosynthetic pathways.

readily detected in many tissue types and is thought responsible for “housekeeping” activities, such as gastrointestinal cytoprotection, renal blood flow regulation, and platelet aggregation. COX-2, in contrast, is considered the inducible isoform. It is generally not detected in most tissues but can be found in large amounts in macrophages and other inflammatory cell types following exposure to cytokines, growth factors, and mitogens (Simmons *et al.*, 1992; Smith and Dewitt, 1996). The alternative pathway (Figure 8.1) for inflammation is mediated by 5-lipoxygenase. Lipoxygenases (LOXs) comprise a family of non-heme iron-containing dioxygenases, representing the key enzymes in the biosynthesis of leukotrienes that have been postulated to play an important role in the pathophysiology of several inflammatory and allergic diseases. The products of LOXs catalyzed oxygenation (hydroperoxyeicosatetraenoic acids (HPETE), hydroxyeicosatetraenoic acids (HETE), leukotrienes, and lipoxins) are the important inflammatory mediators. According to the currently used nomenclature, the LOXs are classified with respect to their positional specificity of arachidonic acid oxygenation (5-LOX, 9-LOX, 12-LOX, 15-LOX) (Yamamoto, 1992).

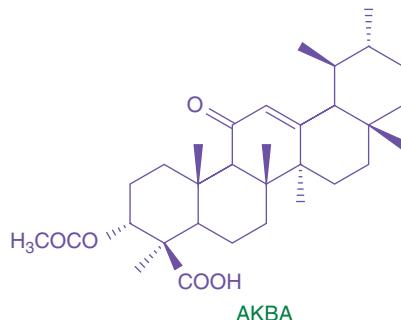
8.2 *Boswellia serrata*

Gum resin of *Boswellia serrata*, most commonly known as Indian frankincense, has been used traditionally for the treatment of a wide range of indications including rheumatism, menstrual pain, and wrinkles (Ethan *et al.*, 2004). It was an important anti-inflammatory agent in Ayurveda, the traditional Indian system of Medicine (Chatterjee, 1984). Many *in vitro* and *in vivo* experiments have supported the traditional claims on *Boswellia* products. The ethanolic extract of the gum resin, for example, inhibited neutrophils (Ammon *et al.*, 1991) *in vitro* and inhibited carrageenan-induced paw edema (Singh and Atal, 1986) in rats on par with phenylbutazone. Oral administration of boswellic acids to mice, after intra-pleural injection of carrageenan inhibited polymorphonuclear infiltration into the pleural cavity, a response similar to indomethacin (Ammon, 2002).

Boswellia gum resin and its extracts also demonstrated significant therapeutic improvements in human clinical trials (Kimmakar *et al.*, 2003; Gupta *et al.*, 1997, 2001). A randomized, double blind, placebo controlled crossover clinical trial with *Boswellia* extract on a group of patients with osteoarthritis of knee exhibited statistically significant mean improvements

with respect to the reduction in pain, decreased swelling, and increased knee flexion (Kimmatkar *et al.*, 2003). In an open non-randomized equivalence study, 30 patients with chronic colitis were administered either *Boswellia* gum (300 mg three times daily) or sulfasalazine (1 g three times a day) and the therapeutic effects shown by *Boswellia* were comparable to those exhibited by sulfasalazine (Gupta *et al.*, 2001). In a similar study with 42 ulcerative colitis patients, *Boswellia* gum and sulfasalazine also showed comparable improvements in abdominal pain, diarrhea, and histopathology without any statistically significant difference between the two treatment groups (Gupta *et al.*, 1997). In an another equivalent study, *Boswellia* standardized extract exhibited therapeutic improvements comparable to, or better than, mesalazine in a randomized, double-blind study on patients with active Crohn's disease (Gerhardt *et al.*, 2001). *Boswellia* gum resin also showed statistically significant improvement in patients with bronchial asthma in a 6-week double blind, placebo controlled study (Gupta *et al.*, 1998).

8.3 Mechanism of Action



The source of the anti-inflammatory actions of *Boswellia* gum resin has been attributed to a group of triterpene acids (Safayhi and Sailer, 1997) called boswellic acids, isolated (Pardhy and Bhattacharya, 1978) from the gum resin of *Boswellia serrata*. These compounds exert anti-inflammatory activity by inhibiting 5-lipoxygenase (5-LOX). In addition to their 5-lipoxygenase inhibitions, boswellic acids also inhibit human leukocyte elastase (HLE), an enzyme of different pro-inflammatory pathway (Safayhi *et al.*, 1997). Catechins, quercetin, and boswellic acids are some of the important naturally occurring 5-lipoxygenase inhibitors. Although quercetin is a potent inhibitor, it belongs to the redox type of 5-lipoxygenase inhibitor. Redox type inhibitors are not selective and thus interfere with some useful biochemical pathways causing undesirable side effects. Boswellic acids, on the other hand, are selective non-redox type 5-lipoxygenase inhibitors.

Drugs that target lipoxygenases and leukotrienes have recently become the emerging therapies for inflammatory diseases and cancer (Poff and Balazy, 2004). A detailed study on the structural requirements for boswellic acids indicated that, of all the six acids, AKBA shows most pronounced inhibitory activity against 5-LOX (Safayhi *et al.*, 1992; Sailer *et al.*, 1996) with an IC₅₀ of 1.5 μM (Sailer *et al.*, 1996) against 5-lipoxygenase enzyme in intact cells. AKBA acts by a unique mechanism, binding to 5-LOX in a calcium-dependent and reversible manner, and inhibits its activity as an allosteric regulator and not as a redox-type or competitive inhibitor (Sailer *et al.*, 1998). Other boswellic acids inhibited 5-LOX only partially and incompletely, whereas, the non-inhibitory triterpenoid constituents in the extract such as amyrin and its derivatives antagonized the biological activity of AKBA (Safayhi *et al.*, 1995; Sailer *et al.*, 1996). AKBA is a minor constituent in the natural *Boswellia serrata* extracts; though some of the partially active boswellic acids are present in higher proportion (up to 25%). AKBA concentration even in a higher grade commercial material (85% boswellic acids) typically varies in the range of 2–5%. AKBA has thus become the subject of intensive research for many groups across the globe because of its potential for the treatment of chronic inflammatory disorders.

8.4 Development of 5-LOXIN® (BE-30)

5-LOXIN® is a novel *Boswellia serrata* extract selectively enriched in AKBA concentration. 5-LOXIN® contains minimum 30% AKBA (BE-30) and is produced using commercially viable process developed by the researchers at the Laila Impex R&D Center. Its efficacy at the enzyme and cellular level, and in *in vivo* has been clearly established (Roy *et al.*, 2005, 2006).

The safety of it has been proven by a whole spectrum of safety studies (Lalithakumari *et al.*, 2006). Finally, the clinical efficacy of 5-LOXIN® was established in a double blind placebo controlled human clinical study (Sengupta *et al.*, 2008a). Keeping in perfect consonance with its higher AKBA content, 5-LOXIN® (BE-30) exhibited significantly better inhibitory activity against 5-lipoxygenase ($IC_{50} = 40 \mu\text{g/ml}$), when compared to other commercially available *Boswellia* extracts ($IC_{50}>100\mu\text{g/ml}$). It has also been found that 5-LOXIN® (BE-30) is more efficacious as an antibacterial and anti-proliferative agent compared to extracts containing lower concentrations of AKBA.

8.4.1 Genetic Basis for Efficacy of 5-LOXIN® (BE-30)

Tumor necrosis factor α (TNF α) is a pleotropic inflammatory cytokine produced by the immune system that suppresses tumor cell proliferation. Subsequent studies established that TNF α is a key mediator of inflammation (Sullivan, 2003; Strieter *et al.*, 1993; Warren *et al.*, 1988). TNF α is produced in endothelial cells (EC). Endothelial cells (EC) are squamous epithelial cells that form thin lining to the interior surface of the vasculature and helps reduction of turbulence to the blood flow. EC are crucial elements in the inflammatory etiology, which participate through production and secretion of several pro-inflammatory cytokines including interleukins, M-CSF, G-CSF, GM-CSF, MCP, and so on. EC also expresses a series of glycoproteins and cell surface proteins, which helps recruitment of leucocytes to the site of inflammation by binding to circulating leucocytes. Inflammatory response in EC is potentially induced by TNF α (Pober, 2002). Genomics has recently become the cutting edge tool in inflammation research. With the advent of microarray technology, the study of the amelioration of the genes associated with inflammation has become faster and economical. Thus the genetic basis for the anti-inflammatory effects of the standardized extract, 5-LOXIN® (BE-30) was tested in a system of TNF α induced gene expression in human microvascular endothelial cells (HMEC). This was the first whole human genome screen to delineate the genetic basis of the anti-inflammatory efficacy of a medicinal plant derivative.

8.5 Gene Chip Probe Array Analysis

GeneChip™ analysis was done to identify the sets of genes in HMEC that are sensitive to TNF α . The HMEC cells were grown in MCDB-131 under standard cultured conditions (Roy *et al.*, 2005). Twenty four hours after the seeding, the HMEC cells were treated with 5-LOXIN® (BE-30) 25 $\mu\text{g}/\text{mL}$; or Vehicle (DMSO). Forty eight hours after treatment, the cells were challenged with 50 ng/ml of human recombinant TNF α for 6 h. The cells were harvested and subjected to RNA extraction. The RNA fraction was purified from DNA contamination using DNA-free™ kit (Ambion, Austin, TX). After the quality assessment, the RNA samples were hybridized to probes using affymetrix human genome arrays U 133 Plus 2.0 containing 47000 transcripts including the entire human genome. After hybridization at 45°C for 16 h, the arrays were washed, stained and scanned using Affymetrix Gene Chip Scanner 3000. Affymetrix micro array suite 5.0 and data mining tool 2.0 were used for raw data analysis. dChip software was used for processing additional data and to obtain mean change of each gene expression by hierarchical clustering (Li and Wong, 2001). 5-LOXIN® (BE-30) sensitive TNF- α inducible genes were processed using comparative analysis approach.

In nine out of nine pair-wise comparisons, TNF α upregulated 522 genes and downregulated 141 genes (Figure 8.2). Of the 522 genes induced by TNF α in HMEC, 113 genes were clearly sensitive to 5-LOXIN® (BE-30) treatment. BE-30 sensitive TNF α inducible genes that exhibited significant response were subjected to further processing for identification of signaling pathways using Affymetrix gene ontology mining tools KEGG (Kyoto Encyclopedia of Genes and Genomes), Gen-MAAP, DAVID (Database for Annotation, Visualization, and Integrated Discovery Verification), GO (gene ontology), and Locus Link. The genes that were significantly modulated in the treatment group supplemented with both TNF α and 5-LOXIN® (BE-30) compared to the control group treated with TNF α alone are summarized along with their functional category (Table 8.1). These genes were identified to be directly related to inflammation, cell adhesion (ICAM-1, VCAM-1), proteolysis, peroxisome proliferation, fatty acid metabolism, angiogenesis, and so on. The down regulation of significant number of inflammatory genes induced by TNF α in HMEC by AKBA enriched *Boswellia* extract (BE-30) strongly supports the traditional claims listed on *Boswellia*.

More importantly, TNF α -inducible expression of ICAM-1 and VCAM-1 was significantly sensitive to BE-30 treatment. Cell Adhesion Molecules (CAMs) are proteins expressed on the cell surface helps binding with other cells or with the extra-cellular matrix (ECM). CAMs enables extravasation of leucocytes during physiological and more evidently in pathological processes. Three families of CAMs (selectins, integrins, and immunoglobulins) play important role in leucocyte endothelial interactions. Inter cellular adhesion molecule-1 (ICAM-1 or CD54), vascular cell adhesion molecule-1 (VCAM-1 or CD106)

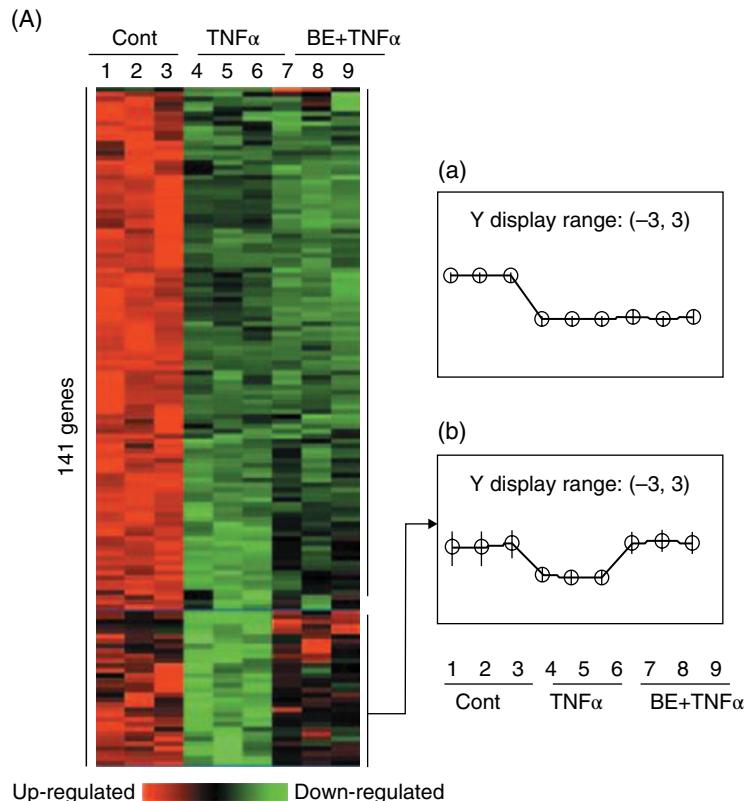


Figure 8.2 Hierarchical cluster images illustrating BE sensitive genes induced or downregulated by TNF α in human microvascular endothelial cells. For a clear graphic display of the specific clusters of genes showing a decrease (A) or an increase (B) in expression following TNF α treatment to HMEC cells, a count percentage analysis was performed. Genes that were found up or downregulated in 100% of replicates and all comparisons (nine out of nine) following TNF α treatment were selected. These select candidate genes were subjected to hierarchical clustering to identify clusters of genes that are induced/down regulated by TNF α and are sensitive to Boswellia. The changes in shading represent higher to lower expression signals.

are induced by cytokines and aids in recruitment of circulating leucocytes to the inflamed site through adhesion and emigration (Albelda *et al.*, 1994; Barreiro *et al.*, 2002). These cell adhesion molecules are directly implicated in the pathogenesis of inflammation and their expression in endothelial cells and lymphocytes is regulated by the inflammatory cytokines. VCAM-1 plays a key role in recruiting leukocytes to the site of inflammation. Further studies quantitatively established the effect of 5-LOXIN® (BE-30) on TNF α -inducible expression of VCAM-1 manifested that the upregulated VCAM-1 expression was completely abrogated in the human microvascular endothelial cells pretreated with 5-LOXIN® (BE-30). This further supported the anti-inflammatory properties of *Boswellia* extracts in general and enriched extract 5-LOXIN® (BE-30) in particular.

8.6 Proteomics

GeneChip study on *Boswellia* products further revealed that Matrix metalloproteinases (MMP) are a prominent class of TNF α -inducible genes sensitive to *Boswellia* extracts. Matrix metalloproteinases (MMPs) are a large family of zinc dependent metallo-endopeptidases that degrade various components of the extra cellular matrix (ECM) in both normal and diseased tissues. The first MMP discovered was a collagenase in the tail of a tadpole undergoing metamorphosis. The MMP family consists of more than 64 members, out of which 23 were identified in humans. These enzymes include collagenases and elastases, which were segregated into three groups based on their substrate preference. Collagenases degrade connective

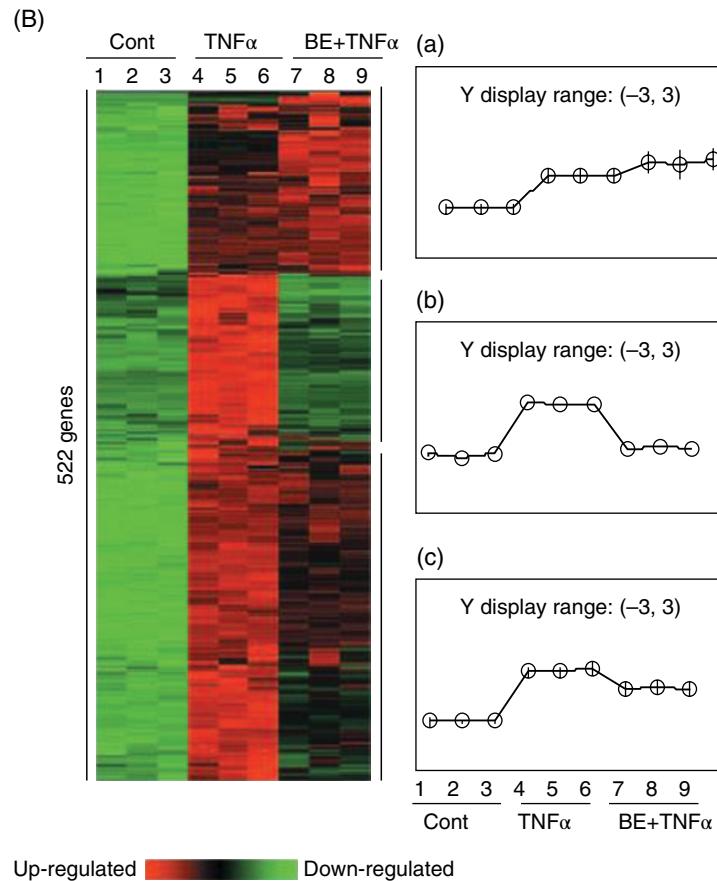


Figure 8.2 (Continued)

tissue collagen, whereas gelatinases degrade basement membrane collagens. However, the targets of stromelysins are ECM proteoglycans, laminin, fibronectin, and gelatin. MMPs are expressed at low levels in normal adult tissues and are upregulated during different physiological and pathological remodeling processes such as embryonic development, tissue repair, inflammation, rheumatoid arthritis, angiogenesis, tumor invasion, and metastasis. Inhibitors are predicted to have clinical benefits in arthritis and metastasis. Most MMPs are constitutively expressed *in vitro* at low levels by different cell types, such as keratinocytes, fibroblasts, macrophages, endothelial cells, mast cells, eosinophils, and neutrophils (Herouy, 2004; Massova *et al.*, 1998).

Hence, the efficacy of 5-LOXIN® (BE-30) was further studied in human microvascular endothelial cells against TNF α -inducible MMP expression (Roy *et al.*, 2006). The robust 5-LOXIN® (BE-30) sensitive candidate genes were then subjected to further processing for the identification of BE-30 sensitive signaling pathways. The use of resources such as GenMAPP, KEGG, DAVID, and GO led to the recognition of the primary 5-LOXIN® (BE-30) sensitive TNF α -inducible pathways. Members of the MMP family of genes were subjected to hierarchical clustering. MMP3, MMP10, MMP12, and MMP19 were recognized as TNF α inducible genes that were sensitive to 5-LOXIN® (BE-30) (Figure 8.3). A quantitative real-time PCR approach was used in this study to follow up on the results from the gene microarray study. TNF α caused a dose-dependent induction of MMP3, MMP10, and MMP12 in HMEC. Pre-treatment of HMEC for 2 days with 5-LOXIN® (BE-30) potently prevented TNF α -induced expression of MMP3, MMP10 as well as MMP12 mRNA in HMEC (Figure 8.4). HMEC were treated with 25 μ g/ml Boswellia extracts (BE-3 or BE-30) for 48 h. BE were prepared in DMSO at concentrations such that the final concentration of the solvent in cell suspension never exceeded 0.1% v/v. Controls were treated with matching volume of DMSO. After the treatment period, cells were activated with recombinant human TNF α (50 ng/ml) for 6 h. Expression of MMP3, -10, and -12 mRNA was determined

Table 8.1 *TNF α* -inducible genes sensitive to 5-*loxin*[®] (BE-30).

SC Probe set	Gene ^b	TNF α Mean ^a	TNF α SD	BE-30 +TNF α Mean ^a	BE-30 +TNF α SD	Functional category
203868_s_at	VCAM1	172.74	5.83	22.23	4.90	Cell adhesion/inflammation
205681_at	BCL2A1	26.18	1.80	8.14	0.36	antiapoptosis
1555799_a_at	CCL5	26.19	2.04	6.09	0.36	Chemokine
1405_I_at	CCL5	32.21	2.12	5.64	0.53	Chemokine
205890_s_at	UBD	83.34	13.26	4.45	2.16	Proteolysis
204932_at	TNFRSF11B	11.86	0.72	3.80	0.30	Inflammation
205828_at	MMP3	16.76	2.40	3.77	0.43	Proteolysis
229437_at	BIC	13.74	0.61	3.43	0.35	Carcinogenesis
221371_at	TNFSF18	8.87	0.67	2.97	0.89	Inflammation
223484_at	NMES1	15.82	1.04	2.95	0.12	Tumor suppressor
219209_at	MDA5	7.25	0.16	2.82	0.41	Differentiation
206211_at	SELE	11.21	0.60	2.62	0.76	Cell adhesion
207339_s_at	LTB	10.03	1.08	2.58	1.00	Inflammation
202023_at	EFNA1	7.82	0.31	2.39	0.15	Signal transduction
206553_at	OAS2	12.29	0.75	2.27	1.70	RNA binding
204655_at	CCL5	8.75	0.84	2.22	0.11	Chemokine
205680_at	MMP10	8.44	0.19	2.20	0.14	Proteolysis
229450_at	IFIT4	5.62	0.40	1.98	0.13	Immune/inflammation
24393_x_at	DNAH5	3.29	0.43	1.95	0.53	Microtubule
213338_at	RISI	3.68	0.07	1.94	0.14	Apoptosis
221085_at	TNFSF15	5.68	0.34	1.92	0.46	Inflammation
226847_at	FST	4.56	0.08	1.78	0.17	TGF-Beta signaling
211122_s_at	CXCL11	5.26	0.59	1.64	0.76	Chemokine
204897_at	PTGER4	3.19	0.07	1.60	0.07	G-protein receptor
212448_at	NEDD4L	3.40	0.35	1.58	0.53	Proteolysis
232517_s_at	PRIC285	2.90	0.32	1.56	0.08	Peroxisome proliferation
20474_at	IFIT4	4.26	0.18	1.55	0.25	Immune/inflammation
204298_s_at	LOX	2.90	0.21	1.54	0.05	Collagen metabolism
233500_x_at	LLT1	5.71	0.60	1.51	0.70	Signal transduction
219593_at	SLC15A3	2.73	0.15	1.51	0.01	Transport
206825_at	OXTR	2.83	0.11	1.49	0.08	G-protein receptor
203595_s_at	IFIT5	2.60	0.11	1.49	0.07	Immune/inflammation
203835_at	GARP	3.17	0.21	1.47	0.15	Unknown
38037_at	DTR	2.61	0.06	1.46	0.10	Hypertrophy model
229865_at	FAD104	2.39	0.14	1.41	0.25	Protein-tyrosine phosphatase
221653_x_at	APOL2	2.16	0.26	1.41	0.20	Fatty acid metabolism
226757_at	IFIT2	2.60	0.25	1.40	0.05	Immune/inflammation
201662_s_at	ACSL3	2.88	0.30	1.40	0.16	Fatty acid metabolism
220132_s_at	LLT1	5.21	0.99	1.40	0.09	Signal transduction
225344_at	NCOA7	2.03	0.05	1.34	0.05	Receptor activity
230820_at	SMURF2	2.30	0.24	1.33	0.05	Proteolysis
205596_s_at	SMURF2	2.05	0.06	1.32	0.05	Proteolysis
222062_at	IL27RA	2.54	0.05	1.32	0.18	Immune/inflammation
206995_x_at	SCARF1	2.18	0.20	1.31	0.10	Endothelial scavenger receptor
204533_at	CXCL10	4.63	0.38	1.31	0.04	Chemokine
227489_at	SMURF2	2.25	0.36	1.29	0.03	Proteolysis
209567_at	RRS1	2.04	0.12	1.29	0.12	Ribosome biogenesis
211588_s_at	PML	5.21	0.89	1.29	0.49	Transcription
202086_at	MX1	10.67	0.46	1.28	0.41	Immune/inflammation
208075_s_at	CCL7	4.06	0.75	1.25	0.68	Chemokine
201645_at	TNC	2.35	0.08	1.23	0.09	Adhesion molecule

(Continued)

Table 8.1 (Continued)

SC Probe set	Gene ^b	TNF α Mean ^a	TNF α SD	BE-30 +TNF α Mean ^a	BE-30 +TNF α SD	Functional category
202464_s_at	PFKFB3	2.04	0.16	1.23	0.08	RNA binding
224013_s_at	SOX7	2.04	0.11	1.22	0.05	Development
201661_s_at	ACSL3	2.57	0.17	1.21	0.18	Fatty acid metabolism
209277_s_at	TFPI2	2.76	0.26	1.21	0.11	Protease inhibitor
200704_at	LITAF	2.36	0.05	1.19	0.03	Inflammation
227020_at	YPEL2	2.41	0.04	1.18	0.05	Unknown
201660_at	ACSL3	2.55	0.07	1.16	0.06	Fatty acid metabolism
219522_at	FJX1	2.29	0.20	1.16	0.13	Differentiation
219279_at	DOCK10	2.75	0.12	1.16	0.15	Cell cycle
210001_s_at	SOCS1	2.37	0.33	1.15	0.23	Signal transduction
228607_at	OAS2	2.90	0.42	1.14	0.19	RNA binding
206133_at	HSXIAPAF1	3.07	0.84	1.14	0.21	Anti-apoptosis
209969_s_at	STAT1	2.15	0.15	1.14	0.11	TGF β signaling
204580_at	MMP12	16.42	0.56	1.13	0.42	Proteolysis
204702_s_at	NFE2L3	2.42	0.22	1.13	0.07	Transcription
218400_at	OAS3	2.55	0.19	1.12	0.13	RNA binding
241916_at	PLSCR1	2.27	0.37	1.12	0.09	Clearance of apoptotic cells
201150_s_at	TIMP3	2.19	0.18	1.12	0.08	Proteolysis
237169_at	TNC	3.58	0.27	1.11	0.33	Cell adhesion
226436_at	RASSF4	2.26	0.10	1.11	0.15	Signal transduction
236471_at	NFE2L3	2.46	0.09	1.11	0.06	Transcription
225163_at	FRMD4	2.15	0.03	1.11	0.08	Cytoskeletal binding
204972_at	OAS2	3.27	0.29	1.09	0.17	RNA binding
232020_at	SMURF2	2.62	0.07	1.06	0.03	Proteolysis
220104_at	ZC3HAV1	2.17	0.18	1.06	0.07	Immune
204994_at	MX2	2.58	0.12	1.05	0.20	Immune
221898_at	T1A-2	2.11	0.07	1.03	0.04	Angiogenesis
213506_at	F2RL1	2.01	0.07	1.02	0.10	G-protein receptor
208461_at	HIC1	2.21	0.19	1.01	0.14	Transcription
202446_s_at	PLSCR1	2.20	0.06	0.99	0.05	Clearance of apoptotic cells
237206_at	MYOCD	3.18	0.37	0.98	0.18	Transcription
204879_at	T1A-2	2.19	0.30	0.98	0.19	Angiogenesis
222912_at	ARRB1	2.12	0.11	0.98	0.12	Signal transduction
214059_at	IFI44	2.91	0.25	0.97	0.04	Immune
206757_at	PDE5A	3.11	0.72	0.97	0.17	Purine metabolism
205483_s_at	G1P2	2.24	0.09	0.96	0.02	Immune
205660_at	OASL	2.10	0.16	0.94	0.12	RNA binding
218995_s_at	EDN1	2.12	0.15	0.93	0.19	Signal transduction
228617_at	HSXIAPAF1	4.99	0.26	0.91	0.62	Anti-apoptosis
241986_at	BMPER	2.47	0.29	0.91	0.21	Differentiation
226722_at	FAM20C	2.26	0.26	0.90	0.05	Unknown
218832_x_at	ARRB1	2.28	0.39	0.89	0.07	Signal transduction
242726_at	ACSL3	2.02	0.13	0.85	0.15	Fatty acid metabolism
219211_at	USP18	2.80	0.11	0.84	0.19	Deubiquitination
210797_s_at	OASL	2.32	0.11	0.80	0.26	RNA binding
227657_at	RNF150	2.10	0.12	0.80	0.07	Unknown
201601_x_at	IFITM1	2.11	0.31	0.77	0.09	Cell cycle
222802_at	EDN1	2.21	0.09	0.77	0.07	Signal transduction
205552_s_at	OAS1	4.12	0.21	0.74	0.23	RNA binding
230746_s_at	STC1	2.21	0.19	0.74	0.12	Signal transduction
214453_s_at	IFI44	3.48	0.18	0.71	0.13	Immune/inflammation
214022_s_at	IFITM1	2.78	0.10	0.57	0.04	Cell cycle
212977_at	CMKOR1	2.60	0.13	0.52	0.06	G-protein receptor

Table 8.1 (Continued)

SC Probe set	Gene ^b	TNF α Mean ^a	TNF α SD	BE-30 +TNF α Mean ^a	BE-30 +TNF α SD	Functional category
206801_at	NPPB	2.02	0.03	0.44	0.04	Cardiovascular homeostasis
202869_at	OAS1	3.81	0.13	0.43	0.02	RNA binding
235276_at	EPSTI1	2.34	0.16	0.42	0.13	Unknown
203153_at	IFIT1	2.11	0.06	0.33	0.03	Immune/inflammation
206385_s_at	ANK3	2.62	0.33	0.27	0.15	Signal transduction
214329_x_at	TNFSF10	2.64	0.19	0.27	0.10	Apoptosis

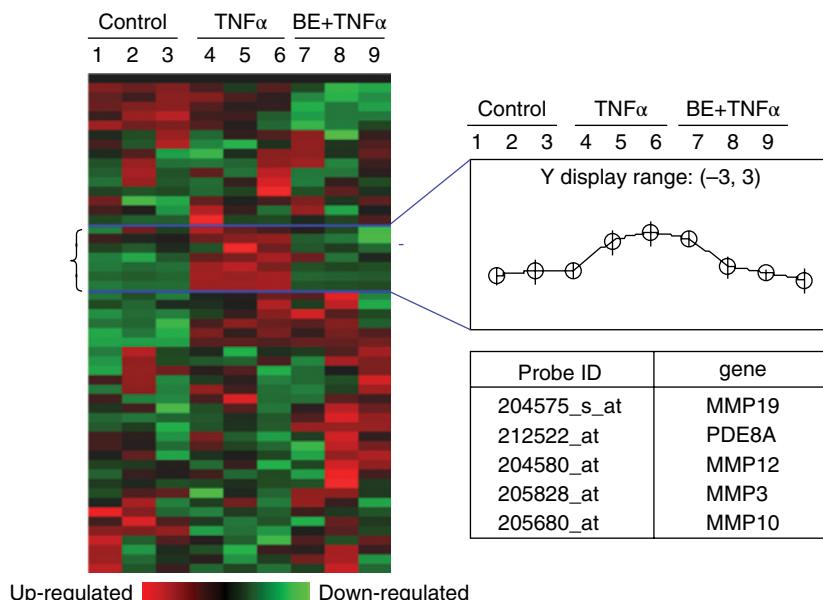


Figure 8.3 TNF α induced *Boswellia* sensitive genes of the MMP family in HMEC. GeneChip data were subjected to hierarchical clustering to visualize expression pattern of BE-sensitive MMP-family genes. TNF α , 50 ng/ml, 6 h; TNF α + *Boswellia* extract, 48 h pre-treatment with BE-30 (25 μ g/ml) followed by TNF α for 6 h. The line graphs show average pattern of gene expression in corresponding cluster graph. The shading represents higher to lower expression signals. MMP19, matrix metalloproteinase 19; PDE8A, phosphodiesterase 8A; MMP12, matrix metalloproteinase 12 (macrophage elastase); MMP3, matrix metalloproteinase 3 (stromelysin 1, progelatinase); MMP10, matrix metalloproteinase 10 (stromelysin 2).

using real-time PCR. The data presented are normalized for β -actin (housekeeping gene) expression. Pre-treatment of HMEC for 2 days with 5-LOXIN $^{\circ}$ (BE-30) potently prevented TNF α -induced expression of MMP3, MMP10, as well as MMP12 mRNA (Figure 8.5).

The observed effects of 5-LOXIN $^{\circ}$ (BE-30) on TNF α -induced MMP3 gene and mRNA expression was further tested at the level of protein expression (MMP3) using ELISA. To establish qualitatively the correlation between AKBA concentration and the efficacy, the observed outcome with BE-30 was compared with that of the commercial extract containing 3% AKBA (BE-3). Consistent with the findings with quantitative gene expression, BE-30 potently inhibited TNF α -induced expression of MMP3 protein. The concentration of AKBA in the extracts was found to have an influence on the observed efficacy. 5-LOXIN $^{\circ}$ (BE-30) was significantly more potent than BE-3 in preventing TNF α -induced MMP3 protein expression (Figure 8.6).

To further validate the effect of 5-LOXIN $^{\circ}$ (BE-30) on TNF α -induced MMP3 expression, the effect of 5-LOXIN $^{\circ}$ on TNF α -induced MMP3 activity was determined using Biotrak MMP3 activity assay system (Amersham Biosciences, USA). The HEMCs were treated with 25 μ g/ml of BE-3, BE-30, or control (DMSO). The cells were then treated with

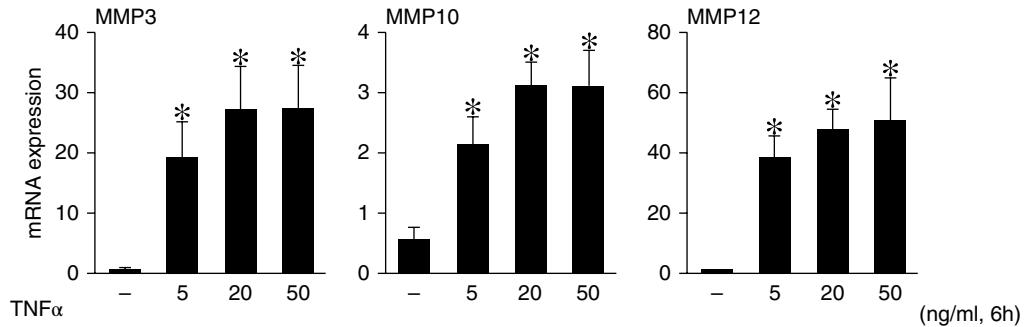


Figure 8.4 *TNF α -induced MMP gene expression.* HMEC were activated with various concentrations (5–50 ng/ml) of recombinant human TNF α for 6 h. Expression levels of MMP-3, -10, and -12 mRNA were determined using real-time PCR. The data presented are normalized for β -actin. Each bar represents a mean \pm SD. *p < 0.001, significantly different compared to control (untreated) sample.

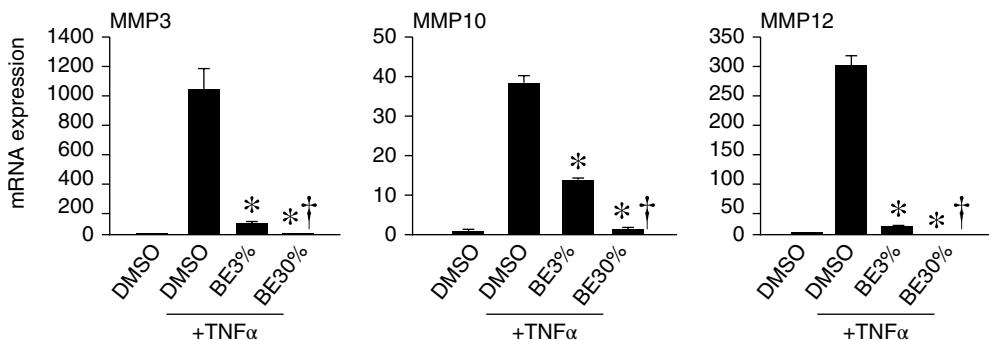


Figure 8.5 *Boswellia extracts significantly downregulated TNF α -induced MMP mRNA expression.* HMEC were pre-treated with 25 μ g/ml Boswellia extracts (BE-3 or BE-30) for 48 h. BE were prepared in DMSO at concentrations such that the final concentration of the solvent in cell suspension never exceeded 0.1% v/v. Controls were treated with equivalent volume of DMSO. After the treatment duration, cells were activated with recombinant human TNF α (50 ng/ml) for 6 h. Expression of MMP-3, -10, and -12 mRNA was determined using real-time PCR. The data presented are normalized for β -actin (housekeeping gene) expression. Each bar represents a mean \pm SD. * p < 0.05, significantly lower compared to TNF α treated sample. † p < 0.05, significantly lower compared to BE-3 treated samples.

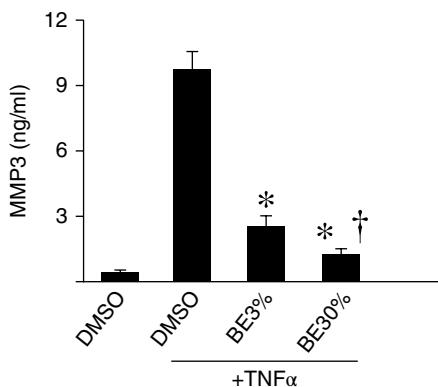


Figure 8.6 *Boswellia extracts inhibited TNF α -induced MMP-3 protein expression.* HMEC were treated with 25 μ g/ml Boswellia extracts (BE-3 or BE-30) for 48 h. After the treatment period, cells were activated with recombinant human TNF α (50 ng/ml) for 24 h. Pro-MMP-3 levels in culture media were determined using ELISA. Data (mean \pm SD) are presented as actual MMP-3 levels (ng/ml) in the culture media. *p < 0.05, significantly lower compared to the TNF α treated sample. † p < 0.05, significantly lower when compared to BE-3 treated samples.

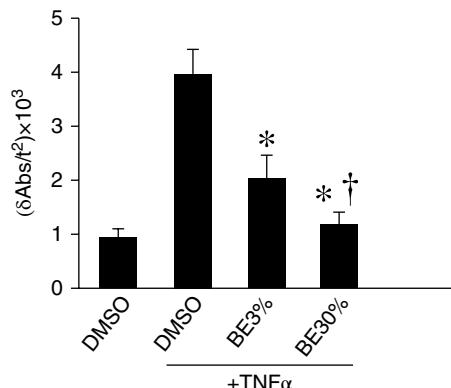


Figure 8.7 *Boswellia* extracts downregulated TNF α -induced MMP-3 activity. HMEC were treated with 25 $\mu\text{g/ml}$ *Boswellia* extracts (BE-3 or BE-30) for 48 h. After the treatment period, cells were activated with recombinant human TNF α (50 ng/ml) for 24 h. Pro and active MMP-3 activity was determined using a Biotrak MMP-3 activity assay system. Data (mean \pm SD) are presented as actual MMP-3 activity in the culture media. The MMP-3 activity is represented by the rate of change of absorbance at 405 nm, that is, $\delta\text{Abs}405/t_2$, where t is the incubation time in hours. Data are expressed as $\{(\delta\text{Abs}405/t_2) \times 10^3\}$. * $p < 0.05$, significantly lower compared to the TNF α treated sample. † $p < 0.05$, significantly lower compared to BE-3 treated samples

recombinant human TNF α for 24 h. The total (pro and active) MMP3 activity was then measured. This assay system comprises of pro form of a detection enzyme that can be activated by captured active MMP3, and converted into an active detection enzyme, through a single proteolytic event. The results showed significant induction of MMP3 activity in HMECs after TNF α treatment. However, such inducible MMP3 activity was significantly inhibited by both the *Boswellia* extracts. 5-LOXIN $^{\circledR}$ (BE-30) showed significantly more potent MMP3 inhibitory activity when compared to BE-3 in HMECs (Figure 8.7).

The MMP3 inhibitory potential of 5-LOXIN $^{\circledR}$ (BE-30) was further evaluated in Phorbol-12-Myristate-13-Acetate (PMA)-induced A2058, human melanoma cells, (unpublished). The supernatants obtained from control, treated cells were used to measure MMP3 production by the MMP3 ELISA Development Kit (R&D System, Minneapolis, MN, USA). Cell culture experiments show BE-30 is able to inhibit MMP3 production in PMA induced human melanoma cells in a dose dependent manner. Quantitative measurement of MMP3 in the culture supernatants revealed an IC₅₀ of 5-LOXIN $^{\circledR}$ (BE-30) for MMP3 production is 25 $\mu\text{g/ml}$ (Sengupta *et al.*, 2008b).

8.7 Molecular Basis of Anti-Inflammatory Properties of 5-LOXIN $^{\circledR}$

The enzyme 5-Lipoxygenase (5-LOX) and 5-lipoxygenase-activating protein (FLAP) are required for the synthesis of leukotrienes in intact cells, which lead to inflammatory response via arachidonate pathway (Dixon *et al.*, 1990). In response to inflammatory stimulation, production and secretion of pro-inflammatory cytokines from macrophages or monocytes are essential phenomena. Among pro-inflammatory cytokines, TNF α holds the key role in the inflammatory process. Sengupta *et al.* (2009) investigated the molecular mechanism of anti-inflammatory activity of 5-LOXIN $^{\circledR}$ on lipopolysaccharide (LPS) stimulated THP-1 human monocytic cells (Sengupta *et al.*, 2009). 5-LOXIN $^{\circledR}$ (BE-30) inhibited TNF α production in LPS-induced THP-1 human monocytes cells in a dose dependent manner (Sengupta *et al.*, 2009). The half maximal inhibitory concentration (IC₅₀) of 5-LOXIN $^{\circledR}$ for TNF α production are $4.61 \pm 0.87 \mu\text{g/ml}$. This investigation also unveils several important molecular cross-talks which provide the explanation for anti-inflammatory properties of 5-LOXIN $^{\circledR}$. These are; (1) 5-LOXIN $^{\circledR}$ completely abrogates the over production of 5-lipoxygenase and its activator protein, that is, 5-lipoxygenase-activating protein (FLAP) production in LPS-induced THP-1 cells; (2) 5-LOXIN $^{\circledR}$ inhibits the LPS-induced activation of serine/threonine kinases of mitogen-activated protein kinase family, which are the key players responsible for a variety of cellular responses, including inflammation; and (3) 5-LOXIN $^{\circledR}$ inhibits I κ B α phosphorylation and p65 translocation to the nuclear compartment of THP-1 monocytes; thereby, it blocks LPS-induced NF κ B activation.

8.8 *In vivo* Studies

The *in vitro* evidences including gene expression and protein expression studies motivated us to test *in vivo* anti-inflammatory properties of BE-30 in rat models of inflammation and experimental arthritis. 5-LOXIN® (BE-30) significantly inhibited carrageenan-induced rat paw inflammation in Albino Wistar Rats (Roy *et al.*, 2005). Animals were orally supplemented with BE-30 in 1% CMC (25, 50, and 100 mg per kg) or ibuprofen (50 mg/kg body weight; positive control) for 30 days. The control group received same volume of vehicle (1% CMC) orally. Following treatment for 30 days, 1 h after the last dose 0.1 ml of 1% solution of carrageenan was injected subcutaneously in the subplantar region of left hind paw. The paw volume was measured using a plethysmometer before and after 4 h of the subcutaneous injection of carrageenan. The difference in paw volume between 4 h after the injection and the initial paw volume was noted as edema volume. Percentage inhibition in edema was calculated by comparing mean edema of control group and the treated groups. 5-LOXIN® (BE-30) exhibited dose dependent reduction in paw edema. It showed 18.23, 23.65, and 27.07% inhibition at 25, 50, and 100 mg per kg body weight, respectively (Figure 8.8).

5-LOXIN® (BE-30) also exhibited significant therapeutic effects against Freund's adjuvant-induced arthritis in Wistar Albino rats (Roy *et al.*, 2006). Oral supplementation of 5-LOXIN® (BE-30) offered dose dependent and statistically significant reduction in paw edema and showed 49.3, 56.7, and 68% reduction in paw edema, respectively, at 25, 50, and 100 mg per kg body weight. Prednisolone 10 mg/kg showed similar (55.7%) protection to that of BE-30 at 50 mg/kg dose. 5-LOXIN® showed significantly higher inhibition against adjuvant-induced inflammatory response compared to BE-3 (Figure 8.9).

8.9 Safety of 5-LOXIN®

A broad spectrum safety studies of 5-LOXIN® (BE-30) conducted in rats and rabbits indicated its safety (Lalithakumari *et al.*, 2006). No acute oral toxicity was observed in either male or female Sprague-Dawley rats supplemented with BE-30 at a dose of 5 g/kg body weight, a dose level considered to be several fold higher than the recommended daily human dose (100 mg/day).

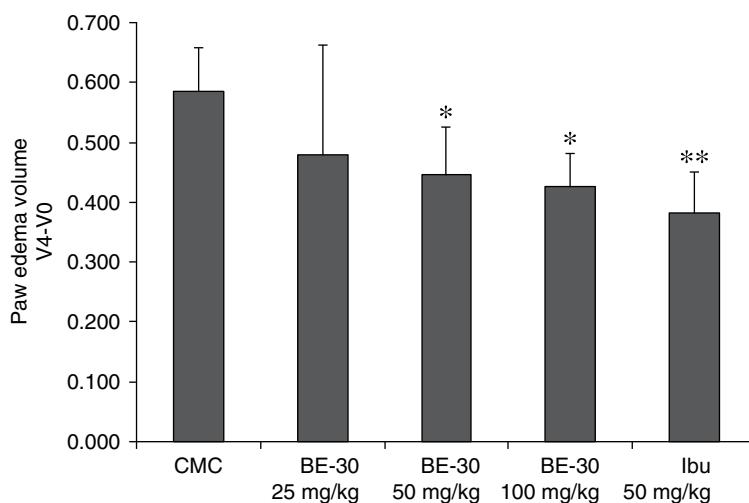


Figure 8.8 *Boswellia* extracts inhibit carrageenan-induced rat paw inflammation. Wistar Albino rats were orally supplemented with BE in 1% CMC (25, 50, and 100 mg per kg) or ibuprofen (50 mg/kg body weight; positive control) for 30 days. The control group received the same volume of vehicle (1% CMC). Following treatment for 30 days, 1 h after the last dose 0.1 ml of 1% solution of carrageenan was injected subcutaneously in the subplantar region of left hind paw. The difference in paw volume between 4 h after the injection and the initial paw volume was noted as the edema volume. Percentage inhibition in edema was calculated by comparing mean edema of control group and the treated groups. Each bar represents a mean \pm SD. *P < 0.05 significantly different compared to the CMC treated control group. **P < 0.005 significantly different compared to CMC treated control group. Ibu, Ibuprofen (oral 50 mg/kg body weight for 30 days); BE (oral 25, 50, and 100 mg/kg body weight for 30 days); CMC, oral 1% carboxymethylcellulose for 30 days.

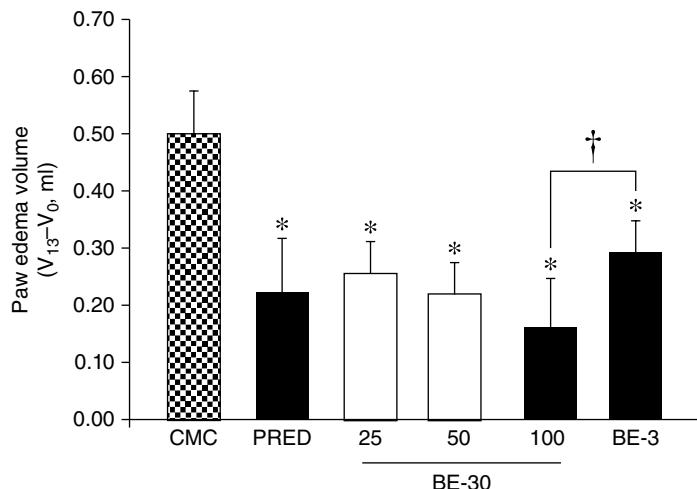


Figure 8.9 *Boswellia extracts inhibit adjuvant induced rat paw edema. Boswellia extract protects against Freund's adjuvant-induced arthritis in rats. Wistar Albino rats (n = 6) were supplemented with BE-30 (25, 50, and 100 mg/kg body weight oral), BE-3 (100 mg/kg body weight oral) or prednisolone (PRED, 10 mg/kg body weight oral) in 1% carboxymethylcellulose (CMC) for 30 days. The control group (marked CMC) received the same volume of vehicle (i.e., 1% CMC) orally. On day 31, 50 μ l of complete Freund's adjuvant was injected subcutaneously in the sub-plantar region of the left hind paw. Paw edema = $V_{13} - V_0$. V₀ and V₁₃ represent the paw volume on days 0 and 13, respectively, after adjuvant injection.*

5-LOXIN® was classified as practically non irritating to skin at a dose of 2.0 g/kg when topically applied to skin in acute dermal toxicity study conducted on New Zealand albino rabbits and showed no irritation to eye in primary eye irritation test performed also on New Zealand albino rabbits. A dose dependent 90-day sub-chronic toxicity study was conducted on male and female Sprague-Dawley rats. A comprehensive perusal of the safety data indicated that the no observed adverse effect level (NOAEL) for male and female Sprague-Dawley rats supplemented with 5-LOXIN® (BE-30) *ad libitum* is presumed to be at least 20.0 g per day human equivalent dose (HED). Finally, the genotoxic effect of 5-LOXIN® was evaluated using the Bacterial Reverse Mutation Test (AMES test) and the results showed that 5-LOXIN® was non mutagenic up to the highest tested concentration of 3000 μ g/plate (Indrani, 2006). 5-LOXIN® (BE-30) or did not exhibit clastogenic potential to induce micronucleated reticulocytes of mouse peripheral blood in micronucleus assay in BALB/c mice. Also, 5-LOXIN® (BE-30) did not induce structural chromosome aberration both with and without metabolic activation in Chinese hamster cells (Chang, 2007). These studies further confirm the non genotoxic nature of 5-LOXIN® (BE-30).

Safety Data

Acute Dermal Toxicity:	LD50 > 2000 mg/kg
Acute Oral Toxicity:	LD50 > 5000 mg/kg
Primary Skin Irritation:	Nonirritating to skin
Primary Eye Irritation:	Mildly irritating to eye
Chronic Toxicity:	LD50 > 2.5 gm/kg

8.10 Clinical Efficacy of 5-LOXIN® in the Management of Osteoarthritis

Osteoarthritis is a common, chronic, progressive, degenerative disorder, which commonly affects the knee joint and can disable the elderly. Osteoarthritis is a growing health concern that has become a major challenge to the health professionals. In Western populations it is one of the most frequent causes of pain, loss of function, and disability in adults. Significant efficacy of 5-LOXIN® (BE-30) in downregulation of key inflammatory genes including adhesion molecules and MMPs was observed in GeneChip studies and reduction in expression of MMPs and inhibiting the activity of MMP3 were observed in MMP expression and activity studies. These results were corroborated with *in vivo* efficacy

studies in inflammation and arthritis rat models. These results prompted us to evaluate the efficacy of 5-LOXIN® in a human clinical study.

A 90-day double blind, placebo controlled human clinical study to evaluate the efficacy and tolerability of 5-LOXIN® in the treatment of osteoarthritis of knee was conducted at Alluri Sitarama Raju Academy of Medical Sciences (ASRAM), Eluru, AP, India (Sengupta *et al.*, 2008a). The study protocol was approved by IRB of ASRAM (IRB# 06 001) and the trial was registered in clinical trial registry (ISRCTN 5212803). It was a three arms study with placebo and two doses of 5-LOXIN® (100 and 250 mg per day).

The clinical study was conducted on 75 subjects with mild to moderate osteoarthritis of knee, who were screened and recruited according to inclusion-exclusion criteria of the American College of Rheumatology. All the selected subjects were undergone base line evaluation and completed a questionnaire, providing demographics, medical history, and nutritional status. Widely acceptable and validated pain and physical function scores including Western Ontario and McMaster Universities (WOMAC) Index of Osteoarthritis (Bellamy *et al.*, 1988), Lequesne functional index (LFI) (Lequesne *et al.*, 1987) and visual analog scale (VAS) (Chapman *et al.*, 1985; Takahashi *et al.*, 2004) were used to evaluate the efficacy of 5-LOXIN®. In addition, blood and urine samples were collected from all the subjects for the evaluation of serum biochemical parameters, hematology, and urinalysis.

At both doses (100 and 250 mg/day), 5-LOXIN® conferred clinically and statistically significant improvements in pain, joint stiffness, and physical function scores in osteoarthritis patients. Interestingly, significant improvement in pain scores was observed in both the treatment groups at as early as 7 days. 5-LOXIN® at 100 mg daily dose showed 48.83% ($P < 0.001$), 23.79% ($P < 0.036$), 39.61% ($P = 0.009$), 42.5% ($P = 0.120$), and 28.62% ($P = 0.100$) improvements, respectively, in VAS, LFI, WOMAC pain, WOMAC stiffness, and WOMAC functional ability scores. 5-LOXIN® at 250 mg showed 65.94% ($P < 0.001$), 31.34% ($P < 0.017$), 52.05% ($P < 0.001$), 62.22% ($P = 0.014$), and 49.34% ($P = 0.002$) improvements, respectively, in VAS, LFI, WOMAC pain, WOMAC stiffness, and WOMAC functional ability scores. In corroboration with the improvements in pain scores in the treatment groups, significant reduction of synovial fluid MMP3, a potent cartilage-degrading enzyme, was also documented. Under normal physiological conditions degeneration and synthesis of cartilage macromolecules are under equilibrium state. Synovial and serum MMP levels are well correlated with collagen degeneration and clinical symptoms. MMP3 plays a central role in arthritis etiology due to its capability of degrading various macromolecules of joint cartilage, collagen, and other connective tissues (Indrani, 2006). The MMP3 data for the placebo group and the two treatment groups are summarized in the (Figure 8.10).

Compared to the base line data, both the treatment groups showed highly significant reductions in synovial fluid MMP3 concentrations at the end of 90-day study period. The MMP3 level in the placebo group virtually unchanged

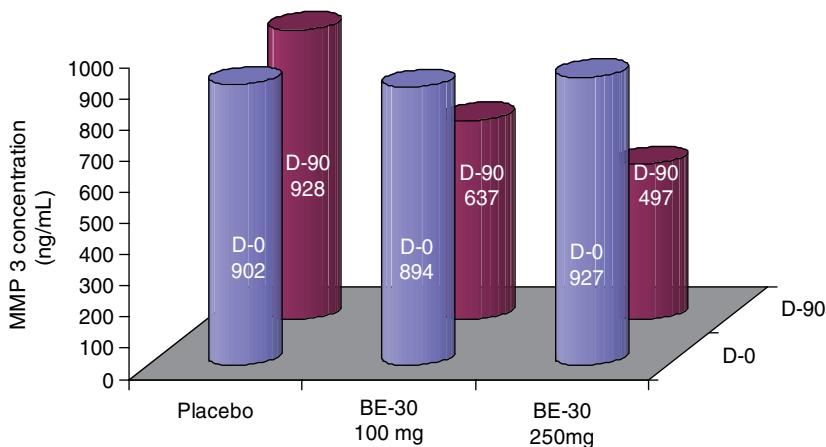


Figure 8.10 Synovial MMP-3 levels. Each bar represents a mean concentration of MMP-3 (ng/ml) in synovial fluid. Bars represent mean matrix metalloproteinase (MMP)-3 levels in synovial fluid collected from 5-LOXIN® (BE-30) and placebo supplemented human subjects with osteoarthritis of knee. At day 90 there was no significant change in MMP-3 concentration in the placebo group compared with baseline. In comparison with the placebo group, at the end of the study the groups receiving 100 mg/day and 250 mg/day BE-30 showed 31.37% ($P = 0.002$), and 46.4% ($P < 0.001$) reductions in MMP-3 concentration, respectively.

at the end of 90 day period, when compared to the baseline value. When compared to the placebo group the low-dose (100 mg) and high-dose (250 mg) 5-LOXIN® supplemented groups showed 31.37% ($P = 0.002$) and 46.4% ($P < 0.001$) reductions in MMP3 concentrations, respectively. The high-dose group also showed significant reduction ($P < 0.0001$) in synovial MMP3 concentration when compared to the low-dose group (Sengupta *et al.*, 2008a). These clinical findings further substantiated the data obtained from gene chip studies, which showed down regulation of genes having anti-inflammatory and proteolytic functions. The safety parameters were virtually unchanged in the treatment groups, when compared with those in the placebo group. This further confirms safety and tolerability of 5-LOXIN®.

8.11 An Advanced 5-LOXIN®: Aflapin®

Recently we have developed Aflapin®, a proprietary composition containing *B. serrata* extract with at least 30% AKBA and *B. serrata* non-volatile oil, which synergistically improved the anti-inflammatory efficacies *in vitro* and *in vivo* models. This synergistic composition of *B. serrata* extract also enhanced AKBA bioavailability, when compared with 5-LOXIN®. Enzymatic assays in a cell free system demonstrated that Aflapin® was 21.06% ($P = 0.0001$) more effective in inhibiting 5-LOX activity in comparison with 5-LOXIN®. Studies in Freund's Complete Adjuvant (FCA) induced inflammation model of Sprague-Dawley rats demonstrated that Aflapin® provided significantly ($P = 0.0102$) better protection than 5-LOXIN® (Sengupta *et al.*, 2011). A randomized double blinded placebo controlled clinical study demonstrated that in comparison with 5-LOXIN®, Aflapin® conferred prompt and higher improvement in pain relief, physical ability and quality of life in OA subjects (Sengupta *et al.*, 2010).

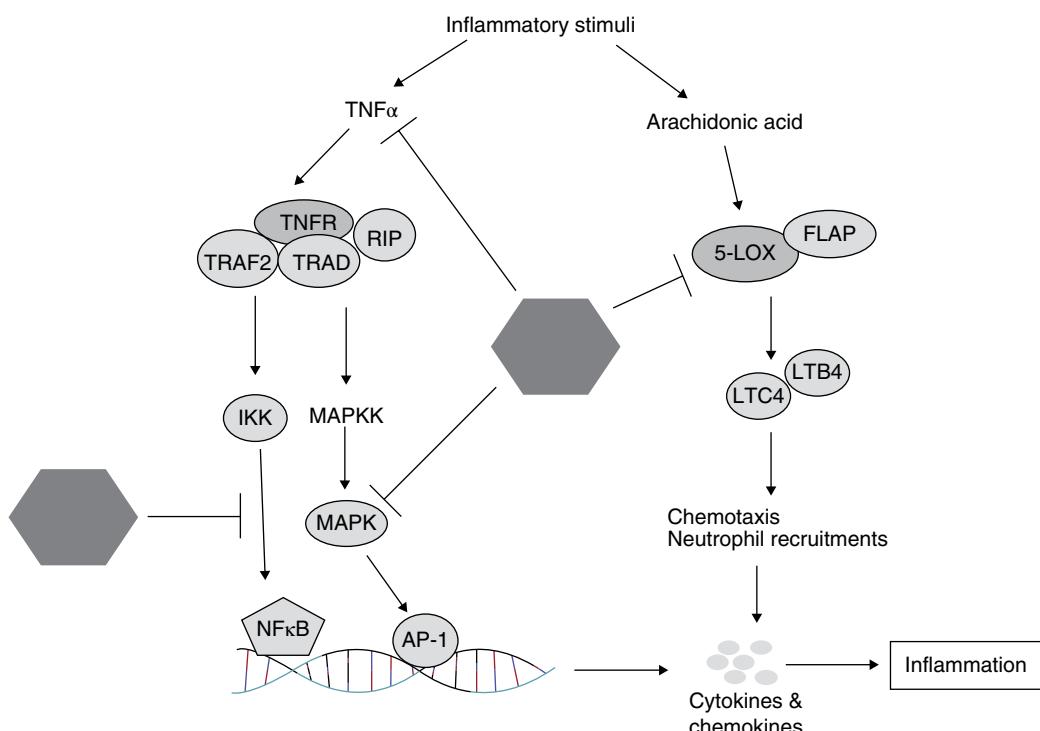


Figure 8.11 Overview of anti-inflammatory properties of 5-LOXIN. Flow diagram represents simplified summary on molecular events in inflammatory responses and major sites of actions of 5-LOXIN®. (T) indicates inhibition or blockade. 5-LOX, 5-Lipoxygenase; FLAP, 5-Lipoxygenase Activating Protein; LTB4, Leukotriene B4; LTC4, Leukotriene C4; TNFR; TNF α Receptor.

8.12 Conclusion

5-LOXIN® (BE-30) represents the first plant derived product that underwent whole genome screening and had its molecular basis of anti-inflammatory properties established through detailed gene expression and proteomic studies. The sensitivity of the inducible expression of many inflammatory genes including TNFR1, ICAM-1, and VCAM-1 to 5-LOXIN® offers a mechanistic rationale for the observed efficacy of *Boswellia* extracts. The inflammatory properties exhibited by 5-LOXIN® in the GeneChip study were supported by potent efficacy shown by 5-LOXIN® in many *in vitro* and *in vivo* models of inflammation. Different, true validation of GeneChip study findings, however, came from clinical human studies. This placebo controlled, double blind clinical human study not only improved the joint function significantly, but also significantly reduced the concentration of cartilage degrading enzyme MMP3 in synovial fluid consistent with the observed sensitivity of the inducible expression of MMP3, MMP10, and MMP12 to 5-LOXIN® in human microvascular endothelial cells. This chapter reviewed the detailed studies carried out by ours and other previous workers relating the molecular basis of anti-inflammatory properties of AKBA and its enriched product 5-LOXIN®. Based on the previous, 5-LOXIN® can be used as a potential therapeutic candidate against various types of inflammatory manifestations. Further studies suggest that 5-LOXIN® reduces the inflammatory responses by decreasing pro-inflammatory cytokine production and by inhibiting several important molecular events which lead to cellular inflammatory responses. Figure 8.11 illustrates a schematic diagram showing the molecular targets of 5-LOXIN® for anti-inflammatory properties. Further, we have developed Aflapin®, a novel synergistic composition to enhance its anti-inflammatory efficacy, in comparison with 5-LOXIN®. Results from cell based experiments, a series of preclinical and clinical studies, suggest that these novel formulations derived from *Boswellia serrata* extracts provide efficient management of osteoarthritis in human subjects.

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9

Cancer Chemopreventive Phytochemicals Targeting NF-κB and Nrf2 Signaling Pathways

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9.1 Introduction

The successful completion of human genome projects early in the twenty-first century led to confirmation and discovery of distinct set of genes implicated in the pathogenesis of many human diseases. The majority of human disease genes that have been identified and characterized are now known to cause monogenic diseases (e.g., scurvy, sickle cell anemia, phenylketonuria, etc.). In the case of cancer, however, abnormalities (e.g., mutation, amplification, and deletion) of multiple genes, especially oncogenes and tumor suppressor genes, are involved in each stage of carcinogenesis. Moreover, there are complex interactions between particular genes and environmental factors in the natural history of cancer. Such complexity in the genetic and epigenetic basis of cancer may explain why it is difficult to cure this dreaded disease, compared with most monogenic disorders. So, despite an enormous progress in the development of a vast variety of anti-cancer drugs and strategies, we do not have a magic bullet or a wonder drug that can completely and selectively destroy malignant cells. In this context, more attention should be paid to the prevention of cancer. One promising and generally acceptable approach to reduce the risk of cancer is “chemoprevention”, which is an attempt to use of nontoxic or relatively safe chemical substances or their mixtures to intervene in the progress of carcinogenesis.

It has been estimated that at least one third of cancer-related death is associated with our diet. Recently, much attention has been focused on common dietary substances that act, either directly or indirectly, on the human genome, thereby altering the structure and expression of particular genes. This may influence the onset, incidence, progression, and/or severity of chronic diseases, including cancer. The science that studies the interface between the nutritional environment and cellular/genetic processes is termed “nutrigenomics”. A vast variety of dietary chemical substances derived from a plant-based diet (fruits, vegetables, nuts, legumes, etc.), collectively called “phytochemicals” (*phyto* in Greek means plants), possess substantial cancer chemopreventive activities. Figure 9.1 illustrates the chemical structures of some representative edible phytochemicals that have been known to possess chemopreventive potential.

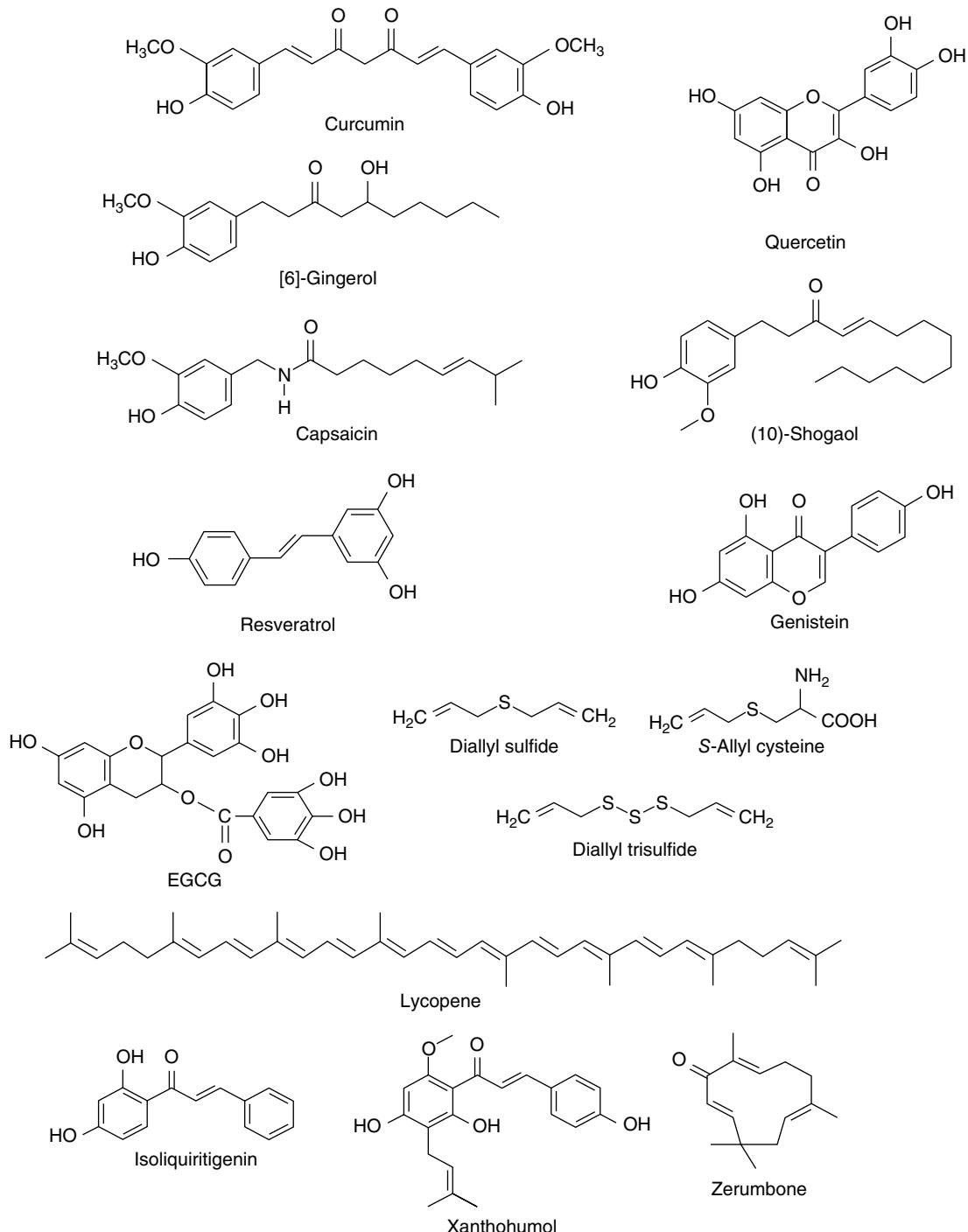


Figure 9.1 Chemical structures of representative chemopreventive phytochemicals with anti-inflammatory and/or antioxidant activities.

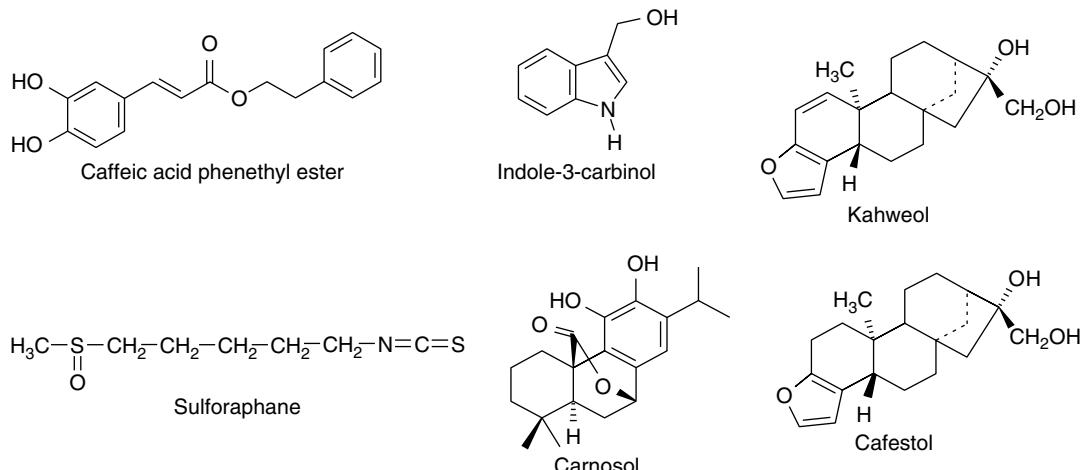


Figure 9.1 (Continued)

Plant-based products are, in general, inexpensive and relatively safe compared to synthetic agents. Therefore, a series of human intervention trials are being considered with individual phytochemicals or their combination with other chemopreventive agents. Based on preclinical results, selected phytochemicals can be evaluated in clinical interventions for various cancers (Greenwald *et al.*, 2002). However, precise assessment of underlying mechanisms of individual components is necessary before undertaking large-scale human trials.

9.2 Molecular-Based Cancer Chemoprevention

Carcinogenesis is a complex process that involves a series of individual steps, each of which accompanies distinct molecular and cellular alterations. Initiation is a rapid and irreversible process that involves a chain of extracellular and intracellular events. These include the initial uptake or exposure to a carcinogenic agent, its distribution and transport to organs and tissues where metabolic activation can occur, and the covalent interaction of ultimate electrophilic species with target cell DNA that causes genotoxic damage. In contrast to initiation, tumor promotion is considered to be a relatively lengthy and reversible process in which actively proliferating preneoplastic cells accumulate. Progression, the final stage of neoplastic transformation, involves the growth of a tumor with increased invasiveness and metastatic potential.

The chemopreventive effects of most edible phytochemicals are likely to be the sum of several distinct mechanisms. These include blockade of metabolic activation and subsequent DNA binding of carcinogens, stimulation of detoxification, maintenance or potentiation of DNA repair capacity, inhibition of cell proliferation, suppression of metastasis or angiogenesis, and induction of differentiation or apoptosis of precancerous or malignant cells (Surh, 2003). Since the cellular signal transduction network often goes awry in carcinogenesis, it is fairly rational to target intracellular signaling cascades for achieving chemoprevention. Components of signaling networks include protein kinases, such as the family of proline-directed serine/threonine kinases named mitogen-activated protein (MAP) kinases, protein kinase C (PKC), phosphatidylinositol-3-kinase (PI3K), protein kinase B/Akt, glycogen synthase kinase (GSK), and their downstream targets, including redox-sensitive transcription factors. Many chemopreventive phytochemicals can work as modifiers of aforementioned signaling molecules to elicit their beneficial effects (Surh, 2003).

Inflammation and oxidative stress are two major culprits implicated in the pathogenesis of the majority of human cancer. Inflammatory tissue damage can generate the reactive oxygen species (ROS) that can directly cause DNA damage, thereby initiating carcinogenesis or stimulate the proliferation of mutated cells. Conversely, oxidative stress can cause or accelerate inflammation. Therefore, a vicious cycle may exist between inflammation and oxidative stress. Two major redox-sensitive transcription factors that have opposite roles in regulating the interplay between inflammation and oxidative stress are nuclear factor-kappa B (NF- κ B) and the nuclear factor erythroid-2-related factor-2 (Nrf2).

In general, pro-inflammatory gene expression is mainly under the control of NF- κ B that is activated by ROS generated as a consequence of oxidative stress. In contrast, Nrf2 plays a role in cellular protection against oxidative stress and inflammatory insult.

Therefore, Nrf2 counteracts the NF- κ B-mediated signal transduction, and *vice versa*. In this context, a possible cross-talk seems to exist between Nrf2 and NF- κ B. Many chemopreventive phytochemicals have been found to activate Nrf2-regulated antioxidant/anti-inflammatory signaling with concurrent inhibition of NF- κ B-mediated proinflammatory signaling.

This chapter deals with some chemopreventive phytochemicals that have ability to modulate the activation of NF- κ B and/or Nrf2.

9.3 Nuclear Factor-Kappa B (NF- κ B)

Although inflammation acts as an adaptive host defense against infection or injury, inadequate resolution of inflammatory responses often ends up with various chronic diseases, including cancer. Mounting evidence from preclinical and clinical studies suggests that chronic inflammation plays a multifaceted role in carcinogenesis. It is estimated that 15–20% of human malignancies are associated with chronic inflammation as a consequence of persistent pathogen infections (Kuper *et al.*, 2000). In response to pro-inflammatory stimuli, activated inflammatory/immune cells produce ROSs, which can function as chemical effectors in inflammation-driven carcinogenesis. Thus, one of the plausible mechanisms by which chronic inflammation causes malignant transformation includes oxidative DNA damage leading to activation of oncogenes and/or inactivation of tumor suppressor genes. Furthermore, a wide array of DNA-binding proteins is aberrantly activated in response to inflammatory stimuli, which can cause inappropriate induction of various proinflammatory genes.

NF- κ B, a ubiquitously expressed eukaryotic transcription factor, regulates the expression of genes encoding those proteins that are essential for inflammatory responses, cell survival, immune reactions, and cell proliferation. Activation of NF- κ B occurs in response to diverse stimuli including ligation of innate immune receptors, antigen-receptor engagement, and pro-inflammatory cytokines (Gilmore, 2006; Hayden and Ghosh, 2006; Hayden *et al.*, 2006). NF- κ B activation upon exposure to external stimuli is typically rapid and transient for timely regulation of target gene expression. However, constitutively overactivated NF- κ B signaling often occurs in many types of cancers, making this transcription factor an attractive target for the development of anticancer as well as anti-inflammatory drugs.

The NF- κ B family contains at least five structurally related members – p50, p52 (the N-terminal fragments of the longer NF- κ B1/p105, and NF- κ B2/p100 proteins, respectively), p65 (RelA), c-Rel, and RelB (Solt and May, 2008). These proteins form dimers and are normally sequestered in the cytoplasm of resting cells by the inhibitors of I κ B family. The I κ B family proteins comprises I κ B α , I κ B β , I κ B γ , and the C-terminal of p105 and p100. A prototypic NF- κ B-I κ B complex expressed in the majority of cell types is a heterodimer of p50 and p65 associated with I κ B α as an inactive complex in cytoplasm. Upon stimulation with mitogens, pro-inflammatory cytokines, ROS, UV radiation, viral infection, and bacterial toxins, I κ B α becomes phosphorylated by activated I κ B kinases (IKKs). Phosphorylated I κ B α , upon ubiquitination, is directed to proteasomes for degradation. The degradation of I κ B α allows NF- κ B to translocate to the nucleus, and to bind to a - κ B element located in the promoter regions of various proinflammatory genes including *cox-2*, thereby controlling their expression.

A pivotal regulator of all inducible NF- κ B signaling pathways is IKK complex that consists of two kinases (IKK α and IKK β) and a regulatory subunit named NF- κ B essential modulator (NEMO)/IKK γ . The IKKs functions as a bridge between inflammation and cancer (Karin, 2008). IKK β plays a role in tumor promotion while IKK α is mainly involved in metastatogenesis (Karin, 2008). Two major pathways, the classical (or canonical) and non-canonical mechanisms, of NF- κ B activation have been identified. The activation involves NEMO/IKK γ - and IKK β -dependent I κ B phosphorylation and degradation, liberating NF- κ B complexes typified by the p50-p65 heterodimer (Figure 9.2). Classical NF- κ B signaling is induced by tumor necrosis factor (TNF), interleukin-1 (IL-1), lipopolysaccharide (LPS), and antigen-receptor engagement. The classical pathway regulates the vast majority of genes, including those encoding pro-inflammatory and immunomodulatory cytokines (e.g., IL-1, IL-2, IL-6, and TNF), chemokines (e.g., CXCL8, CCL2, and CCL3), leukocyte adhesion molecules (e.g., E-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1), and various pro-survival and anti-apoptotic genes (e.g., Bcl-2, Bcl-X_L, and XIAP) (Bonizzi and Karin, 2004; Hayden and Ghosh, 2006). The non-canonical pathway is absolutely dependent upon an upstream kinase named NF- κ B inducing kinase (NIK) that phosphorylates and activates IKK α , thereby directly phosphorylating NF- κ B2/p100 at C-terminal domain. This results in the generation of p52 bound to RelB, and p52-RelB dimer translocates to the nucleus. NIK is rapidly turned over in resting cells through the ubiquitin ligase activity of the TRAF3 adaptor protein that ubiquitinates NIK triggering its proteasomal degradation (Liao *et al.*, 2004). Ligation of non-canonical pathway-inducing receptors sequesters TRAF3, leading to the accumulation of newly synthesized NIK and activation of IKK α (Liao *et al.*, 2004). Non-canonical NF- κ B signaling is induced by ligation of a subset of TNF-receptor family members including the lymphotoxin- β receptor, CD40, receptor activator of NF- κ B, and

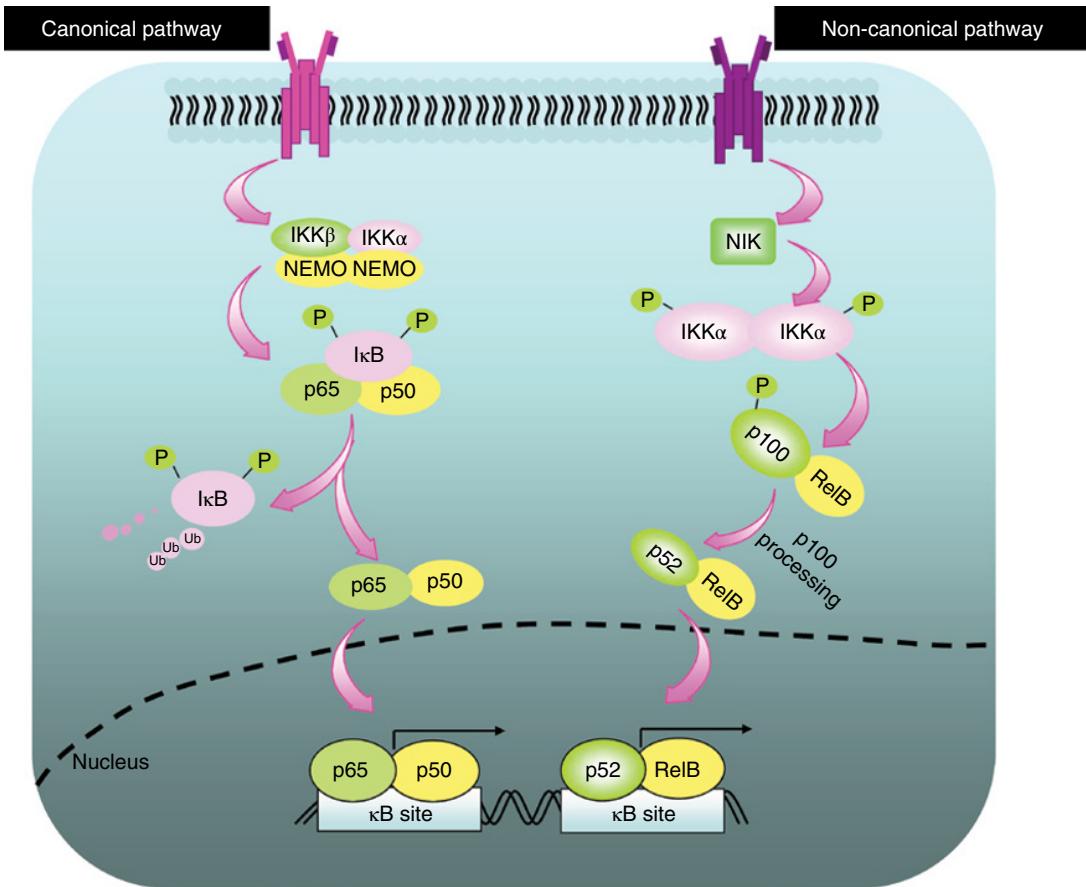


Figure 9.2 Mechanisms of NF-κB activation.

B cell-activating factor receptor. Most stimuli that activate the classical pathway do not stimulate the non-canonical pathway. However, non-canonical stimuli can activate both NF-κB pathways.

Functional loss of NF-κB and its regulator is associated with reduced susceptibility to carcinogenesis. Knock-out of IKK β in liver and hematopoietic cells substantially reduced diethylnitrosamine-induced elevation of TNF- α and IL-6, and suppressed tumorigenesis in mice (Maeda *et al.*, 2005). Inactivation of NF-κB in multi-drug resistance-2 (mrd2)-null mice by overexpressing a super-repressor of I κ B α enhanced apoptosis of transformed hepatocytes, and attenuated tumorigenesis (Pikarsky *et al.*, 2004). In addition, LPS-induced colon adenocarcinoma progression was regressed after deletion of NF-κB (Luo *et al.*, 2004). Thus, the NF-κB represents an important target for cancer prevention.

Several dietary phytochemicals – such as curcumin, [6]-gingerol, capsaicin, resveratrol, quercetin, sulforaphane, genistein, epigallocatechin gallate (EGCG), myricetin, guggulsterone, zerumbone, indole-3-carbinol, ellagic acid, lycopene, caffeic acid phenethyl ester, emodin, and S-allyl cysteine – are natural chemopreventive agents that have been found to be potent inhibitors of NF-κB (Aggarwal and Shishodia, 2006; Surh, 2003). These phytochemicals may block one or more events in the NF-κB signaling pathway, such as stimulation of I κ B α , translocation of NF-κB into the nucleus, DNA binding of the dimers, or interactions with the basal transcriptional machinery.

9.3.1 Curcumin

Curcumin, a yellow coloring agent contained in turmeric (*Curcuma longa* L., Zingiberaceae), has been reported to possess strong anti-tumor promotional as well as anti-inflammatory and antioxidant activities. Curcumin and structurally related curcuminoids from plants of the ginger family mediate their therapeutic effects by suppressing the activation

of NF- κ B and expression of NF- κ B regulated gene products, cyclooxygenase-2 (COX-2), cyclin D1, adhesion molecules, metalloproteinases (MMPs), inducible nitric oxide synthase (iNOS), Bcl-2, and Bcl-X_L (Shishodia *et al.*, 2005). Topically applied curcumin inhibited activation of NF- κ B and expression of COX-2 in mouse skin stimulated with the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) (Chun *et al.*, 2003). Curcumin blocked TPA⁻, TNF- α ⁻, or fecapentaene-mediated NF- κ B transactivation by inhibiting the NIK/IKK signaling in colon epithelial cells (Plummer *et al.*, 1999). Curcumin induced apoptosis in mouse melanoma cells (Marin *et al.*, 2007) and human neuroblastoma cells (Freudlsperger *et al.*, 2008) via inactivation of NF- κ B. In addition, curcumin also blunted radiation-induced NF- κ B activation in human neuroblastoma cells (Aravindan *et al.*, 2008) and colorectal cancer xenografts (Kunnumakkara *et al.*, 2008). Moreover, curcumin treated to mouse leukemia cells suppressed the expression of P-glycoprotein associated with multidrug resistance by targeting NF- κ B (Choi *et al.*, 2008). Nonrandomized, open-label, phase II clinical trial with curcumin was conducted for 25 pancreatic cancer patients. The patients took capsules containing curcumin for 8 weeks without any apparent toxicity (Dhillon *et al.*, 2008). Oral administration of curcumin downregulated the expression of COX-2 and phosphorylation of signal transducer and activator of transcription 3 in peripheral blood mononuclear cells from the majority patients, but the compound showed poor bioavailability (Dhillon *et al.*, 2008). A synthetic monoketone compound termed 3,5-bis(2-fluorobenzylidene) piperidin-4-one (EF24) exhibited potent anticancer activity through direct suppression of IKK activity (Kasinski *et al.*, 2008).

9.3.2 [6]-Gingerol

[6]-Gingerol, a pungent phenolic substance derived from the root of ginger (*Zingiber officinale* Roscoe, Zingiberaceae), inhibited TPA-induced TNF- α production, ornithine decarboxylase activity, and skin tumor promotion in female ICR mice (Park *et al.*, 1998). Topically applied [6]-gingerol inhibited TPA-induced phosphorylation of p65 at Ser 536 and its interaction with the coactivator cAMP response element binding protein-binding protein (CBP/p300) in mouse skin, thereby rendering NF- κ B transcriptionally inactive (Kim *et al.*, 2005). In addition, [6]-gingerol has been shown to inhibit UVB-induced activation of NF- κ B and COX-2 expression in hairless mouse skin and also in an immortalized human keratinocytes cell line (Kim *et al.*, 2007). [6]-Gingerol induced apoptosis in the gastric cancer cells by blocking TRAIL-induced NF- κ B activation (Ishiguro *et al.*, 2007).

9.3.3 Capsaicin

Capsaicin, a major pungent principle of hot chili pepper (*Capsicum annuum* L., Solanaceae) with potential anti-inflammatory and anti-tumor promoting properties also suppressed TPA-induced activation of NF- κ B in mouse skin *in vivo* (Han *et al.*, 2001) as well as in cultured human promyelocytic leukemia (Han *et al.*, 2002) and human myeloid ML-1a cells (Singh *et al.*, 1996). Capsaicin, by inhibiting TNF- α stimulated proteasome activity, abrogated the degradation of I κ B α , preventing the activation of NF- κ B in prostate cancer cells (Mori *et al.*, 2006). In another study, capsaicin inhibited the degradation of I κ B α , resulting in the decrease of NF- κ B/p65 DNA binding activity and Bcl-2 expression in T-cell leukemia cells (Zhang *et al.*, 2003).

9.3.4 Resveratrol

Resveratrol, a phytoalexin present in grapes and red wine, inhibited TPA-induced phosphorylation of I κ B α and subsequent p65 nuclear translocation in mouse skin by blocking IKK α and IKK β (Kundu *et al.*, 2006). Resveratrol attenuated the TPA-induced NF- κ B activation and NF- κ B-dependent luciferase activity in human fibrosarcoma HT1080 cells (Park *et al.*, 2009). Resveratrol inhibited hyaluronan/CD44-mediated β -catenin/NF- κ B p65 acetylation via suppression of NF- κ B activation, which was associated with a significant decrease in both P-glycoprotein and Bcl-xL gene expression and enhancement of caspase-3 activity and chemosensitivity in breast cancer MCF-7 cells (Bourguignon *et al.*, 2009). Resveratrol enhanced the radiosensitivity of human non-small cell lung cancer (Liao *et al.*, 2005) and TRAIL-resistance of melanoma cells (Ivanov *et al.*, 2008) via NF- κ B inactivation. Resveratrol induced apoptosis of human multiple myeloma cells and sensitized these cells to chemotherapeutic agents. These effects were attributed to its inhibition of constitutively active NF- κ B through suppression of IKK and the phosphorylation of I κ B α and of p65 (Bhardwaj *et al.*, 2007). Piceatannol (3,4,3',5'-tetrahydroxy-*trans*-stilbene), a hydroxylated analog of resveratrol, has been reported to have anti-proliferative, anti-inflammatory, and antioxidant properties. Piceatannol suppressed TNF-induced NF- κ B activation, I κ B α phosphorylation, IKK activation, p65 phosphorylation, and p65 nuclear translocation, but had no effect on degradation of I κ B α in

myeloid cells (Ashikawa *et al.*, 2002). Piceatannol abrogated the expression of a TNF-induced NF-κB dependent reporter gene and of MMP-9, COX-2, and cyclin D1 (Ashikawa *et al.*, 2002). In another study, piceatannol inhibited migration and anchorage-independent growth of human mammary epithelial cells (MCF-10A) and suppressed the activation of NF-κB and expression of COX-2 in these cells when stimulated with TPA (Son *et al.*, 2010). It is speculated that piceatannol may directly modify IKK β , presumably at the cysteine 179 residue, thereby blocking NF-κB signaling for COX-2 induction in TPA-treated MCF-10A cells (Son *et al.*, 2010).

9.3.5 Quercetin

Quercetin is an herbal flavonoid derived from various foods of plant origin and has been widely used as a major constituent of nutritional supplements. Quercetin inhibited I κ B degradation and NF-κB activity in pulmonary epithelial cells treated with IL-1 (Ying *et al.*, 2009). Quercetin decreased the gene expression and production of TNF- α , IL-1 β , IL-6 and IL-8, and these effects appears to be associated with downregulation of NF-κB in TPA-stimulated human mast cells (Min *et al.*, 2007).

9.3.6 Sulforaphane

Sulforaphane, [1-isothiocyanato-(4R,S)-(methylsulfinyl)butane], a representative isothiocyanate present in broccoli and other cruciferous vegetables, exerts its chemopreventive effects in various tumor models. Sulforaphane inhibited LPS-induced activation of NF-κB and COX-2 expression in cultured mouse macrophages (Heiss *et al.*, 2001). Interestingly, sulforaphane-induced suppression of NF-κB was associated with neither degradation of I κ B nor nuclear translocation of NF-κB, but rather attributable to its direct binding to essential thiol groups of p50 subunit of NF-κB. Contrary to these findings, treatment of human mammary epithelial cells with sulforaphane inhibited TPA-induced COX-2 expression by blocking expression of NF-κB-activating kinase, IKK activities and subsequent phosphorylation and degradation of I κ B α , leading to suppression of NF-κB activation (Kim *et al.*, 2014). Alternatively, sulforaphane may interact with glutathione (GSH) or other redox regulators like thioredoxin and Ref-1, resulting in perturbation of an intracellular reducing milieu required for the proper DNA binding of NF-κB (Heiss and Gerhäuser, 2005).

9.3.7 Genistein

Genistein, an isoflavonoid present in soybeans, exhibits anti-carcinogenic effects. Several studies have shown that genistein inhibits cancer cell proliferation and triggers apoptosis in various human cancers. Exposure of breast cancer MDA-231 cells to genistein caused a concentration-dependent decrease in NF-κB/p65 protein levels and DNA-binding activity of NF-κB, which may account for its inhibition of cell growth and induction of apoptosis (Li *et al.*, 2008). Genistein inhibited the radiation-induced activation of NF-κB in prostate cancer cells (Raffoul *et al.*, 2006; Singh-Gupta *et al.*, 2009). In addition, suppression of NF-κB activity by genistein abolished drug resistance induced by chemotherapeutic agents such as erlotinib (El-Rayes *et al.*, 2006), cisplatin (Ali *et al.*, 2009; Banerjee *et al.*, 2007), and docetaxel (Li *et al.*, 2006).

9.4 Nrf2

Environmental-related factors (e.g., tobacco smoking, poor diet, alcohol, ionizing radiations, biocides, pesticides, and viral infections) and other health-related factors (e.g., obesity or the aging process) capable of generating ROS can cause oxidative stress (Mena *et al.*, 2009). Epidemiological observations have shown that oxidative stress is one of the major causes of carcinogenesis (Toyokuni, 2008). Oxidative stress occurs as a consequence of an imbalance between ROS production and cellular capability to inactivate or detoxify these reactive species.

Therefore, the protection against oxidative cell damage confers the first line of defense against carcinogenic insults. This can be achieved either by blocking the formation of ROS or stimulating their elimination. Likewise, neutralization or elimination of carcinogens is often mediated by a series of phase II detoxifying enzymes, rendering the carcinogens water-soluble, thereby facilitating their excretion out of the body. The induction of phase II detoxifying or antioxidant enzymes is one of the most important components of cellular cytoprotection whereby a diverse array of toxic oxidants or electrophiles are eliminated from the cell before they damage the target cell DNA (Kong *et al.*, 2003).

The redox-sensitive transcription factor, Nrf2 plays a key role in regulating induction of phase II detoxifying or anti-oxidant enzymes via interaction with a *cis*-acting DNA element called antioxidant-response element (ARE) or the

electrophile-responsive element (EpRE) (Kong *et al.*, 2003). These include NAD(P)H:quinone oxidoreductase 1 (NQO1), glutathione S-transferase (GST) Ya subunit, glutamate cystein ligase (GCL), and heme oxygenase-1 (HO-1). The involvement of this redox-sensitive transcription factor in regulating the induction of antioxidant and carcinogen detoxifying enzymes has been directly evidenced by disruption of the *nrf2* gene in mice (Ramos-Gomez *et al.*, 2001; Xu *et al.*, 2006). Nrf2 knockout mice are predisposed to developing the chemically-induced DNA damage and exhibited a higher gene mutation frequency in the lung than did the wild-type mice (Aoki *et al.*, 2007). Likewise, *nrf2*-null mice developed the much larger number of tumors in the forestomach (Ramos-Gomez *et al.*, 2001), liver (Kitamura *et al.*, 2007), urinary bladder (Iida *et al.*, 2007), skin (Xu *et al.*, 2006), and colon (Khor *et al.*, 2006) than did the wild-type mice after treatment with a carcinogen. Therefore, Nrf2 is considered as another important molecular target for cancer prevention (Lee and Surh, 2005).

Kelch-like erythroid CNC homolog (ECH)-associated protein 1 (Keap1) is a member of a large family of proteins containing an N-terminal region (two cysteines), Broad complex, Tramtrack, and Bric-a-brac (BTB) region (three cysteines), linker region (eight cysteines), and Kelch repeat region (DGR, nine cysteines, and six Kelch motifs), and C-terminal region (three cysteines) (Adams *et al.*, 2001). Keap1 functions as a substrate adaptor protein for a Cul3-dependent E3 ubiquitin ligase complex (Zhang *et al.*, 2004). The cytoplasmic protein Keap1 interacts with Nrf2 and represses its function (Motohashi and Yamamoto, 2004).

While the molecular mechanisms involved in the Nrf2-derived transcriptional activation are not fully understood, the dissociation of Nrf2 from Keap1 as a consequence of Keap1 cysteine thiol modification is considered as a plausible mechanism underlying Nrf2 activation by electrophiles and prooxidants (Eggler *et al.*, 2005). According to the new paradigm, the direct interaction of the highly reactive cysteine residues of Keap1 with phase II enzyme inducers as well as electrophiles causes conformational changes of this repressor protein, which abrogate the capability of Keap1 to aid proteasomal degradation of Nrf2. In addition to activation of Nrf2 through oxidation or chemical modification of Keap1 cysteine thiols, phosphorylation of specific serine (Huang *et al.*, 2000) or threonine (Lo *et al.*, 2006) residues of Nrf2 may facilitate the release of Nrf2 from the Keap1 repression and its subsequent nuclear translocation. As a result, Nrf2 forms a heterodimer with a small Maf protein (e.g., Marf K, Marf G or Marf F) and binds to ARE sequences in the promoter region of the genes encoding many antioxidant enzymes (Figure 9.3). Activation of several upstream kinases, such as MAP kinases (Xu *et al.*, 2006b), PI3K/Akt (Rojo *et al.*, 2008), PKC (Huang *et al.*, 2000, 2002) and casein kinase-2 (CK-2) (Apopa *et al.*, 2008) has been considered to facilitate nuclear translocation and transcriptional activation of Nrf2. On the other hand, GSK3 β negatively regulates Nrf2 signaling via phosphorylation of Nrf2 at tyrosine (Jain and Jaiswal, 2007) or serine (Salazar *et al.*, 2006) residues. Nrf2 activation by dietary chemopreventives through modulation of one or more of the upstream kinases or thiol modification has been reviewed (Chen and Kong, 2004).

9.4.1 Sulforaphane

Sulforaphane has been known to induce genes encoding phase 2 detoxifying and antioxidant enzymes through activation of Nrf2. Sulforaphane upregulated the expression of detoxifying enzymes including NQO1, GST and GCL in the small intestine (Thimmulappa *et al.*, 2002) and liver (Hu *et al.*, 2006 of *nrf2*-wild-type mice, while the *nrf2*-null mice displayed lower levels of these enzymes. Sulforaphane induced thioredoxin in murine retina (Tanito *et al.*, 2005) and human adenocarcinoma Caco-2 cells (Jakubikova *et al.*, 2006), which appeared to be mediated via Nrf2-ARE binding. Sulforaphane induced Nrf2-driven phase II enzyme expression by modulating the activation of MAP kinases (Keum *et al.*, 2006; Kong *et al.*, 2001; Jakubikova *et al.*, 2005; Juge *et al.*, 2007; Manandhar *et al.*, 2007). Sulforaphane and its sulfide analogue, erucin elevated the mRNA expression of NQO1, UDP-glucuronosyltransferases 1A1, and multidrug transporter (MRP)2 in Caco-2 cells by activating PI3K/Akt- or MAP kinase kinase (MEK)/extracellular signal-regulated kinase (ERK)-mediated signaling (Jakubikova *et al.*, 2005). Involvement of ERK1/2 and c-Jun-N-terminal kinase (JNK) in sulforaphane-induced ARE-transcription activities was also observed in murine keratinocytes (Manandhar *et al.*, 2007). Sulforaphane induced HO-1 expression in HepG2 cells by down-regulating p38 MAP kinase, thereby activating the Nrf2-ARE signaling (Keum *et al.*, 2006).

In addition to the modulation of upstream kinases, the mechanism of Nrf2 activation by sulforaphane involves a direct modification of cysteine residue(s) present in Keap1, facilitating dissociation of Nrf2 from Keap1. Zhang and Hannink (2003) have suggested that sulforaphane inhibits the Keap1-dependent ubiquitination of Nrf2, which increases steady-state levels of Nrf2, leading to enhanced nuclear localization and transcriptional activity of this transcription factor. Particularly, modification of Cys151 of Keap1 by sulforaphane is required for its suppression of Keap1-dependent Nrf2 ubiquitination (Zhang and Hannink, 2003). Sulforaphane may alter the redox state of Cys 151 and reduces the ability of Nrf2-bound Keap1 proteins to associate with the Cul3-Rbx1 core complex, thereby increasing the stability and accumulation of Nrf2.

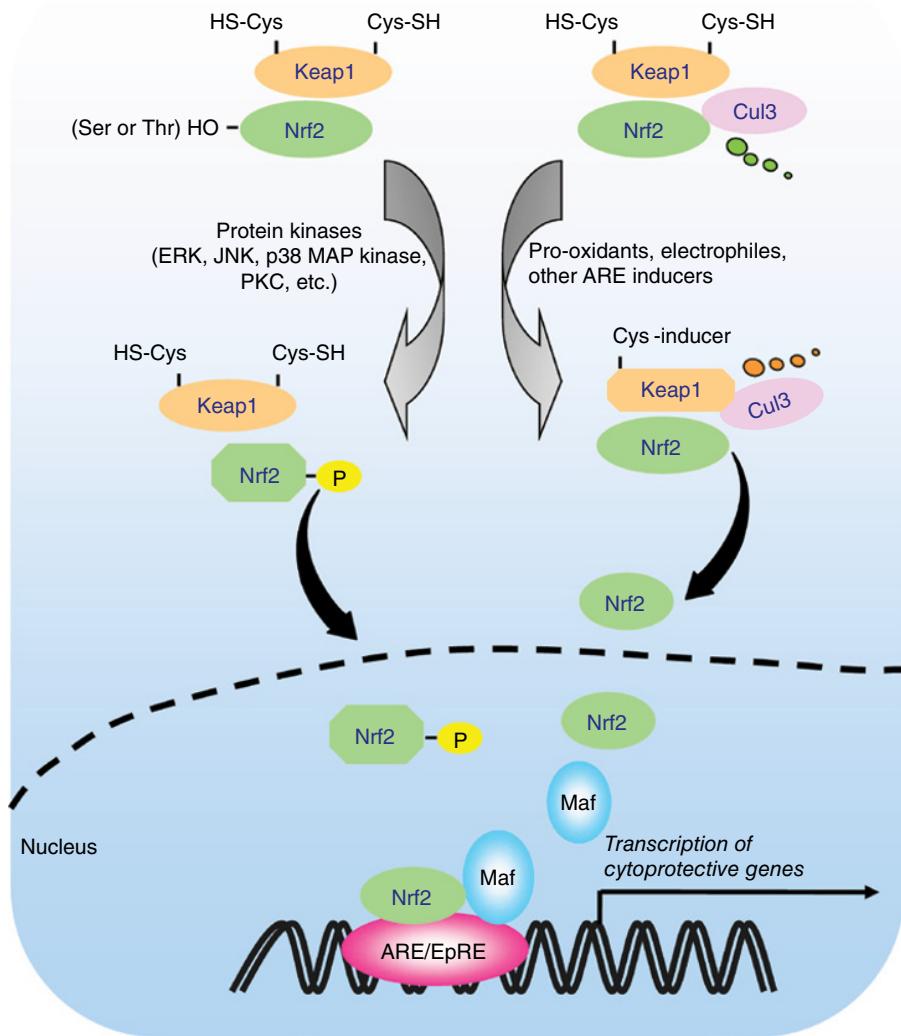


Figure 9.3 Proposed pathways for Nrf2-ARE activation.

As sulforaphane is an electrophile, it can react with cysteine thiols of Keap1 to form thionoacyl adducts, thereby inducing stability of Nrf2 (Hong *et al.*, 2005).

Chemopreventive effects of sulforaphane are mediated, at least in part, through Nrf2 activation. The gastrointestinal GPx (GI-GPx) is a selenium-dependent protein and involved in the control of inflammation and malignant growth (Banning *et al.*, 2005). It has been reported that gastrointestinal tumor formation was enhanced in *gpx2/gpx1* double knockout mice, corroborating the anticarcinogenic activity of GI-GPx (Brigelius-Flohe and Banning, 2006). Sulforaphane induced Nnf2 binding to the ARE site located in the GI-GPx promoter in human hepatoma (HepG2) or human colon cancer (Caco-2) cells (Banning *et al.*, 2005). Topical application of sulforaphane (100 nmol) for 14 consecutive days inhibited skin carcinogenesis induced by 7,12-dimethylbenz[a]anthracene plus TPA in C57BL/6 mice, whilst no such chemopreventive effects of sulforaphane were elicited in the *nrf2*-deficient mice (Xu *et al.*, 2006).

A sulforaphane analog, 6-methylsulphanylhexylisothiocyanate isolated from Japanese wasabi was also found to induce cytoprotective gene expression via the Nrf2-ARE signaling (Morimitsu *et al.*, 2002). Likewise, phenethyl isothiocyanate PEITC

induced HO-1 expression and ARE activity in human prostate cancer (PC3) cells via ERK- and JNK-mediated phosphorylation of Nrf2 and subsequent Nrf2 nuclear translocation (Xu *et al.*, 2006b).

9.4.2 Curcuminoids

The chemopreventive effects of curcuminoids have been extensively investigated and well defined (Surh and Chun, 2007; Thangapazham *et al.*, 2006). As part of its chemopreventive mechanism, curcumin targets the Nrf2-ARE signaling pathway to induce phase II detoxifying and antioxidant enzymes. Given *ad libitum* for 16 days, dietary curcumin (0.05%) enhanced not only the Nrf2 levels but also its nuclear translocation and the ARE binding in liver and lung of mice (Garg *et al.*, 2008). Dietary curcumin enhanced the expression of carcinogen detoxifying enzymes such as GST isoforms and NQO1 in parallel with the activation of Nrf2, leading to increased detoxification of benzo[a]pyrene (Garg *et al.*, 2008). Oral administration of curcumin at 200 mg/kg for four consecutive days resulted in enhanced nuclear accumulation and the ARE-binding of Nrf2 and HO-1 upregulation in rat liver, and these effects account for cytoprotective effect against liver toxicity (Farombi *et al.*, 2008).

Curcumin induced nuclear localization of Nrf2 and HO-1 expression effectively in wild-type mouse embryonic fibroblasts, but not in those from *nrf2*-deficient mice (Andreadi *et al.*, 2006). Many cytoprotective genes were induced in liver and small intestine of wild-type C57BL/6J, but not in C57BL/6J/*nrf2*(-/-) mice, given a single oral dose of curcumin (1,000 mg/kg) (Shen *et al.*, 2006). Curcumin also induced expression of GSTP1, GCL, and HO-1 via Nrf2 activation in various cells (Andreadi *et al.*, 2006; Balogun *et al.*, 2003; Dickinson *et al.*, 2003; Nishinaka *et al.*, 2007). The curcumin-induced expression of antioxidant enzyme via Nrf2-ARE signaling was mediated by activation of PKC delta, PI3K, and p38 MAPK (Kang *et al.*, 2007; Rushworth *et al.*, 2006). In addition, curcumin-induced HO-1 expression and Nrf2 activation were ROS dependent (McNally *et al.*, 2007).

Structurally, curcumin has two α,β unsaturated carbonyl groups and can hence act as a Michael reaction acceptor, thereby causing thiol modification of Keap1. Consistent with this notion, tetrahydrocurcumin, which lacks an electrophilic α,β -unsaturated carbonyl functional moiety, failed to induce Nrf2-ARE binding as well as HO-1 induction when given orally to rats (Farombi *et al.*, 2008). Demethoxycurcumin and *bis*-demethoxycurcumin induced expression of HO-1, GCL and NQO-1 mRNA, and *HO-1* promoter activity, and activated Nrf2 more effectively than curcumin in mouse pancreatic- β (MIN6) cells (Pugazhenthi *et al.*, 2007). However, demethoxycurcumin and *bis*-demethoxycurcumin exhibited almost similar potency to induce QR activity as compared to that observed with curcumin (Dinkova-Kostova and Talalay, 1999). Oral administration of curcumin protected against dimethylnitrosamine-induced hepatic injury in rats, which appeared to be attributable, at least in part, to its activation of Nrf2 and subsequent induction of HO-1 protein expression as well as activity (Farombi *et al.*, 2008).

9.4.3 EGCG

EGCG, the major active catechin component of green tea, has been known to possess antioxidant, anti-inflammatory, and chemopreventive properties (Na and Surh, 2006; Park and Surh, 2004). EGCG was found to be the most potent Nrf2 activator among the green tea polyphenols, as evidenced by its pronounced ability to induce ARE-luciferase reporter gene transactivation (Chen *et al.*, 2000). EGCG has been reported to activate Nrf2 and induce expression of HO-1 in endothelial cells (Wu *et al.*, 2006) and B-lymphoblasts (Andreadi *et al.*, 2006). While EGCG-induced HO-1 expression was attributed to activation of Akt and ERK1/2 in endothelial cells (Wu *et al.*, 2006), p38 MAP kinase as well as Akt is involved in HO-1 induction and Nrf2 nuclear translocation in B lymphoblasts treated with EGCG (Andreadi *et al.*, 2006). Activation of ERK1/2 and Akt induced by EGCG was involved in HO-1 expression in human mammary epithelial MCF-10A cells (Na *et al.*, 2008). Similarly, a nontoxic dose of EGCG increased the ARE-luciferase activity and the expression of ARE-regulated genes by activating MAP kinases in HepG2 cells (Chen *et al.*, 2000). EGCG inhibited the growth and liver/pulmonary metastasis of colon tumor implanted orthotopically in the cecum of nude mice, and this anticancer effect was proposed to be partly mediated by activating the Nrf2-UGT1A signal pathway (Yuan *et al.*, 2007). Oral administration of EGCG at 200 mg/kg induced the Nrf2-dependent gene expression in the liver and small intestine of Nrf2 wild type mice (Shen *et al.*, 2005).

9.4.4 Allyl Sulfides

Garlic oil contains several organosulfur compounds, such as diallyl sulfide (DAS), diallyl disulfide (DADS), and diallyl trisulfide (DATS), capable of inducing carcinogen-detoxifying enzymes. Chen *et al.* examined Nrf2-driven ARE activity and antioxidant gene expression by garlic organosulfur compounds in HepG2 cells (Chen *et al.*, 2004). Of the three allyl

sulfides derived from garlic, DATS was most potent in terms of inducing Nrf2 activation and expression of cytoprotective enzymes, such as HO-1 and NQO1. In addition, DAS induced NQO1 5-fold in wild-type mice, whereas induction was completely absent in *nrf2* (-/-) mice (Fisher *et al.*, 2007). DAS induced HO-1 expression, Nrf2 protein expression, nuclear translocation, and DNA-binding activity in HepG2 cells (Gong *et al.*, 2004). Both ERK and p38 pathways appeared to be involved in DAS-induced Nrf2 nuclear translocation and HO-1 gene expression (Gong *et al.*, 2004), whereas MAP kinases induced by DATS did not affect the ARE activity (Chen *et al.*, 2004). DAS- and DATS-induced Nrf2 activation is presumably mediated by generation of ROS. The thiol antioxidant N-acetyl-L-cysteine (NAC) and catalase blocked not only DAS-induced ROS production but also ERK activation as well as nuclear translocation of Nrf2, and also HO-1 expression. Moreover, co-treatment with thiol antioxidants NAC and GSH inhibited the ARE activity and the Nrf2 accumulation induced by DATS (Chen *et al.*, 2004). DAS-treatment rendered the HepG2 cells resistant to oxidative stress caused by hydrogen peroxide or arachidonic acid, and this was attributable to its induction of HO-1 as pharmacologic inhibition of HO-1 activity blunted the cytoprotective effects of DAS (Gong *et al.*, 2004). It is noteworthy that the pro-oxidant activity of DATS contributes to Nrf2-driven antioxidant enzyme induction, which conferred the protection against oxidative cell death. Three major MAP kinases, that is, ERK, JNK, and p38, were activated by DATS treatment. However, the inhibition of these enzymes did not affect DATS-induced ARE activity. Likewise, the PKC pathway was not directly involved in DATS-induced ARE activity, but instead the calcium-dependent signaling pathway might be responsible for the DATS-induced cytoprotective effect. DATS administration by gavage increased the expression of HO-1 and NQO1 in C57BL/6 mouse stomach. Treatment with DATS increased the accumulation of Nrf2 in the nucleus of cultured AGS cells and in mouse stomach *in vivo*. It has been suggested that DATS may directly interact with the Cys288 residue of Keap1, which partly accounts for its ability to induce Nrf2 activation and upregulate defensive gene expression (Kim *et al.*, 2014).

9.4.5 Resveratrol

Resveratrol exerts antioxidant, anti-inflammatory, and chemopreventive activities by modulating various events in cellular signaling (Kundu and Surh, 2004). Resveratrol prevented chemically induced tumorigenesis in many experimental models (Aziz *et al.*, 2005; Banerjee *et al.*, 2002; Jang *et al.*, 1997; Li *et al.*, 2002). As a mechanism of carcinogen detoxification and cellular antioxidant defense, resveratrol induced NQO1 activity in murine hepatoma cells (Gerhauser *et al.*, 2003) and human K562 cells (Gerhauser *et al.*, 2006). The stimulation of NQO1 gene expression by resveratrol involved the stimulation of ARE signaling, which was accompanied by an increase in the state of phosphorylation of Nrf2 and its re-distribution to the nucleus (Gerhauser *et al.*, 2006). The compound was found to induce HO-1 expression and activity in human aortic smooth muscle (Gerhauser *et al.*, 2005) and rat pheochromocytoma (PC12) cells (Chen *et al.*, 2005) via activation of NF-κB and Nrf2, respectively.

Treatment of human primary small airway epithelial and human alveolar epithelial (A549) cells with cigarette smoke extract (CSE) dose dependently decreased GSH levels and GCL activity, effects that were associated with enhanced production of ROS (Kode *et al.*, 2008). Resveratrol restored CSE-depleted GSH levels by upregulation of GCL via activation of Nrf2 and also quenched CSE-induced release of ROS.

9.4.6 Pungent Vanilloids

Capsaicin induced expression of HO-1 by activating PI3K/Akt-mediated activation of Nrf2 signaling in a ROS-dependent manner in HepG2 cells (Joung *et al.*, 2007). It was suggested that a quinone metabolite or other reactive forms of capsaicin would covalently modify NQO-1, and inhibit its activity, leading to production of ROS. The resulting overproduction of ROS is speculated to stimulate PI3K/Akt-mediated activation of Nrf2 (Joung *et al.*, 2007). (10)-Shogaol, a pungent ingredient of ginger (*Zingiber officinale* Roscoe, Zingiberaceae) has been reported to interact with the cysteine 151 residue of human Keap1 to form an alkylated adduct (Luo *et al.*, 2007). The alkylation of Keap1 by this electrophilic natural product may contribute to its antioxidant, anti-inflammatory and chemopreventive properties.

9.4.7 Lycopene

Lycopene, a natural antioxidant present predominantly in tomato products, has been reported to exert chemopreventive activity, especially against prostate and mammary carcinogenesis. The antioxidant properties of lycopene are thought to be primarily responsible for its chemopreventive effects (Hwang and Bowen, 2002). Lycopene elevated GCL promoter activity

in MCF-7 cells (Ben-Dor *et al.*, 2005). GCL-ARE and NQO1-ARE reporter activities were induced in HepG2 cells as well (Ben-Dor *et al.*, 2005). Lycopene elevated the mRNA and/or protein levels of GCL and NQO1, enhanced the cellular GSH level and reduced ROS generation in MCF-7 and HepG2 cells. In addition, the induction of NQO1 and GCL by lycopene was diminished in HepG2 cells ectopically expressing a dominant negative mutant Nrf2, suggesting that lycopene induces these antioxidant enzymes via Nrf2 activation (Ben-Dor *et al.*, 2005). Treatment of both cells with lycopene also lowered the intracellular ROS level. Lycopene mitigates the nephrotoxic effect of cisplatin in rats, presumably through activation of Nrf2-mediated induction of HO-1 expression, while it suppresses the NF-κB p65 signaling (Sahin *et al.*, 2010).

9.4.8 Coffee-Derived Diterpenes

Epidemiological studies have revealed an inverse relationship between coffee consumption and the risk of certain types of cancer (Cavin *et al.*, 2002). Dietary administration of coffee (3 or 6%) for 5 days showed significantly elevated expression of mRNA transcripts of NQO1 and GSTA-1 in liver and small intestine, and that of UGTA-6 and GCLC in small intestine of *nrf2*^{+/+} mice as compared to *nrf2*^{-/-} animals (Higgins *et al.*, 2008). The coffee-derived diterpenes, cafestol, and kahweol, when treated to embryonic fibroblasts isolated from *nrf2*^{+/+} mice, increased NQO1 mRNA expression to a greater extent than that achieved with embryonic fibroblasts from *nrf2*^{-/-} mice.

Coffee extracts of different proveniences and selected constituents were tested for their effects on the Nrf2/ARE signaling in human colon carcinoma cells (HT29). In addition to the known Nrf2 activator 5-*O*-caffeoylquinic acid, pyridinium derivatives were identified as potent activators of Nrf2 nuclear translocation and ARE-dependent expression of selected antioxidative Phase II enzymes (Boettler *et al.*, 2011).

9.4.9 Carnosol

Carnosol, an orthophenolic diterpene present in rosemary (*Rosmarinus officinalis*, Lamiaceae), induced HO-1 expression at both protein and mRNA levels by increasing the binding of Nrf2 to ARE and induced Nrf2-dependent activation of *HO-1* promoter in PC12 cells via upregulation of MAP kinases [133]. Cinnamaldehyde, another dietary diterpene present in dried stem bark of *Cinnamomum cassia* Presl. (Lauraceae), induced HO-1 protein expression, increased Nrf2 nuclear translocation, and ARE-luciferase reporter activity in human endothelial cells (Liao *et al.*, 2008).

9.4.10 Xanthohumol

Xanthohumol, a chemopreventive sesquiterpene derived from hops (*Humulus lupulus* L.), exhibited capability to induce expression of antioxidant enzymes. Pretreatment of hepa1c1c7 cells with xanthohumol diminished menadione-induced DNA damage via upregulation of NQO1 (Dietz *et al.*, 2005). Xanthohumol induced NQO1 expression in an ARE-dependent manner, partly through alkylation of a cysteine residue in the Keap1 (Dietz *et al.*, 2005). Luo and colleagues also suggested that xanthohumol alkylated the cysteine 151 (located in BTB domain), and 319 (located in central linker domain) and 613 (located in Kelch repeat domain residues), thereby contributing to the Nrf2-dependent ARE activation (Luo *et al.*, 2007). Likewise, isoliquiritigenin derived from licorice has also been shown to alkylate cysteine 151 residue of Keap-1, thereby inducing the ARE activity (Luo *et al.*, 2007). Cumulatively, the data suggest that xanthohumol upregulates the transcription of ARE-mediated detoxifying genes by directly binding to Keap1 protein.

Xanthohumol induced expression of some phase II enzymes in concert with p53 induction in immortalized normal THLE-2 hepatocytes, which may account for the molecular mechanism underlying chemopreventive activity of this compound (Krajka-Kuzniak *et al.*, 2013).

9.4.11 Zerumbone

Zerumbone, a sesquiterpene occurring in zingiberaceous plants in Southeast Asian countries, has been shown to have anti-inflammatory effects in several independent experimental studies (Murakami *et al.*, 2004). Zerumbone enhanced the cellular GSH level and induced a battery of antioxidant enzymes, such as GSTP-1, GCL, GPx, and HO-1 in normal rat liver epithelial (RL34) cells (Nakamura *et al.*, 2004). Treatment of RL34 cells with zerumbone (25 μM) showed increased nuclear accumulation of Nrf2, while its non-electrophilic analogues such as α-humulene or 8-hydroxy-α-humulene failed to activate Nrf2 and induce aforementioned antioxidant enzymes, suggesting that the α,β-carbonyl moiety at the 8 position is crucial for Nrf2 activation and antioxidant enzyme induction by zerumbone.

Topical application of zerumbone onto dorsal skin of hairless mice induced activation of Nrf2 and expression of HO-1 (Nakamura *et al.*, 2011), which provides a mechanistic basis for the chemopreventive effects of this sesquiterpene on mouse skin carcinogenesis. Nrf2-deficient mice expressed HO-1 protein to a much lesser extent than the wild-type animals following topical application of zerumbone. Treatment of mouse epidermal JB6 cells with zerumbone markedly enhanced nuclear translocation of Nrf2 followed by the promoter activity of HO-1. Notably, α -humulene and 8-hydroxy- α -humulene, the structural analogues of zerumbone that lack the α , β -unsaturated carbonyl group were unable to activate Nrf2 and induce HO-1 expression (Nakamura *et al.*, 2011).

9.4.12 Chalcones

Chalcone, an α , β -unsaturated flavonoid, possesses anti-inflammatory properties. Chalcone upregulated the nuclear levels of Nrf2 and increased the ARE-luciferase activity and also the thioredoxin reductase promoter activity in bovine aortic endothelial cells (Liu *et al.*, 2007). It also induced expression of thioredoxin reductase as well as HO-1 in the same cells. Some synthetic chalcone derivatives, such as 4',5',3,4,5-hexamethoxychalcone diminished NF- κ B activation, whereas they induced HO-1 expression (Alcaraz *et al.*, 2004; Lee *et al.*, 2007).

2',3'-Dihydroxy-4',6'-dimethoxychalcone isolated from green perilla (*Perilla frutescens* var. *crispa* f. *viridis*) extract exhibited high Nrf2-ARE activity, and induced the expression of antioxidant enzymes, such as NQO1 and HO-1 (Izumi *et al.*, 2012).

9.5 Interplay/Crosstalk between Nrf2 and NF- κ B Signaling Pathways

A compelling amount of evidence supports the fact that Nrf2-ARE signaling is associated with the cellular defense against inflammation. Thus, the Nrf2-ARE pathway has been proposed to be a promising target for the prevention of inflammation-associated cancer (Surh *et al.*, 2005). According to a previous report by Khor *et al.*, the aggravation of DSS-induced colitis in *nrf2*^{-/-} mice was associated with decreased expression of HO-1, NQO-1, UGT1A1, and GST μ -1 (Khor *et al.*, 2006). Levels of proinflammatory mediators, such as COX-2, iNOS, IL-1 β , IL-6, and TNF- α , were significantly elevated in the colonic tissues of *nrf2*^{-/-} mice as compared to their wild-type counterparts (Khor *et al.*, 2006). In a subsequent study, the tumor incidence, the multiplicity, the size, and the stage of progression were found to be increased in *nrf2*-deficient mice in an azoxymethane plus DSS-induced colon carcinogenesis (Khor *et al.*, 2008). Inflammatory cytokines, through induction of oxidative stress, suppressed NQO1 activity in both cholangiocarcinoma cells and normal HeLa Chang liver cells (Prawan *et al.*, 2009). Moreover, activation of the NF- κ B pathway by tumor promoter could be attenuated by diverse Nrf2 activators, such as sulforaphane, curcumin, and PEITC. These results indicate that dysfunction of Nrf2 seems to accelerate NF- κ B-mediated pro-inflammatory reactions, directly or indirectly (Li *et al.*, 2008b). Proper activation of Nrf2 signaling hence represents a promising strategy for prevention of inflammation-associated cancer. Therefore, Nrf2-mediated anti-tumorigenic effects are likely to be achieved not only by potentiation of antioxidant machinery but also through suppression of pro-inflammatory pathways mediated by NF- κ B signaling.

The molecular mechanisms underlying suppression of NF- κ B and activation of Nrf2-ARE are independent of each other or may involve a crosstalk between two signaling pathways. Liu *et al.* (2008) have suggested that overexpression of p65 liberates CBP from Nrf2 by competitive interaction with the CH1-KIX domain of CBP, which resulted in inactivation of Nrf2. The status of p65 phosphorylation was not associated with obstruction of MafK-mediated recruitment of CBP to ARE. Overexpression of p65 facilitated the recruitment of HDAC3, the repressor of ARE by promoting the interaction of HDAC3 with either CBP or Mafk, leading to local histone hypoacetylation, thus, serving as a negative regulator of Nrf2-ARE signaling. Recent studies have revealed that cysteine thiols present in various transcription factors, such as NF- κ B, AP-1, and p53 function as redox sensors in fine-tuning of transcriptional regulation of many genes essential for maintaining cellular homeostasis. It is noticeable that NF- κ B binds to DNA preferentially when these cysteines are reduced and that redox-related modifications of cysteine thiols, such as the formation of disulfides (Matthews *et al.*, 1992) or nitrosothiol (Matthews *et al.*, 1996), disrupts DNA binding ability of NF- κ B. Liu *et al.* (2007) reported that chalcone abrogated the activation of NF- κ B on IL-6-and LPS-treated endothelial cells (Liu *et al.*, 2007). Furthermore, chalcone upregulated the levels of Nrf2 in nuclear extracts and increased the ARE activity, while it suppressed both IL-6 and LPS-induced signaling pathways by disrupting the thiol-dependent intracellular redox state. In contrast, Nrf2 signaling is activated by a compound possessing an α , β -unsaturated carbonyl moiety capable of modifying the cysteine thiols present in Keap1. Therefore, thiol modification by electrophilic phase II inducers may result in opposite effects in the NF- κ B- and Nrf2-mediated signal transducing pathways (Figure 9.4).

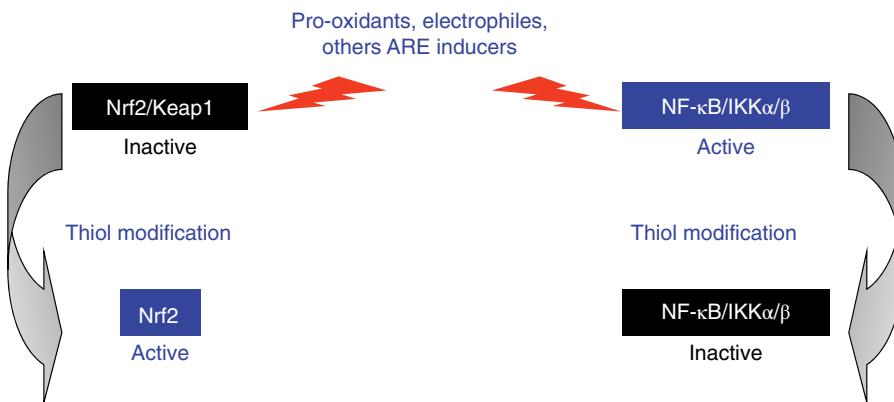


Figure 9.4 Differential effects of thiol modification on IKK-NF-κB- and Nrf2-mediated signal transduction.

Nair *et al.* (2008) performed multiple sequence alignment of Nrf2 and NF-κB genes in five mammalian species, including human, chimpanzee, dog, mouse, and rat to explore conserved biological features. The comparative analyses of transcription factor-binding sites in these two gene promoters revealed that many matrix families were conserved between Nrf2 and NF-κB promoter regions in both humans and mice. In addition, a conserved transcription factor-binding site for NF-κB itself was found to be present in murine promoter regions of Nrf2 and NF-κB, lending further support to the idea of possible crosstalk between these two transcription factors. Curcumin-induced aldose reductase via activation of Nrf2 was abolished by a pharmacological NF-κB inhibitor Bay 11-7082 or siRNA knock-down of the *p65* gene, suggesting the involvement of NF-κB in the cellular response to oxidative stress and toxic aldehydes.

Recently, a double-edged sword nature of the Nrf2-HO-1 axis attracts much attention (Na and Surh, 2014). Nrf2 enhances resistance of cancer cells to chemotherapeutic drugs including tamoxifen (Kim *et al.*, 2008; Wang *et al.*, 2008). It has been shown that genetic alteration or mutation of Keap 1 confers constitutive activation of Nrf2 in breast and gallbladder cancer. Based on these findings, Nrf2 has different roles in cancer chemoprevention and carcinogenesis. The intercellular network between Nrf2 and NF-κB signaling may also be differentially regulated in the normal and cancer cells.

Sulforaphane has been reported to inhibit NF-κB activation in human colon cancer HT-29 (Jeong *et al.*, 2004) and human prostate cancer PC-3 (Xu *et al.*, 2005) cells. It selectively reduced DNA binding of NF-κB without blocking LPS-induced degradation of IκB or nuclear translocation of NF-κB (Heiss *et al.*, 2001). The sulforaphane-mediated attenuation of NF-κB DNA binding activity was prevented by the sulphydryl-reducing agent mercaptoethanol, suggesting that sulforaphane could either directly inactivate NF-κB subunits by binding to essential cysteine residues or indirectly interacts with GSH or other redox regulators like thioredoxin and Ref-1 essential for optimal NF-κB function. In contrast, increased nuclear accumulation of NF-κB as well as Nrf2 was observed in human adenocarcinoma Caco-2 cells after sulforaphane treatment (Jakubikova *et al.*, 2006).

9.6 Conclusion

Oxidative stress and inflammatory tissue injuries are two of the most critical factors that are implicated in multistage carcinogenesis. Therefore, suppression of abnormally amplified inflammatory signaling and restoration/potentiation of anti-oxidant machinery that is improperly working or repressed represent important strategies for chemoprevention. NF-κB and Nrf2 are major redox-sensitive transcription factors that are involved in regulating pro-inflammatory and antioxidant genes, respectively. Many chemopreventive phytochemicals with anti-inflammatory activities have shown to inhibit NF-κB activation via multiple mechanisms. Some antioxidative phytochemicals not only scavenge ROS but also induces Nrf2-driven synthesis of antioxidant or phase II detoxification enzymes, thereby fortifying inherent cellular defense capacity against oxidative or electrophilic insults. Interestingly, the majority of chemopreventive phytochemicals possess both anti-inflammatory and antioxidant properties. In consideration of the close association between anti-inflammatory and antioxidant effects mediated by the same phytochemicals, it is worthwhile identifying molecules that mediate the cross-talk

between NF-κB and Nrf2 signaling. Cysteine thiols present in redox-sensitive transcription factors and their regulators (e.g., IKK and Keap1) are recognized as functioning as redox sensors involved in fine-tuning of transcriptional regulation of many genes essential for maintaining cellular homeostasis. Thus, oxidation or covalent modification of thiol groups present in redox-sensitive transcription factors and their regulating molecules can constitute an essential component of molecular target-based chemoprevention and cytoprotection with anti-inflammatory and antioxidant phytochemicals (Na and Surh, 2006b; Surh *et al.*, 2005).

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10

The Beneficial Health Effects of Fucoxanthin

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10.1 Introduction

Carotenoids are natural pigments produced by plants, algae, and photosynthetic bacteria. The number of naturally occurring carotenoids reported continues to rise and has now reached more than 700. Animals, including humans, are incapable of synthesizing carotenoids. They use carotenoids derived from their diet for skin pigmentation. More than 40 carotenoids are absorbed, metabolized, and used by the human body. Carotenoids such as α - and β -carotene and β -cryptoxanthin, which can be converted to retinal and then retinoic acid, are known as provitamin A carotenoids (Figure 10.1). Retinoic acid in its all-*trans* or 9-*cis* configuration is an activator of the retinoic acid receptor (RAR) and the retinoid-X receptor (RXR). The transcription of retinoid response genes is activated by the binding of retinoic acid to either RAR or RXR (De Luca, 1991). In addition to their provitamin A activity, carotenoids also play a role in the prevention of common chronic diseases such as cardiovascular disease and age-related macular degeneration (Cooper *et al.* 1999). In addition, epidemiological studies have established a positive correlation between carotenoid consumption and a reduced risk of cancer (Riboli and Norat 2007; Willett 2001).

Carotenoids have a light harvesting function that allows the blue and green wavelengths from sunlight to be converted to chemical energy. This makes carotenoids essential to plants and algae during photosynthesis. Carotenoids also protect photosynthetic membranes from oxidative and photo-oxidative damage by quenching free radicals and reactive oxygen species (ROS) that are produced during photosynthesis (Edge *et al.* 1997). Because ROS and oxidative damage are thought to be involved in the initiation and progression of chronic diseases such as cancer and cardiovascular disease, one of the protective mechanisms of dietary carotenoids may be their antioxidant properties. There is little doubt that under the right conditions, carotenoids can protect cells, tissues, and other structures, such as lipoproteins, against oxidative damage. However, to act as a meaningful antioxidant in these tissues, carotenoids must be present in sufficient concentrations and the right location where the ROS and/or free radicals are generated (Sies 2007).

Several non-provitamin A carotenoids reportedly decrease the risk of disease by reducing oxidative stress in target tissues. For example, lutein and zeaxanthin (Figure 10.1) are known to improve eye health (Ma and Lin 2010). The protective effects of both provitamin A and non-provitamin A carotenoids may include their antioxidant properties and their ability to filter blue light. There have been several studies on the relationship between the antioxidant activity of astaxanthin (Figure 10.1) and the prevention of cardiovascular disease (Fassett and Coombes

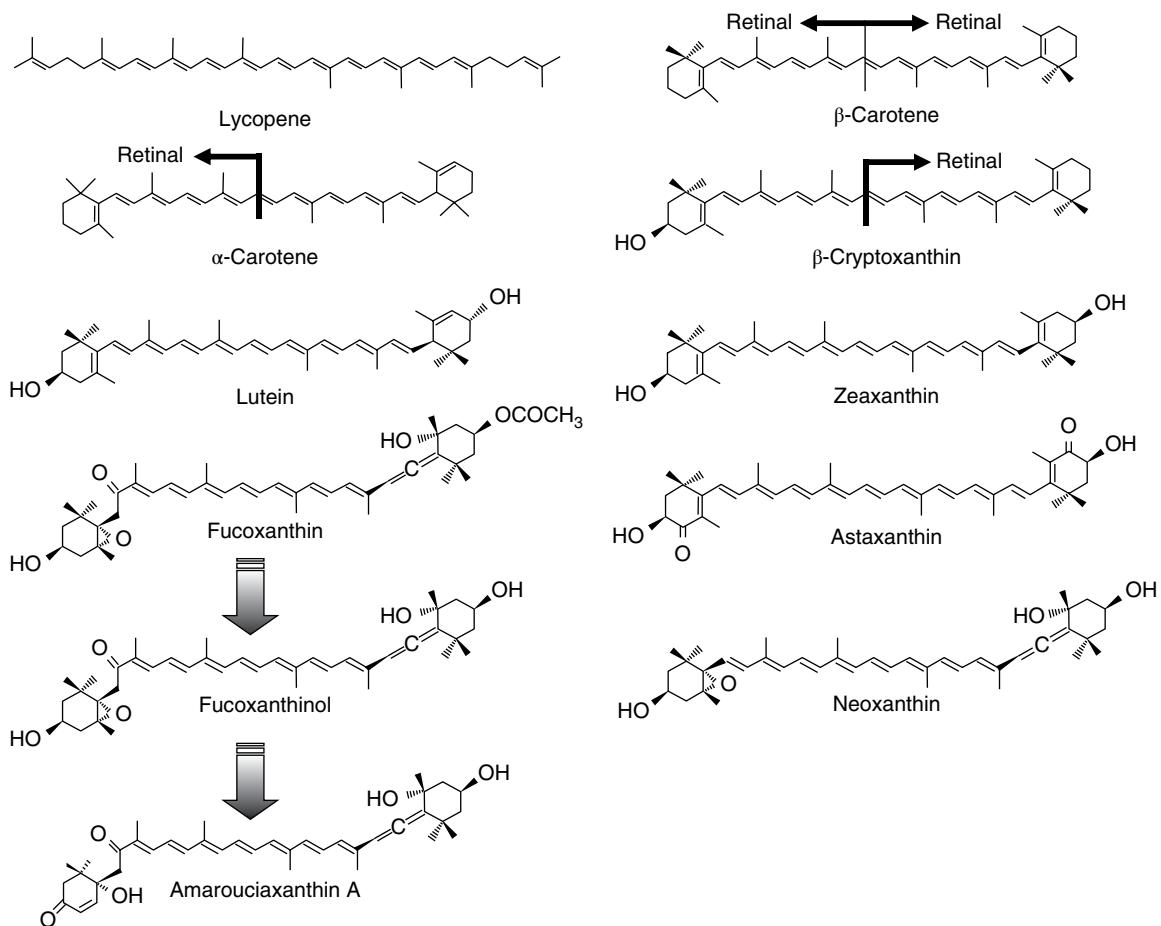


Figure 10.1 Chemical structures of major carotenoids.

2009; Fredric *et al.* 2008; Yuan *et al.* 2011). Prevention of atherosclerosis by astaxanthin intake can be explained by the ability of astaxanthin to protect low density lipoprotein (LDL) and vein endothelial cells against oxidative injury and dysfunction.

Lycopene, the main carotenoid in human plasma, is especially interesting because its antioxidant activity exceeds that of β -carotene and α -tocopherol (Di Mascio *et al.* 1989; Miller *et al.* 1996) (Figure 10.1). Skin lycopene has been reported to be more sensitive to UV light stress than β -carotene. However, Erdman *et al.* (2009) question whether the antioxidant activity of lycopene is its major mechanism of protection against disease because there is an insufficient concentration of lycopene in target tissues for antioxidant activity to be the prevalent protective mechanism. They propose that there are other protective mechanisms that lycopene has that need to be researched further. As with aging, many chronic diseases, including cardiovascular disease, cancer, diabetes, and eye diseases, are the result of long-term oxidative stress and the antioxidant properties of carotenoids remain important to combating and preventing these types of chronic disease. However, all the physiological effects of carotenoids cannot be explained only by their antioxidant activity. In some cases, the modulating effect of carotenoids on specific gene and protein expression in biological systems may be the more important mechanism for the beneficial health effects of carotenoids (Chew and Park 2004).

The purpose of this chapter is to review the physiological activity of fucoxanthin, a marine carotenoid (Figure 10.1), from a nutrigenomics point of view. Fucoxanthin has beneficial regulatory effects on gene and protein expression pathways that are important in chronic diseases such as obesity and diabetes.

10.2 The Beneficial Health Effects of Carotenoids as Antioxidants

Free radicals and ROS such as $\bullet\text{O}_2^-$ (superoxide anion), H_2O_2 (hydrogen peroxide), $\bullet\text{OH}$ (hydroxyl radical), and $^1\text{O}_2$ (singlet oxygen) are produced in the body and oxidize polyunsaturated lipids, proteins, and DNA. Carotenoids protect lipids, proteins, and DNA from oxidative damage by deactivating singlet oxygen and scavenging free radicals. The inactivation of free radicals by carotenoids occurs when carotenoids form adducts with free radicals, transfer electrons to free radicals, and donate hydrogens to free radicals (Krinsky and Yeum 2003).

The quenching of singlet oxygen by carotenoids has been attributed mainly to the transfer of excess energy from singlet oxygen to the carotenoid molecule (Böhm *et al.* 2012; Namitha and Negi 2010; Stahl and Sies 2012). The transfer of energy excites the carotenoid to the triplet state. Without changing its chemical structure, the carotenoid molecule relaxes to the singlet state by losing the excess energy as heat. The efficacy of different carotenoid molecules in quenching singlet oxygen is related to the number of conjugated double bonds present in the molecule. As the number of conjugated double bonds in the carotenoid molecule increases, the triplet energy of the excited state decreases, resulting in an increased ability of the carotenoid molecule to quench singlet oxygen (Conn *et al.* 1991; Edge *et al.* 1997). The singlet oxygen quenching ability of carotenoid molecules also varies with chain structure, functional groups, and solvent viscosity (Hirayama *et al.* 1994; Mascio *et al.* 1991). In biological systems, the singlet oxygen quenching ability of carotenoid molecules also depends on the concentration of carotenoids in cell membranes, the localization of active groups within the membrane, the solubility of the generation site of singlet oxygen in membranes, and the mobility of carotenoids in membranes (Cantrell *et al.* 2003).

Disruption of the equilibrium between ROS and endogenous antioxidants causes an increase in oxidative stress and initiates sub-cellular changes that lead to pathological conditions (Lau *et al.* 2008; Nakamura and Lipiton 2009; Paravicini and Touyz 2008). The antioxidant properties of carotenoids are expected to reduce disease risk by their ability to scavenge ROS and free radicals. On the other hand, it is well-known that healthy cells provide effective mechanisms to protect oxidized substrate against the biological oxidants, maintain a low intracellular concentration of oxygen, and keep a fine balance between ROS or free radicals and endogenous antioxidants. Oxidative stress occurs more easily in disrupted than in healthy tissues. Therefore, to be effective antioxidants, sufficient carotenoids must be present at the target location where ROS and free radicals are overproduced.

However, most dietary carotenoids may not exist at meaningful concentrations in the tissues. Of all of the carotenoids measured in serum from populations in the US, the concentration of lycopene is highest, although the level is much lower than that of α -tocopherol (Erdman *et al.* 2009). Furthermore, α -tocopherol concentrations are 162-, 17-, 53-, and 269-fold higher than lycopene in human prostate, LDL, plasma, and skin tissues (Freeman *et al.* 2000; Peng *et al.* 1995; Romanchik *et al.* 1995). On the basis of the measured lycopene concentrations, Erdman *et al.* (2009) demonstrate that the beneficial health effects of lycopene are not due to its antioxidant activity. Instead, the beneficial health effects of lycopene are due to lycopene metabolites, known as lycopenoids, which are postulated to alter gene expression *in vivo*.

Fucoxanthin, a carotenoid found in brown seaweeds, shows *in vivo* antioxidant activity in the KK- A^y mouse model of obesity and diabetes (Iwasaki *et al.* 2012). In the diabetic condition, fucoxanthin intake normalizes blood glucose levels and thus decreases the generation of ROS caused by hyperglycemia (Andrikopoulos 2010; Grattagliano *et al.* 2008; Miyashita *et al.* 2011, 2013; Shi and Pan 2012; Vincent and Taylor 2006; Vincent *et al.* 2007; Yang *et al.* 2011). The obese and diabetic conditions of KK- A^y mice result in an increase in lipid hydroperoxides in the liver and abdominal WAT of obese/diabetic KK- A^y mice compared with normal ICR mice (Iwasaki *et al.* 2012) (Figure 10.2). Fucoxanthin supplementation reduces glucose and lipid hydroperoxide levels in obese/diabetic KK- A^y mice to the levels found in control ICR mice. However, the *in vivo* antioxidant activity of fucoxanthin is found only in KK- A^y mice and not in normal ICR mice (Figure 10.2). These results suggest that the *in vivo* antioxidant activity of fucoxanthin is not due to its ability to scavenge active oxygen species and/or free radicals but is due to its ability to regulate glucose and lipid hydroperoxide levels and prevent the generation of ROS and free radicals.

While the antioxidant activity of dietary carotenoids has been historically recognized as the protective mechanism of action against oxidative stress in pathological conditions, it is now evident that the regulatory effects of carotenoids on gene and protein expression via RAR and RXR may, instead, be the main mechanism of action.

10.3 Anticancer Activity of Fucoxanthin

There have been many studies on the modulation of transcriptional activity by carotenoids and their anti-proliferative effect on cancer cells. Fucoxanthin has been reported to be more effective than other carotenoids in inducing apoptosis in GOTO, HL-60, Caco-2, HepG-2, Neuro2a, DU145, PEL, PC-3, HeLa, H1299, HT-29, and DLD-1 cancer cells (Hosokawa *et al.*

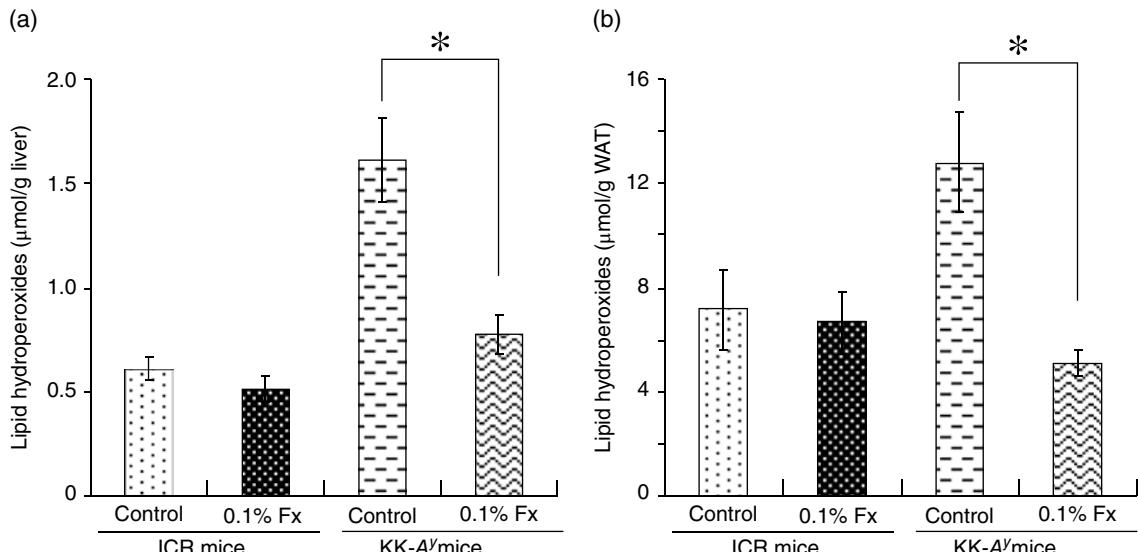


Figure 10.2 Effect of fucoxanthin (Fx) supplementation on lipid hydroperoxide levels in liver (a) and WAT (b) of normal ICR and obesity/diabetes KK-A γ mice. Values represent means \pm SD of six mice per group. *Significantly different from corresponding control ($P < 0.01$). (Adapted from Iwasaki, S., Airanthi, M.K.W.A., Koide, A., Kaga, T., Nakano, S., Beppu, F., Hosokawa, M., Miyashita, K. 2012, In vivo antioxidant activity of fucoxanthin on obese/diabetes KK-A γ mice. Food Nutr. Sci. 3, 1491–1499).

2004; Ishikawa *et al.* 2008; Jaswir *et al.* 2011; Kotake-Nara *et al.* 2001, 2005a; Liu *et al.* 2009; Okuzumi *et al.* 1990; Yamamoto *et al.* 2011). The chemical structure of fucoxanthin contains a 5/6-monoepoxide group and a rare allenic bond that only 43 out of 700 carotenoids share (Figure 10.1). Allenic carotenoids have a strong inhibitory effect on the growth of human prostate cancer cells (Kotake-Nara *et al.* 2001). Out of 15 dietary carotenoids, phytoene, phytofluene, lycopene, β -carotene, β -cryptoxanthin, α -carotene, canthaxanthin, astaxanthin, capsanthin, lutein, zeaxanthin, violaxanthin, neoxanthin, and fucoxanthin, the main, naturally occurring allenic carotenoids, fucoxanthin, and neoxanthin (Dembitsky and Maoka 2007) (Figure 10.1), were found to cause the strongest reduction in PC-3, DU 145, and LNCap prostate cancer cell growth compared with non-allenic carotenoids. In prostate cancer cell lines exposed to fucoxanthin, a dose-dependent decrease in cell viability was observed along with morphological changes such as rounding up, detachment, reduction of cell volume, and apoptotic bodies. The DNA fragmentation observed in cells treated with fucoxanthin suggests that apoptosis is the cause of suppression of cell viability. In a separate study on the effect of neoxanthin and fucoxanthin on PC-3 prostate cancer cells, Kotake-Nara *et al.* (2005b) report decreased cell viability, rounding up, reduced cell volume, chromatin condensation, nuclei fragmentation, and formation of apoptotic bodies in addition to the apoptotic DNA ladder, indicating apoptosis in the cells.

In Caco-2, HT-29 and DLD-1 colon cancer cell lines fucoxanthin significantly decreased cell viability compared with β -carotene and astaxanthin (Hosokawa *et al.* 2004). Ganeshan *et al.* (2011) report that fucoxanthin and siphonaxanthin, another marine carotenoid, inhibit growth and induce apoptosis in HL-60 leukemia cells. Moreover, fucoxanthin treatment reduced cell viability and increased apoptosis as measured by DNA fragmentation and chromatin condensation.

Dietary fucoxanthin is hydrolyzed to fucoxanthinol (Figure 10.1) in the gastrointestinal tract by the digestive enzymes lipase and cholesterol esterase (Sugawara *et al.* 2002). When a duodenal infusion of 1 ml of test oil emulsion with 2 mg of fucoxanthin was administered to portal vein cannulated rats through the lymph duct, intact fucoxanthin was not detected in either lymph fluid or portal blood at any time point, whereas fucoxanthinol was detected in the lymph but not in portal blood (Matsumoto *et al.* 2010). This result indicates a quick conversion of fucoxanthin to fucoxanthinol during the lymphatic absorption from the intestine. Fucoxanthinol can be converted to amarouciaxanthin A (Figure 10.1) in the liver (Asai *et al.* 2004, 2008; Tsukui *et al.* 2009). In humans, a daily intake of cooked edible brown seaweed, *Undaria pinnatifida* (Wakame), containing 9.26 mmol of fucoxanthin, for 1 week, produced a 0.8 pmol/ml concentration of fucoxanthinol in plasma (Asai *et al.* 2008). Fucoxanthinol retains the allenic bond (Figure 10.1) and has strong anti-proliferative effects

on cancer cells (Yamamoto *et al.* 2011). Fucoxanthinol has a more potent anti-proliferative effect on HL-60, MCF-7 and Caco-2 cancer cells than fucoxanthin (Konishi *et al.* 2006).

The anti-proliferative activity of fucoxanthin on cancer cells involves cell cycle arrest and the induction of apoptosis. The arrest of the cell cycle in the G₀/G₁ stage by fucoxanthin has been observed in different cell lines (Das *et al.* 2005, 2008; Ishikawa *et al.* 2008; Kim *et al.* 2013; Okuzumi *et al.* 1990; Satomi 2012; Satomi and Nishino 2007, 2009; Yamamoto *et al.* 2011). Liu *et al.* (2009) report the anti-proliferative effect of fucoxanthin with enhanced gap junction intracellular communication (GJIC) and increased intracellular calcium ions. They suggest that the enhanced expression of connexin genes and GJIC may increase intracellular calcium levels resulting in cell cycle arrest and apoptosis. Cell cycle arrest by fucoxanthin has been reported to be accompanied by alterations in the expression of many genes and proteins such as the growth arrest and DNA damage 45 (GADD45) proteins. GADD45 proteins are involved in cell cycle arrest at the G2/M and G1 stages, DNA repair, cell survival, and apoptosis and are known to interact with members of the mitogen-activated protein kinase (MAPK) family. Fucoxanthin can induce cell arrest by a GADD45- dependent pathway where GADD45A expression and G1 arrest are negatively regulated by p38 mitogen-activated protein kinase (MAPK) and positively regulated by the stress-activated protein kinase/c-Jun NH(2)-terminal kinase (SAPK/JNK) pathway (Satomi and Nishino 2009). Fucoxanthin induced cell cycle arrest also changed the expression of PIM 1, interferon-related developmental regulator gene 1 (IFRD1), p21 and p27. Das *et al.* (2005) found continuous cell cycle arrest at G₀/G₁ phase at lower concentrations of fucoxanthin (25μM), followed by apoptosis at higher concentrations (>50μM) with increased numbers of cells in sub G₁ phase (index of apoptotic DNA fragmentation) and fragmentation of nuclei.

The B-cell lymphoma 2 (Bcl-2) and caspase protein families are closely associated with the induction of cancer cell apoptosis. Bcl-2 proteins include anti-apoptotic members such as Bcl-2, Bcl-x_L, A1, Bcl-w, and Boo and pro-apoptotic members such as Bax and Bak, Bok, Bcl-xs, Bim, Bad, Bid, Bik, Bmf, Puma, Noxa, and Hrk (Adams and Cory 2007; Ola *et al.* 2011). Fucoxanthin has been reported to show apoptotic activity by the regulation of anti-apoptotic and pro-apoptotic Bcl-2 protein ratios and activation of caspase pathways (Adams and Cory 2007; Ganesan *et al.* 2011; Hosokawa *et al.* 2004; Ishikawa *et al.* 2008; Kim *et al.* 2010, 2013; Konishi *et al.* 2006; Kotake-Nara *et al.* 2005a, b; Liu *et al.* 2013; Wang *et al.* 2012; Yamamoto *et al.* 2011; Zhang *et al.* 2008).

Although considerable *in vitro* studies have been conducted on the effects of fucoxanthin on cancer cell viability, limited studies are available on the anti-tumor effects of fucoxanthin in animal models. Fucoxanthin supplementation has been reported to significantly decrease the percentage of skin tumor bearing mice and the number of tumors per mouse (Nishino 1995; Okuzumi *et al.* 1990, 1993). Fucoxanthin intake also resulted in significant decreases of sarcoma weight and aberrant crypt foci formation in rats (Das *et al.* 2006; Kim *et al.* 1998; Wang *et al.* 2012). The main fucoxanthin metabolite, fucoxanthinol, delayed tumor growth, decreased in tumor volume, and increased apoptosis (Ishikawa *et al.* 2008). More detailed evidence of the anti-proliferative and anti-tumor effects of fucoxanthin on *in vivo* is required.

10.4 Anti-Obesity Effects of Fucoxanthin

Fucoxanthin exerts an anti-obesity effect in obese/diabetic mice that was first observed using brown seaweed lipids containing 10% fucoxanthin (Beppu *et al.* 2013; Hosokawa *et al.* 2010; Maeda *et al.* 2005, 2007a, b). Body weight gain in obese/diabetic KK-A^y mice fed brown seaweed lipids rich in fucoxanthin (Maeda *et al.* 2007a) or purified fucoxanthin (Maeda *et al.* 2007b) is significantly reduced compared with control mice and is consistent with the decrease in the weight of uterine, mesentery, perirenal, and retroperitoneal white adipose tissue (WAT) normalized for body weight. Brown adipose tissue (BAT) weight normalized for body weight increases with fucoxanthin intake. Fucoxanthin supplementation also significantly suppresses body weight gain and attenuates excess fat accumulation in the abdominal WAT of normal animals fed high fat diets (Hu *et al.* 2012; Kang *et al.* 2012; Maeda *et al.* 2009; Woo *et al.* 2009). Excess fat accumulation in abdominal WAT results in hyperglycemia, hyperinsulinemia, and hyperleptinemia in the mouse model. These perturbations were completely normalized in the fucoxanthin supplementation. Animal experiments using both obese model KK-A^y mice and lean C57BL/6J mice indicated the specificity of fucoxanthin. Fucoxanthin attenuates the excess fat accumulation in abdominal WAT of obese KK-A^y mice but has no effect in lean C57BL/6J mice fed a normal fat diet (Hosokawa *et al.* 2010). However, fucoxanthin significantly suppresses abdominal WAT weight of C57BL/6J mice fed a high-fat diet to the same level of that found in the normal dietary group (Maeda *et al.* 2009). These results suggest that the suppressive effect of fucoxanthin on WAT weight gain is specific to adiposity in the development of obesity.

The anti-obesity effect of fucoxanthin has also been reported using adipose cell models. When various carotenoids were screened for potential suppression effects on adipocyte differentiation (Maeda *et al.* 2006; Okada *et al.* 2008; Yim *et al.* 2011),

only fucoxanthin, neoxanthin, and two fucoxanthin metabolites (fucoxanthinol and amarouciaxanthin A) showed a suppressive effect on the differentiation of 3T3-L1 adipose cells, the inhibition of intercellular lipid accumulation, a decrease in glycerol-3-phosphate dehydrogenase activity, and a decrease in the expression of CCAAT/enhancer binding protein α (C/EBP α) and the peroxisome proliferator-activated receptor γ (PPAR γ). An examination of structure and function suggests that carotenoids containing an allene bond may show suppressive effects on adipocyte differentiation in 3T3-L1 cells.

The potential biological mechanisms for obesity control include increased energy expenditure, increased fat oxidation, decreased fat absorption, and increased satiety through the regulation of related signaling pathways and specific molecular targets, mainly in adipose tissue and liver. In mice given purified fucoxanthin, more than 80% of the fucoxanthin metabolites accumulated in abdominal WAT indicating that abdominal WAT is the main target tissue for fucoxanthin (Airanthi *et al.* 2011). The main mechanism for the anti-obesity effect of fucoxanthin is induction of the uncoupling protein 1 (UCP1) in abdominal WAT. UCP1 is a key factor in the thermogenic process that occurs in BAT (Collins *et al.* 2010; Kozak 2010; Nedergaard *et al.* 2001; Rothwell and Stock 1979; Seale, 2010; Smith and Horwitz 1969). The presence of UCP1 allows BAT to dissipate the electrochemical gradient that is normally used to drive adenosine triphosphate synthesis (ATP). UCP1 allows for the reentry of protons into the mitochondrial matrix thereby uncoupling oxidative phosphorylation, and releasing excess dietary energy intake as heat, preventing weight gain. UCP1 expression levels in BAT are controlled by the sympathetic nervous system via noradrenaline that is stimulated by cold, adrenergic stimulation, β 3-agonists, retinoids, and thyroid hormone (Argyropoulos and Harper 2002; Del Mar Gonzalez-Barroso *et al.* 2000; Nedergaard *et al.* 2001; Mozo *et al.* 2005; Silva and Rabelo 1997). This adrenergic signaling pathway includes a G-protein receptor mechanism. A β 3-adrenoreceptor receptor (β 3Ad) is coupled to the heterotrimeric G-protein Gs and to adenylyl cyclase (AC). Stimulation of the receptor results in elevated levels of intracellular cAMP, activation of cAMP-dependent protein kinase (PKA), and of cyclic AMP response element binding protein (CREB). PKA activation induces hormone-sensitive lipase (HSL) expression that stimulates lipolysis. The liberated fatty acids serve as substrates in BAT thermogenesis. PPAR γ and peroxisome proliferator-activated receptor gamma co-activator 1 (PGC-1) are key molecules in UCP1 induced BAT thermogenesis (Lowell and Spiegelman 2000). PGC-1 binds to a variety of nuclear receptors including the retinoic acid and thyroid hormone receptors, both of which positively regulate expression of UCP-1. Moreover, mitochondrial biogenesis is induced by PGC-1 activation.

Fucoxanthin increased BAT weight and decreased abdominal WAT weight in mice (Hosokawa *et al.* 2010; Maeda *et al.* 2005, 2007a, b). Interestingly, fucoxanthin supplementation also induced UCP1 expression in both BAT and abdominal WAT (Maeda *et al.* 2005, 2007a, b). These findings show that the decrease in abdominal WAT weight observed in mice fed fucoxanthin is due to the upregulation of thermogenesis through UCP1 expression both in BAT and abdominal WAT. UCP1 is expressed in BAT through the upregulation or stimulation of several key biomolecules such as β 3Ad, PGC-1, and PPAR γ . UCP1 is generally considered to be exclusively expressed in BAT. However, UCP1 has been found in WAT of mice over-expressing forkhead box protein C2 (FoxC2) (Cederberg *et al.* 2001). Additionally, the differentiation pathway for brown adipocytes has been found in white fat depots in mice overexpressing FoxC2 (Cummings *et al.* 1996; Soloveva *et al.* 1997). These results suggest that inducing UCP1 in WAT may be developed into a therapy for obesity. It should be noted that fucoxanthin induces both protein and mRNA expressions of UCP1 in abdominal WAT (Maeda *et al.* 2005, 2007a, b). Although the mechanism for UCP1 expression in abdominal WAT by fucoxanthin supplementation is not clear, upregulation of several factors such as β 3Ad and PGC-1 by fucoxanthin, as found in BAT would be a likely explanation (Maeda *et al.* 2009; Nishikawa *et al.* 2012) (Figure 10.3). The UCP1-expressing brown-like adipocytes can be recruited in WAT by prolonged cold exposure or by treatment with β -adrenergic agonists (Himms-Hagen *et al.* 2000). Fucoxanthin may be related to the induction of this brown-adipose like cell formation in abdominal WAT.

Fucoxanthin supplementation in obese model mice effectively decreases excess fat accumulation in abdominal WAT when fucoxanthin intake is more than 60 mg intake/kg mouse/day (Airanthi *et al.* 2011). In humans, an intake of less than 0.024 mg/kg/day fucoxanthin (2.4 mg intake/day for volunteers with 100 kg average weight) demonstrated a significant reduction of abdominal WAT in obese female volunteers (Abidov *et al.* 2010). This difference in the effectiveness of fucoxanthin on reducing abdominal WAT between rodents and human may be due to a difference in fucoxanthin absorption rates and/or sensitivity to fucoxanthin.

10.5 Anti-Diabetic Effects of Fucoxanthin

When fucoxanthin is given to obese/diabetic KK-A y mice, it significantly decreases blood glucose and plasma insulin concentrations compared with control KK-A y mice (Maeda *et al.* 2007b). The same reduction in blood glucose and plasma insulin levels was obtained in normal mice (C57Bl/6J mice) fed a high fat diet and supplemented with fucoxanthin

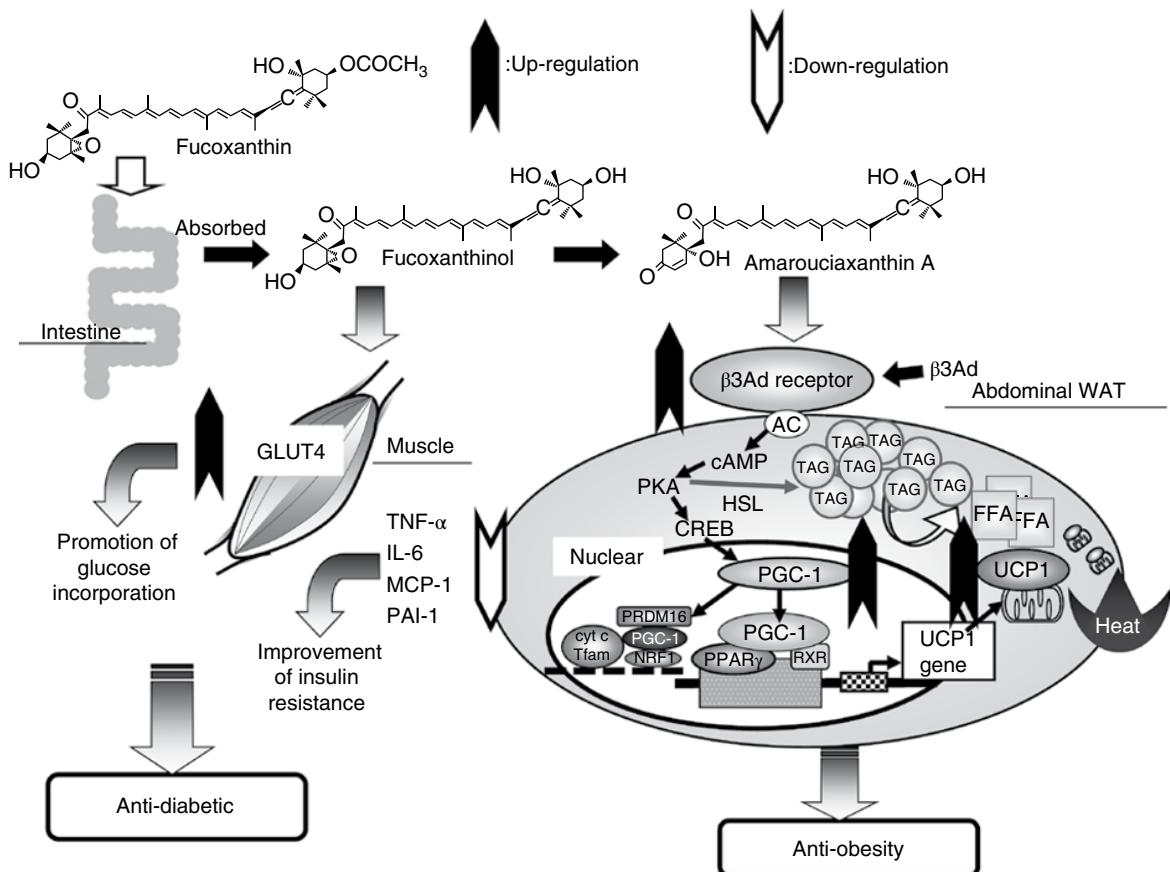


Figure 10.3 Possible mechanism for the anti-obesity and anti-hyperglycemic effects of fucoxanthin.

compared to normal mice only fed a high fat diet (Maeda *et al.* 2009). Interestingly, the effect of fucoxanthin on decreasing blood glucose levels is specific to the diabetic condition. When fucoxanthin was given to two types of mice, obese/diabetic KK-A γ mice and lean C57BL/6J mice, the blood glucose level of the obese/diabetic mice decreased to the same level as that in control C57BL/6J mice, whereas fucoxanthin did not affect blood glucose levels in C57BL/6J lean mice (Hosokawa *et al.* 2010). Fucoxanthin metabolites accumulate in visceral WAT and regulate and release adipokines and/or upregulate glucose transporter 4 (GLUT4) expression and its translocation to the cell membrane thereby exerting an anti-diabetic effect (Miyashita *et al.* 2011, 2013).

Abdominal obesity is the most obvious symptom of metabolic syndrome, a disease characterized by a cluster of risk factors including Type 2 diabetes and cardiovascular disease (CVD) (Gade *et al.* 2010). The increasing global prevalence of Type 2 diabetes is tied to rising rates of obesity (Teixeira and Budd 2010). With the exception of adiponectin and adiponectin (complement factor D), most other adipokines have been implicated in obesity. Type 2 diabetes, hypertension, inflammation, and CVD (Deng and Scherer 2010). Excessive fat accumulation in abdominal WAT induces overproduction of pro-inflammatory adipokines such as tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), chemoattractant protein-1 (MCP-1), and plasminogen activator-1 (PAI-1). Fucoxanthin intake can downregulate the overexpressions of these pro-inflammatory adipokines. In the obese/diabetic KK-A γ mouse model, leptin and TNF- α mRNA expression in abdominal WAT are significantly decreased by fucoxanthin intake (Maeda *et al.* 2007b). Additionally, increased expression of MCP-1 mRNA expression is observed in normal mice on a high-fat diet but is normalized by fucoxanthin intake (Maeda *et al.* 2009).

Development of obesity induces macrophage infiltration into the abdominal WAT, resulting in the interaction between the macrophage and adipocyte. Macrophage infiltration upregulates pro-inflammatory adipokines and downregulates anti-inflammatory adiponectin resulting in chronic low-grade inflammation. Pro-inflammatory adipokines can also induce insulin resistance. In the abdominal WAT of obese/diabetic KK-A^y mice, fucoxanthin supplementation significantly inhibits the infiltration of monocytes into the adipose tissues, the differentiation of monocytes to macrophages, and reduces pro-inflammatory adipokine (TNF- α , IL-6, MCP-1, and PAI-1) inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) expression (Hosokawa *et al.* 2010). Suganami *et al.* (2005) demonstrated that a paracrine loop involving TNF- α and free fatty acids (FFA) exists between infiltrated macrophages and adipocytes. Adipose cells and macrophages secrete large amounts of TNF- α . TNF- α and other pro-inflammatory adipokines promote lipolysis of triacylglycerols and the release of FFA. These FFA induce macrophages infiltration into WAT and then enhance TNF- α production (Kennedy *et al.* 2008). Fucoxanthin effectively halts this paracrine signaling loop between adipose cells and macrophages (Hosokawa *et al.* 2010).

The regulatory effect of fucoxanthin on GLUT4 expression in skeletal muscle (Maeda *et al.* 2009) is strongly related to its anti-diabetic effect because the skeletal muscle accounts for up to 40–60% of body mass, expresses GLUT4, and is therefore the major tissue involved in glucose homeostasis. GLUT4 is the predominant isoform of the glucose transporters and is expressed abundantly in skeletal muscle and adipose tissue (Joost *et al.* 2002); therefore, many studies have been done for the mechanisms regulating the expression of GLUT4. Studies using transgenic mouse models demonstrate that modulating GLUT4 expression profoundly affects the effect of insulin on the body and, consequently, glucose and lipid metabolism (Charron and Katz 1998; Minokoshi *et al.* 2003). Mice with the over-expressed GLUT4 gene in skeletal muscle or adipose tissue have an enhanced peripheral glucose use and an enhance response to insulin (Charron *et al.* 1999). In contrast, skeletal muscle tissue from mice with metabolic disorders such as hyperlipidemia has a significant reduction of GLUT4 protein and mRNA levels (Kahn and Pedersen 1993; Kim *et al.* 1994). Thus, the regulation of the GLUT4 gene is of clinical relevance for whole-body glucose homeostasis and insulin sensitivity.

For example, when mice were fed high fat (HF) or normal fat (NF) diets, the HF group resulted developed hyperglycemia, hyperinsulinemia and hyperleptinemia with a significant decrease in GLUT4 mRNA levels in skeletal muscle compared with the NF group (Maeda *et al.* 2009). However, the addition of fucoxanthin to the HF diet completely attenuated the hyperglycemia, hyperinsulinemia, and hyperleptinemia and restored GLUT4 mRNA to levels observed in the NF group. An increase in GLUT4 levels and upregulation of PGC-1 α expression in the extensor digitorum longus (EDL) muscles of obese/diabetic KK-A^y mice fed fucoxanthin intake has also been reported (Nishikawa *et al.* 2012). PGC-1 α is an important activator of GLUT4 that has been implicated in the regulation of mitochondrial biogenesis (Bonnen 2009; Michael *et al.* 2001). A significant increase in GLUT4 expression and in insulin-stimulated glucose uptake was, therefore, found in the muscle over-expressed PGC-1 α (Benton *et al.* 2008). Fucoxanthin intake upregulates GLUT4 expression by increasing PGC-1 α levels.

The upregulation of GLUT4 translocation to the cell membrane by fucoxanthin is one of the major mechanisms for its anti-diabetic activity (Nishikawa *et al.* 2012). The phosphoinositide 3-kinase (PI3K) signaling pathway is initiated by insulin binding to the insulin receptor substrate (IRS) family (Lee and Pilch 1994; Myers and White 1996; Saltiel and Kahn 2001) and the pathway is responsible for GLUT4 translocation in muscle (Leto and Saltiel 2012) (Figure 10.4). The Tyr-phosphorylated IRS proteins interact with and activate PI3K and result in the synthesis of phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P3) from phosphatidylinositol-4,5-bisphosphate (PI(4,5)P2) at the plasma membrane. PI(3,4,5)P3 in turn serves as a docking site for several PH domain-containing Ser/Thr kinases, such as phosphoinositide-dependent kinase 1 (PDK1) and AKT (Protein Kinase B) that are implicated in glucose uptake. PDK1 and rapamycin complex 2 (mTORC2) activate AKT through dual Ser/Thr phosphorylation. Activated AKT can regulate the trafficking of GLUT4 from intracellular stores to the plasma membrane.

In Type 2 diabetes mellitus, insulin signaling is impaired, and GLUT4 translocation to the plasma membrane is attenuated (Shepherd and Kahn 1999). When fucoxanthin is given to KK-A^y mice, GLUT4 translocation to plasma membranes from cytosol is significantly upregulated in the soleus muscle of KK-A^y mice (Nishikawa *et al.* 2012). Upregulation of GLUT4 translocation has also been found in EDL muscle, though the effectiveness of fucoxanthin on EDL muscle was lower than that on the soleus muscle. The expression levels of insulin receptor mRNA and phosphorylation of Akt, which are upstream of the insulin signaling pathway regulating GLUT4 translocation, were also enhanced in the soleus and EDL muscles of the mice fed fucoxanthin.

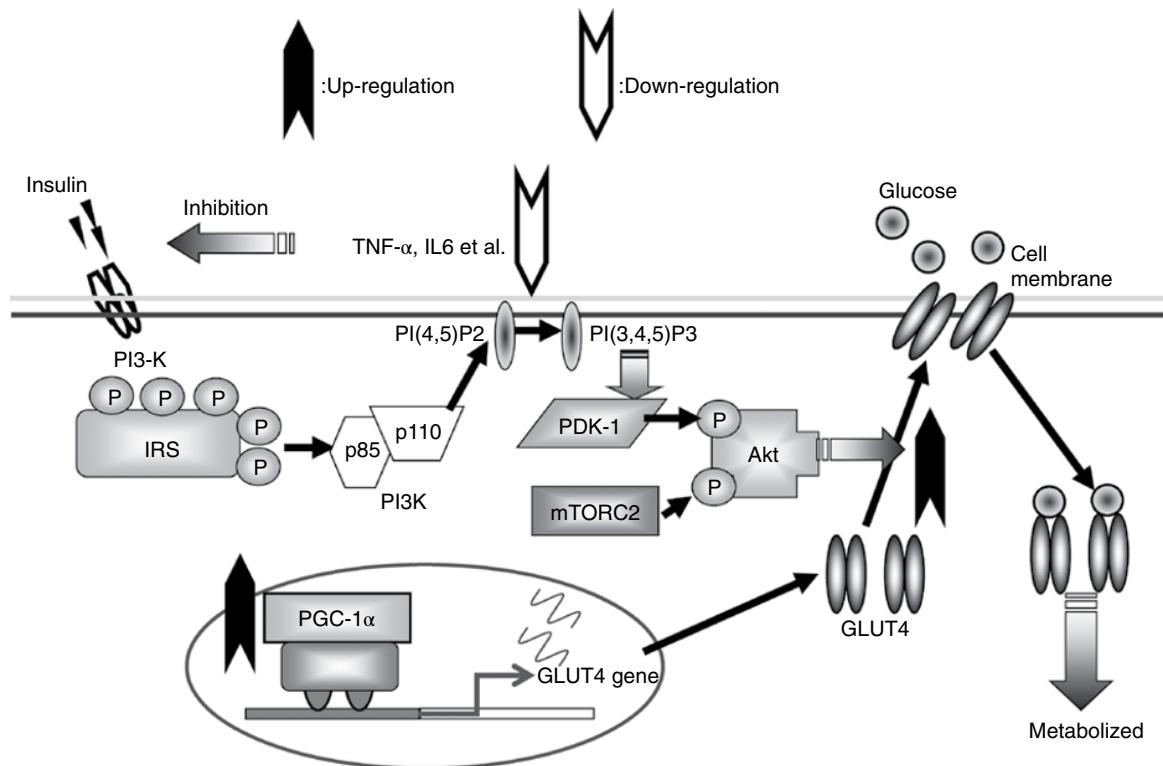


Figure 10.4 Signaling pathway of insulin-stimulated glucose uptake into muscle.

10.6 Conclusion

Epidemiological studies have established a positive correlation between vegetable and fruit consumption and a reduced risk of chronic disease. Decreases in oxidative stress by antioxidants are supposedly one of the major mechanisms for the beneficial health effects in fruits and vegetables. However, the regulatory effects on specific gene and protein expression of carotenoids may play a larger role in the beneficial effects on human health. On the other hand, the health effects of “antioxidants” have also been made clear on the basis of their regulatory effects on specific gene and protein expression but not much on their antioxidant activities. Some carotenoids, such as fucoxanthin, can improve pathological conditions such as obesity and hyperglycemia through specific molecular mechanisms, resulting in a decrease in *in vivo* oxidative stress. A greater focus on nutrient-gene interactions is necessary in future research on carotenoids.

Fucoxanthin shows a significant reduction of oxidative stress based on the regulation of specific gene and protein expressions. These effects can be found only in pathological conditions such as obesity and diabetes, where oxidative stress is high. The most interesting physiological effect of fucoxanthin is the induction of UCP1 in abdominal WAT mitochondria that leads to the oxidation of fatty acids and heat production in the WAT. It is apparent that there are only two ways to treat obesity: reduction of energy intake or increase in energy expenditure. Lifestyle interventions such as changed dietary habits and increased physical activity are important for obesity therapy (Saha *et al.* 2010). In addition there is an increased focus on metabolically active food compounds that can address the energy imbalance issue (Hursel and Westerterp-Plantenga 2010; Kovacs and Mela 2005; St-Onge 2005). There is a great deal of interest in food components that induce thermogenesis through UCP1 activation in adipose tissues. Fucoxanthin is the most promising nutraceutical for activating UCP1 in abdominal WAT and is an ideal target for obesity therapy. More studies in cells, animals, and humans are required to determine the mechanistic details by which fucoxanthin might be developed into a therapy to combat obesity and other metabolic disorders.

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11

Nutrition, Genomics, and Human Health: A Complex Mechanism for Wellness

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11.1 Introduction

The interplay between an individuals' genetic makeup can be critical to the outcome of therapy and to successful management of disease condition and proper maintenance of health and hence wellness. Adverse drug reactions (ADRs), is one of the leading cause of death globally and the causes for ADRs may include product defects, medication errors, differences in drug exposure, and potentially genomic dispensations. The study of pharmacogenetics can be broadly described as that focused on genetic causes of individual variations in drug response and pharmacogenomics more broadly involves genome-wide analysis of the genetic determinants of drug efficacy and toxicity and ability to properly metabolize foods.

While medicine and its practice in disease management continues to advance, the context of personalized medicine approach presents a new way of dealing with individual health, individualized by the patients genetic makeup. Such drug effects are not only confined to individual but may vary between racial and ethnic populations (Aruoma and Bahorun, 2010; Docherty *et al.*, 2014; Fernald *et al.*, 2011; Pacanowski and Zineh, 2012; Schröder *et al.*, 2013).

Single nucleotide polymorphisms are now recognized as the main cause of human genetic variability and are already a valuable resource for mapping complex genetic traits. The identification and validation of accurate biomarkers of individual responses to drug or biologic treatment remain prerequisite conditions ascribed to the development of PM and other evolving therapeutic strategies. Thus sequence variations in the genes for proteins involved in drug disposition can alter the pharmacokinetics of a drug, while sequence variations in drug target genes can change the pharmacodynamics of the drug (see Figure 11.1). The fact that pharmacogenomics connects genotype to patient-specific treatment intrinsically implies that individuals have variations in the composition of their genetic characteristics (factored on strategies that embrace testing for candidate-genes and genome-wide association) that will affect the availability of functional proteins, which ultimately impacts upon functional homeostasis and the outcome of drug therapy. Primary candidate genes include those encoding for drug receptors, metabolizing enzymes, and transporters. However, a selection of optimal drug therapies may also involve disease susceptibility genes indirectly affecting drug response. Figure 11.1 is illustrative of the emerging viewpoints, as discussed by Fernald *et al.* (2011).

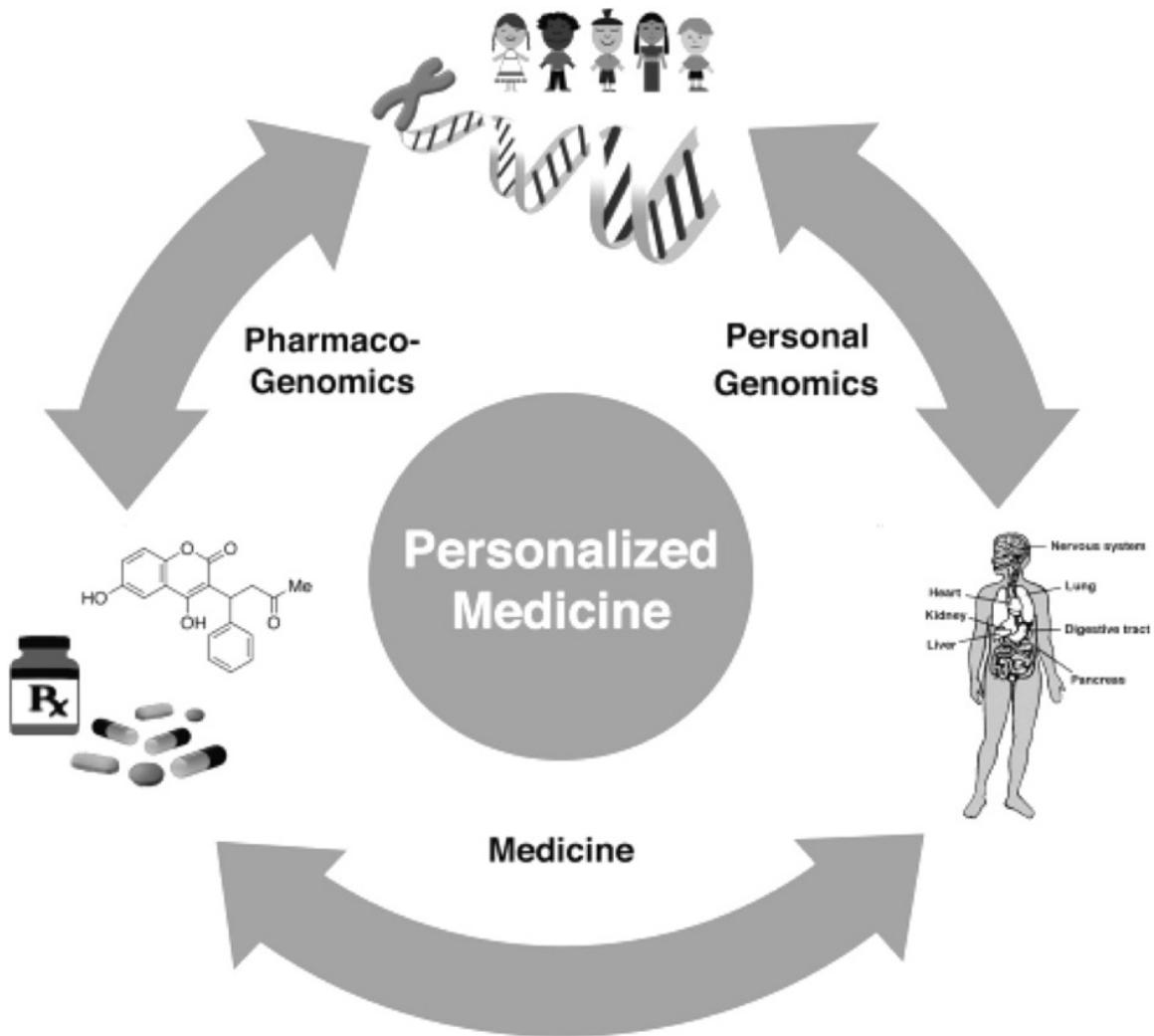


Figure 11.1 Personal genomics connect genotype to phenotype and provide insight into disease. Pharmacogenomics has helped us to understand some of the factors responsible for ADRs caused by high exposures and factors associated with the mechanism-of-action of the drug and examples continue to emerge where genetic markers identified patients at risk for serious, often life-threatening ADRs before administration of drugs. (The reader is referred to Fernald et al. (2011). Bioinformatics challenges for personalized medicine. *Bioinformatics* 27: 1741–1748; and to the US Food and Drug Administration's (US FDA) website: www.fda.gov/drugs/scienceresearch/researchareas/pharmacogenetics, for pertinent literature on pharmacogenomics).

11.2 Nutrition Sciences and Clinical Applications in Nutritional Genomics

Nutritional genomics is the study of the effects of foods and food constituents on gene expression. Nutritional genomics aims to develop a rational means to optimize nutrition through the identification of the person's genotype and this defines the relationship between nutrients and human health. Individuals cannot change their genetics but they can eat the right foods to support genetic predispositions, take the right supplements to support gene variations, and promote normal cell function and structure. Indeed, poor diet can be a risk factor of disease. Given that dietary components can alter gene expression, the degree to which diet influences health and disease depends upon an individual's genetic make-up. Herein, the advancement in the use of pharmacogenomics technology should be well defined and embrace diagnostic, prognostic,

predictive characteristics. This would warrant a clear statement on the goals and population targeted. This said, little progress has been reported for clinically useful predictive markers, for instance, in cancer therapy. The context of structured scientific research process using proteomics, nutrigenomics, metabolomics, bioinformatics, and genetic susceptibility, extensively contributed to another area of biomarker research, *nutritional biomarker research*. The human body is able to deal with chemical entities irrespective of their origin, and the pharmaceutical terms “absorption, distribution, metabolism, and excretion” have their equivalents where biomarkers are concerned. Let us take a closer look and consider the learning points from Figures 11.2 and 11.3. Polymorphisms in drug metabolizing enzymes, transporters, and/or pharmacological targets of drugs may profoundly influence the dose-response relationship between individuals. Carbohydrate metabolism is but one component of energy production and storage. In fact, a much larger percentage of the total energy reserves in animals are lipids in the form of fat deposits consisting of energy-rich fatty acids. As can be seen in Figure 11.2, there are three basic sources of fatty acids in animals that can be used for energy conversion processes; fatty acids present in triacylglycerols obtained from the diet, fatty acids stored as triacylglycerols in adipose tissue that are released by hydrolysis following hormone stimulation (glucagon or epinephrine signaling), and fatty acids synthesized in the liver from excess carbohydrates and exported as triacylglycerols. These processes are controlled by enzymes and they have to be present in their functional entity to effectively mediate the various processes involved. Thus, nutrigenetics/nutrigenomics conceptualizes the research into the “relationship between genes and nutrients from basic biology to clinical practice”. By understanding how genes alter the body’s response to nutrition or how nutrition alters the body’s response to defective genes, scientists are unlocking the codes to health and longevity. Profiling of genetic nutritional responses can help in the determination of which specific foods that give the best biological response, based on an individual’s DNA. Fatty acids in dietary triacylglycerols are transported from the intestines to the rest of the body by large lipoprotein particles called chylomicrons. Hormone signaling releases fatty acids from adipose tissue that bind to an abundant transport protein in serum called albumin. The fatty acids that are synthesized in the liver are carried through the body as triacylglycerols by very low density lipoprotein particles. Fat is stored in fat cells (adipocytes). Obesity, especially childhood obesity, can be due to both, that is, more fat storage per cell,

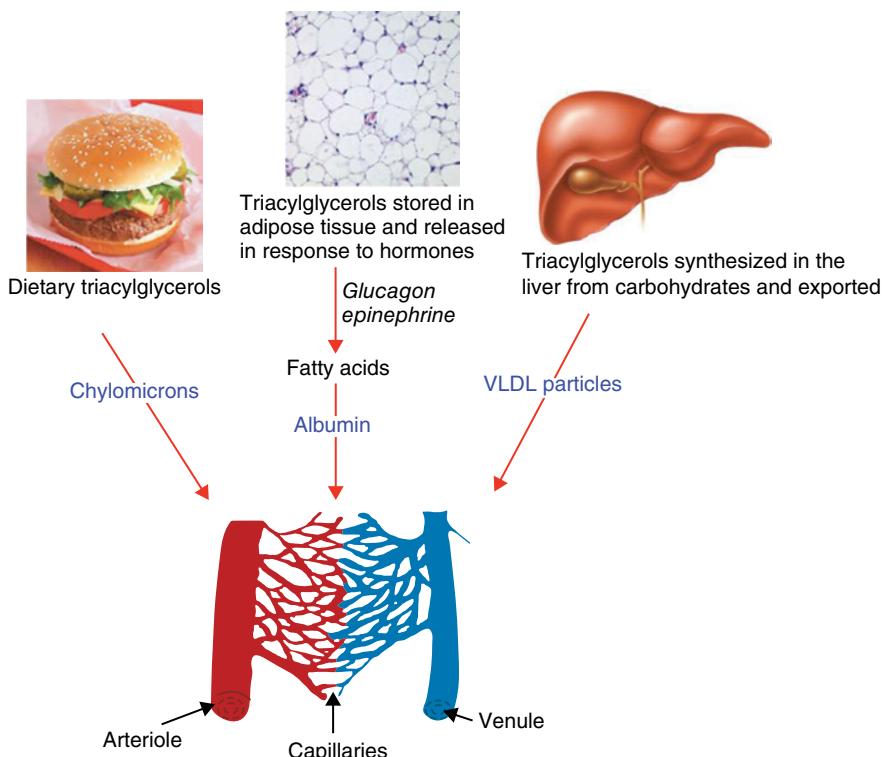


Figure 11.2 Fatty acid storage and energy production components: A large percentage of the total energy reserves in animals is lipids in the form of fat deposits consisting of energy-rich fatty acids.

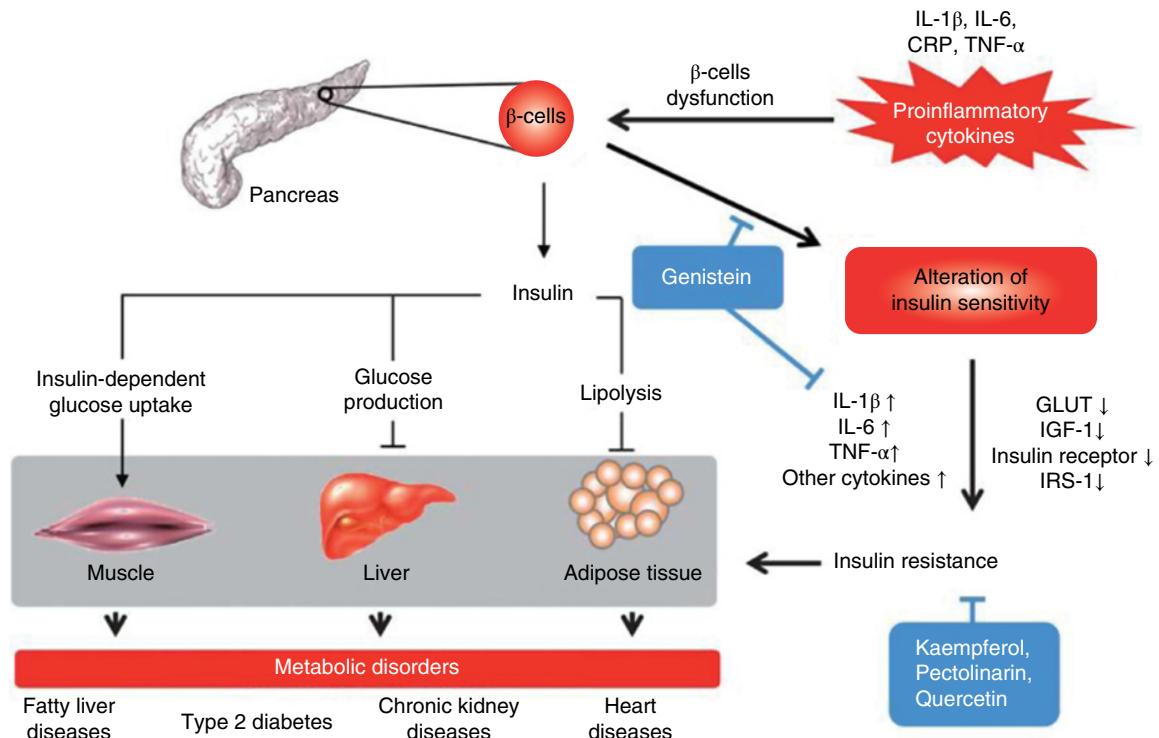


Figure 11.3 Metabolism and potential impact on disease of over inflammation by bioactive component or food compound or nutrients (genistein, anthocyanins, quercetin, kaempferol) and insulin secretion or beta-cell function or beta-cell preservation, diabetes (Type 1 and 2) or metabolic syndrome, or insulin resistance. Flavonoids are antioxidants, and have anti-inflammatory and protective effects on metabolic diseases.

and to a larger number of adipocytes (Figure 11.2). In contrast, in normal healthy adults, the onset of old age and reduced metabolic rates leads to weight gain resulting primarily from storing more fat per cell (although adults can also add more fat cells if they become obese). (The thematic review by Saini-Chohan *et al.* 2012 is worth perusing by the reader regarding fatty acid metabolism.) Thus, the genomic disposition of the individual has a direct bearing in the control of metabolism which is nicely illustrated here. Because of this, nutritional genomics offers insights into ways to tailor the diets of individuals and populations. Personalized nutrition, like its parallel in medicinal approaches, presents a new way of dealing with individual nutritive health, using a “personalized” approach sustained by high throughput technologies including pharmacogenetics, pharmacogenomics, and epigenetics interlinked with genomic medicine. Given that many factors can influence the application of pharmacogenetic discoveries to patient care, the influence of a drug dose, determination of an appropriate dose of a particular drug for a given patient will eventually require knowledge about both genetic and nongenetic factors that affect drug disposition and pharmacodynamics. A patient’s genetics are consulted only for a few diagnoses and treatment plans. The limitations doctors have in access to their patients’ genomes resides on the context that only a small percentage of the genome could be used because such data come from association studies, which tend to identify variants with small effect sizes and have limited applications to healthcare. Individuals have variations in the composition of their genetic characteristics (factored on strategies that embrace testing for candidate genes and genome-wide association) that will affect the availability of functional proteins, which ultimately impacts functional homeostasis and the outcome of drug therapy. The NIH generically defines biomarkers as “characteristics that can be objectively measured as indicators of a biological or pathological process or pharmacological response to a therapeutic intervention”, qualifying them to be potentially used across the whole translational medical research process. Biomarkers are therefore touted as the next frontier in the realm of modern medicine as they would represent the essentials in guiding treatment decisions that could enable complementary matching of specific drugs with individual patients, effective patient therapeutic dose and management of drug-related risks (Aruoma and Bahorun, 2010).

The human body is able to deal with chemical entities irrespective of their origin, and the pharmaceutical terms “absorption, distribution, metabolism, and excretion” have their equivalents when biomarkers are concerned. The fact that pharmacogenetic factors operate at pharmacokinetic as well as pharmacodynamic levels (the two components of the dose-response curve of a drug are elegantly captured in the view of Shah 2005), indicates that polymorphisms in drug metabolizing enzymes, transporters, and/or pharmacological targets of drugs may profoundly influence the dose-response relationship between individuals. The context shown in Figure 11.3 shows the potential bioefficacy of dietary phenolic compounds that would have been either consumed by an individual as a supplement or through food. Anthocyanins and anthocyanidins are responsible for a variety of colors – including red, blue, and purple – in fruits, vegetables, and flowers, and are prevalent in the human diet. Genistein is the most studied isoflavone with respect to diabetes. Genistein is found in a number of plants including lupine, fava beans, soybeans, and soybean products. Quercetin is a natural polyphenolic flavonoid found in a wide variety of plant-based foods, which displays anti-diabetic properties *in vivo*. These components would have been absorbed and, following distribution and metabolism, will have impact on the various targets. Shah elegantly highlighted the clinical consequences for drug metabolism involving phenotypes of CYP2D6. Drug metabolizing enzymes are generally expressed in genetically variant forms with altered functional properties. Glucuronidation is by far the most important conjugation pathway in humans. Indeed, a multigene family encodes the UGTs and a relatively small number of human UGT enzymes catalyze the glucuronidation of a wide range of structurally diverse endogenous (bilirubin, steroid hormones, and biliary acids) and exogenous chemicals. The seminal reviews by Schröder *et al.* (2013) and Epstein and Teagarden (2010), focus on the genetics of inter-individual variability of gene expression with emphasis on pharmacogenomics personalized medicine and are both worth perusing. The guidelines pertinent to pharmacotherapy, drug development (and with a context that impacts nutrition) and pharmacogenomics, can be found on the US FDA website (by following the link: www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM337169.pdf). The document refers to the presence of pharmacogenomics in premarket investigations and drug labeling and provides guidance and recommendations on when and how genomic information should be considered to address questions arising during drug development and regulatory review, including study design, data collection, and data analysis in early-phase trials. The guidelines of the European Medicines Agency pertaining to pharmacogenomics can be found on their website (www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/02/WC500121954.pdf).

Whilst the promise of pharmacogenomics in global healthcare needs the identification of suitable targets for drug discovery and development, as is now becoming apparent, there is a need to ensure pharmacogenomic literacy. The net outcome is that nutritionists, dietitians, doctors (physicians), nurses, pharmacists, and all healthcare professionals will have the knowledge base to both counsel and advise patients. The vein is to provide individuals with lifestyle recommendations, particularly in nutrition, which will help them enjoy optimal health and the highest possible quality of life. This is the basis of wellness.

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12

Application of Genomics and Bioinformatics Analysis in Exploratory Study of Functional Foods

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12.1 Introduction

The wealth of genomic information, genomics-based technologies, and model systems available provide a spectrum of new tools for use in human nutrition and food science. These new technologies are being used to study the molecular basis of the interaction of individual food constituents with both the genome and the metabolism of the human consumer. For historical reasons, nutrition and food science are not well prepared to exploit the genomics technologies, primarily because of the lack of appropriate teaching of human genetics, genomics, and molecular biology in most university programs.

However, these deficits have been recognized and, in response, numerous initiatives have been launched recently in Europe, Asia, and the USA under the heading of “Nutrigenomics”. Although to some, nutrigenomics might represent just another “-omic”, it will change the face of research in nutrition and food science by moving the genome into the center of all processes that essentially determine mammalian metabolism in health and disease (Muller and Kerten, 2003; van der Werf *et al.*, 2001; Rist *et al.*, 2006).

According to Kussmann *et al.* (2008), “nutritional genomics” is the collective term that covers the three subdisciplines of transcriptomics, proteomics, and metabolomics, and describes the use of medium- to high-throughput profiling technologies to assess the response of a cell or organism to dietary treatment or particular foods or food constituents (Kussmann *et al.*, 2008).

Microarray-based transcriptome analysis may be considered the first mature genome-wide profiling technology. Consequently, it is also used widely and applications of transcriptomics in nutritional studies seem unlimited when it comes to basic and preclinical research in either cell culture systems or animal models. The mRNA profiling techniques have the potential to easily identify specific transcript changes that respond to a given nutrient, non-nutrient compound, treatment, or diet in a well-defined experimental setting. This might not mean that the changes in mRNA level can be taken as a causal marker; it might rather be a pattern of expressed mRNAs that changes in a characteristic and reproducible way. Because the technology has the character of a screening process covering thousands of potentially affected indicators of the

metabolic status simultaneously, it also reveals often totally unexpected findings (Kussmann *et al.*, 2008). In the infrastructure of microarray experiments, commercial platforms such as those of Affymetrix and Agilent are high quality and are provided with a manual, annotation file and other services (Affymetrix, Inc., 2015; Agilent Technologies, 2015a).

For the food industry, particularly functional food or dietary supplements, these new technologies look attractive to their business. Most industries conduct research on their most important ingredient that is important to human health. They hope to find new and novel functions for their items. A comprehensive approach may give them clue as to new ideas. There is no reason we do not conduct microarray experiments. Actually, wet experiments such as labeling fluorescent dyes, hybridization, or scanning of spots are not difficult to do. However, most beginners may be stalled by the analysis of immense amounts of data. Therefore, the bioinformatics/dry experiment technique, as well as the wet experiment technique, are both very important in study of functional foods.

In this chapter, we present useful data analysis tools of microarray-based transcriptome for beginners. In addition, we show a practical analysis with our original data.

12.2 Analysis Tools

Microarrays have become a standard tool for gene expression measurement in biology, medicine, and nutrition. Although microarrays are widely used, a fundamental challenge is to cope with the immense amount of data generated. Therefore, special software packages have been developed that are capable of handling the analysis of microarray data.

The common challenge faced by researchers is translating lists of differentially regulated genes into a better understanding of the underlying biological phenomena. Generally, the analysis flow is explained by two steps.

The first step is making a “gene list”. This step can be the translation of the list of differentially expressed genes into a functional profile able to offer insight into the cellular mechanisms relevant in the given condition.

Gene lists are made by two methods that are gene expression-based and bioinformatics-based. The former is extracted by experimental data; for example, threshold of fold ratio or change pattern of gene expression on time course or treatment conditions.

The latter is extracted by known bioinformatics technologies. In this case, the researcher must have target fields before analysis.

The second step is filtering by gene list A and gene list B (and lists C, D, etc.) that are made in the first step.

Each operation is simple but takes a lot of time. It is inefficient to use Microsoft Excel for making gene lists because the computational load is too large.

12.2.1 GeneSpring GX

The most typical software is GeneSpring GX (Agilent) (Agilent Technologies, 2015b). Other statistical analysis software has developed add-in packages to support microarray data. However, these are not dedicated software for analysis of microarray data. GeneSpring GX is a gene expression software tool enabling analysis, comparisons, visualization, and management of gene expression data. The best feature of this software is its easy-to-use interface.

12.2.2 Bioconductor

We recommend Bioconductor for the R-user (Bioconductor, 2015). The Bioconductor toolkit is among the most sophisticated free software for microarray data analysis, based on the *R* statistical programming language (R Core Team, 2015). Most algorithms developed for microarray data analysis are available within this package. Unfortunately, Bioconductor is a text-driven command line tool and does not provide an easy-to-use graphical interface. Therefore, it offers advanced analysis methods and the possibility of easy extension only for professional users, and it is difficult to use for people unskilled in *R*.

12.2.3 Others

Other tools, EXPANDER (Shamir *et al.*, 2005) and TM4 (Saeed *et al.*, 2003), are well known as installed local machine software. The web-based tools Expression Profiler (Kapushesky *et al.*, 2004) and GEPAS (Herrero *et al.*, 2003) are widely used for microarray data analysis.

These programs share a focus on data analysis, but most of them lack tools for the interpretation of the result. That is the biggest issue for beginners.

12.3 Interpretation Tools

After making a list of differentially expressed genes from analysis tools GeneSpring GX and Bioconductor, the researcher conducts interpretation analysis using biological information.

Biological information for interpretation is grouped into three categories, Gene Ontology (GO), Pathway, and Association Network. Outlines of the three categories are explained in the following.

12.3.1 Go Analysis Tools

The Gene Ontology (GO) project is a collaborative effort to address the need for consistent descriptions of gene products in different databases (The Gene Ontology Consortium, 2015). The GO project has developed three structured controlled vocabularies (ontologies) that describe gene products in terms of their associated biological process, cellular component, and molecular function in a species-independent manner. These are called “GO Term”. There are three separate aspects to this effort: first, the development and maintenance of the ontologies themselves; second, the annotation of gene products, which entails making associations between the ontologies and the genes and gene products in the collaborating databases; and third, development of tools that facilitate the creation, maintenance, and use of ontologies.

In 2002, an automatic ontological analysis approach using GO was proposed to help with this task (Khatri *et al.*, 2002). From 2003 to date, many tools, including Onto-Express (Khatri and Draghici, 2005), DAVID (Database for Annotation, Visualization, and Integrated Discovery) Dennis *et al.*, 2003), BinGO (The Biological Networks Gene Ontology) (Maere *et al.*, 2005), and AmiGO (Carbon *et al.*, 2009), have been proposed for this type of analysis, and more tools appear every day. Currently, this approach is the *de facto* standard for the secondary analysis of high-throughput experiments, and a large number of tools have been developed for this purpose. Although these tools use the same general approach, they differ greatly in many respects that influence the results of the analysis. GO mining tools and their detailed information are available at the GO project website. You can get GO analysis results using your gene list by typing UniGene, EntrezGene, or GeneBank as a query.

12.3.2 Pathway Analysis Tools

Pathway maps, especially metabolic pathway maps, are familiar to researchers. Most researchers are confident interpreting their own data on a pathway map. Pathways focus on physical and functional interactions between genes rather than the gene-centered view of GO-based analyses. Therefore, it is intriguing to map the list of significantly regulated genes onto precompiled pathways to elucidate the whole chain of events observed in a microarray experiment. However, not all pathways are equally suitable for microarray analysis. For instance, metabolic pathways are controlled largely by protein-based events, which are not observable in microarrays because only steady-state levels of mRNAs are monitored. Kinase-based signaling cascades also do not necessarily involve changes in mRNA levels. The best case for microarray-based pathway analysis is that transcriptional signaling pathways are directly coupled to *de novo* transcription. Although they contain post-transcriptional steps, there is usually enough transcriptional feedback regulation of pathway-related genes to allow for the identification of the pathways via alterations in mRNA level. Most pathway-analysis tools relying on precompiled databases of pathways derived from large-scale literature analysis require constant updating because of the continuous growth of the literature.

Many software tools capable of analyzing microarray data within the context of biological pathways have been developed. Recently released commercial software packages including PathwayAssist (Nikitin *et al.*, 2003), PathArt, the Ingenuity Pathways Analysis tool (QIAGEN, 2015), and MetaCore (GeneGo, Thomson Reuters, 2015) also compete in the field of pathway-based microarray analysis. These tools provide an assortment of interfaces for the visualization of gene networks, natural language processing (NLP) extracted or hand-curated biological pathway/association network databases, and they accept gene list-based data input. Each of these tools has one or more unique features that distinguishes it from the others. Some open-source or publicly accessible software, such as GenMAPP (Dahlquist *et al.*, 2002) and Pathway Processor (Grosu *et al.*, 2002) display microarray data within the context of pathways annotated in the *Kyoto Encyclopedia of Genes and Genomes (KEGG)* pathways (Kanehisa *et al.*, 2002) and provide statistical assessment of the reliability of each differentially expressed gene.

KEGG is a widely used database of biological systems, consisting of genetic building blocks of genes and proteins (*KEGG GENES*), chemical building blocks of both endogenous and exogenous substances (*KEGG LIGAND*), molecular wiring diagrams of interaction and reaction networks (*KEGG PATHWAY*), and hierarchies and relationships of various

biological objects (*KEGG BRITE*). KEGG provides a reference base for linking genomes to biological systems and to environments by the processes of PATHWAY mapping and BRITE mapping.

12.3.3 Association Network Analysis Tools

Protein interaction information is essential for systems-level understanding of cellular behavior, and it is needed to place the molecular functions of individual proteins into their cellular context. The database Web tool STRING (Search Tool for Retrieval of Interacting Genes/Protein) (Jensen *et al.*, 2009) is developed to collect, predict, and unify most types of protein–protein associations, including direct and indirect associations. It is a metaresource that aggregates most of the available information on protein–protein associations, scores and weights, and results of automatic literature-mining searches.

The most advanced point of STRING is the result of network analysis; it understands researchers intuitively. STRING analysis is simple; one can get network analysis results by using a gene list and writing biological common IDs, UniGene, EntrezGene, GeneBank..., as queries.

We have explained three categories of biological information. Many convenient tools are also present that refer to you.

In addition, some analysis tools integrate interpretation tools, for example, GeneSpring GX bundles the GO analysis tool, GEPAT (Genome Expression Pathway Analysis Tool) combines GO and KEGG analysis functions. These analysis programs and databases are no more than tools. Other tools such as TM4 also bundle a protein–protein interaction analysis function.

What follows is an example of how to apply these tools for the study of the effect of kale on alcohol metabolism.

12.4 Application Example of Kale (*Brassica oleracea L. Var Acephala DC*)

Kale (*Brassica oleracea L. var acephala DC*) is a form of cabbage, green in color, in which the central leaves do not form a head (Olsen *et al.*, 2009). It is more closely related to wild cabbage than most domesticated forms. Until the end of the Middle Ages, kale was one of the most common green vegetables in Europe. Curly-leaved varieties of cabbage already existed along with flat-leaved varieties in Greece in the fourth century BC. These forms, which were referred to by the Romans as Sabellian kale, are considered the ancestors of modern kale. Today, one may differentiate among different varieties according to the low, intermediate, or high length of the stem, with varying leaf types.

The species *Brassica oleracea* (Cruciferae family) contains a wide array of vegetables including broccoli, cauliflower, collard greens, and Brussels sprouts. Epidemiological studies suggest that cruciferous vegetable intake may lower overall cancer risk. One such family of chemoprotective constituents are isothiocyanates (ITC), which are formed by glucosinolates (Clarke *et al.*, 2008). Within the plant, glucosinolate content can vary greatly between and within members of the Cruciferae family depending on cultivation environment and genotype. There are more than 120 glucosinolates in the various varieties of cruciferous vegetables, each yielding different aglycone metabolic including ITC. The general structure of glucosinolate consists of a beta-D-thioglucose group, a sulfonated oxime group, and a variable side chain. Many of the anticancer effects observed from cruciferous vegetables have been attributed to the ITCs rather than to their parent glucosinolates. Two important and well-studied isothiocyanates derived from cruciferous vegetables are sulforaphane (SFN) and indole-3-carbinol.

The glucosinolate precursor to SFN, glucoraphanin, is abundant in kale, broccoli, cauliflower, and cabbage. Hydrolysis of glucoraphanin to its aglycone product SFN requires the activity of myrosinase enzymes released from the plant during consumption and other myrosinase enzymes present in human gut. The structures of glucoraphanin and SFN are shown in Figure 12.1. The mechanisms of SFN chemoprevention have been well studied and reveal diverse responses depending upon the stage of carcinogenesis. SFN can function by blocking initiation via inhibiting Phase 1 enzymes that convert procarcinogens to proximate or ultimate carcinogens, and by inducing Phase 2 enzymes that detoxify carcinogens and facilitate their excretion from the body. Recently, kale juice has become popular as a healthy vegetable juice in Japan. Kale juice is squeezed from fresh kale leaves, and the slight bitter taste is caused by ITC. The studies of kale as a functional food have shown beneficial properties such as antitumor activity, anti-hypercholesterolemia, antihyperglycemia, improvement in constipation, antipigmentation, and antiallergic effects. Incidentally, kale is effective in preventing a hangover in popular folklore; however, scientific evidence of this was not reported. Therefore, we investigated the effect of kale on alcohol and related metabolisms by microarray study.

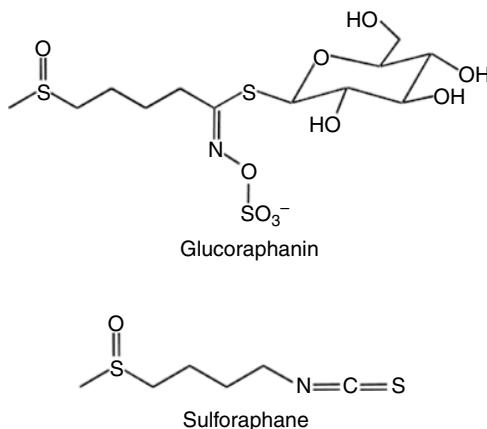


Figure 12.1 Structures of glucosinolate precursor glucoraphanin and its isothiocyanate hydrolysis product sulforaphane.

12.4.1 Animal Study and DNA Microarray Analysis

Sixteen male ddY mice aged 3 weeks were used for animal experiments. Mice were divided into two groups of eight: control group and kale group.

Kale was administered in the form of mixed food prepared by adding hot water extracted kale at 5% (w/w) in MF powder, a standard laboratory rodent diet (Oriental Yeast, Tokyo). The diet and drinking water were *ad libitum*. Fifteen weeks after kale supplementation, the mice were sacrificed and their livers were collected. There were no significant differences in body and liver weights between the kale treatment and control groups. Total RNA was extracted from the livers of eight mice fed on each diet, pooled and subjected to a DNA microarray analysis by whole mouse genome (Agilent). The number of probes consisted of 22,963 mouse genes. There were 17,472 genes that passed the selection criteria and subsequently used in the gene list for exploratory data analysis.

12.4.2 Data Analysis

12.4.2.1 Expression Data

We conducted data analysis by filtering and selection. The analysis software used was GeneSpring GX. At first, it required preparation of gene lists. We first identified 1682 genes whose expression was increased by twofold or decreased by <0.5-fold, and it was designated List A. We then extracted alcohol metabolism-related genes from Entrez Gene and STRING.

12.4.2.2 Search by Entrez Gene

An ethanol metabolism-related gene list was extracted by the search query “ethanol metabolism” limited to *Mus musculus*. A total of 44 genes were identified by Entrez Gene search and annotated in a file named “Agilent whole mouse genome” (shown in Table 12.1).

12.4.2.3 Search by STRING

A gene list from STRING 8 was made with four queries, “alcohol dehydrogenase 1”, “aldehyde dehydrogenase 2”, “cyp2e1”, and “catalase”. The search condition was as follows:

Required confidence (score): high confidence (0.07)

Interactors shown: no more than 0 interactors

Additional (white) nodes: 0

or Network depth: 1

Table 12.1 The list of ethanol metabolism genes extracted by the Entrez Gene.

Gene Symbol	Genbank ID	Description
Adh1	NM_007409	Alcohol dehydrogenase 1 (class I)
Adh4	AK004863	Alcohol dehydrogenase 4 (class II), pi
Adh4	NM_011996	Alcohol dehydrogenase 4 (class II), pi
Aldh2	NM_009656	Aldehyde dehydrogenase 2, mitochondrial
Avpr1a	NM_016847	Arginine vasopressin receptor 1a
Al326910	NM_145429	Arrestin, beta 2
Bdnf	NM_007540	Brain-derived neurotrophic factor
Creb1	NM_025702	cAMP responsive element binding protein 1
Creb1	NM_009952	cAMP responsive element binding protein 1
Cat	NM_009804	Catalase
Cdc42	NM_009861	Cell division cycle 42 homolog (<i>S. cerevisiae</i>)
Cyp2e1	NM_021282	Cytochrome P450, 2, subfamily e, 1
Dbh	NM_138942	Dopamine beta hydroxylase
Gad1	NM_008077	Glutamic acid decarboxylase 1
Gad2	NM_008078	Glutamic acid decarboxylase 2
Gad2	AK018118	Glutamic acid decarboxylase 2
Gpt1	NM_182805	Glutamic pyruvic transaminase 1, soluble
Gpx1	NM_008160	Glutathione peroxidase 1
Il6	NM_031168	Interleukin 6
Lep	NM_008493	Leptin
Lpl	NM_008509	Lipoprotein lipase
Mapk14	NM_011951	Mitogen-activated protein kinase 14
Mapk8	NM_016700	Mitogen-activated protein kinase 8
Mapk8	AK047936	Mitogen-activated protein kinase 8
Maoa	NM_173740	Monoamine oxidase a
Nos1	NM_008712	Nitric oxide synthase 1, neuronal
Nos3	NM_008713	Nitric oxide synthase 3, endothelial cell
Ppar, PARalpha	NM_011144	Peroxisome proliferator-activated receptor alpha
Prkcd	NM_011103	Protein kinase c, delta
Rxra	NM_011305	Retinoid X receptor alpha
Spp1	NM_009263	Secreted phosphoprotein 1
Sirt1	NM_019812	Sirtuin 1 (silent mating type information regulation 2, homolog) 1
Slc18a2	AK035644	Solute carrier family 18 (vesicular monoamine), subfamily 2
Shh	NM_009170	Sonic hedgehog
Scd1	NM_009128	Stearoyl-Coenzyme A desaturase 1
Scd1	NM_009127	Stearoyl-Coenzyme A desaturase 1
Sod1	BC057592	Superoxide dismutase 1, soluble
Sod1	NM_011434	Superoxide dismutase 1, soluble
Sod1	AK080908	Superoxide dismutase 1, soluble
Trp53	NM_011640	Transformation related protein 53
Tnf	NM_013693	Tumor necrosis factor

The search result is illustrated as a network view. The search result of alcohol dehydrogenase 1 is shown in Figure 12.2 as network view example.

12.4.2.4 Filtering

Filtering was conducted by the Venn diagram function on GeneSpring GX.

Each gene list, extracted either from Entrez Gene or STRING 8, was written in common name, gene symbol, gene bank ID, and so on, for gene identity on GeneSpring GX. The result of filtering is shown in Figure 12.3. We extracted 14 genes showing significant changes related to alcohol metabolism by kale treatment. For interpretation of the 14 genes, STRING search was used. The result of network analysis of remarkable genes is shown in Figure 12.4.

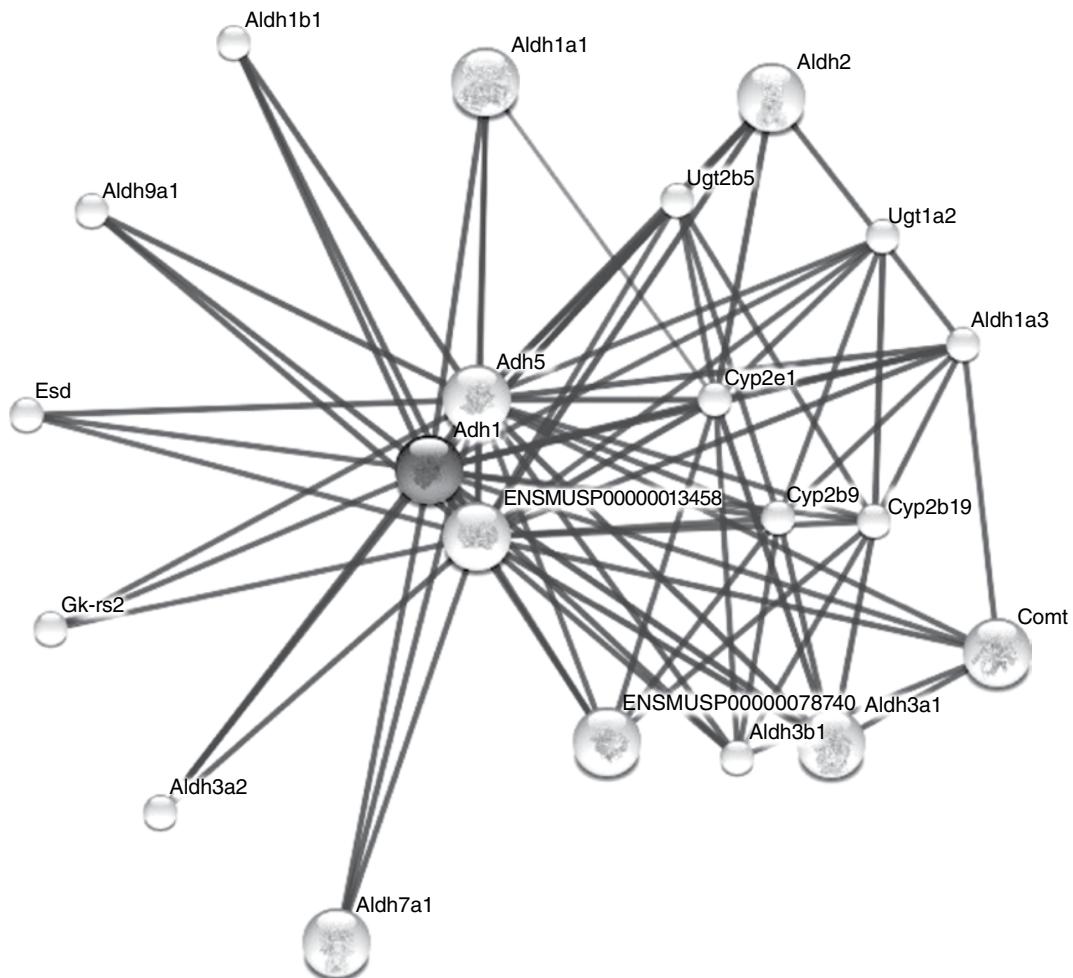


Figure 12.2 Gene networks analysis of alcohol dehydrogenase 1 by STRING 8.0.

12.4.3 Result

The identified genes were used to construct a network comprising alcohol metabolism, fatty acid metabolism, glycolysis/glucogenesis, tryptophan metabolism, and propanoate metabolism-related factors. The other gene network was MAPK signaling pathway-related factors.

In general, the main pathway for ethanol oxidation involves hepatic alcohol dehydrogenase, a cytosolic enzyme that catalyzes the transformation of ethanol into acetaldehyde, which is in turn converted to acetic acid through aldehyde dehydrogenase. Ethanol can also be metabolized by peroxisomal catalase and an alcohol-inducible isoform of P450, CYP2e1, in the microsomal ethanol-oxidizing system (Lieber, 1997).

Alcohol dehydrogenase 1(Aldh1), Cyp2e1, and catalase (Cat) exhibited remarkable alteration in gene expression (fold ratios were 2.99, 5.91, 3.10, respectively). However, aldehyde dehydrogenase 2 (Aldh2), the main acetaldehyde dehydrogenase, was shown to be slightly changed (fold change: 1.6). Meanwhile, aldehyde dehydrogenase 1 (Aldh1), an isoform of Aldh2, was shown to change markedly (fold ratio: 3.05).

Approximately 60% of acetaldehyde was metabolized by Aldh2 and Aldh1 whereas 20% was metabolized by Cyp2e1. The pathway via Aldh1 and Cyp2e1 bypasses that expressed under excess alcohol intake. Taken together, we hypothesized

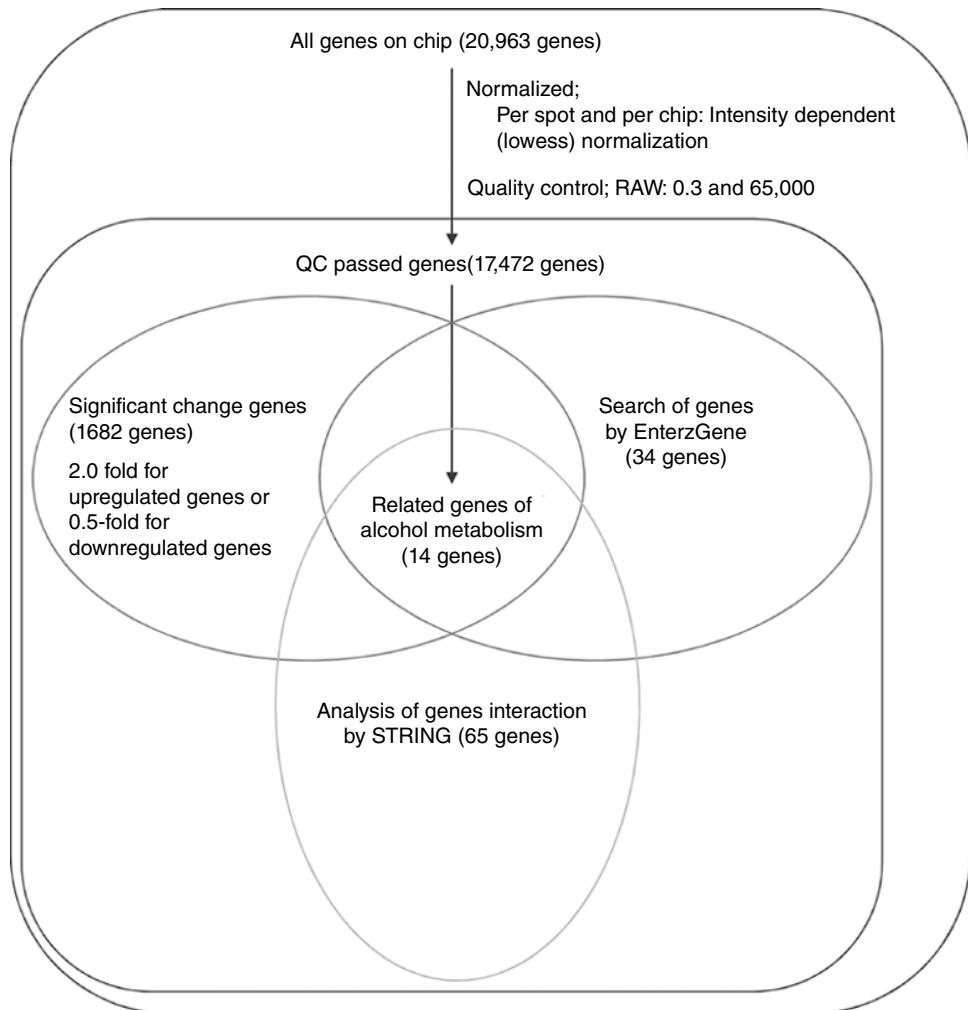


Figure 12.3 Scheme of identification of regulatory genes by *Brassica oleracea* var. *acephala* DC.

that the mechanism of alcohol metabolism of kale is as follows. Kale upregulates Adh1 and alcohol is promoted by the conversion of ethanol to acetaldehyde. Acetaldehyde is converted to acetic acid by up-regulated Aldh1, Cyp2e1, and Cat supported to Aldh2 (Figure 12.5). Fatty acid metabolism and glycolysis/glucogenesis are also known to relate to alcohol metabolism.

This study is preliminary and there are outstanding issues in proving the effect of kale on alcohol metabolism. For example, efficacy of kale on alcohol-loading animals, gene expression profile on time course (including early response gene expression), dose dependency, protein expression analysis, and so on. Analysis of gene expression profile on time course may show an intriguing hint for understanding the mechanism of kale.

These results provided useful information for basic research of kale function. After this microarray study, we conducted a clinical study for evaluating alcohol metabolism improvement. In healthy subjects, the level of serum alcohol and acetaldehyde decreased when drinking alcohol with kale. Thus, the microarray study coupled with bioinformatics is efficient for exploratory study.

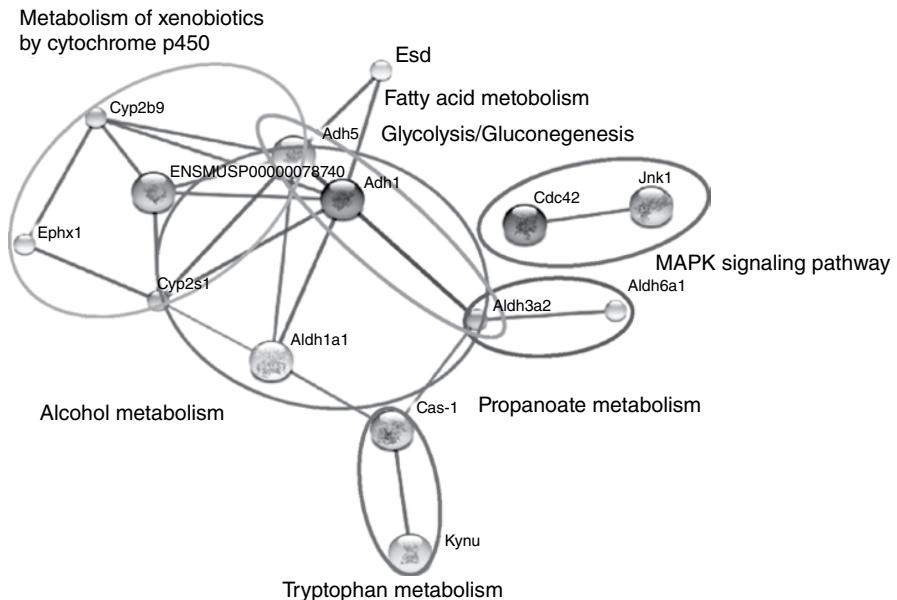


Figure 12.4 Genes network that shows remarkable change by *Brassica oleracea* var. *acephala* DC.

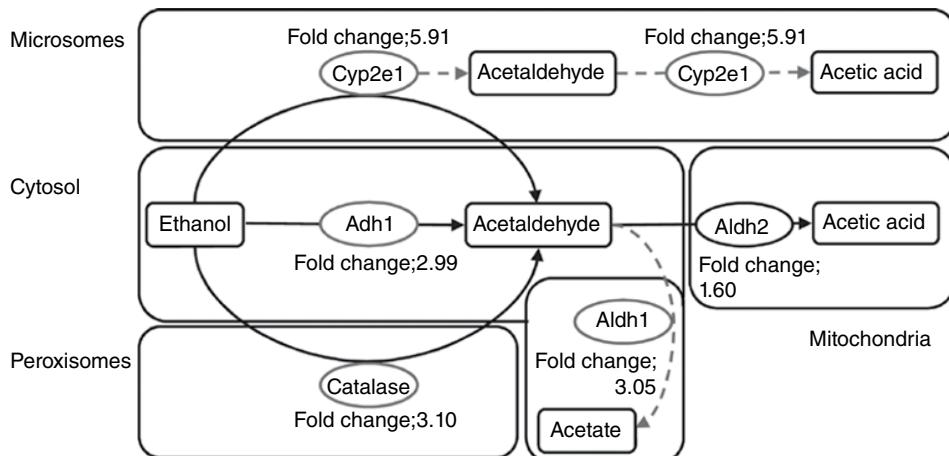


Figure 12.5 Effect of *Brassica oleracea* var. *acephala* DC on alcohol metabolism.

12.5 Conclusion

In this chapter, we introduced the use of bioinformatics databases for interpretation of microarray data for the novice. These methods may not be conventional or standard practice; however, we believe they are useful methods as an initial approach to microarray analysis. Moreover, construction of gene lists from databases is an efficient and effective method.

In our application example, we used Entrez Gene and STRING to obtain information not only related to alcohol metabolism but also related to other metabolisms. It is more efficient to combine the existing databases when analyzing microarray data.

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13

Omics Analysis and Databases for Plant Science

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13.1 Introduction

Big data offers new opportunities and challenges for the understanding of biological mechanisms at the molecular to individual and/or population levels, including interactions among different species and environments. In the plant sciences, big data consist of information in the literature, bioresources for experiments, historical and meteorological factors, and experimental omics data. In particular, omics data, including data on genome sequences, gene expression, compounds, interactions of proteins, and other information, are huge. Comprehensive omics analysis has been vigorously pushed forward by many projects (Ikeda *et al.* 2013; Kaever *et al.* 2014; Kudo *et al.* 2013; Suzuki *et al.* 2013). Whereas big data provide much information, the enormousness makes it difficult to easily and quickly analyze and interpret. To efficiently analyze large-scale omics data, maximizing valuable information, enhancement of proper interpretation of the results, and advances in biostatistics methodology, bioinformatics tools, and databases are required.

The exponential growth of omics data in the plant sciences has been mainly brought about by high-throughput DNA sequencing technologies. For example, next-generation sequencing (NGS) technologies have been widely employed in genome sequencing and mRNA sequencing (mRNA-Seq), not only for model plants such as *Arabidopsis* and rice, but also for other crops such as tomato and potato (Ahmadvand *et al.* 2014; Kobayashi *et al.* 2014; Osaka *et al.* 2013; Pacurar *et al.* 2014). The amount of sequence data obtained by NGS technologies has accumulated rapidly in public sequence databases; namely, SRA (NCBI Resource Coordinators 2014; <http://www.ncbi.nlm.nih.gov/Traces/sra/>), DDBJ DRA (Kaminuma *et al.* 2011) and ENA (Pakseresht *et al.* 2014; <http://www.ebi.ac.uk/ena/about/statistics>). In order to take advantage of NGS data, the sequencing length and quality for each read (sequence) should be controlled. Most reads generated by NGS technologies are shorter and lower in quality than ones obtained by Sanger technology. Sequence regions with low quality must be trimmed before sequence analyses such as mapping and assembling. Short reads raise another problem in mapping analysis. Due to the short length of NGS reads, some sequences have no specific sequence pattern. Since each such sequence will show the highest sequence similarity to multiple sites among the reference sequences, the unique best-hit position of each read cannot be determined. In single nucleotide polymorphisms (SNP) mining or expression analysis using mapping methods, NGS technologies tend to show a lower mapping rate of reads that uniquely and accurately map to reference sequences than Sanger technology. Although NGS technologies pose problems

in length and quality, the high throughput of sequencing data assists in identifying false-positives (such as mapping errors). A new platform to provide reads with average lengths of more than 4 kb has also been developed, such as the single molecule, real-time (SMRT) DNA sequencing system (see Section 13.3 and Section 13.8). In this chapter, analysis methods using NGS data are introduced.

In this big data era, databases providing biological information in the plant sciences are essential in molecular biology, genetics, physiology, population genetics, and other fields. Biological databases store information covering the literature, genomic DNA, expressed genes, biological functions of genes and proteins, metabolism, experimental resources, and so on. Besides information for laboratory research, statistical data on agricultural production are also available from FAOSTAT (Tubiello *et al.* 2013). With such a database infrastructure, biologically important information of interest can be easily retrieved.

Most databases have been designed by bioinformaticians; each database has biological data and enhanced functions installed to efficiently search and retrieve particular information. Since each database has its own search functions and data structure design, it is important to be familiar with the common basics of search functions and database contents. For example, functional annotations of genes are accessible from many databases. Most databases provide functional annotations predicted by bioinformatics tools such as BLAST (Boratyn *et al.* 2013) and InterPro (Hunter *et al.* 2012). While bioinformatics tools enable genome-wide analysis, the results obtained by computational analysis must be confirmed by manual curation or validation studies. When annotations predicted by only computational tools are employed, their reliability should always be assessed and taken into account. The reliability of annotations is available in some cases. The Gene Ontology Consortium (The Gene Ontology Consortium 2013) provides annotations with Gene Ontology (GO) terms. It assists in quickly understanding biological functions of genes and proteins as well as the reliability of the annotations. In GO annotations, an evidence code indicating reliability is assigned to each annotation. While manual curation is often labor intensive and time consuming, biological annotation based on manual curation of the literature offers higher reliability. The UniProt Knowledgebase (UniProtKB) has provided an excellent resource for functional annotation of proteins by manual curation (The UniProt Consortium 2014). In this chapter, the main public databases available in the plant sciences are also described.

13.2 NGS Technologies and Data Processing

The so-called NGS technologies are becoming indispensable to future plant omics analysis and databases. Originally, they were designed and implemented to decipher the sequences of DNA polymers, but in combination with reverse transcription, chromatin immunoprecipitation or bisulfite modification methodologies, multi-omics layer analyses can be performed. More applications of NGS technologies are emerging, making them invaluable for comprehensive plant omics.

The first NGS implementation launched was the 454 Sequencing System by Roche Diagnostics Corporation. Within a few years, the Illumina SBS System and Life Technologies SOLiD System had been released. Each has its own features, for example regarding sequence length, sequence quality, error characteristics, data throughput, and cost-to-performance ratio. Here it is important to catch up on the latest trends in NGS technologies, because they have been continuously upgraded, and will rarely be discontinued (the Roche 454 will be discontinued in the coming years).

Compared with the conventional Sanger DNA sequencers, those NGS instruments output relatively short, low-quality but ultrahigh-throughput DNA sequence reads. In terms of genomics, due to their features in reads, NGS technologies are quite suitable for polymorphism detection by a conventional resequencing strategy (see Section 13.4). But recent advancement of bioinformatics and rich computer resources, particularly in terms of fast memory modules, have made it possible to reconstruct the vast majority of large and complicated plant genomes (see Section 13.3).

For any goal of NGS sequencing, data preprocessing to clean up each read is a key process. Each read should be checked for sequence quality (quality value or QV), adapter/linker contamination, and dubious read elimination according to the k-mer distribution. Tools for preprocessing are summarized in Table 13.1. For the latest SMRT technology by Pacific Biosciences (PacBio), the error correction step is also critical to the quality of final outcome (see Section 13.3).

13.3 *De novo* Plant Genome Assembly by NGS

13.3.1 Basics of Plant Genome Assembly

Plant genomes are known to be large, repeat-rich, and sometimes highly redundant (due to large-scale genome duplications, or multiploidy). The early challenges to determining plant genomes were steadily addressed with the strategy of

BAC-by-BAC sequencing with Sanger technology (Arabidopsis Genome Initiative 2000; International Rice Genome Sequencing Project 2005). Each BAC fragment was located on the physical map of the genome, and small-scale assembly was performed within each BAC clone. This procedure makes the final result firm, but simultaneously tends to be a cost- and time-consuming framework.

13.3.2 Plant Genome Assembly by NGS Short Reads

In this NGS era, many challenges exist to deciphering plant genomes mainly with cost-effective NGS technologies. A number of plant genomes have been sequenced by NGS technologies (sometimes in combination with Sanger technology), and whole genome sequencing has been feasible with mainly NGS technologies (Tomato Genome Consortium 2012; Chen *et al.* 2013; International Wheat Genome Sequencing Consortium 2014). Each project was carried out with a unique strategy (NGS, NGS-Sanger combination, and NGS-other technology combination). In many cases, it has proven quite difficult to finalize the assembly only with the NGS reads, and another method to locate all contig/scaffold sequences onto the genome's physical map has been required. Tools for genome assembly are summarized in Table 13.2.

13.3.3 Hybrid-Type Assembly

As shown in Section 13.2, there are multiple technology options for whole plant genome assembly. Among the major three early technologies (454, Illumina, and SOLiD), 454 has a unique feature in that it achieves relatively long sequence reads (up to ~1000 bp). Therefore, it has been combined with one of the other two technologies for better genome

Table 13.1 Preprocessing tools.

Tool name	Application	URL
FastQC	Quality controlling	http://www.bioinformatics.babraham.ac.uk/projects/fastqc/
FASTX-Toolkit	Removing adapter sequences and low quality bases, etc	http://hannonlab.cshl.edu/fastx_toolkit/
cutadapt	Removing adapter sequences	https://code.google.com/p/cutadapt/

Table 13.2 De novo assemblers.

Tool name	Application (read length) (●: short read, ○: long read, ○: both short and long read)	Application (experiment type) (●: Genome-Seq, ○: mRNA-Seq, ○: both Genome-Seq and mRNA-Seq)	URL
Velvet	○	●	http://www.ebi.ac.uk/~zerbino/velvet/
SOAPdenovo	●	●	http://soap.genomics.org.cn/soapdenovo.html
ABYSS	●	●	http://www.bcgsc.ca/platform/bioinfo/software/abyss
Ray	○	●	http://denovoassembler.sourceforge.net/
Platanus	●	●	http://platanus.bio.fitech.ac.jp/
Oases	○	○	http://www.ebi.ac.uk/~zerbino/oases/
SOAPdenovo-Trans	●	○	http://soap.genomics.org.cn/SOAPdenovo-Trans.html
Trans-ABYSS	●	○	http://www.bcgsc.ca/platform/bioinfo/software/trans-abyss
GS De Novo Assembler(Newbler)	○	○	http://www.454.com/products/analysis-software/
Trinity	○	○	http://trinityrnaseq.github.io/
MIRA4	○	○	http://sourceforge.net/p/mira-assembler/wiki/Home/

assembly (Chagné *et al.* 2014; Garcia-Mas *et al.* 2012; Schmutz *et al.* 2014). Recently, PacBio SMRT technology was released, and methods to correct the erroneous PacBio long reads with Illumina reads (<https://github.com/jgurtowski/ectools>), or by bootstrapping PacBio reads (<http://www.pacb.com/devnet/>; <http://zombie.cb.k.u-tokyo.ac.jp/sprai/index.html>) were developed.

13.4 Plant Genome Resequencing by NGS

The rationale of resequencing by NGS is the detection of differences in nucleotide positions between the reference genome and a subject individual (e.g., a related species, a subspecies, a cultivar, an individual in the same species) genome. The subject genome is sequenced by NGS technology, short reads are then mapped onto the reference genome according to the sequence similarities, and finally, the differing nucleotide positions (SNPs) are detected as high-quality mismatched nucleotides.

13.4.1 Conventional Resequencing Technologies

SNPs (and sometimes indels) among individuals or cultivars are beneficial as genetic markers for breeding. QTL studies and genome-wide association studies (GWAS) enable prediction of gene loci according to the statistical associations between the phenotypic data and genotypic markers. The cost of genome resequencing by read mapping is more affordable than that of *de novo* assembly. At least ten times the amount of read data to the whole genome size is recommended for genome resequencing. If the amount of read data is insufficient, the quality of genotype estimation will be low. The Burrows–Wheeler Aligner (BWA) and Bowtie2 are commonly used computational tools for read mapping (Langmead and Salzberg 2012; Li and Durbin 2009, 2010). These mapping tools map reads on a reference genome sequence, taking mismatches and gaps into account. Results of read mapping are output in Sequence Alignment/Map (SAM) format or a compressed binary version of SAM format (BAM). Consequently, SAMtools and Genome Analysis Toolkit (GATK) estimate SNPs and indels from SAM or BAM data (DePristo *et al.* 2011; Li *et al.* 2009; McKenna *et al.* 2010; Van der Auwera *et al.* 2013). Information on variations is output in Variant Call Format (VCF) by SAMtools or GATK. Conventional mapping tools and polymorphism calling tools are listed in Tables 13.3 and 13.4.

Table 13.3 Read mappers.

Tool name	Application (read length) (●: short read, ○: long read, ◎: both short and long read)	Application (experiment type) (●: Genome-Seq, ○: mRNA-Seq)	URL
BWA	○	●	http://bio-bwa.sourceforge.net/
Bowtie2	●	●	http://bowtie-bio.sourceforge.net/bowtie2/index.shtml
TopHat2/Bowtie2	●	○	http://ccb.jhu.edu/software/tophat/index.shtml

Table 13.4 Miscellaneous tools.

Tool name	Application	URL
SAMtools	Variant detection with Genome-Seq map results, etc	http://www.htslib.org/
GATK	Variant detection with Genome-Seq map results, etc	https://www.broadinstitute.org/gatk/
Stacks	Variable loci detection with Genome-Seq map results or raw reads	http://creskolab.uoregon.edu/stacks/
Cufflinks	Transcript assembly, differential expression, and differential regulation for mRNA-Seq	http://cole-trapnell-lab.github.io/cufflinks/

13.4.2 GBS/RAD-Seq

Here, we review the latest Genome-Seq method, genotyping by sequencing (GBS)/restriction-site associated DNA sequencing (RAD-Seq) technology, which improves the cost-effectiveness of conventional Genome-Seq (Baird *et al.* 2008). By taking advantage of GBS/RAD-Seq, deeper Genome-Seq data are available with a relatively small number of short reads, because the technology concentrates the short reads in particular restriction enzyme flanking regions. At first, genomic DNA is digested by one or two restriction enzymes. To obtain genome-wide markers, it is recommended that the selected restriction sequences be widely distributed in the genome. Next, multiplex adapters are ligated to DNA fragments, and multiple samples are sequenced simultaneously by NGS sequencing. Each read among samples is classified according to index sequences inscribed within the multiplex adapters and used as barcode sequences to determine the origin of each read. Finally, read mapping and polymorphism calling can be performed as well as conventional genome resequencing as mentioned previously (see Section 13.4.1). If a reference genome is not available, Stacks (Table 13.4) software is useful to create markers from GBS/RAD-Seq (Catchen *et al.* 2011). Stacks software is able to build loci by *de novo* assembly and compare loci among multiple samples.

13.5 Plant Transcriptome Analysis by NGS

13.5.1 Transcriptome Analysis with Reference Genome Sequences

Big transcriptome data are now available by sequencing cDNA with NGS technology. If a reference genome sequence is available, gene expression levels can be estimated by the read mapping strategy. In this regard, short-read sequencing (e.g., using the Illumina or SOLiD system) rather than long-read sequencing (e.g., using the 454 or PacBio platform) is preferable in terms of cost-effectiveness for estimating gene expression levels because short-read sequencing outputs a larger number of reads than long-read sequencing. Bowtie2/TopHat2 (Table 13.3) is a major mapping tool for short mRNA-Seq reads (Kim *et al.* 2013). It aligns mRNA-Seq reads, considering splice junctions between exons. Cufflinks (Table 13.4) estimates gene structure, transcript isoforms and gene expression levels from the mapping results of Bowtie2/TopHat2 (Trapnell *et al.* 2010). The expression level of each transcript is shown as an FPKM value, which is an expression level normalized by the total number of mapped fragments (read-pairs) and exon length per gene (<http://cole-trapnell-lab.github.io/cufflinks/>; Mortazavi *et al.* 2008).

13.5.2 Reference-Free Transcriptome Analysis

If mRNA-Seq reads are obtained in an organism for which no reference is known, *de novo* assembly is usually performed to obtain expressed gene sets at first. For *de novo* assembly of mRNA-Seq reads, various assemblers are available with some limitations in read length (Table 13.2). For example, Oases, SOAPdenovo-Trans and Trans-ABYSS are specialized for short read assembly (Robertson *et al.* 2010; Schulz *et al.* 2012; Xie *et al.* 2014), while GS De Novo Assembler (Newbler), Trinity and MIRA4 are able to deal with both short reads and long reads (Chevreux *et al.* 1999; Grabherr *et al.* 2011). Some assemblers estimate translated sequences, transcription isoforms and RPKM/FPKM values. Properties of these assemblers are summarized in Table 13.2. For evaluation, in contrast to Genome-Seq assembly, the N50 values should not be considered because the length distribution of mRNA contigs will be skewed. To evaluate assembled transcripts, homology detection between the assembled transcripts and known proteins, for example, UniProtKB or NCBI non-redundant protein sequences obtained by a BLAST search, is suitable. Usually, several frameworks of annotations are assigned to the transcripts. The InterProScan integrated protein domain database (www.ebi.ac.uk/interpro/download.html) is a nice tool that annotates a transcript with functional domains, GO annotations, pathway annotations, and other information (Jones *et al.* 2014).

13.6 Plant Genome and Annotation Databases

13.6.1 TAIR (Arabidopsis)

TAIR (The Arabidopsis Information Resource) (Lamesch *et al.* 2012) is a comprehensive web database of the major dicot model plant, *Arabidopsis thaliana*. It consists of whole genome sequences, whole genome annotations (gene structures and gene functions), and a broad variety of omics data, and is one of the most comprehensive plant genome information databases. It features curated gene annotation with GO compliance, an intuitive user interface and broad-range omics

information. Currently the organization has a funding problem and is changing the access policy from free to a paid subscription. Now the potential users of TAIR must pay particular attention to the charging policy of the organization.

13.6.2 RAP-DB (Rice)

The RAP-DB (Rice Annotation Project Database) (Ohyanagi *et al.* 2006; Rice Annotation Project 2008; Sakai *et al.* 2013) is an integrated web repository of the model monocot plant, rice (*Oryza sativa* ssp. *japonica* cv. Nipponbare). It serves whole genome sequences and whole genome annotations (gene structures and gene functions) in an integrated manner. It includes manually curated gene annotations by jamboree-style manual curation meetings, and the annotations are kept updated. The current release of the genome data is Os-Nipponbare-Reference-IRGSP-1.0 and the corresponding gene annotation set.

13.6.3 Other Plants

Among the species from the family Solanaceae, tomato (*Solanum lycopersicum*) and potato (*Solanum tuberosum*) are some of the most prominent agricultural crops in human culture and history. In the Solanaceae family, the Sol Genomics Network (SGN) represents a comprehensive genome and annotation database. It stores complete genomic DNA sequences of both potato (Potato Genome Sequencing Consortium 2011) and cultivated tomato (Tomato Genome Consortium 2012). The SGN also serves draft genome DNA sequences of wild tomato, *Tabacum benthamiana* and other species. These genome sequences are distributed with gene annotation information via an FTP server and the GBrowse genome browser system. It also covers resources for the SOL-100 project (a comprehensive genome sequencing project).

In the family Cucurbitaceae, the Cucurbit Genomics Database (CuGenDB) (<http://www.icugi.org/cgi-bin/ICuGI/index.cgi>) is a comprehensive database containing genomic and annotation information. The genomes of cucumber (Huang *et al.* 2009) and watermelon (Guo *et al.* 2013) are available in the CuGenDB. It also covers a massive amount of resequenced cucumber genome information for a broad variety of cucumber lines, deciphered by the Beijing Genomics Institute (BGI; www.genomics.cn/en/index) in late 2013 (Qi *et al.* 2013). The CuGenDB also provides omics information such as annotations, ESTs, pathways, nucleotide variants, SSRs, and genetic maps.

13.7 Plant Omics Databases

In this section, databases providing large-scale information on the transcriptome and metabolome are described. Particularly, in Section 13.7.4, public databases providing omics information in model plants and crops are introduced.

13.7.1 Transcriptome Databases

Transcriptome data include a list of expressed genes, gene expression profiles, sequence data for mRNAs and/or ESTs, and related types of information.

For publications based on microarray experiments, it is nearly mandatory that the experimental data are freely accessible online. The Minimum Information About a Microarray Experiment (MIAME) standard (Brazma *et al.* 2001) gives guidelines for depositing experimental data into public databases such as the NCBI Gene Expression Omnibus (GEO) database (Barrett *et al.* 2013). Using the GEO DataSets database, information on experiments on microarray platforms can be searched and retrieved. Another database, GEO Profiles, provides gene expression profile data with graphical viewers.

Sequence data on expressed genes, cDNAs and ESTs, are stored in nucleotide sequence databases, such as INSDC. In the NCBI, sequence data on full-length cDNAs and mRNAs are available in the Nucleotide database. The EST and UniGene databases of NCBI have stored EST sequence data. In the UniGene database, ESTs and the mRNAs predicted as transcripts originating from each identical locus are categorized into a cluster. By taking advantage of the UniGene database, gene expression profiles estimated from the EST data are also available. The new ESTs (accessions) provided from INSDC are updated in the UniGene database on a monthly basis.

The DFCI Gene Index (Pertea *et al.* 2003) has also provided information on ESTs and clusters of ESTs. While the Gene Index databases are not frequently updated, they provide a consensus sequence (tentative consensus sequence or TC) for each cluster. It allows designing primers, mining TCs with a specific sequence pattern, and similar strategies. The Gene Index databases contain various annotations, such as ORFs, SNPs, metabolic pathways, and GO terms.

13.7.2 Gene Expression Network Databases

Gene expression networks (GENs) assist in identifying genes with similar expression profiles. Similarities in expression profiles suggest the existence of the same mechanism controlling the regulation of gene expression. Similar expression profiles of genes also indicate that they may possess the same biological functions (Yano *et al.* 2006). To efficiently identify genes with similar expression profiles, graphical viewers for GENs, such as CytoScape (Saito *et al.* 2012), Graphviz (Ellson *et al.* 2004), and Pajek (de Nooy *et al.* 2011) have been widely used. Web databases providing information on GENs have also been maintained. ATTED-II (Obayashi *et al.* 2014), OryzaExpress (Hamada *et al.* 2011) and RiceFriend (Sato *et al.* 2013), and TOMATOMICS (Kobayashi *et al.* 2014), respectively, store information on GENs in *Arabidopsis*, rice, and tomato.

13.7.3 Metabolic Pathway Databases

Information on metabolic pathways is available from KEGG (Kanehisa *et al.* 2014), BioCyc (Caspi *et al.* 2014), PlantCyc (Chae *et al.* 2012), and similar databases. KEGG is an integrated database for metabolic pathways of a broad variety of organisms. Metabolic pathways for each species in the KEGG database can be searched. With KAAS (KEGG Automatic Annotation Server), KEGG pathways can be predicted on the basis of sequence similarities.

The KaPPA-View4 database (Sakurai *et al.* 2011) provides information on metabolic pathways associated with genes showing similar expression profiles in major model plants such as *Arabidopsis*, rice, and tomato. Information on enzyme nomenclature is available from the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (Webb 1992). The information page on each enzyme has hyperlinks to web pages in protein and/or metabolic pathway databases, such as BRENDA (Schomburg *et al.* 2013), ExPASy (Artimo *et al.* 2012), KEGG, MetaCyc (Caspi *et al.* 2014), and PDB (Berman *et al.* 2014).

13.7.4 Other Databases for Omics Integration

Information on biological functions of genes and proteins offers important clues to categorize genes and proteins. Although functional annotations for genes and proteins are accessible from many public databases, the vast majority of functional annotations have been obtained by computational tools such as the BLAST program. As mentioned in the Introduction (Section 13.1), computational approaches bring large-scale annotations with low quality. Manual curation offers reliable annotations, though it is labor intensive and time consuming. To solve this problem, bioinformatics approaches have been proposed. The Natural Language Processing (NLP) method, which is one of the solutions, collects information on biological relationships (such as regulation, binding, and control) between genes and compounds by means of literature text mining. The Plant Omics Data Center (PODC) database stores information on GENs and functional annotations obtained by manual curation and NLP (<http://bioinf.mind.meiji.ac.jp/podc/>) (Ohyanagi *et al.* 2015). The current version of the PODC database contains information on omics data in *Arabidopsis*, rice, tomato, sorghum, grape, potato, *Medicago*, and soybean.

13.8 Conclusion

Here, we have summarized the current NGS technologies and omics databases deeply related to the plant sciences. For any plant science goal, the latest NGS technologies will be indispensable to facilitate research. Every year, NGS vendors release upgrades and new applications of technologies, requiring researchers to catch up with them. NGS innovations are always appearing (and sometimes quickly disappearing); for example, SMRT long reads by PacBio (<http://www.pacificbiosciences.com/>), Moleculo technology by Illumina (<http://www.illumina.com/technology/next-generation-sequencing/long-read-sequencing-technology.html>), and strand-specific mRNA sequencing (Zhong *et al.* 2011). The omics databases introduced in this chapter promise to be comprehensive data repositories suitable for offering a panoramic view to a broad range of plant scientists. In this NGS era, data planned to be acquired by future experiments may already have been uploaded in the public domain, meaning all that may be necessary is efficiently searching for it in databases. From this point of view, rather than conventional experimental biological literacy, database literacy may be the first priority of future plant scientists.

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14

Synergistic Plant Genomics and Molecular Breeding Approaches for Ensuring Food Security

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14.1 Introduction

Food security exists when all people, at all times, have physical and economic access to sufficient safe and nutritious food that meets their dietary needs and food preferences for an active and healthy life (FAO 2002). In the advent of the twenty-first century, climate change threatens the agro-economic status and agricultural productivity, leading to food insecurity globally. Different environmental parameters associated with climate change include increasing CO₂ concentration in the atmosphere, water shortages, and rising in atmospheric temperature adversely affect crop productivity. The process of global warming will lead to long term changes in weather conditions. In developing countries like India, it is predicted that for every 2°C rise in temperature (which has been predicted by 2030), the GDP (gross domestic product) will reduce by 5%. (Ranuzzi and Srivastava 2012). Rising temperature, changing precipitation patterns, and an increasing frequency of extreme weather events are thought to be the main reasons for reducing water availability and thus imparting a negative impact on crop production. Several interplaying factors, including increase atmospheric CO₂ concentration and rising atmospheric temperature and moisture content, lead to the emergence of pests and pathogens affecting crop yield. These factors dramatically reduce the crop yield by imposing stress on the crop plant in biotic and abiotic manners. Climate change will affect food security by influencing all components of global, national, and local food production systems, affecting the four dimensions of food security; namely food availability, stability of food supplies, access to food, and food utilization (Schmidhuber *et al.* 2007). In 2014, the world population was expected to be near about 7.5 billion. It will be a challenging task to feed this ever and fast increasing population in an era of climatic variability. To meet this rising food demand, development of high-yielding durable stress tolerant and climate resilient crop varieties is essential.

Most of these agronomic traits including yield, abiotic (drought, salinity, cold, high temperature, acidity, and sodicity), and biotic (evolution and emergence of new races of pathogen due to climate change) stresses are complex and quantitative in nature (Kujur *et al.* 2013a). Many major and several small effect genes/QTLs (quantitative trait loci) are involved in regulating these complex traits (Wang *et al.* 2008a). Henceforth, understanding the molecular basis and dissection of such complex quantitative traits is the prime objective of current plant genomics and molecular breeding research.

In current years, significant progress has been made in plant structural, functional and comparative genomics, epigenomics, metabolomics, and proteomics, which led to generate enormous information pertaining to known/candidate genes, transcription factors, molecular markers, epialleles, proteomes, and metabolites. The significant inputs derived by integrating these omics based approaches/resources are being utilized in genomics-assisted crop genetic enhancement studies, particularly through individual/combined approach of QTL mapping, trait association analysis, and genetical genomics. The process of genomics-assisted breeding has been accelerated with the availability of assays/approaches for large-scale and high-throughput marker genotyping and robust phenotyping of naturally occurring diverse germplasm lines and advanced generation mapping populations. The synergistic plant genomics and molecular breeding approaches thus could be an attractive approach to mine functionally relevant novel genes/QTLs and alleles regulating complex quantitative traits. The molecular tags obtained from such synergistic approaches can subsequently be introgressed into diverse crop genotypes, for instance, through marker-assisted selection, multi-parent advanced generation intercross (MAGIC), and genomic selection for their genetic improvement to develop high-yielding stress tolerant varieties.

14.2 Plant Genomics, Transcriptomics, Proteomics, and Metabolomics Resources

The advancement of sequencing technology enables the scientist community to uncover the hidden information specifically at genome, transcriptome, and epigenome level in a cost- and time-effective manner. Sequencing has traditionally been performed using first generation Sanger sequencing technology. In the early 2000s, the next generation sequencing (NGS) technologies; Roche 454/FLX Pyrosequencer, ABI SOLiD, and Illumina Solexa Genome Analyzer were discovered, which expedited the whole genome sequencing effort in many plant genomes, either individually or used along with Sanger sequencing. One of the biggest constraints in sequencing the genome is the presence of highly repeat-rich region in the genome. To overcome the problem associated with sequencing the repeat-rich regions in the genome, third generation sequencing technologies such as Pacific Bioscience (PacBio) that provide long (more than 5 kb) single molecule reads are expected to improve sequencing and assembly of repeat-rich plant genomes. The whole/draft genome sequencing efforts using the first-generation Sanger sequencing-based clone-by-clone and/or whole genome shotgun (WGS) and NGS-based WGS approaches have been accomplished in diverse crop genotypes. Using these approaches, around 60 plant genomes have been sequenced to date, including cereals (rice, wheat, maize, sorghum, barley, and *brachypodium*), legumes (lotus, *Medicago*, chickpea, pigeonpea, and soybean), vegetables (tomato, potato, melon, cucumber, hot pepper, and watermelon), fruits (banana, grape, papaya, apple, peach, chinese plum, strawberry, and sweet orange), and fiber crops (foxtail millet, mustard, flax, sesame, and cotton) (Michael and Jackson 2013). These complete/draft plant genome sequencing efforts have generated enormous genomic sequence resources, including structurally and functionally annotated protein-coding genes and transcription factors. Next generation sequencing also enables us to re-sequence the genome of diverse crop genotypes leading to generation of a huge number of genomic sequence resources for structural, functional, and comparative genome analysis. The genome sequences also shed light on the evolutionary aspect of the sequenced plants, thus facilitating identification of the genes underlying domesticated traits.

Macro-array analysis (suppression subtractive hybridization: SSH, and cDNA-AFLP: Amplified fragment length polymorphism), array-based whole genome transcriptome profiling (microarray chips, serial analysis of gene expression: SAGE, and massively parallel signature sequencing: MPSS), and currently, the whole genome NGS-based transcriptome sequencing/RNA sequencing (RNA-seq) assayed in different vegetative and reproductive tissues during developmental stages of diverse crop genotypes under normal growth and stress-induced conditions, are all currently underway. These sequencing efforts have expedited the generation of large-scale ESTs (expressed sequence tags), full-length cDNA sequences and unigenes (NCBI GenBank, www.ncbi.nlm.nih.gov) as well as numerous transcript sequences, including differentially expressed transcripts encoding the known/candidate genes (NCBI and the GEO database) globally. The enormous genomic and transcriptomic sequences are available with online public databases (NCBI (www.ncbi.nlm.nih.gov), EMBL (www.embl.de), EBI (www.ebi.ac.uk), DDBJ (www.ddbj.nig.ac.jp), The Institute for Genomic Research (TIGR) (<http://rice.plantbiology.msu.edu>), Phytozome (www.phytozome.org), and TAIR: www.arabidopsis.org) for unrestricted use. The transcriptome atlases for several crop plants, including rice and *Medicago*, have been generated to pave the way of understanding the complex gene expression networks at different developmental stages of crop plants. For instance, a cell type transcriptome atlas that includes 40 cell types from *japonica* rice shoot, root, and germinating seed at several developmental stages has been developed (Jiao *et al.* 2009). Another atlas of reproductive development in Nipponbare has also been developed (Fujita *et al.* 2010). In *indica* rice (IR64), transcriptomic dynamics across various stages of vegetative and reproductive development have been studied using whole genome microarray profiling (Sharma *et al.* 2012).

In the chickpea, to track the tissue specific gene expression, transcriptome dynamics across several tissues, including flower bud, pod, root, and shoot (chickpea transcriptome database: CTDB) have been developed (Garg *et al.* 2011a, b). In *Medicago*, a gene expression atlas that provides a global view of gene expression in all major organ systems of this species, with special emphasis on nodule and seed development, has been developed (Vagner *et al.* 2008).

In recent years, high-resolution maps of plant epigenome have been generated by profiling of all the epigenetic regulators (histone modifications and DNA methylation) across the whole plant genome (Bernstein *et al.* 2007). The combination of chromatin profiling, genomic tiling microarrays, and high-throughput NGS technologies (Eckardt 2009; He *et al.* 2011; Zhang 2008; Zhu 2008) have now expedited the generation of such epigenome maps in crop plants. Among different genome-wide methylation profiling approaches, the bisulfite conversion combined with high-throughput NGS, that is, BS-Seq (bisulfite-sequencing), is regarded as the gold standard for determining the methylation state of any cytosine in genomic DNA sequences of any plant genotype at single-base-pair resolution (Lister and Ecker 2009). Using this BS-Seq approach, for example, extensive genome-wide DNA methylation information has been generated in different tissues and/or developmental stages of diverse rice genotypes (Chodavarapu *et al.* 2012; Feng *et al.* 2010; He *et al.* 2010; Li *et al.* 2012; Yan *et al.* 2010; Zemach *et al.* 2010). These epigenome studies inferred a deep insight pertaining to the diverse role of the epigenetic marks in regulating growth, development, and stress responses in plants. The decoding of genetic information on differential epigenetic modifications of chromatin (histone) structure and DNA methylation patterns have significance to dissect the contribution of epialleles in controlling gene/promoter regulation and differential gene/transcript expression in diverse contrasting crop genotypes. At present, the availability of epigenome information, like DNA methylation pattern and histone acetylation, methylation, phosphorylation, and ubiquitination enrichment sites in diverse genotypes, has enriched the understanding of their epigenetic make-up (He *et al.* 2011; Kohler *et al.* 2012). A number of studies involving the effects of differential DNA methylation in regulation of rice endosperm biogenesis during seed development as well as gametogenesis in plants have been documented (Baroux *et al.* 2011; Zemach *et al.* 2010). The stress-responsive epigenomes have been studied to decipher the role of environmental signals for triggering DNA methylation and nucleosome histone post-translational modifications (Chinnusamy and Zhu 2009; Mirouze and Paszkowski 2011). Hence, the heritable natural epigenetic variants (epialleles) and informative epigenetic markers identified during epigenetic mechanisms can be exploited to broaden the plant phenotypic and genotypic variations for improving its adaptation to abiotic and biotic stresses (Manning *et al.* 2006; Mirouze and Paszkowski 2011; Zhong *et al.* 2013). For instance, a diverse array of rice epigenetic marks/regulators and large-scale epialleles based on genome-wide methylome profiling in target genomic regions of 12 rice chromosomes, and more specifically, in different sequence components, including promoter, genic, and inter-genic regions of genes and transposable elements (TE)-associated genes controlling many developmental-responsive pathway, have been identified. This includes identification of epialleles in *OsSPL14*, a SBP (squamosa-binding protein) like protein-encoding gene for regulating panicle branching and higher grain yield in rice (Miura *et al.* 2011). Recent analysis of cytosine methylation variants in an advanced generation bi-parental mapping population indicated their stable inheritance throughout multiple generations, which lead us to identify methyl-QTL regulating the phenotypic variations for different agronomic traits in rice (Schmitz *et al.* 2013a). The identification of natural epigenomic (methylome) diversity during seed developmental stages of diverse germplasm lines and their use in association mapping has suggested the role of stable and heritable epialleles in controlling phenotypic diversity (Schmitz *et al.* 2013b).

Proteomics is the study of the proteome in a complex biological system at a specific condition. Nowadays, NGS technology enables us to provide deep insight into the complex genomic networks of a plant genome including *Arabidopsis*, rice, wheat, sorghum, and tomato. This sequence information gives us an idea regarding the number of functional genes in a respective sequenced plant genome. The number of genes is found to be much lower compared to the number of proteins present within the same plant species, indicating the involvement of a complex mechanism to produce a greater number of proteins from a lower number of genes. The genomic information is unable to address all the problems arising at biochemical and physiological level of a plant species. Proteomics provides an opportunity to understand the mechanism and generation of different proteins in crop plants at a specific environmental condition. It also provides information about the interacting partners of different proteins, thus facilitating understanding of the genetic regulation and metabolic pathways within a plant species in a better way. These days, several techniques, including two-dimensional (2D) gel electrophoresis, fluorescence 2D difference gel electrophoresis (2DDIGE), isotope-coded affinity tag (ICAT), isobaric tag for relative and absolute quantitation (iTRAQ), and multidimensional protein identification technology (MudPIT) are available to analyze the proteome of an organism. Specifically, among these available assays, 2D gel electrophoresis, along with MALDI-TOF-MS (matrix-assisted laser desorption-ionization time-of-flight mass spectrometry), is widely used for proteome analysis of diverse crop plants. For example, Xu *et al.* identified nine proteins and several differentially expressed proteins during seed germination under salinity stress in soybean using 2D gel electrophoresis and MALDI-TOF-MS analysis (Xu *et al.* 2011a).

In another study, Cheng *et al.* used 2D gel electrophoresis and MALDI-TOF-MS analysis to analyze soybean seed during imbibition at chilling temperature and observed 40 proteins to be differentially expressed (Cheng *et al.* 2010). Using a proteomics approach, Dumont *et al.* identified 68 proteins during a study to decipher chilling response from cold acclimation in the pea (Dumont *et al.* 2010). During a comparative proteomic study to analyze salt tolerance in *Arabidopsis thaliana* and *Thellungiella halophile*, a total of 88 and 37 proteins from *Arabidopsis* and *Thellungiella* were identified using 2D gel electrophoresis and iTRAQ LC-MS (Pang *et al.* 2010). The role of protein phosphorylation in rice embryos during early stages of germination is revealed through a quantitative proteomic approach (Han *et al.* 2014). A total of 107 putative phosphoproteins have been identified in developing chickpea seedlings using the mass spectrometry method (Kumar *et al.* 2014). Sad1/UNC-84 (SUN1) Protein, a novel component in dehydration signaling, has been identified in chickpea by using mass spectrometry and 2D gel electrophoresis (Jaiswal *et al.* 2014). A number of freely accessible proteomics database are now available for retrieving the protein sequences and their related information from diverse cellular components of crop plants. The most widely used proteomics database are: UniProtKB (uniprot knowledgebase, www.uniprot.org/help/uniprotkb), NCBI Protein (www.ncbi.nlm.nih.gov/protein), PIR Protein information resource (<http://pir.georgetown.edu>), neXtprot (www.nextprot.org), HAMAP (high-quality automated and manual annotation of proteins, <http://hamap.expasy.org>), and OWL (www.bioinf.manchester.ac.uk/dbbrower/OWL/index.php).

Metabolomics is a systemic study in which comprehensive analysis is carried out to identify and quantify all the metabolites of a crop plant (Fiehn 2002). Metabolomics provides the opportunity to understand the interactions of different components in a biological system, thus facilitating the characterization of the biochemical response of a plant to a conditional perturbation. Metabolomics, in association with genomics, proteomics, and transcriptomics, can be considered to be the potential functional genomics tool to correlate the genotype with the phenotype for a particular trait of interest. Around 2×10^6 diverse metabolites are found to be present within the plant kingdom (Pichersky *et al.* 2000) creating a large scale biochemical diversity. It is, therefore, a challenging task to develop a complete metabolite profile at a genome wide level in plant. In order to address these problems, several cutting edge technologies, including thin layer chromatography, optical spectroscopy, nuclear magnetic resonance, and mass spectrometry, have been developed to identify and characterize the metabolites. For instance, in a study, Yang *et al.* identified 36 specialized metabolites from rice using MS/MS (tandem mass spectrometry) and NMR (nuclear magnetic resonance) analysis (Yang *et al.* 2013). Barros *et al.* used metabolomics tools to compare two GM (genetically modified) maize varieties with a near-isogenic non-GM variety and found 15 metabolites to be differentially expressed (Barros *et al.* 2010). The function of tryptophan in a genetically modified rice species was also revealed through the metabolic approach and the involvement of tryptophan in inter-tissue translocation was observed (Matsuda *et al.* 2010). A comparative metabolic profiling was performed in pigmented rice by using GC-TOF-MS (gas chromatography-time of flight-mass spectrometry) to find the correlation between primary and secondary metabolites and 52 metabolites, including seven secondary metabolites that were identified (Kim *et al.* 2011). Matsuda *et al.* conducted metabolic quantitative trait loci (mQTL) analysis in rice grains using inbred lines, and determined few loci affecting the levels of metabolites (Matsuda *et al.* 2012). A total of 121 metabolites in mature seeds of a wide panel *Oryza sativa japonica* and *indica* cultivars have been identified revealing the correlations between the metabolic phenotype and geographic origin of the rice seeds (Hu *et al.* 2014). A number of metabolomics databases with a rich repository of metabolomic information have been developed for many crop plants. The most widely used plant metabolomics database are AraCyc (www.arabidopsis.org/tools/aracyc/), ArMet (www.armet.org/), DOME (<http://medicago.vbi.vt.edu/dome.html>), MetaCyc (<http://metacyc.org/>), MapMan (<http://gabi.rzpd.de/projects/MapMan/>), MetNet (www.public.iastate.edu/wmash/MetNet/homepage.html), and MMCD (<http://mmcd.nmr.fam.wisc.edu/>).

14.3 Molecular Markers in Plant Genome Analysis

Molecular markers can be defined as the differences in the nucleotide sequence of DNA at corresponding sites on the homologous chromosomes that follow a simple Mendelian pattern of inheritance. DNA-based markers are advantageous over conventional ones and biochemical markers have the potential to provide a relatively unbiased estimation of genetic diversity and tag the genes/QTLs governing important agronomic traits in crop plants (Bennett 1994; Soller and Beckmann 1983). Molecular markers are a powerful tool for fingerprinting and genetic diversity analysis, tagging of useful genes/QTLs, construction of high-density comparative physical and genetic linkage maps, evolutionary studies, fine-mapping and map-based isolation/positional cloning of genes/QTLs, marker-assisted selection, and gene pyramiding in crop plants (Agarwal *et al.* 2008; Gupta and Rustgi 2004; Varshney *et al.* 2005). Various molecular marker technologies have been utilized to visualize DNA polymorphism (Staub *et al.* 1996). The most common molecular markers include Restriction

Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeat (ISSR), Amplified fragment Length Polymorphism (AFLP), Simple Sequence Repeat (SSR), Sequence Tagged Sites (STS), Cleaved Amplified Polymorphic Sequence (CAPS), and Single Nucleotide Polymorphism (SNP) (Semagn *et al.* 2006). Among these, PCR-based random molecular markers such as RAPD and ISSR are more commonly used in species in which there is a lack of DNA sequence information. RFLP is the first molecular marker to be developed and used for plant genome mapping (Weber and Helentjaris 1989). This marker revolutionized plant genome analysis by providing unlimited and high polymorphism rate, locus specificity and co-dominant inheritance with extensive genome coverage. However, due to certain constraints like low amenability to automation, requirement of larger quantity of DNA per assay, use of hazardous radioisotopes, and greater demands on time and labor, RFLP markers are currently not being used so frequently in gene mapping and marker-assisted selection programs. In spite of this, RFLP remains the most informative marker in comparative mapping of plant genomes (Guimaraes *et al.* 1997; Lagercrantz and Lydiate 1996). RAPD is a dominant marker-based PCR technique, which employs a single decamer primer of arbitrary sequences for differential amplification of genomic DNA from the genome (Rafalski and Tingey 1993; Williams *et al.* 1990). Being based on random primers, it does not require prior knowledge of sequence information for its design. However, utility of a desired RAPD marker can be increased by converting it into more reproducible informative marker (Paran and Michelmore 1993) termed the Sequence Characterized Amplified Region (SCAR). To overcome the limitation of reproducibility associated with RAPD, the AFLP marker system was developed by selective amplification of DNA fragments obtained by restriction enzyme digestion (Vos *et al.* 1995). The requirement of significant technical skills, laboratory facilities, financial resources, and high quality genomic DNA for complete restriction digestion and dominant inheritance has limited the use of AFLP markers in plant genetic analysis.

The available enormous genomic and transcriptomic sequences represent a valuable and cost-effective resource for identification and characterization of different kinds of informative sequence-based robust molecular markers; namely, STS, CAPS, SSRs, and SNPs by employing several computational software tools (Parida *et al.* 2006, 2009, 2010; Temnykh *et al.* 2001; Varshney *et al.* 2005). The STS marker was first conceived and used in genome analysis, including tagging and marker-assisted selection of useful genes/QTLs of crop plants (Gopalakrishnan *et al.* 2008; Huang *et al.* 1997; Murai *et al.* 2001; Singh *et al.* 2001). STS markers are considered robust and efficient in terms of their functionality, when they are derived from the expressed component of the genome such as gene sequences/ESTs/unigenes. The STS markers that are specifically derived from the transcribed sequence component of the genome usually show low polymorphic potential among genotypes. This problem, however, is circumvented by restriction digestion of the STS amplified PCR products, which detects polymorphism in the target site of a restriction enzyme internal to the two primer binding sites. This has led to development of a new marker system called the cleaved amplified polymorphic sequence (CAPS, Konieczny and Ausubel 1993). Among various kinds of sequence-based markers, the microsatellite and SNPs have been the preferred ones and most widely used in diverse applications of plant genetics, genomics, and breeding.

14.3.1 Microsatellite Markers

Microsatellites, or simple sequence repeats (SSRs), are 1–6 long nucleotide DNA motifs repeated in tandem up to 100 times at a locus (Litt and Luty 1989; Tautz 1989; Tautz and Schlotterer 1994; Weber and May 1989). SSRs are ubiquitous and present in both coding and non-coding regions of all prokaryotic and eukaryotic genomes (Toth *et al.* 2000; Zane *et al.* 2002). The sequences flanking the SSR repeat-motifs are unique in nature and thus used to design primers for amplification of specific locus. Variation in the number of repeat-units among individuals is due to unequal crossing over and/or replication slippage (Levinson and Gutman 1987) and results in different size amplicons and thus a hypervariable class of PCR based DNA markers. SSR markers are reliable, abundant, co-dominant, multi-allelic, chromosome-specific, and highly informative genetic markers that are well established for genetic analysis in crop plants. These markers are amenable to high-throughput genotyping and thus suitable for variety identification, molecular diversity analysis, population genetic applications, construction of genetic linkage maps, gene/QTL mapping, and marker-assisted selection (MAS) (Ellis and Burke, 2007; Garris *et al.* 2005; Nordborg *et al.* 2005; Ostrowski *et al.* 2006; Sharopova 2008). The presence of microsatellite repeat-motifs in plant was first reported by Condit and Hubbell (1991) in soybean (Akkaya *et al.* 1992) and wheat (Bryan *et al.* 1997), which opened a new source of PCR-based DNA markers for genome mapping. The microsatellite genetic linkage maps have been developed in many plant species including major cereals namely, wheat (Roder *et al.* 1998), rice (McCouch *et al.* 2002), maize (Sharopova *et al.* 2002), sorghum (Bhatramakki *et al.* 2000), and barley (Varshney *et al.* 2007). These markers were found suitable for many applications in plant structural, functional, and comparative genomics (Abe *et al.* 2003; Chabane *et al.* 2007; Garris *et al.* 2005; Gopalakrishnan *et al.* 2008; Gupta and

Varshney 2000; Han *et al.* 2006; Hanai *et al.* 2007; Hayden *et al.* 2006; Kohli *et al.* 2004; Mohan *et al.* 1997; Saal *et al.* 2001; Schmidt *et al.* 2001; Song *et al.* 2005; Suwabe *et al.* 2002; Xue *et al.* 2008) and thus are highly preferred for genetic and evolutionary studies in crop plants.

For the crop plants for which no sequence information is available, the use of selective hybridization-based microsatellite enrichment method namely, size fractionated genomic and cDNA libraries and microsatellite-enriched genomic libraries, is found to be expedient (Parida *et al.* 2009). This method is extremely popular and widely adopted for designing genomic microsatellite markers for several small and large genome crop species, including rice (Cho *et al.* 2000), wheat (Roder *et al.* 1998), maize (Sharopova *et al.* 2002), sorghum (Bhatramakki *et al.* 2000), barley (Ramsay *et al.* 2000), chickpea (Sethy *et al.* 2006), and *Brassica* (Suwabe *et al.* 2002). The advantages of SSR markers were partially offset by difficulties in the marker development, as laborious iterations of genomic DNA library screening with SSR probes and sequencing of a large number of SSR positive clones were involved (Zane *et al.* 2002). Amplification of SSR loci using conserved sequences across genera and species has been demonstrated in plants (Eujayl *et al.* 2004; Gutierrez *et al.* 2005). Cross-species amplification of tomato SSR sequences in potato (Provan *et al.* 1996) has suggested the utility of orthologous SSR loci as an additional source of markers for genetic improvement of minor or under-funded crop species. The useful characteristics of genic SSR markers have helped to identify SSR marker-based conserved orthologous set (COS) markers (Parida *et al.* 2006) and thus would extend the accessibility of such COS markers to other orphan crop species. These identified SSR-COS markers would be useful for further studies on comparative genome analysis, phylogenetics and development of syntenic networks for understanding the evolution of genes and genomes. However, the utilization of SSR-COS markers from one species generally results in lower level of amplification and polymorphism in other closely related species/genera and thus has limited the use.

The enormous genomic and transcript sequences (differentially expressed transcripts/genes) generated from diverse crop genotypes have the potential to develop a large number of informative genomic and genic SSR markers *in silico* at a genome-wide scale (Grover *et al.* 2007; Lawson and Zhang 2006; Parida *et al.* 2006, 2009) by specifically employing perl script module/ programs such as MISA (*MIcroSATellite*, <http://pgrc.ipk-gatersleben.de/misa/>) and the Simple Sequence Repeat Identification Tool (SSRIT, www.gramene.org/db/searches/ssritool). A web-based freely accessible relational database, *EuMicroSatdb* (*Eukaryotic MicroSatellite database*) has recently been developed (Aishwarya *et al.* 2007) for easy and efficient positional mining of SSRs from the sequenced eukaryotic genomes. For instance, about 18,828, 94,000, 71,000, 111,845, and 309,052 SSR markers have been developed from whole genome sequences including annotated protein-coding genes of rice, wheat, sorghum, chickpea, and pigeonpea, respectively (IRGSP 2005; Jain *et al.* 2013; McCouch *et al.* 2002; Paterson *et al.* 2009; Brenchley *et al.* 2012; Varshney *et al.* 2012, 2013). A large number of SSR markers have been successfully developed from the EST/gene sequences in several plant genomes, including rice (La Rota *et al.* 2005; Temnykh *et al.* 2000), wheat (Chabane *et al.* 2007; Wang *et al.* 2007), barley (Thiel *et al.* 2003; Varshney *et al.* 2006), and soybean (Choi *et al.* 2007). The unigene sequences have been used to develop a novel class of genic microsatellite markers designated as UniGene derived MicroSatellite (UGMS) markers for five cereal genomes; namely, rice, wheat, barley, maize, and sorghum (Parida *et al.* 2006). The utility of these markers in cross-transferability, physical, and comparative genome mapping, studying functional molecular diversity and establishing evolutionary relationships, specifically among monocot and dicot plant species, has been evaluated. A web-based freely accessible relational database *UgMicroSatdb* (*Unigene MicroSatellite database*) has been developed (Aishwarya and Sharma 2007) for mining and designing SSR markers from the multiple unigene input sequences covering 80 plant and animal genomes. The detailed comparative analysis of genomic/genic sequences generated from diverse crop genotypes has enabled us to produce a larger set of *in silico* polymorphic SSR markers for their immense use in large-scale genotyping applications. For example, *in silico* polymorphic genomic and genic SSR markers have been developed by comparing the genome and transcript sequences of *indica* 93–11 and *japonica* Nipponbare rice (~52,485 markers) and diverse *desi*, *kabuli*, and wild chickpea (~5000 markers) genotypes for their wider use in large-scale genotyping applications of crop plants (Garg *et al.* 2011a, b; Hiremath *et al.* 2011; Jhanwar *et al.* 2012; Zhang *et al.* 2007).

Alteration in the SSR repeats derived from the protein coding and non-coding untranslated regions (UTRs) of ESTs/genes might have significant consequences with regard to gene function (Young *et al.* 2000). The waxy gene in rice for instance, has been found to contain poly (CT)_n SSR in the 5'UTRs, whose length polymorphism is associated with amylose content (Bao *et al.* 2002). Variation in the length of SSR repeats in the promoters, UTRs, and coding sequences of genes has been associated with different agronomic traits including sugar metabolism in sugarcane, 100-seed weight in chickpea, protein content in maize, and light and stress tolerance in *Brassica* and *Arabidopsis* (Dresselhaus *et al.* 1999; Kujur *et al.* 2013b, 2014; Parida *et al.* 2010; Zhang *et al.* 2006). The SSR markers based on such sequence motifs could be “functional genetic markers” and thus useful for various applications in plant genetics, genomics, and breeding, including identification of genes/QTLs regulating important agronomic traits for crop improvement program.

14.3.2 Single Nucleotide Polymorphism (SNP) Markers

Polymorphism observed among accessions may arise either due to insertion/deletion of multiple nucleotide bases or single nucleotide substitutions. The detection of such variation has led to the development of a novel sequence-based molecular marker called single/simple nucleotide polymorphism (Wang *et al.* 1998). In the case of plant genomes, SNPs are the most basic unit of genetic variation and represent the commonest class of DNA based genetic markers (Rafalski 2002). The markers have gained considerable importance in plant genetics and breeding because of their wide genomic distribution, co-dominant inheritance, high reproducibility, and chromosome-specific location. The detection and assay of SNPs are highly amenable to automation (Gupta *et al.* 2001) and have the ability to reveal hidden polymorphisms where other methods fail (Bhatramakki *et al.* 2002) and thus useful for high-throughput genotyping in a far shorter period than the other markers. The SNPs are excellent genetic markers for various genotyping applications (Bhatramakki *et al.* 2002), including assessment of genetic diversity (Hamblin *et al.* 2007; Monna *et al.* 2006; Varshney *et al.* 2008), evolutionary studies (Caicedo *et al.* 2007; Varshney *et al.* 2007), construction of high resolution genome maps (Kim *et al.* 2007), detection of genome-wide linkage disequilibrium (Kim *et al.* 2007; Mather *et al.* 2007), patterns and population substructure (Schmid *et al.* 2006; Caicedo *et al.* 2007), association mapping of genes/QTLs controlling complex traits (Li *et al.* 2008; Ravel *et al.* 2006), and marker-assisted breeding (Dracatos *et al.* 2008; Li *et al.* 2008; Van *et al.* 2008) in many plant species. A number of SNP markers in plants have been identified to be associated with genes regulating various agronomic traits such as *betaine aldehyde dehydrogenase-2* gene for the fragrance trait (Bradbury *et al.* 2005), *starch synthase IIa* gene for starch gelatinization temperature (Bao *et al.* 2006; Waters *et al.* 2005), and *qSH1* (Konishi *et al.* 2006) and *Sh4* (Li *et al.* 2006) genes underlying QTLs with the seed shattering in rice, the *teosinte branched 1* gene for plant or inflorescence architecture in maize (Weber *et al.* 2007), *Lr1* (Tyrka *et al.* 2004), and *Pm3* (Tommasini *et al.* 2006) genes for leaf rust and powdery mildew resistance, respectively, in wheat, β -*amylase* gene for enzyme thermostability in barley (Chiapparino *et al.* 2003; Paris *et al.* 2001) and *Dwarf8* gene for flowering time in maize (Anderson *et al.* 2005). The SNP markers are useful for isolation of these economically important candidate genes harboring QTLs through map-based cloning (Tander *et al.* 2002).

The freely accessible whole genome sequences/pseudomolecules and transcriptomic sequence resources of multiple crop genotypes enabled rapid discovery and development of genomic and genic SNP markers *in silico* at a genome-wide scale. More than five million SNPs (www.ncbi.nlm.nih.gov/snp/?term=oryza) have been discovered by comparing the whole genome sequences of *indica* and *japonica* rice for high-throughput genotyping applications. Moreover, the whole genome resequencing of ~1000 rice germplasm lines and transcriptome sequencing of diverse rice accessions have led to the identification of 1.6–8 million SNPs (Huang *et al.* 2012b; Lu *et al.* 2010a, b; McNally *et al.* 2009; Xu *et al.* 2011a). Likewise, the comparison of genome and transcript sequences of diverse genotypes have identified about 1.32, 3.3, 15, 0.13, 3.67, and 5.4 million SNPs to date in wheat, maize, barley, chickpea, potato, and tomato, respectively, for their use in genome analysis (Brenchley *et al.* 2012; International Barley Genome Sequencing Consortium 2012; Jain *et al.* 2013; Potato Genome Sequencing Consortium 2011; Schnable *et al.* 2009; TGC 2012; Varshney *et al.* 2013). Specifically, in the chickpea, about 4.4 million SNPs, including ~76,084 SNPs in 15,526 genes, have been mined by *genome resequencing*-based genotyping of 90 *desi*, *kabuli*, and wild accessions (Varshney *et al.* 2013). The continuously growing EST databases have provided the opportunity for detecting SNPs in the transcribed regions of the genome either by exploiting the redundancy of gene sequences/assembled contigs or the diversity of genotypes represented within a database (Picoult-Newberg *et al.* 1999; Kota *et al.* 2008). Using the redundancy-based approach, a large number of SNPs have been successfully discovered in the EST sequences of several crop species, including soybean (Van *et al.* 2004; Zhu *et al.* 2003), maize (Batley *et al.* 2003), wheat (Somers *et al.* 2003), and barley (Rostoks *et al.* 2005) and utilized for genetic diversity and genome mapping studies. A major route for SNP discovery in genic sequence starts with an *in silico* comparison of either homologous gene or EST sequences and STS fragments from two or more closely related representatives of a given species (Bundock *et al.* 2006; Gupta and Rustgi 2004; Varshney *et al.* 2007).

A large number of different SNP genotyping assays have been developed based on various methods of allelic discrimination and detection platforms (Rafalski 2002; Tost and Gut 2005). Broadly, the majority of SNP genotyping assays are classified into gel- and non-gel based assays. Gel-based assays include use of PCR-RFLP, PCR-STS, and PCR-CAPS (Neff *et al.* 2002; Thiel *et al.* 2004; Varshney *et al.* 2007), single-strand confirmation polymorphism (SSCP, Ovesna *et al.* 2003), and sequencing of allele-specific amplicons (Caicedo *et al.* 2007; Hayashi *et al.* 2006; Varshney *et al.* 2007). The non-gel based assays of genotyping specifically include Denaturing High Performance Liquid Chromatography (DHPLC, Sivakumaran *et al.* 2003; Wolford *et al.* 2004), Primer extension as single base (SNuPE, Batley *et al.* 2003;

SNPlex, Lijavetzky *et al.* 2007), pyrosequencing (Ablett *et al.* 2006; Cordeiro *et al.* 2006), and mass spectrophotometry (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, MALDI-TOF)/Good assay. The non-gel based SNP genotyping assays usually have their own advantages or disadvantages, but they are highly automated with capacity for multiplexing and are thus useful for various high-throughput genotyping in many plant species. Among the available diverse non-gel based SNP genotyping assays used until now, the Illumina GoldenGate genotyping assay (www.illumina.com) relying on bead array technology and allele-specific extension adapter ligation has demonstrated its enormous applicability for high-throughput genotyping of SNPs in many crop plants, including small diploid barley (Sato and Takeda 2009) and maize (Jones *et al.* 2009), and the larger, more complex soybean (Hyten *et al.* 2008) and wheat (Akhunov *et al.* 2009) genomes.

14.4 Identification of Functionally Relevant Molecular Tags Governing Agronomic Traits

To expedite the identification of potential trait-influencing genes, QTLs, alleles, and haplotypes through genomics-assisted breeding for crop genetic enhancement, the use and/or integration of strategies like genetic/QTL mapping and association analysis have been considered. To achieve those, the large-scale validation and high-throughput genotyping of sequence-based robust genic and genomic SSR and SNP markers in natural germplasm collections (association panel) and advanced generation bi-parental mapping/mutant populations and their further integration/correlation with multi-locations/years, replicated field phenotyping data and has been initiated in many crop plants using the modern high-throughput genotyping assays and phenotyping platforms.

14.4.1 Plant Genetic Resources Rich in Trait Diversity

The germplasm resources, including cultivated varieties, breeding lines, landraces, and wild accessions representing diverse agro-climatic regions of the world available for diverse crop species, have been stored efficiently in different National and International germplasm repository centers, including the International Rice Research Institute (IRRI), the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), the National Bureau of Plant Genetic Resources (NBPGR), the International Centre for Agricultural Research in Dryland areas (ICARDA), and the National Plant Germplasm System-United States Department of Agriculture (NPGS-USDA). For example, about 102547 accessions of *Oryza sativa*, 1651 accessions of *O. glaberrima* and 4508 accessions of 22 wild ancestors of rice (McNally *et al.* 2009) and more than 20,000 germplasm lines of chickpea (Gaur *et al.* 2012) are now available at these centers. According to FAO reports (2012–2013), about 85,6158, 235,688, 466,531, 40,820, and 98,285 accessions of wheat, sorghum, barley, pigeonpea, and potato, respectively, are now accessible in different germplasm resource centers developed around the world for their large-scale phenotyping and genotyping. Considering the difficulties involved in genotypic and phenotypic characterization of these huge sets of available germplasm resources of crop species, efforts have been made currently to constitute the core and mini-core collections in several crops by identifying the largest amount of genetic diversity with a minimum number of accessions. As a result of the efforts of International institutes like IRRI, ICRISAT, and USDA, a set of 932, 242, 211, 238, 146, and 184 germplasm lines belonging to the core/min-core collections of rice, sorghum, chickpea, pearl millet, pigeonpea, and groundnut have been constituted from 55,908, 37,904, 16,991, 21,594, 13,632, and 15,490 accessions available for these respective crop species (Upadhyay *et al.* 2001, 2002; Zhang *et al.* 2011) utilizing both marker-based genotyping and phenotyping strategies and different precise statistical measures. These readily available core/min-core germplasm resources of many crop plants have been phenotyped at different geographical locations (multi-environment) for several years in fields for diverse important agronomic traits, including yield component and stress tolerance traits. Based on the phenotypic and genotypic characterization of germplasm lines, genotypes contrasting for different agronomic traits, including yield component and stress tolerance traits have been selected and utilized as parents for generation of advanced bi-parental and back-cross mapping populations, RILs (recombinant inbred lines), NILs (near isogenic lines), and DHs (double haploids) in many crop plants. Some of these selected contrasting accessions have been induced with different mutagens, including EMS (ethyl methanesulfonate) and γ -ray and generated mutant lines of diverse crop genotypes to identify functional mutation sites for qualitative and quantitative trait regulation. For instance, about 66,891 EMS, MNU (N-methyl-N-nitroso urea), sodium azide and γ -ray irradiated mutant lines (Till *et al.* 2007; Wu *et al.* 2005) of rice and 10000 EMS-induced mutant lines of chickpea are currently available (<http://tilling.ucdavis.edu>; www.iris.irri.org) for mining of novel trait-influencing alleles for their genetic improvement.

14.4.2 High-Throughput Phenotyping

To expedite the process efficient and precise phenotyping, a larger set of natural/mutant and mapping populations generated for many crop plants have recently been phenotyped for diverse complex yield, and stress component traits using automated modern high-throughput phenotyping and E (environmental)-typing platforms (Mir *et al.* 2012; Xu *et al.* 2012b). For high-throughput and precise phenotyping of complex quantitative traits in many crop plants, an International Plant Phenomics Network (IPPN) has been developed (Clark *et al.* 2011).

14.4.3 High-Throughput Marker Genotyping

The allele mining, inter-varietal validation, and high-throughput genotyping of sequence-based markers are the expedient approaches and thus extensively utilized at present to accelerate genomics-assisted crop improvement. The diverse mutant populations and natural germplasm collections (core and mini-core) available for many crop plants have been assayed through TILLING (targeting induced local lesions in genomes, Till *et al.* 2007) and EcoTILLING (Raghavan *et al.* 2007) to mine novel functional allelic variants in the known/candidate genes associated with agronomic traits. Moreover, high-throughput genotyping of sequence-based informative markers (SSRs and SNPs) in a larger set of core/mini-core germplasm lines, mapping populations, and mutant collections has been hastened, currently using various array-based next-generation sequencing and marker genotyping technologies like fluorescent dye-labelled automated fragment analyzer, TILING array, Illumina GoldenGate and Infinium assays, Fluidigm dynamic array, KASP (KBioScience Allele-Specific Polymorphism) profiling, MALDI-TOF, Affymetrix GeneTitan array, Reduced Representation library (RRL), and the Genotyping-By-Sequencing (GBS) assay. The automated fragment analyzer, MALDI-TOF, Illumina GoldenGate and Infinium assays, and KASP profiling have been considered to be very advantageous and utilized widely for high-throughput genotyping of prior mined SSR and SNP markers in many crop plants, including rice and chickpea (Gaur *et al.* 2012; Hiremath *et al.* 2012; Parida *et al.* 2012) with sub-optimal use of resources. The GBS assay has now been extensively utilized for simultaneous genome-wide discovery and genotyping of SNPs in diverse plant species (Morris *et al.* 2013; Poland *et al.* 2012; Sonah *et al.* 2013; Spindel *et al.* 2013). Thus, it has expedited the mining of novel functional allelic variants and their large-scale validation and genotyping at whole genome level for constructing high-resolution genome map as well as in efficient QTL and trait association mapping of diverse small and large genome crop plants.

14.4.4 Identification and Mapping of QTLs/Genes

Realizing the advantages of sequence-based robust SSR and SNP markers, high-throughput genotyping of these markers in advanced generation bi-parental mapping populations enabled us to construct high-density genetic linkage and functional transcript maps and hasten the process of identification and mapping of genes/QTLs associated with agronomic traits in many crop plants. For instance, about 4861, 388, 122, and 530 QTLs associated with yield component and stress (abiotic and biotic) tolerance traits have been identified and mapped in rice, wheat, chickpea, and tomato, respectively (Figure 14.1) by utilizing inter-/intra-specific high-density SSR and SNP marker-based genetic linkage maps (<http://archive.gramene.org/qlt>; <http://solgenomics.net/search/phenotypes/qlt>; Suresh *et al.* 2014; Varshney *et al.* 2013). The marker-based genetic linkage maps constructed and trait-specific QTLs identified and mapped on chromosomes of different crop species have now become a resource for generating more high-resolution integrated genetic, physical and genome maps (Varshney *et al.* 2014) as well as fine mapping and map-based cloning/positional cloning of trait-influencing genes/QTLs. These approaches traditionally been proved to be the most powerful tools for gene isolation and dissection of the complex quantitative yield and stress tolerance traits in crop plants. For constructing SSR and SNP marker-based high-density and integrated genetic linkage/transcript maps in several crop species, high-throughput next-generation whole genome and transcriptome sequencing have been successfully applied at present (Gaur *et al.* 2012; Hiremath *et al.* 2012; Huang *et al.* 2009; Xie *et al.* 2010). The constructed high-density genetic linkage maps have been integrated with sequence-based physical map and improved the resolution and accuracy of trait-specific genes/QTLs identification (Wang *et al.* 2011) by additional genome/gene-based fine-mapping and thus significantly expedited the process of fine mapping and map-based gene isolation and positional cloning of genes/QTLs in crop plants. Utilizing such strategies, for example, about 50 genes harboring the major QTLs associated with agronomic traits including grain size, heading date, plant height, prostrate growth, cold, salinity, lodging and submergence tolerance, and disease resistance, have all been cloned and characterized so far in rice (Miura *et al.* 2011). For many other crop species, the identification of genes/QTLs regulating traits of agricultural importance utilizing the fine mapping and positional cloning approach is underway. However, with the applications of NGS approaches,

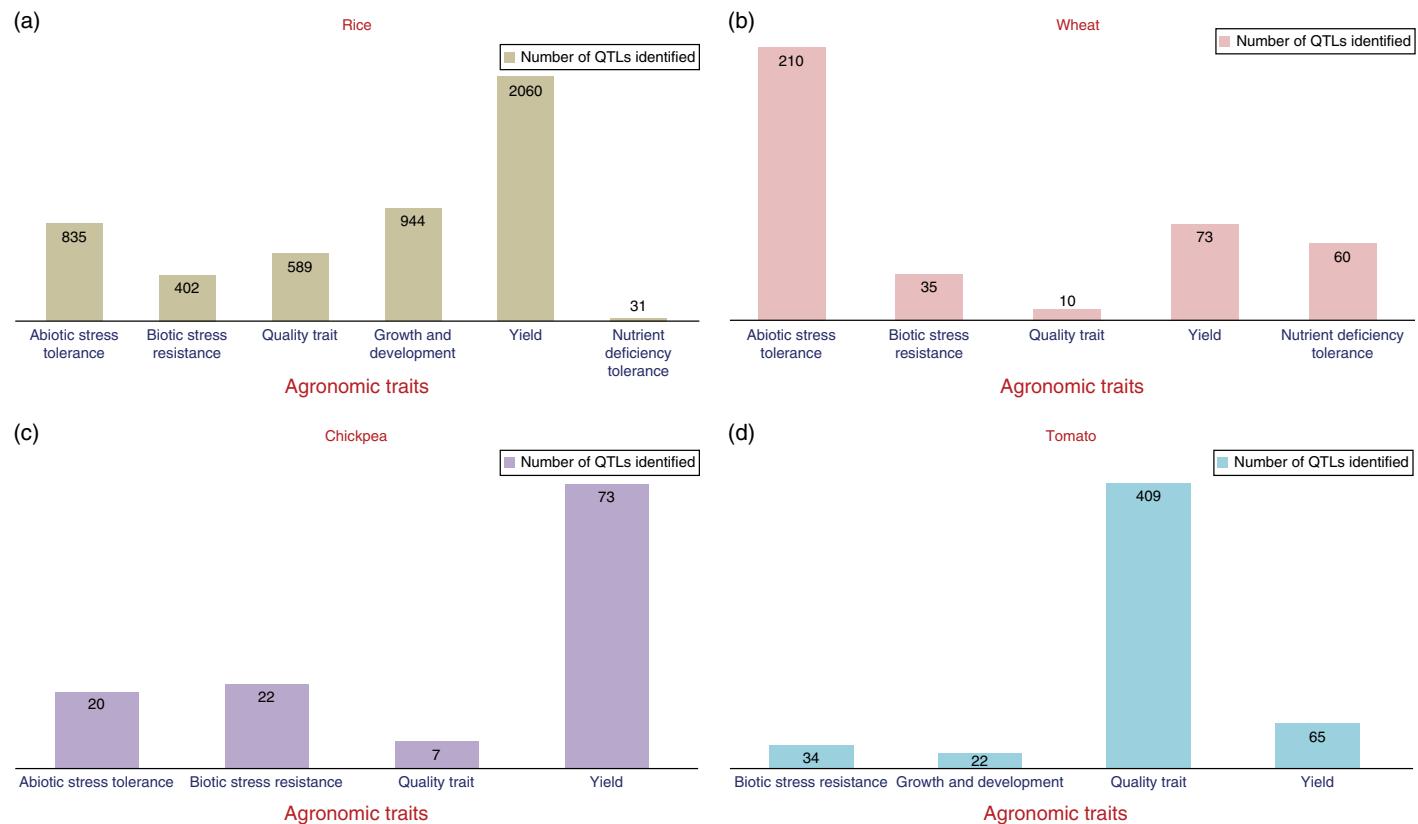


Figure 14.1 QTLs governing important agronomic traits, including yield component and abiotic/biotic stress tolerance traits identified and mapped in rice (a), wheat (b), chickpea (c), and tomato (d).

it is now possible to accelerate the identification and mapping of genes underlying the major as well as minor QTLs. An integrated high-throughput whole-genome resequencing and genotyping of large-scale SNPs in RIL mapping population has now found to be an effective approach for identification of candidate genes/QTLs associated with complex traits in crop plants based on recombination break-points determination (Huang *et al.* 2009; Yu *et al.* 2011a, b). Recently, a rapid method called “QTL-seq” has been developed for mapping of major genes/QTLs associated with blast disease resistance and seedling vigor in rice by whole genome NGS based resequencing of DNA from their two bulked RIL populations (Takagi *et al.* 2013). To identify candidate genes encoding transcripts and its regulatory sequences (transcription factors) involved in expression of quantitative traits in crop plants, the “genetical genomics”/“expression genetics” integrating the genetic or QTL mapping with transcript profiling was developed (Emilsson *et al.* 2008). The transcripts showing differential expression either by traditional macro-/micro-arrays or next-generation transcriptome sequencing to the whole genome and their correlation with QTL mapping enabled to identify “expression QTLs” (eQTLs) involved in the *cis*- and *trans*-trait regulation.

14.4.5 Trait Association Mapping

The candidate gene-based association mapping and genome-wide association study (GWAS) relying on the large-scale genotyping of informative SSR and SNP markers and robust field phenotyping information of naturally occurring core/mini-core germplasm lines (association panel) has now been considered to be an effective approach for identification of major and minor genes/QTLs and alleles regulating the simple qualitative and complex quantitative traits in crop plants (Li *et al.* 2011a; Zhao *et al.* 2011) (Table 14.1). The candidate gene-based association mapping done by utilizing the genotyping information of SNPs in different coding and regulatory sequence components of genes among a trait-specific association panel has the ability to identify genes/QTLs controlling yield contributing and stress tolerance traits in crop plants (Fan *et al.* 2009; Kharabian-Masouleh *et al.* 2012; Mao *et al.* 2010; Negrao *et al.* 2013; Parida *et al.* 2012; Sweeny *et al.* 2007). With the availability of huge high-throughput genome-wide SSR and SNP marker-based genotyping information of germplasm lines belonging to an association panel, the GWAS has now become a routine approach for high-resolution scanning of the whole genome to identify target genomic regions, including genes/QTLs (major and minor QTLs) associated with traits of agricultural importance in many crop species (Huang *et al.* 2010, 2012; Zhao *et al.* 2011). However, the integration of trait association mapping with traditional bi-parental linkage/QTL mapping has recently been implemented to identify functionally relevant robust genes/QTLs for dissecting the complex quantitative yield and stress component traits in crop plants. It is quite evident from the study of *GS3* (Wang *et al.* 2011) and *GS5* (Li *et al.* 2011b) genes/QTLs for grain size trait regulation and the metal transporter gene regulating aluminum tolerance (Famoso *et al.* 2011) in rice and acid phosphatase gene governs low-phosphorus tolerance in soybean (Zhang *et al.* 2014). An integrated approach by combining candidate gene-based association mapping with QTL mapping, differential transcript profiling, and LD (linkage disequilibrium)-based gene haplotyping has been developed recently to identify functionally relevant transcription factor genes and QTLs controlling 100-seed weight/seed size in the chickpea (Kujur *et al.* 2013b, 2014b). The trait-influencing molecular tags identified in diverse crop plants have the significance to be utilized for genomics (marker)-assisted crop improvement program.

14.5 Genomics-Assisted Crop Improvement

The functionally relevant molecular tags regulating the qualitative and complex quantitative traits, identified individually and/or integrated approach of traditional bi-parental linkage/QTL mapping, fine mapping/positional cloning, whole genome and candidate gene-based association mapping, and genetical genomics/eQTLs have now all been utilized for introgression, combining, and pyramiding into selected crop genotypes of interest through traditional and advanced genomics-assisted breeding approaches to develop superior high-yielding stress tolerant crop varieties. The introgression of functional natural genetic variations and favorable genes/QTLs/chromosomal segments identified from a larger set of germplasm lines, including landraces and wild species, particularly for yield and stress component traits, have been transferred into the cultivated genetic background for their crop improvement by employing approaches like introgression lines (ILs), advanced-backcross QTL (AB-QTL) analysis, association genetics, and multi-parent advanced generation intercross (MAGIC) population (Huang *et al.* 2012a; McCouch *et al.* 2007; Tan *et al.* 2008; Tian *et al.* 2006). The molecular tags showing major effects on qualitative and quantitative trait regulation have now been transferred into diverse crop genotypes for their genetic enhancement through marker-assisted selection (MAS), including marker-assisted back-crossing

Table 14.1 Significant progress made in association mapping for dissecting complex quantitative traits in crop plants.

Crop plants	Association panel	Size of association panel	Markers used	Candidate gene-based association mapping (CGAM)/ genome-wide association study (GWAS)		Associated traits	References
				GWAS	42		
Arabidopsis	Diverse accessions	95	Sequences	GWAS	4	Flowering time/ pathogen resistance	Aranzana <i>et al.</i> (2005)
	Diverse accessions	–	SSRs/SNPs	GWAS	–	Multiple traits	Ersoz <i>et al.</i> (2007)
	Natural accessions	184	SNPs	GWAS	42	Flowering time	Brachi <i>et al.</i> (2010)
	Diverse accessions	473	SNPs	GWAS	12	Climate-sensitive QTL	Li <i>et al.</i> (2010)
	Landraces	96	SNPs	GWAS	19	Downy mildew	Nemri <i>et al.</i> (2010)
	Diverse accessions	96	SNPs	CGAM	27	Flowering time	Ehrenreich <i>et al.</i> (2009)
	Diverse accessions	201	SNPs	GWAS	1	Root development	Meijón <i>et al.</i> (2014)
	Diverse accessions	950	SNPs	GWAS	18	Flowering time and grain yield	Huang <i>et al.</i> (2012b)
Rice	Diverse accessions	413	SNPs	GWAS	–	34 yield traits	Zhao <i>et al.</i> (2011)
	Diverse accessions	383	SNPs	GWAS	8	Aluminum tolerance	Famoso <i>et al.</i> (2011)
	Landraces	517	SNPs	GWAS	37	Agronomic trait	Huang <i>et al.</i> (2010)
	Diverse accessions	395	SNPs	CGAM	6	Amylose content and grain length	Zhao <i>et al.</i> (2010)
	Diverse accessions	392	SNPs	CGAM	11	Salinity tolerance	Negrao <i>et al.</i> (2013)
	Diverse accessions	89	SNPs	CGAM	43	Biotic and abiotic stress tolerance	Parida <i>et al.</i> (2012)
	Breeding lines	233	SNPs	CGAM	31	Starch synthesis	Kharabian-Masouleh <i>et al.</i> (2012)
	Cultivars	180	SSRs/CAPS	CGAM	1	Grain length	Fan <i>et al.</i> (2009)
Wheat	Diverse accessions	440	InDels	CGAM	2	Pericarp color	Sweeny <i>et al.</i> (2007)
	Cultivars	95	SSRs	GWAS	62	Kernel size and milling quality	Breseghello and Sorrells (2006)
	Diverse accessions	1055	DArTs	GWAS	48	Aluminum resistance	Raman <i>et al.</i> (2010)
	Breeding lines	276	DArTs	GWAS	15	Stem rust resistance	Yu <i>et al.</i> (2011a, b)
	Diverse accessions	235	SNPs	CGAM	19	Flowering time	Rousset <i>et al.</i> (2011)
	Germplasm accessions	108	SSRs	GWAS	14	Agronomic trait	Yao <i>et al.</i> (2009)
	Cultivars	372	SSRs	GWAS	90	Tan spot resistance	Kollers <i>et al.</i> (2014)
	Diverse accessions	287	DArTs	GWAS	11	Yield and yield component	Edae <i>et al.</i> (2014)

(Continued)

Table 14.1 (Continued)

Crop plants	Association panel	Size of association panel	Markers used	Candidate gene-based association mapping (CGAM)/ genome-wide association study (GWAS)			Associated traits	References
				GWAS	Markers associated with traits	Associated traits		
Maize	Inbred lines	282	SNPs	CGAM	6	Aluminum tolerance	Krill <i>et al.</i> (2010)	
	Inbred lines	305	SNPs	CGAM	9	Drought tolerance	Lu <i>et al.</i> (2010b)	
	Inbred lines	5000	SNPs	GWAS	36	Northern leaf blight	Poland <i>et al.</i> (2011)	
	Inbred lines	5000	SNPs	GWAS	51	Southern leaf blight	Kump <i>et al.</i> (2011)	
	Inbred lines	5000	SNPs	GWAS	785	Leaf architecture	Tian <i>et al.</i> (2011)	
	Elite inbred lines	553	SNPs	GWAS	1	Oleic acid content	Belo <i>et al.</i> (2008)	
	Inbred lines	282	SSRs	CGAM	4	Lycopene epsilon cyclase (<i>lcyE</i>)	Harjes <i>et al.</i> (2008)	
	Inbred lines	368	SNPs	GWAS	74	Oil biosynthesis	Li <i>et al.</i> (2013)	
Sorghum	Diverse accessions	107	SSRs	GWAS	14	Morphophysiological trait	Shehzad <i>et al.</i> (2009)	
	Germplasm accessions	206	SSRs	GWAS	4	Plant height	Wang <i>et al.</i> (2012)	
	Diverse accessions	242	SSRs	GWAS	43	Tiller number and kernel weight	Upadhyaya <i>et al.</i> (2012)	
	Germplasm accessions	202	SSRs	GWAS	13	Saccharification	Wang <i>et al.</i> (2011)	
	Diverse accessions	125	SSRs	GWAS	6	Plant height and brix	Murray <i>et al.</i> (2009)	
	Landraces	209	SNPs	CGAM	1	Aluminum tolerance	Caniato <i>et al.</i> (2014)	
	Diverse accessions	971	SNPs	GWAS	10	Agronomic trait	Morris <i>et al.</i> (2012)	
	Minicore collections	242	SNPs	GWAS	16	Maturity and plant height	Upadhyaya <i>et al.</i> (2013)	
Barley	Germplasm accessions	220	SSRs	CGAM	1	Flowering time	Stracke <i>et al.</i> (2009)	
	Inbred lines	102	SNPs	GWAS	85	Growth habit	Rostoks <i>et al.</i> (2006)	
	Cultivars	500	SNPs	GWAS	15	Anthocyanin pigmentation	Cockram <i>et al.</i> (2010)	
	Breeding lines	148	SNPs	GWAS	–	Winter hardiness	Von Zitzewitz <i>et al.</i> (2011)	
	Germplasm accessions	210	SNPs	CGAM	4	Agronomic trait	Xia <i>et al.</i> (2013)	
	Advanced hulled and hulless lines	329	SNPs	GWAS	15	Agronomic trait	Berger <i>et al.</i> (2013)	
Germplasm accessions	Germplasm accessions	184	SNPs	GWAS	19	Frost tolerance	Visioni <i>et al.</i> (2013)	

Table 14.1 (Continued)

Crop plants	Association panel	Size of association panel	Markers used	Candidate gene-based association mapping (CGAM)/ genome-wide association study (GWAS)			References
				GWAS	Associated with traits	Associated traits	
Chickpea	Germplasm accessions	242	SSRs/SNPs	CGAM	62	Seed and pod trait	Kujur <i>et al.</i> (2013b)
Soybean	Breeding lines	139	SSRs	CGAM	24	Iron deficiency chlorosis	Wang <i>et al.</i> (2008b)
	Germplasm accessions	96	SSRs	GWAS	11	Seed protein content	Jun <i>et al.</i> (2008)
	Cultivars	188	SSRs	GWAS	2	Aluminum tolerance	Korir <i>et al.</i> (2013)
Potato	Germplasm accessions	130	SNPs	GWAS	3	<i>Sclerotiana</i> <td>Bastien <i>et al.</i> (2014)</td>	Bastien <i>et al.</i> (2014)
	Diverse cultivars	221	AFLPs	CGAM	68	Quality traits	D'hoop <i>et al.</i> (2008)
	Diverse cultivars	123	NBS	CGAM	2	Disease resistance	Malosetti <i>et al.</i> (2007)
Tomato	Cultivars	205	SSRs/AFLPs	GWAS	32	Agronomic trait	D'hoop <i>et al.</i> (2014)
	Diverse collections	188	SNPs	GWAS	15	Fruit quality	Xu <i>et al.</i> (2013)
	Germplasm accessions	90	SNPs	GWAS	20	Fruit weight, soluble solid content, and locule number	Ranc <i>et al.</i> (2012)
Cotton	Diverse accessions	59	SSRs	GWAS	29	Morpho-physiological trait	Mazzucato <i>et al.</i> (2008)
	Diverse cultivars		SSRs	GWAS	20	Fiber quality	Abdurakhmonov <i>et al.</i> (2009)
	Germplasm accessions	329	SSRs	GWAS	42	<i>Verticillium</i> wilt resistance	Zhao <i>et al.</i> (2014)
Cultivars		356	SSRs	GWAS	17	Yield trait	Mei <i>et al.</i> (2013)

(MABC)/marker-assisted foreground and background selection. The genetic improvement of Basmati rice for yield, quality, and resistance to bacterial leaf blight and blast diseases has been performed by pyramiding the multiple genes/QTLs through MAS and MABC (Gopalakrishnan *et al.* 2008; Joseph *et al.* 2004; Singh *et al.* 2011; Sundaram *et al.* 2008). The sub-mergence tolerance in swarna using the *Sub1* QTL (Sequiningsih *et al.* 2009), drought tolerance in Nagina22 rice using *DTY1.1* QTL (Vikram *et al.* 1999), and drought tolerance and biotic stress tolerance in ICC 4958 and C 214 chickpea by using QTLs associated with root architecture and *Fusarium* and *Ascochyta* blight resistance (Varshney *et al.* 2013, 2014), have been enhanced through MAS. It has suggested the implications of using MAS for introgression of trait-influencing major effect molecular tags into selected crop genotypes for their genetic enhancement.

The complications in genetic background effects/epistasis and linkage drag of QTLs, as well as minor effects of minor and major QTLs/genes on complex trait regulation, have impeded the use of traditional MAS (QTL-MAS) approach to genetic enhancement of crop plants for complex quantitative traits. To overcome these intricacies, many novel advanced genomics-assisted breeding approaches such as marker-assisted recurrent selection (MARS), MAGIC and genomic/genome-wide

(haplotype) selection have been emerged currently in transferring and pyramiding the favorable alleles of minor effect genes/QTLs controlling the complex quantitative traits for genetic enhancement of crop plants for yield and stress tolerance (Chia and Ware 2011; Jannink 2010; Meuwissen *et al.* 2001). The available traditional and novel genomics-assisted breeding approaches provide clues for quantitative dissection of complex trait regulation and thus have the potential to expedite the complex trait genetic enhancement studies in diverse crop species (Figure 14.2).

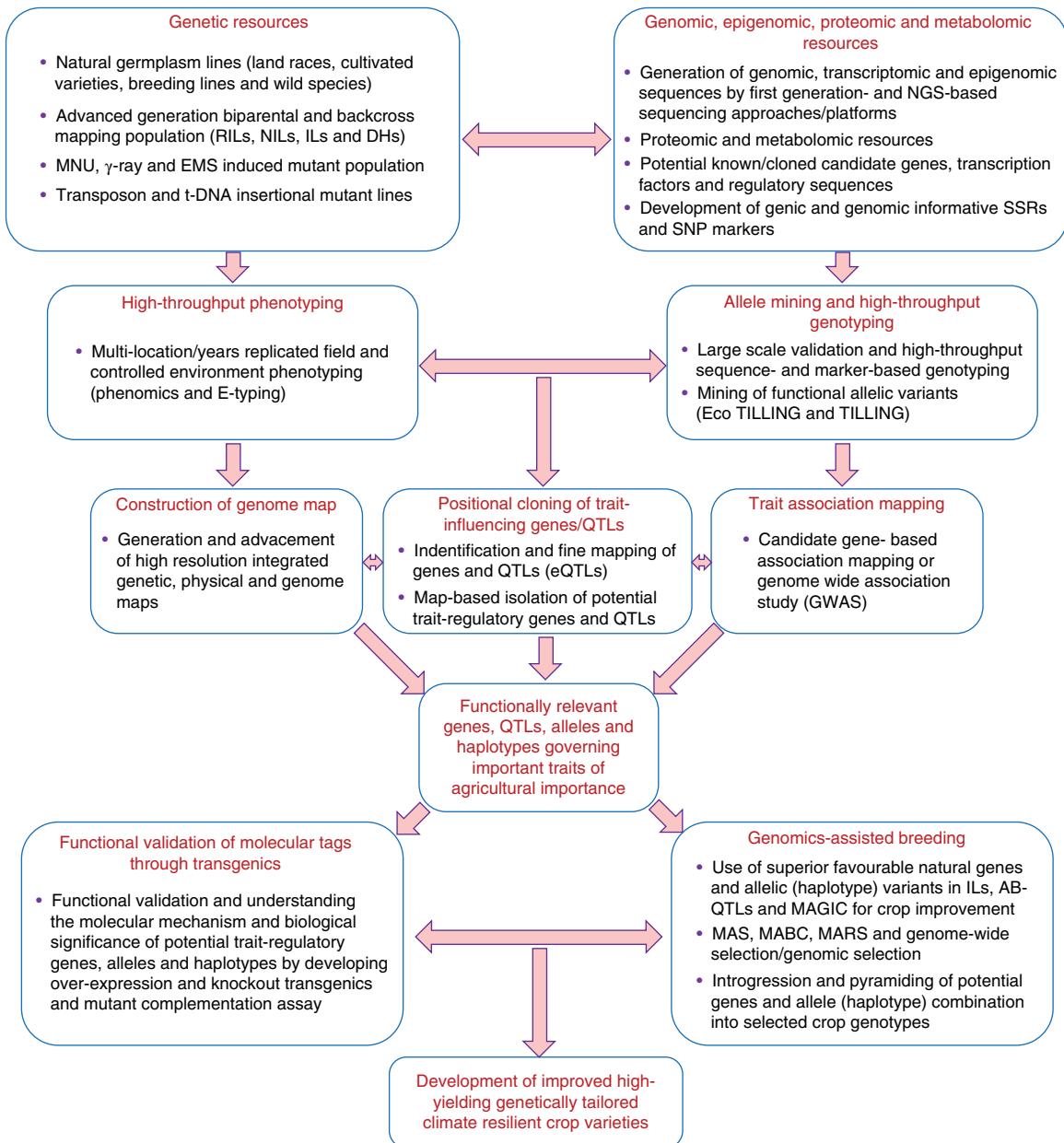


Figure 14.2 An integrated genomics-assisted breeding and transgenics approach for quantitative dissection of complex traits and genetic enhancement of crop plants.

Significant efforts have been made for the functional validation and understanding of the molecular mechanisms/biological significance of potential trait-regulatory genes, alleles, and haplotypes by developing over-expression and knockout transgenics, as well as t-DNA and transposon-mediated mutant complementation assays in crop plants. The integration of genomics-assisted breeding and transgenics has now proven to be the most promising approach for genetic enhancement of crop plants by manipulating diverse complex yield-contributing and stress-responsive traits. The diverse aspects specifically pertaining to genomics, epigenomics, proteomics, metabolomics, and genomics-assisted breeding, as highlighted in this review, can be applied individually and/or an integrated manner at different time points of study for effective genetic and molecular dissection of complex quantitative traits in crop plants. The inputs obtained from these combined strategies can be used further in various marker-assisted genetic improvement studies for developing stress tolerant high-yielding varieties in diverse crop plants.

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15

Combinatorial Approaches Utilizing Nutraceuticals in Cancer Chemoprevention and Therapy: A Complementary Shift with Promising Acuity

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15.1 Introduction

Cancer is among the leading causes of human death. According to an estimate, 14.1 million new cancer cases and 8.2 million cancer associated deaths occurred in 2012, compared with 12.7 million and 7.6 million, respectively, in 2008 (Bray *et al.*, 2013). Further, a substantive increase to 19.3 million new cancer cases per year by 2025 is projected. In the year 2012, more than half of all cancers incidences (56.8%) and deaths (64.9%) occurred in less developed regions of the world, and these proportions will increase further by 2025 (Bray *et al.*, 2013).

Among the causes of cancer both (1) internal factors (e.g., inherited mutations, hormones, and immune conditions) and (2) environmental factors (e.g., tobacco, diet, radiation, and infectious organisms) are involved. Several proposed hypotheses have supported that diet and environment greatly influence cellular function and health (Fernandes, 1989) and more than two-thirds of human cancers could be prevented through suitable lifestyle adaption. Furthermore, the association between diet and cancer is shown by the large variation in rates of specific cancers in different countries and by the observed changes in the incidence of cancer incidence in migrating. According to the World Cancer Research Fund, Asians have a 25 and 10 times lower incidence of prostate and breast cancer, respectively, than do citizens of Western countries, and the incidence rates for these cancers are enhanced significantly in Asians after they migrate to Western countries (www.dietandcancerreport.org). The importance of lifestyle factors in the development of cancer was also revealed by Lichtenstein *et al.* (2000). Inherited genetic flaw contribute only 5–10% of cancers (Hahn and Weinberg, 2002). Most genetic cancers are reported to be evolved by a series of mutations which resulted into interaction between gene and environment (Czene and Hemminki, 2002; Mucci *et al.*, 2001). These observations indicate that most cancers are not of hereditary origin and that individuals' lifestyle factors have intense influence on cancer incidence (Irigaray *et al.*, 2007). Although hereditary factors cannot be modified, lifestyle and environmental factors are potentially modifiable. The lesser hereditary influence of cancer and the modifiable nature of the environmental factors point to the preventability of most human cancers.

A diet rich in fruits and vegetables is strongly linked with reduced risk of cancer (Block *et al.*, 1992; Freedman *et al.*, 2008; Genkinger *et al.*, 2004). According to Doll and Peto (1981) ~35% of human cancer mortality is attributable to diet and could be evaded through precise lifestyle and dietary amendment. Epidemiological data determined predominant forms of cancer and related deaths (those of the lung and bronchus, breast, colorectal, and prostate cancers) are more prevalent in the western parts of the world and are much lower in Asian countries (Jemal *et al.*, 2010, 2011). This is due to prevalence of diet rich in vegetables and fruits with less fat/meat intake in many Asian countries (Jemal *et al.*, 2010, 2011). In conclusion, increasing consumption of fruits and vegetables is among one of the practical strategies to reducing the incidence of cancer. Fruits and vegetables provide a range of nutrients along with bioactive compounds or “nutraceuticals”, including vitamins (vitamin C, folate, and provitamin A), minerals (potassium, calcium, and magnesium), phytochemicals (flavonoids, phenolic acids, alkaloids, and carotenoids, etc.), and fibers (Table 15.1) (Liu, 2004). Phytochemicals reported to mediate their positive health benefits directly, by affecting specific molecular targets such as genes, or indirectly as stabilized conjugates affecting various metabolic pathways (Greenwald, 2004; Surh, 2003; Wattenberg, 1985).

Table 15.1 Dietary nutraceuticals and their natural source(s).

Phytochemicals class	Bioactive neutraceuticals	Food source	Molecular formula*
Alkaloid	Caffeine	Cacao, tea, coffee	C ₈ H ₁₀ N ₄ O ₂
	Theophylline	Cacao, tea, coffee	C ₇ H ₈ N ₄ O ₂
	Piperine	<i>Piper nigrum</i> , <i>Piper longum</i>	C ₁₇ H ₁₉ NO ₃
Monoterpenes	Trigonelline	<i>Trigonella foenum-graecum</i>	C ₇ H ₇ NO ₂
	Limonene	Citrus oils from orange, lemon, mandarin, lime, and grapefruit	C ₁₀ H ₁₆
Organosulfides	Indole-3-carbinol	Cruciferous vegetables	C ₉ H ₉ NO
	Phenylethyl isothiocyanate	Cruciferous vegetables	C ₉ H ₉ NS
	3,3'-diindolylmethane	Cruciferous vegetables	C ₁₇ H ₁₄ N ₂
	Sulforaphane	Cruciferous vegetables	C ₆ H ₁₁ NOS ₂
	Allixin	Allium vegetables	C ₆ H ₁₀ OS ₂
	Diallyl sulfide	Allium vegetables	C ₆ H ₁₀ S
	Allyl methyl trisulfide	Allium vegetables	C ₆ H ₈ S ₃
	S-allyl cysteine	Allium vegetables	C ₆ H ₁₁ NO ₃ S
Carotenoids	Beta-Carotene	Tomatoes	C ₄₀ H ₅₆
	Lycopene	Orange/yellow vegetables	C ₄₀ H ₅₆
	Lutein	Dark green vegetables	C ₄₀ H ₅₆ O ₂
Flavonoids	Epigallocatechin-3-gallate	Green tea	C ₂₂ H ₁₈ O ₁₁
	Quercetin	Black tea	C ₂₁ H ₂₀ O ₁₁
	Rutin	Vegetables, fruits	C ₂₇ H ₃₀ O ₁₆
	Curcumin	Turmeric	C ₂₁ H ₂₀ O ₆
	Anthocyanins	Vegetables, fruits, black tea	C ₃₁ H ₂₆ O ₁₂
	Apigenin	Parsley, onions, oranges, tea, chamomile, wheat sprouts	C ₁₅ H ₁₀ O ₅
Phenolic Acids	Capsaicin	Chilli peppers	C ₁₈ H ₂₇ NO ₃
	Ellagic acid	Black berries, raspberry	C ₁₄ H ₆ O ₈
	Gallic acid	Pomegranate, nuts	C ₇ H ₆ O ₅
Stilbenes	Pterostilbene	Blueberries and grapes	C ₁₆ H ₁₆ O ₃
	Resveratrol	Almonds, blueberries, grapes	C ₁₄ H ₁₂ O ₃
Isoflavones	Daidzein	Soy	C ₁₅ H ₁₀ O ₄
	Genistein	Soy	C ₁₅ H ₁₀ O ₅

*Molecular formulas from PUBCHEM COMPOUND Database.

15.2 Nutraceuticals

The term *Nutraceuticals* was first defined in 1989 by Stephen De Felice “as foods, food ingredients or dietary supplements that demonstrate specific health or medical benefits, including the prevention and treatment of disease beyond basic nutritional functions.” Until 1990, the concept of nutraceuticals was considered to be natural foods to provide energy and as recommended daily requirement in the body for health. The importance of nutraceuticals has been realized in recent decades as beneficial for human health in cardiovascular, cancer, and various developmental conditions (Baichwal, 1999). Subsequently, nutraceuticals came into sight as potential cancer preventive sources from diet. Increasing awareness of nutraceuticals as potent therapeutics and/or supplements with an accepted concept of nutraceuticals medicine developed as a new branch of “Complementary and Alternative Medicine”. Since then, major scientific efforts have been devoted to investigate the effects of active nutraceuticals component(s) on inhibition of cancer cell proliferation and oncogenesis (Figures 15.1 and 15.2). Various clinical and epidemiological studies have proven nutraceuticals as valuable diet supplements in cancer chemoprevention (Table 15.2).

Nutraceuticals, natural bioactive chemical compounds, range from isolated nutrients, herbal products, dietary supplements, and diets, to genetically engineered “custom” foods and processed products such as cereals, soups, and beverages (Dureja *et al.*, 2003). Chemically, nutraceuticals may be classified as isoprenoid derivatives (terpenoids, carotenoids, saponins, tocotrienols, tocopherols, terpenes), phenolic compounds (couramines, tannins, lignins, anthrocynins, isoflavones, flavonones, flavonoids), carbohydrate derivatives (ascorbic acid, oligosaccharides, non-starch polysaccharides), fatty acid and structural lipids (n-3 PUFA, CLA, MUFA, sphingolipids, lecithins), amino acid derivatives (amino acids, allyl-S compounds, capsaicinoids, isothiocyanates, indols, folate, choline), microbes (probiotics, prebiotics), and minerals (Ca, Zn, Cu, K, Se) (Malik *et al.*, 2008). The majority of cancer prevention evidence comes from *in vitro* and *in vivo* studies on phytochemicals, such as flavones, phytoestrogens, isoflavonones, genestein, curcumin, capsaicin, epigallocatechin-3-gallate, gingerol,

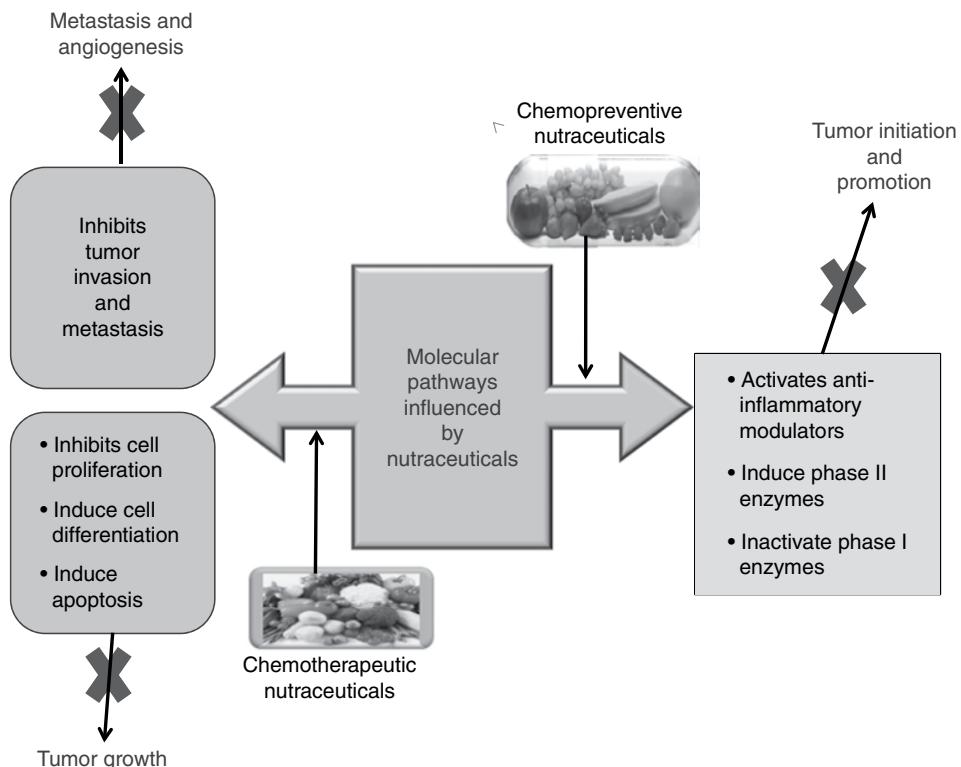


Figure 15.1 Molecular pathways affected by the nutraceuticals. Bioactive compounds in dietary sources are involved in regulating chemopreventive pathways and/or effective in influencing chemotherapeutic pathways.

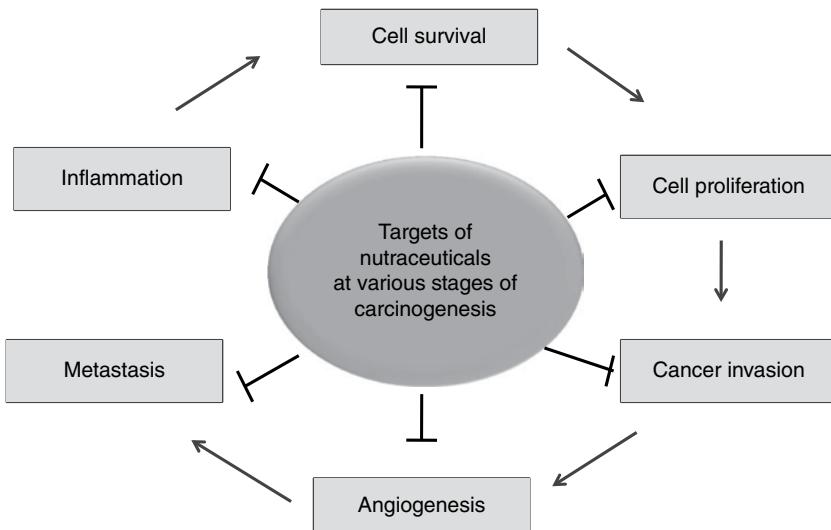


Figure 15.2 Nutraceuticals combinations as discussed in this chapter that can influence different pathways and can have profound effects on tumor growth and inhibition.

Table 15.2 Nutraceuticals in prevention or management of human cancer.

Nutraceuticals	Cancer	References
Soy isoflavones	Bone cancer	Rackley <i>et al.</i> 2006
Lycopene, genistein, red clover isoflavones, carotenoids	Breast cancer	Maskarinec <i>et al.</i> 2005; Powles <i>et al.</i> 2008; Tomar <i>et al.</i> 2008; Vantyghem <i>et al.</i> 2005; Wang <i>et al.</i> 2014
Cruciferous vegetables, carotenoids	Ovarian cancer	Tung <i>et al.</i> 2005; Han <i>et al.</i> 2014
Isoflavones, dietary fibers, cruciferous vegetables	Colon cancer	Ishikawa <i>et al.</i> 2005; Rossi <i>et al.</i> 2006; Sandler <i>et al.</i> 2008; Tse and Eslick, 2014
Citrus fruits, dietary antioxidants	Oesophageal cancer, Gastric cancer	Bjelakovic <i>et al.</i> 2008; Fock <i>et al.</i> 2008; Steevens <i>et al.</i> 2011
Silbinin, citrus flavonoids	Liver cancer	Fullerton <i>et al.</i> 1991; Siegel <i>et al.</i> 2014,
Isoflavones and antioxidants	Pancreatic cancer	Larsson <i>et al.</i> 2006, 2007; Sandler <i>et al.</i> 2008
Lycopene, soy, citrus, green tea, grape seed extract, proanthocyanidin, cruciferous vegetables	Prostate cancer	Rackley <i>et al.</i> 2006; Kirsh <i>et al.</i> 2007; Steinbrecher <i>et al.</i> 2009; Wang <i>et al.</i> 2014
Cruciferous vegetables	Bladder cancer	Parsons <i>et al.</i> 2013

lycopen, antioxidants, and so on (Malik *et al.*, 2008; Tripathi *et al.*, 2005). These days, the focus of most experimental studies is on exploring the molecular pathways by which these dietary nutraceuticals can alter the cancer cell environment.

Though various dietary nutraceuticals belonging to different classes of phytochemicals are evolving as promising cancer therapeutic entities, major obstacles exist to the successful use of individual nutritional compounds as preventive or remedial agents: (1) required high dose, (2) efficacy, and (3) bioavailability. One approach to conquering these problems is to employ the combinations of two or more dietary nutraceuticals with synergistic or additive effects. In fact, human diets can encompass many bioactive molecules, and thus evidence for synergy amid dietary compounds is a promising factor. Recently, emphasis has shifted to the development of novel combination cancer therapies using nutraceuticals with two or more nutratherapy or an effective synthetic drug (Cheah *et al.*, 2009; Saw *et al.*, 2010; Swamy *et al.*, 2008). Numerous studies have been carried out to prove that the use of two or more phytochemicals with different mechanisms of action may be more efficacious in treating the diseases and limiting the side-effects. In this article, the data for using nutraceuticals in combination with another phytomolecule(s) and/or anticancer drug to combat a variety of human cancers is reviewed.

15.3 Nutraceuticals and Key Events in Cancer Development

According to Kelloff and colleagues (2000), a chemopreventive agent act on signal transduction regulation at different levels: modulate hormone/growth factor activity, inhibit oncogenes activity and activate tumor suppressor genes, induce terminal differentiation, activate apoptosis, restore immunoresponses, inhibit angiogenesis, decrease inflammation, and scavenge ROS. In similar way, many dietary compounds have been identified as potential cancer chemopreventive agents, including polyphenols, isoflavins, carotenoids isothiocyanates, and organosulfur compounds. These phytochemicals can be divided into two major groups: (1) cancer-blocking prevents carcinogens to hit their cellular targets by several mechanisms including enhancing carcinogen detoxification, modifying carcinogen uptake and metabolism, scavenging reactive oxygen species (ROS) and other oxidative species, and enhancing DNA repair, and (2) cancer-suppressing agents: inhibiting cancer promotion and progression after the formation of preneoplastic cells has occurred (Figures 15.1 and 15.2).

15.3.1 Inflammation

The link between inflammation and cancers was noticed around 150 years ago; Virchow, in early 1863, indicated that cancers might be predisposed to occur at sites of chronic inflammation (Balkwill and Mantovani, 2001). Lately, developments show that acute inflammation contributes to the regression of cancer (Philip *et al.*, 2004). However, accumulated epidemiologic studies support that chronic inflammatory diseases are frequently associated with increased risk of cancers (Balkwill and Mantovani, 2001; Coussens and Werb, 2003; Philip *et al.*, 2004). Inflammation is linked to carcinogenesis and acts as a driving force for premalignant to malignant transformation of cells (Philip *et al.*, 2004). Available evidence also supports the notion that chronic inflammation is linked to various human cancers, including the skin, stomach, colon, breast, prostate, and pancreas (Karin and Greten, 2005; Li *et al.*, 2005b; Marx, 2004). It has been documented that the development of cancers from inflammation might be a process driven by inflammatory cells as well as a variety of mediators, including cytokines, chemokines, and enzymes, which altogether establish an inflammatory microenvironment (Coussens and Werb, 2002). Although this host response may suppress tumors, it may also facilitate cancer development via multiple signaling pathways (Yang *et al.*, 2005). Reportedly, a well established link between inflammation and cancer is via proinflammatory transcription factor nuclear factor-kappa B (NF- κ B) has been suggested. NF- κ B regulates the expression of genes involved in the transformation, survival, proliferation, invasion, angiogenesis, and metastasis of cancer cells. Several other potential targets, such as inducible ROS, nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and hypoxia-inducible factor-1 α (HIF-1 α), which have functions in both inflammatory responses and cancer development, have also been reported. Numerous nutraceuticals have been shown to exert their chemopreventive/anticancer activity by suppressing these inflammatory molecules.

15.3.2 Oxidative Stress

Oxidative stress is defined as an imbalance between production of free radicals and reactive metabolites (so-called oxidants or ROS), and their elimination by antioxidants. Humans are unwittingly exposed to several environmental abuses caused by chemicals (pesticides, fumes and automobile exhaust, etc.), ionizing and ultraviolet radiations, microbes, viruses and bacteria, and so on, which cause the formation of oxidants by metabolic activity within cells. When in excess, these oxidants cause an imbalance oxidative state and thus leading to oxidative stress by hyper production of ROS (Galli *et al.*, 2005; Reuter *et al.*, 2010). Usually, there is equilibrium between ROS generation and antioxidant defence systems and any imbalance between them leads directly to oxidative stress in cells. Under persistent environmental stress ROS is produced over a long time and thus causes significant damage to cell structure and functions, and may induce somatic mutations and neoplastic transformation (Fang *et al.*, 2009; Khandrika *et al.*, 2009). Indeed, cancer initiation and progression have been linked to oxidative stress by increasing DNA mutations or inducing DNA damage, genome instability, and cell proliferation (Visconti and Grieco, 2009). A variety of transcription factors, including NF- κ B, AP-1, p53, HIF-1 α , PPAR- γ , β -catenin/Wnt, and Nrf2, have also been reported to be activated by oxidative stress situation (Thannickal and Fanburg, 2000). Activation of these transcription factors can lead to the expression of over 500 different genes, including those for growth factors, inflammatory cytokines, chemokines, cell cycle regulatory molecules, and anti-inflammatory molecules. Oxidative stress activates inflammatory pathways leading to transformation of a normal cell to tumor cell, tumor cell survival, proliferation, chemoresistance, radioresistance, invasion, angiogenesis, and stem cell survival. Thus, cancer control can be achieved by decreasing the rate of oxidative stress and enhancing antioxidant defence mechanisms. The potential role of dietary antioxidants present in nutraceuticals in reducing the risk of cancer by suppressing the state of oxidative stress has been documented in the literature (Chu *et al.*, 2002; Dragsted *et al.*, 1993; Liu and Felice, 2007).

15.3.3 Antiproliferation

Dysregulated proliferation becomes visible as a characteristic feature of susceptibility to carcinogenesis. So far, the criteria for most cancer prevention strategies are normally associated with inhibition, reversion, or retardation of hyperproliferation. A large number of nutraceuticals have been proven, to their adorable potential, to inhibit human cancer cell proliferation, while less or non toxic to their regular counterpart (Han *et al.*, 2001; Singh *et al.*, 2011). The molecular mechanism of antiproliferation may involve the inhibition of the prooxidant process that causes tumor promotion, which pursues the initiation stage and is caused by metabolic activation of carcinogens to mutagens. In general, it is supposed that the formation of ROS (growth promoting oxidants) is a major catalytic event of the both tumor promotion and progression stages. Various tumor promoters (phorbol esters, TPA, DMBA, etc.) are reported to be strong activators of pro-oxidant enzymes, such as arachidonate metabolizing enzymes, xanthine oxidase, iNOS, COX, and lipoxygenases. The effectiveness of phytochemicals in inhibiting prooxidant enzymes, and therefore inhibiting tumor cell proliferation, is well documented. In addition, inhibition of polyamine biosynthesis could be a contributing mechanism to the antiproliferative activities of phytochemicals. Ornithine decarboxylase (ODC) is a rate-limiting enzyme in polyamine biosynthesis and has been linked with the rate of DNA synthesis and cell proliferation. Several studies show that dietary phytochemicals can inhibit ODC activity induced by tumor promoters, and thus cause a subsequent decrease in polyamine and inhibition of DNA/protein synthesis. Furthermore, dietary phytochemicals are also effective for inhibiting signal transduction pathways enzymes, for example, protein tyrosine kinase (Ferry *et al.* 1996), protein kinase C (PKC) (Lin *et al.*, 1997), and phosphoinositide 3-kinases (PI3Ks), which are involved in the regulation of cell proliferation.

15.3.4 Cell-Cycle Arrest

Perturbations in cell-cycle progression may account for the anticarcinogenic effects of nutraceuticals. Mitogenic signals commit cells to entry into a series of regulated steps allowing navigate of the cell-cycle. Cyclin dependant kinases (CDKs) have been recognized as key regulators of cell-cycle progression. Alteration and deregulation of CDKs' activity are among the pathogenic hallmarks of neoplasia. A number of cancers are associated with hyperactivation of CDKs as a result of mutation of the CDK genes or CDK inhibitor. Therefore, inhibitors or modulators of CDK(s) would be of interest to explore as novel therapeutic agents in cancer (Lim *et al.*, 2014; Senderowicz, 1999). Checkpoints at both G1/S and G2/M of the cell-cycle in cultured cancer cell lines have been found to be perturbed by phytochemicals such as curcumin, genistein, quercetin, daidzein, luteolin, apigenin, and epigallocatechin 3-gallate. Studies from different laboratories also revealed that dietary phytochemicals could induce cell-cycle arrest during either G1 or G2/M by the inhibition of all CDKs.

15.3.5 Apoptosis

Apoptosis usually occurs during development and aging and as a homeostatic mechanism to maintain cell populations in tissues. Apoptosis also happens as a defense mechanism, such as in immune reactions, or when cells are damaged by disease or noxious agents (Norbury and Hickson, 2001). The equilibrium between cell proliferation and apoptosis is a decisive step in the maintenance of homeostasis. However, there are wide diversities of stimuli and circumstances, both physiological and pathological, which can elicit apoptosis. Defective apoptosis represents a key contributory factor in the development and progression of cancer. Studies have shown an elevated incidence of apoptosis in spontaneously regressing tumors and in tumors treated with anticancer drugs or irradiation (Kerr *et al.*, 1994). Furthermore, the ability of cancer cells to escape engagement of apoptosis plays a significant role in their resistance to conventional therapeutic regimens. Cancer cells can overrun apoptosis mainly via two pathways: extrinsic (receptor mediated) and intrinsic (mitochondria mediated). The extrinsic pathway is triggered by a complex set of antiapoptotic and proapoptotic proteins, including caspase family proteins, Bax, B cell lymphoma (Bcl)-2 family proteins, cytochrome c, apoptotic protease activating factor (Apaf)-1, and death receptors (APO-1/TRAIL). The intrinsic pathway is initiated by cellular developmental signals or as a result of severe cellular stress, including DNA damage. Some antiapoptotic proteins, such as Bcl-2 and Bcl-2 extra large (Bcl-xL), are over-expressed in many cancer types. Therefore, selective downregulation of antiapoptotic proteins and up regulation of proapoptotic proteins in cancer cells offer promising therapeutic interventions for cancer treatment. A number of nutraceuticals, mostly derived phytochemicals from dietary or medicinal plants, have shown prospective to reduce cancer by inducing apoptosis.

15.3.6 Transforming Growth Factor- β (TGF- β)/Smad Signaling Pathway

This acts as negative regulator of cell proliferation and loss of function of its receptor is involved in the carcinogenesis (Tang *et al.*, 1998; de Caestecker *et al.*, 2000; Massague and Blain, 2000). TGF- β receptor activates the SMAD pathway (a complex group of factors: SMAD2–SMAD3–SMAD4 complexes) that, upon phosphorylation and subsequent nuclear translocation, bind co-activators and co-repressors modulating gene transcription (Samanta and Datta, 2012). Similar to the TGF- β receptor, mutations that inactivate Smad signaling pathway are also concerned to cancer development. Therefore, chemopreventive agents able to restore TGF- β receptor and Smad(s) functions are thought to be potential cancer chemo preventers. Further, a link between NF- κ B and TGF- β pathway is symbolized by Smad7 that suppresses TGF- β signaling. Several cytokines are involved in the inflammatory process and promoting carcinogenesis via activating NF- κ B and STAT1 that, in turn, upregulate SMAD7 (Bitzer *et al.*, 2000; Karin *et al.*, 2002; Ulloa *et al.*, 1999). TGF- β signaling has affected Smad-independent pathways, including the Erk, SAPK/JNK, and p38 MAPK pathways.

15.3.7 β -Catenin

β -Catenin is a component of the cell to cell adhesion machinery and represents a key element in cytoskeleton formation linking E-cadherin to actin filaments (Aberle *et al.*, 1996). The involvement of β -catenin in tumorigenesis depends on its nuclear activity as transcription factor (Morin, 1999). In normal conditions, cytoplasmic, β -catenin is rapidly degraded by ubiquitin-mediated proteasomal degradation (Munemitsu *et al.*, 1996; Orford *et al.*, 1997; Rubinfeld *et al.*, 1996). When stabilized following several proliferating stimuli, such as WNT signaling and other growth factors, β -catenin translocates to the nucleus and activates transcription of genes involved in cell cycle control and proliferation. Researchers have been proposed it as a potential phytochemical target in chemoprevention (Surh, 2003).

15.4 Nutraceuticals in Combinatorial Therapy of Human Cancer: A Pledge of the Future

Numerous studies have focused on unravelling the cancer protection properties of dietary phytochemicals independently in various clinical and epidemiological studies. Combination therapies, a new interesting approach to enhancing curative and preventive efficacies of these nutrients, have recently come to light. This approach is based on the factual information available in research data and using the potent properties of one with another to enhance synergistic/additive actions. The additive/and synergistic anticancer effects of nutraceuticals may be attributed to their potent antioxidant potential, and the benefit of a diet rich in fruits and vegetables is credited to the complex mixture of nutraceuticals present in whole food (Liu, 2004; Shukla and George, 2011). In the area of cancer prevention or treatment, combinatorial approaches can be of the following types: (1) a phytochemical and an effective drug, (2) two or more dietary phytochemicals, (3) a synthetic dietary phytochemical and an effective drug, and/or (4) a synthetic phytonutrient and a dietary phytochemical.

The aim of using natural compounds in diets is to render the chemopreventive properties of the compounds to tissues. Numerous studies have shown that a single dosage of compounds used alone is effective for chemoprevention. The problem faced is the inability to achieve high serum concentrations *in vivo*. Although combination studies are just beginning to surface as more prominent approaches in clinical treatment, studies, though limited, have shown that synergistic effects of the compounds achievable at much lower doses than when compounds are used alone. In the context of this, this article summarizes the findings of researchers that have worked with combinations of two or more nutraceuticals belonging to different or same chemical classes of compounds.

15.4.1 Nutraceuticals in Cruciferous Vegetables: Potential for Combination Therapy

Common nutraceuticals present in cruciferous vegetables are indole-3-carbinol (I3C), 3,3'-diindolylmethane (DIM); and the isothiocyanates (ITCs). Recently epidemiological evidence has demonstrated the fact that a reduced risk of human cancers is associated with cruciferous vegetable intake (Egner *et al.* 2014). Follow-up studies have been attributed to the protective activity to the metabolic products of glucosinolates, a class of secondary metabolites produced by crucifers. Indole-3-carbinol (I3C) and isothiocyanate (ITC) are breakdown products of the glucosinolates glucobrassicin and gluconasturtiin, respectively, and are thought to be involved in carcinogen inactivation by P450 enzymes (Plate and Gallaher, 2006). Compared with other families of vegetables, cruciferous vegetables contain an abundance of isothiocyanates

(ITC), which have strong anticarcinogenic potential. ITCs and I3C have been shown to have substantial chemopreventive activity against various human malignancies (Watson *et al.* 2013). Some of the widely studied ITCs that have potent anti-cancer effects are allylisothiocyanate (AITC), benzylisothiocyanate (BITC), phenethylisothiocyanate (PEITC), and sulforaphane (SFN). Cancer cells contain many anomalous signaling pathways that direct to drug resistance and therapy failure. Combination therapy is known to destroy cancer cells more effectively through diverse mechanisms simultaneously. Cruciferous vegetables nutraceuticals exhibit a diverse range of cellular targets for anticancer effects and this potential makes them well deserving candidate(s) for combinatorial therapeutic approaches. Several combination strategies have been tested in various preclinical studies by combining these nutraceuticals with other phytochemicals or among themselves or with conventional anticancer therapies.

15.4.2 Indole-3-Carbinol (I3C) and Combinations

I3C is a substance found in cruciferous vegetables such as broccoli, Brussels sprouts, cabbage, collards, cauliflower, kale, mustard greens, turnips, and rutabagas. I3C is used for prevention of breast cancer, colon cancer, and other types of cancer. The National Institutes of Health (NIH) has reviewed I3C as a possible cancer preventive agent and is now sponsoring clinical research for breast cancer prevention.

15.4.2.1 I3C and Silibinin

Combinations of low doses of I3C with silibinin could inhibit lung tumorigenesis without causing undesirable side effects (Dagne *et al.*, 2011). Exposure of lung cancer cells (A549 and H460 cells) to a mixture of I3C (50 μ M) + silibinin (50 μ M) for 72 h caused inhibition of cell growth and activation ERK and Akt and induction of apoptosis, whereas the individual agents did not have any effect. When I3C (10 μ M/g diet) + silibinin (7 μ M/g diet) were given to mice pretreated with 4-(methylnitrosamino)-1-(3-pyridyl)-1-butane, multiplicities of tumors on the surface of the lung and adenocarcinoma were reduced by 60 and 95%, respectively. Also, the expression of p-Akt, p-ERK and cyclin D1 and cleavage of poly (ADP-ribose) polymerase (PARP) were more robustly altered by the combination than when used alone, suggesting that the chemopreventive activities of the mixture were mediated, via modulation of these proteins.

15.4.2.2 I3C and Resveratrol

A study by Raj *et al.* (2008) revealed a clear synergistic action of I3C+Res used in combination in SK-OV-3 ovarian cancer cells. The cancer cell antigen CA125 was inhibited by either I3C or Res treatments. In contrast, basal nitric oxide production was inhibited by I3C and I3C+RE but not by Res alone.

15.4.2.3 I3C and Genistein

Cotreatment with I3C (300 μ M/l) and genistein (40 μ M/l), synergistically suppressed the viability of human colon cancer cells (HT-29 cells) to 87.0% (after 24 h) and 52.6% (after 48 h) (Nakamura *et al.*, 2009). However, at these concentrations each used alone was ineffective. Researchers suggested that cotreatment with I3C+genistein induces apoptosis through the simultaneous inhibition of Akt activity, downregulation of XIAP and survivin and progression of the autophagic process.

15.4.3 Phenethylisothiocyanate (PEITC) and Combinations

PEITC is mostly found in brassica vegetables such as broccoli, cabbage, Brussels sprouts, and cauliflower. The glucosinolate-derived PEITC has recently been demonstrated to reduce the risk of prostate cancer (Yu *et al.*, 2013). It blocks initiation and post-initiation progression of carcinogenesis (Xue *et al.*, 2014). The major mechanisms by which PEITC protects against prostate cancer involve the induction of cell cycle arrest and apoptosis (Singh *et al.*, 2004; Xiao *et al.*, 2005), inhibition of carcinogen activation via modulation of cytochrome P450-dependent monooxygenases and enhancement of the antioxidant response element dependent carcinogen detoxification enzyme (Yu *et al.*, 2013). This observation has been supported by epidemiological studies showing that consumption of cruciferous vegetables has an inverse effect on prostate cancer risk. From 2012, PEITC was being tested in clinical trials for its ability to prevent the development of lung cancer in smokers (<http://clinicaltrials.gov/ct2/results?term=PEITC+AND+cancer>).

15.4.3.1 PEITC and Curcumin

Cancerous cells express various surface receptors that propagate cellular growth; therefore targeting such receptors can be an effective chemotherapeutic approach. Curcumin (obtained from *Curcumin longa*), has been shown to inhibit the phosphorylation of EGFR, Akt signaling pathway, and negatively regulate NF- κ B. The combined treatment with low doses of PEITC and curcumin has been shown to suppress human prostate cancer cell growth in *in vitro* as well as in *in vivo* mouse models of prostate cancer via inhibition of EGFR, Akt, and NF- κ B signaling pathways (Kim *et al.*, 2006; Khor *et al.*, 2005). Supplementing AIN-76A diet (10–16 weeks) with PEITC (0.05%) or curcumin (2%) both alone and in combination (0.025% PEITC and 1% curcumin) significantly decreased the incidence of prostate tumor formation. Furthermore, a decreased proliferation, increased apoptotic index, and downregulation of the Akt signaling pathway was also noted (Barve *et al.*, 2008). These findings lucidly evidenced the merits of PEITC and curcumin combination in prostate cancer chemoprevention.

15.4.4 Sulforaphane (SFN) and Combinations

SFN is a molecule within the isothiocyanate group of organosulfur compounds and found in cruciferous vegetables such as broccoli, cauliflower, cabbage, and kale. Among the richest source of SFN are sprouts of broccoli and cauliflower. It is a potent antioxidant and stimulator of natural detoxifying enzymes. SFN is one of the most promising chemopreventive agents. It inhibits cell proliferation and induces apoptosis in different cancer cells. Its proapoptotic potential could make it effective either alone or in combination with other therapeutic strategies in reversing chemoresistance. SFN, as broccoli sprout extracts, is now in various clinical trials including a phase II trial for prostate cancer (<http://clinicaltrials.gov/ct2/results?term=Sulforaphane>). A pilot study for evaluating broccoli sprout extract effectiveness in advanced pancreatic cancer cases is also ongoing (Lozanovski *et al.*, 2014).

15.4.4.1 SFN and Epigallocatechin Gallate (EGCG)

EGCG, a green tea agent, has significant anticancer potential. The chemopreventive effects of the combination of SFN + EGG were successfully shown in a transgenic mouse model of prostate cancer and PC-3 cells by Nair *et al.* (2010). AP-1 activation was attenuated by the combinations of SFN (25 μ M/L)+EGCG (20 or 100 μ M/L) in PC-3 cells. Nrf2-dependent genes were downregulated (3–35-fold) after *in vivo* administration of the combination of EGCG (100 mg/kg)+SFN (45 mg/kg). Furthermore, Conserved transcription factor binding sites signatures were also identified in the promoter regions of Nrf2, AP-1, ATF2, and ELK-1 genes suggesting a potential regulatory mechanism of crosstalk between them. In another study, EGCG/SFN doses alone or in combination were used to treat both paclitaxel-sensitive (SKOV3-ip1) and -resistant (SKOV3TR-ip2) ovarian cancer cells. Chen *et al.* (2013a) found that SFN inhibited cell viability of both ovarian cancer cells both time- and dose-dependently and EGCG potentiated the effects of SFN. Cell cycle analysis indicates SFN arrested ovarian cancer cells in the G2/M phase, while EGCG+SFN is arrested in both the S and G2/M phase of the cell cycle. Furthermore, combination treatment induced apoptosis in a paclitaxel-resistant ovarian cancer cell by decreasing hTERT and Bcl-2 levels and enhanced DNA damage response via phosphorylated H₂AX.

15.4.4.2 SFN and Resveratrol

Combination treatment with resveratrol and SFN inhibits cell proliferation and migration, reduces cell viability, induces lactate dehydrogenase release, decreases pro-survival Akt phosphorylation, and increases caspase-3 activation in U251 glioma cells (Jiang *et al.*, 2010).

15.4.4.3 SFN and Quercetin

Quercetin and SFN doses in combination inhibited the proliferation and migration of melanoma (B16F10) cells more effectively than either compound alone (Pradhan *et al.*, 2010). Moreover, this combination also significantly suppressed melanoma growth in a mouse model and effects were predominantly due to decreased MMP-9 expression. Srivastava *et al.* (2011) examined the molecular mechanisms by which SFN inhibits self-renewal capacity of pancreatic cancer stem (CSCs) cells and synergizes with quercetin. SFN inhibited self-renewal capacity of pancreatic CSCs and induced apoptosis by inhibiting the expression of Bcl-2 and XIAP, phosphorylation of FKHR, and activating caspase-3. Moreover, SFN inhibited

expression of proteins involved in the epithelial-mesenchymal transition (β -catenin, vimentin, Twist-1, and ZEB1), suggesting the blockade of signaling involved in early metastasis. Furthermore, the combination of quercetin with SFN had synergistic effects on self-renewal capacity of pancreatic CSCs.

15.4.5 Synergism among Cruciferous Compounds

15.4.5.1 I3C or DIM and ITCs

Synergism was observed after combined treatments of various cruciferous vegetables phytochemicals such as I3C (6.25 μ M) + SFN (1 μ M), I3C (6.25 μ M) + PEITC (1 μ M), and DIM (6.25 μ M) + PEITC (1 μ M), while an additive effect was observed for DIM (6.25 μ M) + SFN (1 μ M) in human hepatoma cells (HepG2-C8) (Saw *et al.* 2011). Induction of endogenous Nrf2, phase II genes (GSTm2, UGT1A1 and NQO1) and antioxidant genes (HO-1 and SOD1) was also observed. Authors concluded that I3C or DIM alone could induce or synergistically induce in combination with the ITCs (SFN or PEITC), Nrf2-ARE-mediated gene expression, which could potentially enhance cancer chemopreventive activity.

15.4.5.2 SFN and DIM

SFN (10 μ M) in combination with DIM (10 μ M) resulted in potent G2M cell-cycle arrest, which was not observed with either compound alone (Pappa *et al.*, 2007). Cytotoxic concentrations of SFN+DIM combinations affect cell proliferation synergistically. Whereas at low concentrations (<20 μ M of each compound), the combined broccoli compounds showed antagonistic interactions in terms of cell growth inhibition.

15.4.5.3 BITC with SFN

According to Hutzén *et al.* (2009) BITC with SFN combination was more effective in preventing pancreatic cancer than the individual treatment. The inhibitory activities of this combination extended to STAT3 activation in pancreatic cancer cells (PANC-1). Study revealed that SFN inhibitory activity was most likely STAT3-independent as it had a minimal effect on the direct inhibition of STAT3 tyrosine phosphorylation. On the other hand, BITC was shown to inhibit the tyrosine phosphorylation of STAT3, but not the phosphorylation of ERK1/2, MAPK and p70S6 kinase. Furthermore, combinations of BITC and SFN inhibited cell viability and STAT3 phosphorylation more potentially.

15.4.5.4 BITC with PEITC

BITC is also a component of cruciferous vegetables and a potent cell cycle inhibitor. The results of a study by Hecht *et al.* (2000) demonstrate that proper doses of PEITC+BITC are effective inhibitors of lung tumorigenesis induced in A/J mice by a mixture of BaP and NNK.

15.4.6 Combinations of Cruciferous Compounds with Conventional Cancer Chemotherapeutics

15.4.6.1 I3C with Chemotherapeutics

TNF-related apoptosis-inducing ligand (TRAIL) is a member of the tumor necrosis factor (TNF) family. It functions as a ligand that induces apoptosis in tumor cells by binding to certain death receptors. Since the mid-1990s it has been used in anticancer therapeutics. Combination of I3C and genistein with TRAIL was synergistically able to induce apoptosis in endometrial cancer cells via a death receptor dependent pathway (Parajuli *et al.*, 2013). Results of Parajuli's study showed significant cell death, sub-G1 arrest, elevated expression of DR4, DR5, cleaved forms of caspase-3, caspase-8, PARP, and downregulated expression of Flip. However, there was no effect in cell growth inhibition and cell-cycle progression in alone or in combination of two. A potent synergistic mechanism between I3C and bortezomib (a proteasome inhibitor with minimal clinical activity as a monotherapy in solid tumours) treatment was also noted. Taylor-Harding *et al.* (2012) evaluated the cotreatment therapeutic potential of bortezomib with I3C, in an ovarian cancer model. Combination treatment led to profound cell-cycle arrest and apoptosis along with disruptions to multiple pathways regulating endoplasmic reticulum stress, cytoskeleton, chemoresistance, and carcinogen metabolism. Importantly, *in vivo* doses of I3C+ bortezomib significantly inhibited tumour growth and reduced tumour weight when compared with either drug alone. Moreover, I3C+bortezomib

co-treatment also sensitized ovarian cancer cells to cisplatin and carboplatin treatment. According to a study by Malejka-Giganti *et al.* (2007), I3C alone was less effective in suppressing mammary carcinogenesis than tamoxifen (Tam); however, the combination of I3C+Tam fostered the benefits of chemoprevention. In this study, 2 weeks after a single oral dose of 7,12-dimethylbenz[a]anthracene (DMBA, 65 mg/kg b.wt.), combination of Tam (10 µg/rat by subcutaneous injection) and I3C (250 mg/kg b.wt. orally) was given to female rats for a period of 20 weeks. Significant decreases in the mean tumor number/rat in Tam+I3C treated rats as compared to Tam or I3C group indicated a combinatorial effect of the two compounds. I3C dependent increases of hepatic cytochrome P450 levels and activities (1A1, 1A2, and 2B1/2) along with increased circulating levels of estrone, was suggested as major contributor to the preventive effects of this combination (Malejka-Giganti *et al.*, 2007).

15.4.6.2 PEITC and Chemotherapeutics

Taxol (Paclitaxel), isolated from plant *Taxus brevifolia*, is the drug of choice with significant antitumor activity toward various cancers (Kohn *et al.*, 1994; Vorobiof *et al.*, 2004; Zanetta *et al.*, 2000) but its major disadvantage is its dose-limiting toxicity and acquired resistance (Horwitz, 1992; Kohn *et al.*, 1994; Vorobiof *et al.*, 2004; Zanetta *et al.*, 2000). Mechanistically, it interferes with the dynamic instability of microtubules and thereby arrests the cell cycle in the G2/M phase, leading to apoptotic cell death (Horwitz, 1992). Combination of PEITC and taxol has been shown to work synergistically to increase apoptosis and cell cycle arrest in breast cancer cells. In a study drug resistant breast cancer cells (MCF7 and MDA-MB-231) were treated with combination of PEITC and taxol (Liu *et al.*, 2013) and a synergistic effect on growth inhibition of breast cancer cells was noted. The combination of PEITC and taxol significantly decreased the IC₅₀ dose of PEITC and taxol over each agent when used alone and also increased apoptosis and arrested cells in G2M phases. A study by Cang and colleagues provided molecular evidence for the mechanism of synergistic action between the PEITC and taxol. Treatment of MCF7 cells with both PEITC and taxol led to a 10.4-fold and 5.96-fold increase in specific acetylation of alpha-tubulin over single agent PEITC and taxol, respectively (Cang *et al.*, 2014). The combination of PEITC and taxol also reduced expressions of cell cycle regulator (Cdk1), and anti-apoptotic (bcl-2), enhanced expression of Bax and cleavage of PARP proteins.

15.4.6.3 BITC and Chemotherapeutics

Doses of BITC sensitized pancreatic cancer cells (adenocarcinoma cell lines with K-Ras12 mutations BxPC3, MiaPaCa2, and Panc-1) to TRAIL-induced apoptosis by dual activation of both the intrinsic and extrinsic pathways (Wicker *et al.*, 2010). A study by Ohara *et al.* (2011) was undertaken to examine the effects of BITC on the radiosensitivity of human pancreatic cancer cells and to gain insights into the underlying molecular mechanism of BITC-induced radiosensitization. Radiosensitization was observed in both PANC-1 and MIAPaCa-2 cells incubated with BITC at 5 to 10 µM and 2.5 to 5 µM for 24 h, respectively. The combination treatments with BITC and X-rays revealed an increased percentage of apoptotic cells and a decrease in the protein levels of the X-linked inhibitor of apoptosis (XIAP), inhibitor of apoptosis (IAP) protein, and in a marked increase in Apaf-1.

15.4.6.4 SFN and Chemotherapeutics

Fimognari and others (2007) investigated the chmosensitization effects of SFN on mouse fibroblasts cells bearing different p53 statuses. In this work p53-knockout fibroblasts from newborn mice transfected with the p53(Ser220) mutation and with a doxorubicin-resistant phenotype, were treated with a combination of doxorubicin and SFN. Taken together, results suggest that a mutated p53 status did not prevent the induction of apoptosis by SFN and that SFN was able to reverse the resistance to doxorubicin. The combination of SFN +doxorubicin may therefore allow doxorubicin to be administered at lower doses, thereby reducing its toxicity. Caco-2 cells were pretreated with increasing concentrations of SFN (1–20 µM) for 24 h followed by oxaliplatin doses (100 nM–10 µM). SFN and oxaliplatin alone inhibited cell growth of Caco-2 cells in a dose-dependent manner and effects were synergistically enhanced when cells were incubated with the combination doses. Researchers further pointed towards the simultaneous activation of both extrinsic and intrinsic apoptotic pathways by the combination (Kaminski *et al.*, 2011).

15.5 Curcumin: Potential for Combination Therapy

Curcumin (diferuloylmethane) is a polyphenol derived from the *Curcuma longa* plant, commonly known as turmeric, and has been used extensively in Indian Ayurvedic medicine system for centuries as it is nontoxic and has a variety of therapeutic properties including antioxidant, analgesic, anti-inflammatory, and antiseptic activity. More recently curcumin

has been found to possess anticancer activities via its effects on a variety of biological pathways involved in mutagenesis, oncogene expression, cell cycle regulation, apoptosis, tumorigenesis, and metastasis. Curcumin has shown an anti-proliferative effect in multiple cancers and is an inhibitor of transcription factor NF- κ B and downstream gene products (including c-myc, Bcl-2, COX-2, NOS, Cyclin D1, TNF- α , interleukins, and MMP-9) as well as AP1. In addition, curcumin affects a variety of growth factor receptors and cell adhesion molecules involved in tumor growth, angiogenesis, and metastasis.

15.5.1 Curcumin with Xanthorrhizol

Combination of xanthorrhizol (a rhizomal sesquiterpenoid of *Curcuma xanthorrhiza*) and curcumin show synergistic growth inhibitory effects towards breast cancer cells (MDA-MB-231 cells) via apoptosis induction (Cheah *et al.*, 2009). A sequential addition of both in breast cancer cells resulted in additive and antagonistic effects depending on which compound was added first to the culture. However, simultaneous addition of the compounds resulted in synergistic effects at lower concentrations and agonistic effects at higher concentrations.

15.5.2 Curcumin with Docosahexaenoic Acid (DHA, Polyunsaturated Fatty Acids Present in Fish Oil)

Since chronic inflammations are associated with several malignancies, it is important to prevent inflammation-mediated carcinogenesis, promotion and/or progression. Therefore, cancer intervention with combination of agents such as curcumin (with potent anti-inflammatory and -oxidative capability) and DHA (anti-inflammatory agents decreasing the production of eicosanoids, cytokines, and ROS) seems more feasible. A synergistic effect was first observed on induction of apoptosis (~6-fold) and inhibition of cell proliferation (~70%) when pancreatic cancer cells (BxPC-3) were exposed to curcumin (5 μ M) + DHA (25 μ M) (Swamy *et al.* 2008). Further, mice bearing pancreatic tumor xenografts were fed with fish oil (15%) and curcumin (2000 ppm), alone and in combination, and showed a significantly reduced tumor volume, 25% and 43%, respectively. Importantly, a combination of curcumin and fish oil diet showed >72% tumor volume reduction (Swamy *et al.*, 2008). Expression and activity of iNOS, COX-2, and 5-LOX are downregulated, and p21 is upregulated in a tumor xenograft fed curcumin plus fish oil diet when compared to individual diets. A study by Saw *et al.* (2010) also clearly showed the synergistic anti-inflammatory as well as the antioxidative stress effects of curcumin and DHA combination. Combinatorial doses of curcumin (5 μ M) and of DHA (25 μ M) were found to have synergistic effects in suppressing LPS-stimulated NO and endogenous NO levels in RAW 264.7 cells. Importantly, very low doses of curcumin (2.5 μ M) and DHA (0.78 μ M) synergistically suppress the LPS-induced prostaglandin E₂ production. Effects of curcumin+DHA combination was also analyzed on five different breast cancer cell lines (SK-BR-3, MDA-MB-231, MDA-MB-361, MCF7, and MCF10AT) expressing different cell surface receptors (Altenburg *et al.*, 2011). SK-BR-3 cells (ER⁻ PR⁻ Her2⁺) exhibited a higher uptake of curcumin in the presence of DHA and the effect was synergistic (Altenburg *et al.*, 2011). Used alone, DHA does not contribute to cell inhibition. Genes involved in cell cycle arrest, apoptosis, inhibition of metastasis, and cell adhesion were upregulated, whereas genes involved in cancer development and progression, metastasis, and cell cycle progression were downregulated (Altenburg *et al.*, 2011). Siddiqui *et al.* (2013) further confirmed that the combination of curcumin and DHA is potentially a dietary supplemental treatment for some breast cancers (ER⁻ PR⁻ Her2⁺), and likely dependent upon the molecular phenotype of the cancer. The curcumin plus DHA combination reduced the incidence of breast tumors, delayed tumor initiation, and reduced progression of tumor growth in DMBA-induced mammary tumorigenesis (ER⁻ and Her-2⁺ phenotype) in mice. Mechanistically synergistic effects also led to increased expression of the pro-apoptotic protein, maspin, but reduced expression of the anti-apoptotic protein, survivin. Curcumin (10 μ M) and DHA (50 μ M) are also reported to block insulin-induced colon cancer cell (MC38 cells) proliferation via a MEK mediated mechanism (Fenton and McCaskey, 2013).

15.5.3 Curcumin and Genistein

Genistein is a phytoestrogen and belongs to the category of isoflavones. Curcumin and genistein compounds in combination drastically affect the development of tumors by mediating growth inhibition. Such changes were observed both in ER-positive and ER-negative cells, indicative of the dual use of such a combination in prevention and therapy. Low concentrations of both compounds are able to inhibit the growth of estrogen-positive human breast MCF-7 cells induced individually or by a mixture of the pesticides endosulfane, DDT, and chlordane or 17- β estradiol (Verma *et al.*, 1997, 1998). When curcumin and genistein were added together to MCF-7 cells, a synergistic effect resulting in a total inhibition of the estrogenic activity of endosulfane/ chlordane/DDT mixtures was noted. These data suggest that the combination of curcumin and genistein in the diet have the potential to reduce the proliferation of estrogen-positive cells induced by pesticides or 17- β estradiol.

15.5.4 Curcumin and Resveratrol

Development and progression of malignancies are associated with activation of multiple signaling pathways. Therefore, the inhibition of these signaling pathways with nontoxic nutraceuticals represents a logical preventive approach. Both curcumin and resveratrol are reported to inhibit the growth of colon cancer, were selected by Majumdar *et al.* (2009) to examine whether combining them would be an effective fighting strategy for colon cancer. In fact, the mixture of was found to be more effective in inhibiting the growth of HCT-116 colon cancer cells (both wild type and mutant p53) both *in vitro* and *in vivo* (in SCID tumor xenografts) than either alone. The documented synergistic responses of combination were associated with the reduction in proliferation and stimulation of apoptosis accompanied by attenuation of NF- κ B activity. *In vitro* studies have further established that the combinatorial treatment caused a greater inhibition of constitutive activation of EGFR and its family members as well as IGF-1R. Furthermore, the combination treatment of curcumin and resveratrol elicited a synergistic antiproliferative effects in Hepa1–6 liver cancer cells (Du *et al.*, 2013). The apoptosis induced by the combination was accompanied by caspase (-3, -8, and -9 activation, intracellular ROS upregulation and XIAP and survivin protein downregulation. Rhabdomyosarcoma and osteosarcoma (tumors of mesenchymal origin) are reported to be highly aggressive pediatric malignancies with a poor prognosis and chemoresistance to chemotherapy. Doses of curcumin induce apoptosis in rhabdomyosarcoma (SJ-RH4, RD/18) and osteosarcoma (Saos-2) cancer cells and that effect was further potentiated when curcumin was combined with resveratrol or diallyl disulfide (Masuelli *et al.*, 2012). Since combination approaches are evolving as the future in the war against several cancers, researchers also evaluated effects of curcumin plus resveratrol combination during mouse lung carcinogenesis. Curcumin and resveratrol offered significant improvement in LPO and GSH levels as well as in the activities of SOD, which was altered during lung carcinogenesis (Malhotra *et al.* 2010). Benzo(a)pyrene (B(a)P) induced premature mitochondria senescence and ultrastructural changes in mouse lungs were also significantly modulated by the combination (Malhotra *et al.* 2012). Decreased micronuclei formation by stimulating apoptosis in BaP treated mice was also noted (Malhotra *et al.*, 2012). Researchers suggested the involvement of modulation of p53 hyperphosphorylation, regulation of caspases (3 and 9) and cellular metabolism enzymes as molecular mechanisms behind the chemopreventive synergism of curcumin plus resveratrol in lung carcinogenesis (Malhotra *et al.* 2014).

15.5.5 Curcumin and EGCG

EGCG and curcumin have been widely investigated worldwide for their anticancer effects. Zhou *et al.* (2013) studied the effect of the combination of EGCG and curcumin on non-small cell lung cancer cells. The results revealed that at low concentrations, the combination of the EGCG and curcumin strongly enhanced cell cycle arrest at the G1 and S/G2 phases and significantly inhibited cell cycle related proteins cyclin D1 and cyclin B1. In a lung cancer xenograft nude mouse model, combination was protective against body weight loss due to tumor burden. Curcumin and tea catechin have synergistic effect on colorectal aberrant crypt foc (ACF) formation and COX-2 mRNA expression in rat colon carcinogenesis, suggesting their potential value in the prevention of human colon cancers too (Xu *et al.*, 2005, 2010).

15.5.6 Curcumin and Citrus Limonoids

Chidambara *et al.* (2013) examined the ability of limonoids, including limonin, limonin glucoside, and curcumin, to inhibit proliferation of human colon cancer (SW480) cells. Combinations of limonoids with curcumin at three different ratios (1:3, 1:1, and 3:1) demonstrated up to 96% inhibition of cell proliferation. Further, incubation of cells with this combination resulted in elevation of total cellular caspase-3 activity by 3.5–4.0-fold along with a 2–4-fold increase in the Bax/Bcl-2 ratio.

15.5.7 Curcumin with Apigenin

Apigenin, a naturally occurring plant flavone (4',5,7,-trihydroxyflavone) abundantly present in fruits and vegetables including parsley, onions, oranges, tea, chamomile, wheat sprouts, and some seasonings. Apigenin has been shown to possess remarkable anti-inflammatory, antioxidant, and anti-carcinogenic properties. Interestingly, apigenin also showed synergistic anticancer effects with curcumin (Choudhury *et al.*, 2013). Apigenin and curcumin synergistically induced cell death and apoptosis and also blocked cell cycle progression at the G2/M phase of A549 cells. Co-activity of apigenin and curcumin was also apparent from their strong depolymerizing effects on interphase microtubules and inhibitory effect of reassembly of cold depolymerized microtubules. Enhanced understanding the mechanism of synergistic effect of apigenin and curcumin could help to develop anticancer combination drugs from dietary nutraceuticals.

15.5.8 Curcumin and Triptolide

A study attempted to clarify that curcumin and triptolide synergistically suppress ovarian cancer cell growth *in vitro*. A study showed that the combination of curcumin and triptolide at lower concentration synergistically inhibit ovarian cancer cell growth, and induce apoptosis, which was accompanied by HSP27 and HSP70 proteins (Cai *et al.*, 2013).

15.5.9 Combinations of Curcumin with Conventional Cancer Chemotherapeutics

Prostate cancer cells are resistant to TRAIL therapy. The potential use of curcumin to sensitize prostate cancer cells (LNCaP, DU145, and PC3 tumor cell lines) for TRAIL-mediated immunotherapy was defined by Deeb *et al.* (2003, 2004, 2005). At low concentrations curcumin (10 μ M) and TRAIL (20 ng/ml) cooperatively interact to promote death and apoptosis of LNCaP cells. Neither of the two agents alone produced significant cytotoxicity at given doses. Both the extrinsic and intrinsic pathways of apoptosis were triggered by combined treatment as evident by induced cleavage of procaspase (-3, -8, and -9), truncation of Bid, and release of cytochrome c (Deeb *et al.*, 2003, 2005). The authors further investigated the mechanism by which curcumin augments TRAIL-induced cytotoxicity in prostate cancer cells and suggested that curcumin enhances the sensitivity of cells to TRAIL by: (1) inhibiting Akt-regulated NF- κ B activation via blocking phosphorylation of I κ B α and its degradation and (2) NF- κ B-dependent antiapoptotic Bcl-2, Bcl-xL, and XIAP (Deeb *et al.* 2004, 2007). The curcumin and TRAIL combination regimen was also the most effective treatment for inhibiting the growth of prostate tumor PC3 and LNCaP xenografts compared to curcumin/TRAIL monotherapy (Andrzejewski *et al.*, 2008; Shankar *et al.*, 2008). The inhibition of PC3 tumors by combined treatment correlated with significant reduction in expression of p-Akt and NF- κ B in tumor tissue and sensitized these tumors to undergo apoptosis by TRAIL (Andrzejewski *et al.*, 2008). Curcumin upregulated the expression of death receptors (TRAIL-R1/DR4, TRAIL-R2/DR5), Bax, Bak, p21/WAF1, and p27/KIP1, and inhibited the activation of NF κ B and its gene products (cyclin D1, VEGF, uPA, MMP-2, MMP-9, Bcl-2, and Bcl-XL), which may have sensitized TRAIL-resistant LNCaP xenografts (Shankar *et al.*, 2008). In another study, chemoresistant ovarian cancer cell lines (SKOV3 and ES-2 cells) were subjected to low doses of curcumin (5–15 μ M) prior to treatment with TRAIL, resulted in markedly enhanced cell death through the activation of both the extrinsic (cleavage of caspase-8) and the intrinsic (cleavage of caspase-9) apoptotic pathways (Wahl *et al.*, 2007). Recently, Park *et al.* (2013) found that the combination of TRAIL with curcumin can synergistically induce apoptosis in TRAIL-resistant breast cancer cell lines via production of ROS and involvement of death receptor pathway.

Many chemotherapeutic drugs including taxol induce the expression of the NF- κ B (Perera *et al.*, 1996), which encourages tumor progression, and curcumin has the potential to inhibit NF- κ B activation (Aggarwal *et al.*, 2005; Bava *et al.*, 2005). A combination of taxol (5 nm) with curcumin (5 μ M) augments anticancer effects more efficiently than taxol alone, as evidenced by increased cytotoxicity and reduced DNA synthesis in HeLa cells (Bava *et al.*, 2005). Evaluation of signaling pathways common to taxol and curcumin reveals that this synergism was in part related to downregulation of NF- κ B and serine/threonine kinase Akt pathways by curcumin. Doses of taxol with curcumin have also been shown to downregulate the expression of NF- κ B and induced apoptosis in breast cancer cells (Aggarwal *et al.*, 2005; Kang *et al.*, 2009; Royt *et al.*, 2011). Together with these, curcumin also suppressed the paclitaxel-induced expression of antiapoptotic (XIAP, IAP-1, IAP-2, Bcl-2, and Bcl-xL), proliferative (cyclooxygenase 2, c-Myc, and cyclin D1), and metastatic proteins (VEGF, MMP-9, and ICAM-1) (Aggarwal *et al.*, 2005). Inhibition of paclitaxel-induced activities of EGFR signaling was suggested as mechanism of synergistic growth inhibition via apoptosis in MCF-7 cells too (Zhan *et al.*, 2013). Moreover, the combination of paclitaxel and curcumin exerted increased anti-tumor efficacy on mouse models of breast cancer (Zhan *et al.*, 2013). The combination of curcumin (20 μ M) and paclitaxel (10 nM) also worked synergistically as a promising therapy for controlling the growth of chemoresistant human brain tumor stem cells (Hossain *et al.*, 2012). Combination therapy inhibited invasion of cells, reduced expression of survival and proliferation factors, and also angiogenic factors.

The evolution of multidrug resistant (MDR) phenotypes is one among many major obstacles in cancer therapy. Andjelkovic *et al.* (2008) tested the potential of sulfinosine (the novel purine ribonucleoside) and curcumin (7.5 or 35 μ M), alone and in combination, for modulating MDR in the human resistant non-small cell lung carcinoma cell line (NCI-H460/R). SF concentrations (5, 10, 15, 20, and 25 μ M) in combination with 15 μ M curcumin displayed synergistic interactions. With high levels of curcumin (55 μ M) the interactions were antagonistic. The most pronounced synergism was obtained with 10 μ M SF+15 μ M curcumin. Altered expression of MDR-related genes mdr1, gsp-pi and topo II alpha was noted in NCI-H460/R cells even in the presence of mutated p53. Along with these, the combination induced a more pronounced cell cycle arrest in S and G(2)/M in NCI-H460/R cells.

Cyclooxygenase-2 (COX-2) expression is central to the carcinogenesis and compounds that regulate the expression or activity of COX-2 in cells may be instrumental in effective chemotherapy. However, long-term use of high concentration of COX-2 inhibitors, such as Celecoxib, is toxic and may be limited due to gastrointestinal and cardiovascular side effects. Curcumin is reported to be augments the growth inhibitory effect of celecoxib in various studies. Exposure to curcumin (10–15 μ M/l) and celecoxib (5 μ M/l) resulted in a synergistic inhibitory effect on colon cancer cell growth via induction of apoptosis and downregulation COX-2 mRNA expression (Lev-Ari *et al.*, 2005). This synergistic effect is clinically important because it can be achieved in the serum of patients receiving standard anti-inflammatory or antineoplastic dosages of celecoxib (Lev-Ari *et al.*, 2005). A combination of celecoxib (0.16%) and curcumin (0.6%) reduced the average number of ACF/rat colon was 24.5 \pm 6 when compared with alone, that is, 39 \pm 5 and 47 \pm 10 for the curcumin and celecoxib-treated group, respectively (Shpitz *et al.*, 2006).

Doxorubicin triggered EMT and resulted in the acquisition of a mesenchymal phenotype in triple-negative breast cancer cells (MDA-MB-231, MDA-MB-468, BT-549, and BT-20 cells). At the molecular level, TGF- β and PI3K/AKT pathways were acquired for doxorubicin induced EMT, combination of doxorubicin with curcumin potentially reverted EMT (Chen *et al.*, 2013b). In gastric cancer cells, curcumin potentiates the antitumor effects of doxorubicin by suppressing NF- κ B and NF- κ B-regulated anti-apoptotic genes (Yu *et al.*, 2011).

Cisplatin, carboplatin, and oxaliplatin are used to treat various types of cancers. However, observed acquired resistance and toxicities limit the therapeutic potential of these compounds. Several pathways and specific targets including NF- κ B, STAT3, COX-2, Akt, and MDR have been identified as assisting curcumin as a chemosensitizer. The addition of curcumin significantly increased cytotoxicity of the anticancer drugs on human colorectal cancer HT-29 cells and produced synergistic (cisplatin and carboplatin) and additivity (oxaliplatin) effects (Wang *et al.*, 2014c). Treatments in combination resulted in induction of apoptosis and G2/M arrest. Elevated expression of nuclear apoptosis-inducing factor (AIF), EndoG and NF- κ B by anticancer drugs was also reversed by curcumin, suggesting the association of EndoG and NF- κ B in curcumin enhanced chemosensitivity (Oiso *et al.*, 2012; Wang *et al.*, 2014c). Combined curcumin and cisplatin treatment markedly inhibited drug resistant cancer cell proliferation, reversed drug resistance, and triggered apoptotic death by promoting HIF-1 α degradation and activating caspase-3, respectively (Ye *et al.*, 2012). Expression of HIF-1 α -dependent P-gp also seemed to decrease as response to curcumin in a dose-dependent manner (Ye *et al.*, 2012). Additionally, reversal of MDR via suppression of MRP1 and Pgp1 by curcumin resulted in sensitization of cervical cancer cells and thus, lowered the chemotherapeutic dose of the drug cisplatin (Roy and Mukherjee, 2014). Hence, intake of foods rich in curcumin or curcumin-containing supplements should be taken into consideration for patients receiving chemotherapy to optimize the outcome of treatments.

15.6 Resveratrol: Potential for Combination Therapy

The cancer preventive properties of grape products such as red wine have been attributed to presence of enriched fraction of polyphenols such as catechin, epicatechin, quercetin, and resveratrol. Grape polyphenols can act as potent antioxidants, antiangiogenics, and selective estrogen receptor modifiers and are therefore, especially relevant for various cancer treatments. The major polyphenols of grapes have been individually shown to have anticancer properties. Resveratrol (3,5,4'-trihydroxy-trans-stilbene) is a stilbenoid, a type of natural phenol and a phytoalexin, produced naturally by several plants when under attack by pathogens such as bacteria or fungi. Majorly, it is found in the skin of red grapes and in other fruits as well as in the roots of Japanese knotweed (*Polygonum cuspidatum*). Resveratrol potential as anticancer and preventive agent has been extensively explored by scientific groups. Several researchers believe that the biggest hurdle in the development of resveratrol as an anticancer drug or preventive agent is its poor bioavailability following oral ingestion, resulting from its rapid metabolism, mainly to its glucuronide and sulfate metabolites. However, researchers have begun to focus on different means of enhancing the bioavailability of resveratrol, as well as developing novel resveratrol analogues with superior efficacy and bioavailability.

15.6.1 Resveratrol and Genistein

In a study, resveratrol and genistein (250 mg/kg each mixed in AIN-76A diet) provided in the diet was found to significantly reduce the most severe grade of prostate cancer in the SV40 Tag rat model (Harper *et al.*, 2009). Regulation of steroid receptor coactivator-3 (SRC-3) and insulin-like growth factor-1 (IGF-1) signaling proteins are consistent with these nutritional polyphenols reducing cell proliferation and increasing apoptosis in the prostate. Dhandayuthapani *et al.*, (2013)

suggested genistein can synergistically enhance the apoptotic potential of resveratrol at doses lower than the usual cytotoxic dose. In this study, lower doses of resveratrol and genistein when combined can induce apoptosis through the activation of a caspases cascade, and by decreasing the expression of HDM2 in cervical cancer cells.

15.6.2 Resveratrol and Piperine

Piperine, an alkaloid from black and long peppers (*Piper nigrum Linn* and *Piper longum Linn*), has been reported to exhibit antitumor activities both *in vitro* and *in vivo*. Piperine has previously been shown to enhance the bioavailability of other polyphenols such as EGCG (Lambert *et al.*, 2004). Piperine has also extensively enhanced resveratrol levels in the blood of mice (Johnson *et al.*, 2011). In a recent study, a resveratrol plus piperine combination was found to act as a sensitizer for ionizing radiation induced apoptotic cancer cell death *in vitro* (Tak *et al.*, 2012). Although these findings are promising, the effects of piperine on resveratrol bioavailability still remains unknown in the human population, so further studies are obligatory.

15.6.3 Resveratrol and Black Tea Polyphenols

The effect of the combination of resveratrol with black tea polyphenol in a two-stage mouse skin carcinogenesis model was determined by us (George *et al.*, 2011). We found that the combination imparts a synergistic tumor suppressive response, compared to either of the agents alone. Results suggested that the observed synergistic response was possibly due to a synergistic action between two agents on same molecular targets. This is an interesting study because a synergistic action of multiple agents on a common pathway(s) can lead to dose-reduction of chemopreventive agents, thereby limiting the chances of side effects.

15.6.4 Resveratrol and Melatonin

Resveratrol was studied in combination with melatonin (a pineal hormone and known antioxidant) for its cancer therapeutic efficacy. Kisková *et al.* recently demonstrated that a combination of resveratrol with melatonin exerts superior chemopreventive effects in *N*-methyl-*N*-nitrosourea (NMU)-induced rat mammary carcinogenesis. The data from this study showed that neither of the two agents alone had any appreciable effect on NMU-induced mammary carcinogenesis, but the combination resulted in a significant decrease in tumor incidence (Kisková *et al.*, 2012). This combination may provide an effective means to treat neurodegenerative disorders (Kwon *et al.*, 2011). Melatonin synergistically enhanced resveratrol-induced heme oxygenase-1, possibly through inhibition of a ubiquitin-dependent proteasome pathway (Kwon *et al.*, 2011). This combination seems to have potential in cancer chemoprevention, possibly via targeting two non-overlapping molecular pathways. Thus, there is a possibility that this combination may lead to a synergistic response to attenuate proliferative signaling and improve cancer chemopreventive response.

15.6.5 Synergism among Resveratrol and Other Grapes' Polyphenols

15.6.5.1 Resveratrol and Quercetin

Both resveratrol and quercetin are polyphenols present in red grapes, red wine, and several other plants. However, the levels of quercetin in red wine are typically around 10-fold higher than the resveratrol levels. In addition, quercetin has also been shown to inhibit sulfation of resveratrol (De Santi, *et al.*, 2000) and can enhance bioavailability, and thus therapeutic efficacy, of resveratrol. Combining resveratrol (50 µM) with quercetin (10, 25, and 50 µM), in concentrations equivalent to that present in red wines, resulted in a gradual and significant increase in cancer cell growth and DNA synthesis (ElAttar and Virji, 1999). Low doses of resveratrol (10 µM) or quercetin (25 µM) separately had no effect on apoptosis induction in glioma cells, but when administered together had a significant effect on caspases activation and Akt phosphorylation reduction (Zamin *et al.*, 2009). A strong synergism in inducing senescence-like growth arrest was also noticed. A recent study showed that resveratrol and quercetin synergistically reduce the extent of restenosis, perhaps by inhibiting vascular smooth muscle cell proliferation and inflammation (Khandelwal *et al.*, 2012). Furthermore, a study by Zhou and colleagues also confirmed synergistic effects between quercetin and resveratrol (Zhou *et al.*, 2012). Gokbulut *et al.* (2013) showed for the first time that combination of resveratrol and quercetin might block chronic lymphocytic leukemia cells growth through inducing apoptosis and cell cycle arrest. Recently, a study by Del Follo-Martinez *et al.* (2013) showed that in combination

resveratrol and quercetin (1:1 ratio) have anticancer activity against colon cancer cells, decreased microRNA-27a, and induced the zinc finger protein. Thus, the combination of resveratrol and quercetin seem to have potential toward cancer management. It seems that additive/synergistic interactions between these two polyphenols may be one of the explanations for the French paradox, especially because both of these agents are present in red wine. However, studies are needed to explore these possibilities.

15.6.5.2 Resveratrol with Quercetin and Catechin

Combined doses of grape polyphenols (resveratrol plus quercetins plus catechin) reduced primary tumor growth of breast cancer xenografts in a nude mouse model (Schlachterman *et al.*, 2008).

15.6.5.3 Resveratrol with Ellagic Acid and Quercetin

Resveratrol with ellagic acid and quercetin, the selected polyphenols that occur in muscadine grapes, interact synergistically in the induction of apoptosis and reduction of cell growth in human leukemia cells (MOLT-4) (Mertens-Talcott and Percival, 2005). This study confirms an annotation that the anticarcinogenic potential of diet containing polyphenols may not be based on the effects of individual compounds, but may involve a synergistic enhancement of the anticancer effects of several nutraceuticals.

15.6.5.4 Combination of Resveratrol and Multiple Nutraceuticals

A few other combinations containing resveratrol have also been investigated for their cancer chemopreventive effects. Bioactive molecules glucan and vitamin C in combination with resveratrol have synergistic anticancer effects along with activation of phagocytosis (Vetvicka and Vetvickova, 2012). A study to determine the inhibitory effects of resveratrol with several phytochemicals (ellagic acid, grape seed extract, and calcium D-glucarate) on chemically induced murine skin tumorigenesis was carried out by Kowalczyk and coworkers (2010). The combination was found to be a very potent inhibitor of mouse skin tumorigenesis, based on the suppression of epidermal hyperplasia as well as on the modulation of cell proliferation, cell survival, inflammation, oncogene mutation, and apoptosis. Slusarz *et al.* (2010) also determined the preventive and therapeutic abilities of a dietary mixture of resveratrol with quercetin, genistein, apigenin, baicalein, curcumin, and EGCG, *in vitro* as well as *in vivo* models. The authors found that all seven compounds, when fed in combination as pure compounds as well as crude plant extracts, inhibited well-differentiated carcinoma of the prostate by 58 and 81%, respectively. *In vitro*, these compounds also inhibit growth in human and mouse prostate cancer cell lines. Mechanistically, researchers propose the Hedgehog signaling pathway to be a direct or indirect target of these compounds mixture. These studies confirmed that the nutraceuticals at pharmacologic concentrations are potentially safer and less expensive alternatives to cyclopamine (antagonizes Hedgehog signaling pathway activity) and its pharmaceutical analogues for cancer therapy.

15.6.6 Resveratrol in Combination with Anticancer Drugs

The prospective use of resveratrol has also been substantially investigated for its potential as an adjuvant in conjunction with cancer chemotherapeutic modalities in order to enhance their efficacy and limit the toxicities (Fulda and Debatin, 2004; Gupta *et al.*, 2011.). *In vitro* and *in vivo* studies have suggested that resveratrol may enhance the effects of chemotherapeutic drugs. Resveratrol either alone or in combination with TRAIL or Smac can be used for the prevention and/or treatment of human prostate cancer. Resveratrol sensitized TRAIL-resistant LNCaP cells via upregulation of the Bax, Bak, PUMA, Noxa, Bim, TRAIL-R1/DR4, and TRAIL-R2/DR5, and downregulation of the Bcl-2, Bcl-XL, survivin, and XIAP expressions (Shankar *et al.*, 2007). Resveratrol has also been shown to overcome chemoresistance in a mouse model of B16/DOX melanoma by inducing cell cycle disruption and apoptosis, which leads to reduced growth of tumor and prolonged survival (Gatouillat *et al.*, 2010). Lin *et al.* (2012) have shown that resveratrol potentiated the therapeutic efficacy of temozolomide (alkylating agent used in cancer therapeutics) in a mouse xenograft model of malignant glioma, through inhibiting ROS/ERK-mediated autophagy and enhancing apoptosis. Additionally a mixture of the grape polyphenols containing resveratrol, quercetin, and catechin was revealed to potentiate the effects of gefitinib in inhibiting mammary tumor growth and metastasis in nude mice (Castillo-Pichardo and Dharmawardhane, 2012). Resveratrol exerts synergistic effects with etoposide on the proliferation of cancer cells when mainly accompanied by p53 activation (Amiri *et al.*, 2013). These studies support the possible use of resveratrol as an adjuvant in combination with chemotherapeutic drugs for cancer treatment.

Conversely, a study by Fukui *et al.* (2010) suggested that resveratrol may weaken the antiproliferative effects of paclitaxel in breast cancer cells. Hence, more preclinical studies in appropriate models to determine the use of resveratrol as an adjuvant are warranted.

15.7 Lycopene (a Carotenoid): Potential for Combinations Therapy

Phytochemical lycopene is a carotenoid pigment found in tomatoes and other red fruits and vegetables, such as red carrots, watermelons, and papaya. Tomatoes and tomato-based products contain the highest concentrations of bioavailable lycopene. Since chemoprevention is one of the most important strategies in the control of cancer development, molecular mechanism-based cancer chemoprevention using carotenoids seems to be an attractive approach. Dietary intake of carotenoids is inversely associated with the risk of a variety of cancers in different tissues (reviewed by Tanaka *et al.*, 2012). Preclinical studies have shown that some carotenoids have potent antitumor effects both *in vitro* and *in vivo*, suggesting potential preventive and/or therapeutic roles for the compounds (Haddad *et al.*, 2013; Gloria *et al.*, 2014). So far, various carotenoids, such as β -carotene, α -carotene, lycopene, lutein, zeaxanthin, β -cryptoxanthin, fucoxanthin, and canthaxanthin have been evaluated for cancer chemopreventive abilities. Several epidemiological studies have linked increased lycopene consumption with decreased prostate cancer risk. A diet-based population study showed an inverse relationship between serum levels of lycopene and prostate cancer risk. The effects of a whole-tomato supplement lycopene on the prostate-specific antigen (PSA) velocity in selected prostate cancer patients were examined by Zhang *et al.* (2014). Administration of lycopene was able to reduce PSA velocity in this study group.

The combination of tomato and broccoli was more effective at slowing tumor growth than either tomato or broccoli alone and supports the public health recommendations to increase the intake of a variety of plant components for men with recurring prostate cancer and rising prostate specific antigen (Canene-Adams *et al.*, 2007).

15.7.1 Lycopene and Genistein

Lycopene and genistein are potent antioxidants and, when given in combination, offer maximum protection against DMBA-induced mammary carcinogenesis (Sahin *et al.*, 2011).

Administration of lycopene and genistein in combination efficiently suppressed breast cancer development (40%) as compared to either lycopene (70%) or genistein (60%). It was associated with a decrease in MDA, 8-isoprostanate, and 8-OhdG levels and with an increase in serum lycopene and genistein levels in animals. Combined tomato and soy products are suggested excellent to reduce the risk of prostate cancer or enhance efficacy of therapy (Grainger *et al.* 2008a; Grainger *et al.* 2008b). The studies suggested that prostate cancer patients will consume diets rich in tomato products and soy with excellent compliance and bioavailability of phytochemicals. Further studies combining tomato and soy foods to determine efficacy for prostate cancer prevention or management are encouraged.

15.7.2 Lycopene and S-allylcysteine

Researchers validated the hypothesis that diet-derived chemopreventive agents such as lycopene and S-allylcysteine (an organosulfur constituent of garlic) in combination may interact synergistically with high efficacy and lessened toxicity against cancer. Lycopene or S-allylcysteine alone significantly suppressed the development of gastric cancer, administration of both in combination was more effective in inhibiting N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)-induced stomach tumors and modulating the redox status in the tumor and host tissues (Velmurugan and Nagini *et al.*, 2005). Furthermore, induction of apoptosis by lycopene and SAC combination represents one of the possible mechanisms that could account for their synergistic chemopreventive activity (Velmurugan *et al.*, 2005b).

15.7.3 Lycopene and 1,25-Dihydroxyvitamin D3

Lycopene has the ability to synergize with various natural anticancer compounds, such as 1,25-dihydroxyvitamin D3, which, when used alone, are therapeutically active only at high and toxic concentrations. The combination of low concentrations of lycopene with 1,25-dihydroxyvitamin D3 exhibited a synergistic effect on cell proliferation and differentiation and an additive effect on cell cycle progression (Amir *et al.*, 1999). Such synergistic anticancer effects of lycopene with other compounds found in the human diet and in plasma might advocate the addition of the carotenoid in the diet as a cancer preventive measure.

15.7.4 Lycopene with Selenium

Selenomethione (a naturally occurring amino acid containing selenium) alone appeared to be chemoprotective, but when used in combination with estrogen, lycopene and thymoquinone (a phytochemical compound found in the plant *Nigella sativa*) caused cellular damage as evidenced by decreased proliferation rate, increased glutathione levels, and increased MDA levels (Brewer *et al.*, 2006). Administration of a combination of micronutrients containing selenium, vitamin E, and lycopene inhibited prostate cancer development in the “Lady” transgenic model (Venkateswaran *et al.*, 2009).

15.7.5 Lycopene and FruHis (Ketosamine)

This combination strongly synergized against the proliferation of the highly metastatic rat prostate adenocarcinoma cells *in vitro*. Additionally, the combination significantly inhibited *in vivo* tumor formation by MAT-LyLu cells in syngeneic Copenhagen rats (Mossine *et al.*, 2008).

15.7.6 Combination of Lycopene with Cancer Chemotherapeutic Drugs

Docetaxel is a potent chemotherapeutic drug that is clinically used to treat patients with advanced metastatic prostate cancers. Although the drug extends survival, it is for a very limited time period and with a poor prognosis. One study tested the possibility to use this combination of compounds to enhance survival of patients that were detected with aggressive, androgen-independent tumors. In a DU145 xenograft tumor model, docetaxel plus lycopene caused tumor regression, with a 38% increase in antitumor efficacy when compared with docetaxel alone (Tang *et al.*, 2011). Analysis of molecular mechanisms revealed that the action of docetaxel was on regulating the insulin-like growth factor receptor (IGFR) pathway by suppressing IGF, and this effect was synergistically enhanced in the presence of lycopene supplementation. Together the molecules asserted negative effects on Akt signaling pathways and suppressed survivin, products of which have been known to maintain tumor growth and metastasis. The enhancement of docetaxel’s antitumor efficacy by lycopene supplementation justifies further clinical investigation of this combination for prostate cancer patients. Patients with IGF-IR-overexpressing tumors may be most likely to benefit from this combination.

15.8 Soy Nutraceuticals: Potential for Combination Therapy

Genistein is a phytoestrogen and belongs to the category of isoflavones. Isoflavones are a class of phytoestrogens-plant-derived compounds with estrogenic activity. Soybeans and soy products are the richest sources of isoflavones in the human diet. Isoflavones such as genistein and daidzein are found in a number of plants including lupin, fava beans, soybeans, kudzu, and psoralea being the primary food source. Genistein has been shown to inhibit the growth of various cancer cells *in vitro* and *in vivo* without toxicity to normal cells. The antitumor effects of genistein could be in part due to inactivation of NF-κB activity (Li *et al.*, 2004, 2005a). Genistein has gained popularity in the fight against cancer within the last decade. Epidemiological evidence indicates that there are positive associations between chemoprevention and the main source of genistein, soy consumption.

15.8.1 Genistein and Daidzein

The reduced incidence of prostate cancer in Asia countries has been attributed to high soy diets, and major soy isoflavones, in particular daidzein and genistein, are thought to be the source of the beneficial and anticancer effects of soy foods. Combination with lower levels of daidzein and genistein was more efficacious and a safer chemopreventive agent for prostate cancer (Dong *et al.*, 2013). Daidzein and genistein (doses from 25 or 50 μM) showed a synergistic effect on inhibiting cell proliferation and inducing apoptosis in cells.

15.8.2 Genistein and 3,3'-Diindolylmethane

The phytochemicals genistein and 3,3'-diindolylmethane (DIM, derived from indole-3-carbinol in cruciferous vegetables), are reported to decrease the risk of prostate cancer (Smith *et al.*, 2008). Evidence suggests that 17beta-estradiol (E2) contributes to the risk of prostate cancer. Genistein and DIM altered major E2 metabolism pathways in LNCaP and PC-3 (E2 insensitive) cells by increasing the expression of the 2-hydroxylation enzyme cytochrome P450 1A1 (CYP1A1) and the O-methylating enzyme catechol-o-methyltransferase.

15.8.3 Genistein and Capsaicin

Suppression of inflammatory events by food origin chemopreventive agents can provide a potential strategy to control carcinogenesis. The effects of genistein alone or in combination with capsaicin on suppression of inflammatory responses induced by TPA were examined (Hwang *et al.*, 2009). Genistein in combination with capsaicin synergistically exerts anti-inflammatory and anticarcinogenic properties through the modulation of AMPK and COX-2, and possibly various MAPKs.

15.8.4 Combination of Genistein with Conventional Cancer Chemotherapeutics

Combination cancer chemotherapeutic strategies have been devised to offer superior tumor response and lower toxicity. In contrast, chemotherapeutic agents unconsciously induce NF- κ B activity, which may lead to chemoresistance. Genistein has been extensively used as an adjunct to chemotherapy to enhance the activity of chemotherapeutic agents without causing increased toxicity. Li *et al.* (2004; 2005a) investigated whether the inactivation of NF- κ B activity by genistein would enhance the efficacy of chemotherapeutic drugs. Pancreatic, lung, prostate, osteosarcoma, and breast cancer cells were pretreated with genistein (15 to 30 μ M/l) for 24 h and then exposed to lower concentrations of chemotherapeutic agents for an additional 24 h. The combination of genistein with docetaxel, doxorubicin, cisplatin, or gemcitabine elicited significant inhibition of cell growth and NF- κ B activity, when compared with either one (Banerjee *et al.*, 2005; Li *et al.*, 2004, 2005a; Liang *et al.*, 2012; Mohammad *et al.*, 2006; Solomon *et al.*, 2008). Carefully designed clinical studies investigating the combination of soy isoflavones and commonly used chemotherapeutic agents for the treatment of human cancers are warranted to explore better possibilities. TRAIL, when used in combination with nontoxic concentrations of genistein, sensitized TRAIL-resistant human hepatocellular carcinoma Hep3B cells to TRAIL-mediated apoptosis (Jin *et al.*, 2009). In addition, p38 MAPK act as key regulators of apoptosis in response to treatment with a combination of genistein and TRAIL. The doses of soy isoflavones (genistein, daidzein, and equol) sensitized TRAIL-resistant prostate cancer cells to apoptotic death (Szliszka *et al.*, 2011). Zhu *et al.* (2012) produced findings that suggest genistein enhanced the antitumor effects of gefitinib in a NSCLC cell line. This synergistic activity was due to increased inhibition of EGFR. In addition, genistein abolished cisplatin-induced MMP-2 expression (Chen *et al.*, 2013c) and exhibited synergistic effects even at relatively low concentrations. Genistein could also enhance the activity of cisplatin via inhibition of NF- κ B and Akt/mTOR pathways (Sahin *et al.*, 2012). Shiau *et al.* (2010) investigated the possible mechanisms of soy phytochemicals on the cell-growth-inhibitory effect of trichostatin A (TSA, a novel anticancer drug). Genistein exerts its effects, by increasing caspase-3 activity and thus enhance TSA-induced cell death. The combination of genistein with gamma-irradiation completely prevented irradiation-induced COX-2 expression and PGE2 production in cervical cancer cells (Shin *et al.*, 2008). Additionally, this combined treatment inhibited proliferation through G2/M arrest and induced apoptosis via ROS modulation in the CaSki cancer cells. Genistein is a promising nontoxic dietary agent that may enhance treatment outcome in cancer patients when given concomitantly with chemotherapy. More experimental data about genistein and its combinations are necessary to test this hypothesis in clinical trials.

15.9 Tea Polyphenols Potential for Combinatorial Therapy

Tea from the *Camellia sinensis* species of the Theaceae family is one of the most ancient and, after water, is the most widely consumed beverage in the world. Tea is rich in polyphenolic constituents, which have strong anti-inflammatory, antioxidant, and anticarcinogenic, as well as antimutagenic properties in a variety of biological systems. Tea polyphenols are also reported to inhibit proliferation and increase apoptosis. Green tea catechins, especially epigallocatechin-3-gallate (EGCG), have been associated with cancer prevention and treatment. On the other hand, there are a number of issues, such as stability, bioavailability, and metabolic transformations under physiological conditions, facing the development of green tea polyphenols into therapeutic agents. The synthetic peracetate of (-)-EGCG has improved stability and better bioavailability than (-)-EGCG itself and can act as pro-drug under both *in vitro* and *in vivo* conditions (Huo *et al.*, 2008). This has resulted in an increased number of studies evaluating the effects derived from the use of this compound in combination with chemo/radiotherapy. Additive and synergistic effects of EGCG when combined with conventional cancer therapies have been proposed, and its anti-inflammatory and antioxidant activities have been related to amelioration of cancer therapy side effects. However, antagonistic interactions with certain anticancer drugs might limit its clinical use (reviewed by Lecumberri *et al.*, 2013).

15.9.1 Green Tea and Quercetin

Metabolic methylation of green tea polyphenols leading to methylated (-)-EGCG may alter the biological activities of EGCG and limit their chemopreventive potential. It has been demonstrated that 50% of EGCG was present in methylated form (4"-MeEGCG) in human prostate tissue, which is less bioactive. In a study, Wang *et al.* (2012a) suggested that a diet supplemented with 0.4% quercetin can increase the intracellular concentration (~4-fold) of EGCG with a decreased methylation rate in different cancer cells. EGCG and quercetin in combination synergistically inhibited cell proliferation, caused cell cycle arrest and induced apoptosis in PC-3 cells whereas in LNCaP cells the observed effects were additive (Wang *et al.*, 2012b). The combination treatment was also associated with a significant increase in the inhibition of androgen receptor and PI3K/Akt signaling (Wang *et al.*, 2014b). In addition to these, EGCG either alone or in combination with quercetin can eliminate cancer stem cell-characteristics. Quercetin synergizes with EGCG in inhibiting the self-renewal properties of prostate cancer stem cell, inducing apoptosis, and blocking cancer cells migration and invasion (Tang *et al.*, 2010). These results provide a novel regimen by combining EGCG and quercetin to improve chemoprevention in a non-toxic manner and warrant future studies in humans.

15.9.2 EGCG and Soy Phytochemical

Both green and black tea combination with soy phytochemicals at low doses are reported to have significantly reduced tumorigenicity rate, primary tumor growth, tumor proliferation index and microvessel density, serum androgen level, and metastases to lymph nodes in androgen-sensitive human prostate tumors in mice than used alone (Zhou *et al.*, 2003). Combination of EGCG and genistein with quercetin exert synergy in controlling the proliferation and expression of androgen receptor and tumor suppressor p53 gene expression in CWR22Rv1 PCa cells (Hsieh and Wu, 2009).

15.9.3 EGCG and Thymoquinone

Administration of EGCG alone or in combination with thymoquinone can limit PANC-1 cell proliferation (Tan *et al.*, 2006). EGCG and thymoquinone may be a potent biologic inhibitor of human pancreatic carcinomas, reducing their propagation activities.

15.9.4 EGCG and Trichostatin A

Trichostatin A (TCA a marine natural compounds with epigenetic activities). DNA methylation and histone deacetylation play important roles in the occurrence and development of cancers by inactivating the expression of tumor suppressors, including p16(INK4a). Cotreatment with EGCG (6 µg/ml) and trichostatin A (TSA, 15 ng/ml) synergistically reactivate p16(INK4a) gene expression in part through reducing promoter methylation, which coincided with increased p16(INK4a) expression and decreased cancer cell proliferation (Wu *et al.*, 2013).

15.9.5 EGCG and Luteolin

A combinatorial approach using two natural dietary polyphenols, luteolin (a flavone found in *Terminalia chebula*) and EGCG was carried out, and it was found that their combination at low doses (at which single agents induce minimal apoptosis) synergistically increased apoptosis (3–5-fold more than the additive level of apoptosis) in both head and neck and lung cancer cell lines (Amin *et al.*, 2010). This combination also significantly inhibited growth of xenografted tumors in nude mice (Amin *et al.*, 2010). This combination treatment strategy has a potential therapeutic advantage for human cancers.

15.9.6 EGCG and Pterostilbene (a Stilbenoid Derived from Blueberries)

EGCG and pterostilbene have additive, antiproliferative effects *in vitro* and alter the apoptotic mechanisms in pancreatic cancer cells by modulation at different points in the apoptotic mechanism (Kostin *et al.*, 2012). The combination induced mitochondrial depolarization and upregulated cytochrome C in pancreatic cancer cells.

15.9.7 EGCG and Panaxadiol

Panaxadiol is a purified sapogenin of ginseng saponins, which exhibits anticancer activity. Significantly enhanced antiproliferative effects were observed in human colorectal cancer cells (HCT-116 and SW-480) when panaxadiol (10 and 20 μ M) was combined with EGCG (10, 20, and 30 μ M) doses (Du *et al.*, 2013). The combination of panaxadiol and EGCG significantly increased the percentage of apoptotic cells compared with alone. The synergistic apoptotic effects were furthermore supported by docking analysis, which demonstrated that PD and EGCG bound in two different sites of the annexin V protein.

15.9.8 Polyphenon E

Polyphenon E (a well-standardized decaffeinated green tea catechin mixture contains about 60% of EGCG) synergistically inhibited growth of the colon cancer cells (Shimizu *et al.*, 2005). Treatment of HT29 cells with EGCG or Poly E caused an increase of cells in G1 and induced apoptosis and subsequently caused a decrease in the phosphorylated forms of EGFR, HER2 extracellular signal-regulated kinase and Akt proteins proteins. Reporter assays indicated that both EGCG and Poly E inhibited the transcriptional activity of the activator protein 1 (AP-1), c-fos, nuclear factor kappaB, and cyclin D1 promoters.

15.9.9 EGCG with Conventional Cancer Chemotherapy

The clinical effect of chemotherapy for cancer is constrained by resistance to drugs and various side effects. To overcome chemoresistance, various modified treatments, including combination therapy, have been used. Since then, nutraceuticals have drawn a great deal of attention toward cancer prevention because of their wide safety margin. However, single agent intervention has failed to bring the expected outcome in clinical trials; therefore, their combinations with anticancer agents are gaining popularity. EGCG may be used as a sensitizer to enhance the cytotoxicity of chemotherapeutic drugs such as tamoxifen, bortezomib, paclitaxel, 5-fluorouracil, doxorubicin, cisplatin, and so on (Chisholm *et al.*, 2004; Kim and Lee, 2014; Liang *et al.*, 2010; Luo *et al.*, 2010; Yang *et al.*, 2012; Wang *et al.*, 2009). The treatment of the multiple myeloma (KM3 cell) with EGCG inhibits cell proliferation and induces apoptosis, and there is a synergistic effect when EGCG and bortezomib are combined (Wang *et al.*, 2009). Further experiments showed that this effect involves the NF- κ B pathway. EGCG also synergistically sensitized breast cancer cells to paclitaxel *in vitro* and *in vivo* (Luo *et al.*, 2010). EGCG impedes proliferation of lung cancer cells including their cisplatin chemoresistant variants through down regulation of Axl and Tyro 3 tyrosine kinases expression (Kim and Lee, 2014). In addition, chemosensitizing effect of EGCG may also occur directly or indirectly by reversal of MDR, involving the suppression of MDR1 expression, or by enhancement of intracellular doxorubicin accumulation, involving inhibition of P-gp function (Liang *et al.*, 2010). TRAIL/Apo2L is a promising candidate for various cancer therapies; however, appearance of drug resistance limits its potential use. A synergistic inhibition in the invasion and migration of LNCaP cells by EGCG and TRAIL dosage was noted. This effect was mediated through inhibition VEGF, uPA MMP(-2, -3, and -9) and angiopoietin (Siddiqui *et al.*, 2008). Further treatment with subtoxic doses of EGCG in combination with TRAIL induces rapid apoptosis in TRAIL-resistant cancer cells (Basu and Haldar, 2009; Siegelin *et al.*, 2008). Furthermore, the effect of EGCG was tested in combination with specific COX-2 inhibitors on the growth of human cancer cells both *in vitro* and *in vivo* (Adhami *et al.*, 2007). EGCG synergistically heighten celecoxib mediated effects and reduces the levels of celecoxib required to elicit favourable effects. Combination of EGCG (10–40 μ M/l) and NS-398 (10 μ M/l) resulted in enhanced cell growth inhibition, apoptosis induction, expression of Bax, pro-caspases (-6 and 9), and PARP cleavage, inhibition of peroxisome proliferator activated receptor gamma; and inhibition of NF κ B compared alone, suggesting a possible synergism (Adhami *et al.*, 2007). *In vivo*, combination treatment resulted in enhanced tumor growth inhibition, lowering of PSA levels, insulin-like growth factor-I levels, and circulating levels of serum insulin-like growth factor binding protein-3 (Adhami *et al.*, 2007). Coincubation of Colo357 cells with celecoxib and EGCG synergistically diminished metabolic activity via apoptosis induction and downregulated release of pro-angiogenic (VEGF) and invasiveness-promoting (MMP-2) molecules. Celecoxib and EGCG also reduced IL-1-induced production of pro-inflammatory IL-6 and pro-angiogenic IL-8 (Härdtner *et al.*, 2012). These findings provided experimental evidence for efficacy of EGCG alone or in combination with drus in cancer therapy. Further pre-clinical studies and clinical trials will hopefully provide answers to the use of continuous low-dose of drugs combination with EGCG in human cancer treatment.

In addition to the previous studies, low-dose metronomic (LDM) chemotherapy represents a new strategy to treat solid tumors by displaying higher anti-angiogenic activity and fewer side-effects, especially in combination with other anti-angiogenic agents. Wu *et al.* (2012a; 2012b) demonstrated that the concurrent administration of EGCG doses with

metronomic capecitabine and docetaxel inhibited angiogenesis, growth of gastric tumor, and improved survival with less toxicity in mice with gastric cancer xenografts. LDM chemotherapy combined with EGCG may be an innovative and promising therapeutic strategy in the experimental treatment of human solid cancer.

Zhang *et al.* (2012) provided the evidence that EGCG potentiated efficacy of radiotherapy in breast cancer patients, and raised the possibility that this tea polyphenol has potential to be a therapeutic adjuvant against human metastatic breast cancer. Administration of EGCG (400 mg/three times daily) to breast cancer patients undergoing treatment with radiotherapy inhibits cell proliferation, invasion, and angiogenesis in breast cancer patients (Zhang *et al.*, 2012). Compared to patients who received radiotherapy alone, treatment with radiotherapy with EGCG for a time period of 2–8 weeks showed significantly lower serum levels of VEGF, hepatocyte growth factor (HGF), and reduced activation of MMP9/MMP2. In addition to this, coculture of MDA-MB-231 breast cancer cells with sera obtained from patients treated with combination therapy resulted in suppressed of cell proliferation and invasion, cell cycles at the G0/G1, reduced activation of MMP9/2, NF- κ B, and Akt.

15.10 D-Limonene: Potential for Combination Therapy

D-limonene is one of the most common terpenes in nature. It is a major constituent in several citrus oils (orange, lemon, mandarin, lime, and grapefruit). It is extensively used as a flavouring agent in common food items such as fruit juices, soft drinks, baked goods, ice cream, and pudding. This has well-established chemopreventive activity against many types of cancer (Gould, 1997). Majorly, chemopreventive activity of limonene during initiation can be attributed to the induction of phase I and phase II enzymes, with resulting carcinogen detoxification (Elegbede *et al.*, 1993). It appears to be as more effective against chemically induced colonic crypt foci by inhibiting the activity of ODC and cell proliferation (Kawamori *et al.*, 1996). Further, a study has shown that D-limonene exhibits antimitogenic activity, and its alcohol-derivatized perillyl alcohol at subtoxic doses has greater inhibitory effects on cell migration in colon cancer cells (Jia *et al.*, 2013). Therefore, D-limonene appears to have potential as a chemopreventive agent in colon carcinogenesis. Substantiation from a phase I clinical trial confirmed a partial response in a patient with breast cancer and stable disease for more than 6 months in three patients with colorectal cancer (Sun, 2007).

15.10.1 D-Limonene and Chemotherapeutic Drugs

A combination of 5% limonene with 4-hydroxyandrostenedione (12.5 mg/kg) resulted in a superior for breast tumour regression (83.3%) than either agent given individually (Chander *et al.*, 1994). Combination of low doses of docetaxel (a synthetic derivative of taxol) and d-limonene has been shown to have anti-prostate carcinogenic effects (Rabi and Bishayee, 2009). The combined treatment enhanced the sensitivity of prostate cancer cells to apoptosis and these effects were mediated through ROS generation and activation of caspases (3 and 9). Such a positive *in vitro* outcome deserves further investigations in *in vivo* models that mimic the progression of the disease, before it can be used in dietary supplements for cancer therapy.

15.11 Miscellaneous: Novel Nutraceuticals Formulation

15.11.1 Collect: A Dietary Supplement

Collect is a combination of nutraceuticals containing curcumin (500 mg), green tea (250 mg), and selenomethionine (100 μ g). Reports have been suggested that these agents can prevent colorectal cancer. 5-aminosalicylic acid (5-ASA), an anti-inflammatory drug, has been in the front line of inflammatory bowel disease (IBD) therapy for more than half a century. Collect has been effective against HT-29 human colon adenocarcinoma grade II cells *in vitro*, and this nutraceuticals complex in combination with 5-ASA has been shown to inhibit the formation or growth of chemically induced aberrant crypt foci (ACF) in rat models (Aroch *et al.*, 2010). The molecular mechanism by which this inhibition is mediated is via the inhibition of COX-2 pathways in HT-29 cells. However, growth inhibition can be affected via COX-2-independent pathways possibly through mechanisms that are regulated by the functional polyphenol presence in collect mixture. Such nutraceutical mixtures are of great clinical significance as a lot of different cellular mechanisms can be regulated by the presence of individual constituents.

15.11.2 BreastDefend: A Natural Dietary Supplement

This contains extracts from medicinal mushrooms (*Coriolus versicolor*, *Ganoderma lucidum*, *Phellinus linteus*), medicinal herbs (*Scutellaria barbata*, *Astragalus membranaceus*, *Curcuma longa*), and purified biologically active nutritional compounds (diindolylmethane and quercetin), inhibits proliferation and metastatic behavior of MDA-MB-231 invasive human breast cancer cells *in vitro*. In the present study, Jiang *et al.* (2012a) evaluated whether BreastDefend suppresses growth and breast-to-lung cancer metastasis in an orthotopic model of human breast cancer cells. Oral application of BD (100 mg/kg of body weight for 4 weeks) did not affect body weight or activity of liver enzymes and did not show any sign of toxicity in liver, spleen, kidney, lung, and heart tissues in mice. Moreover, BreastDefend significantly decreased the change in tumor volume over time compared to the control group ($p = 0.002$). BD treatment also markedly decreased the incidence of breast-to-lung cancer metastasis from 67% (control) to 20% (BD) ($p < 0.05$) and the number of metastases from 2.8 (0.0, 48.0) in the control group to 0.0 (0.0, 14.2) in the BD treatment group ($p < 0.05$). Finally, anti-metastatic activity of BD *in vivo* was further confirmed by the downregulation of expression of PLA2 (urokinase plasminogen activator, uPA) and CXCR4 (C-X-C chemokine receptor-4) genes in breast tumors.

15.11.3 ProstaCaid: A Dietary Supplement

Researchers evaluated the anticancer effects of dietary supplement ProstaCaid, a novel integrative blend contains mycelium from medicinal mushrooms, saw palmetto berry, pomegranate, pumpkin seed, 40% EGCG, 50% resveratrol, extracts of turmeric root, grape skin, pygeum bark, sarsaparilla root, *Scutellaria barbata*, eleuthero root, Job's tears, astragalus root, skullcap, dandelion, coptis root, broccoli, and stinging nettle, with purified vitamin C, vitamin D3, selenium, quercetin, citrus bioflavonoid complex, β sitosterolzinc, lycopene, α lipoic acid, boron, berberine, and DIM. ProstaCaid suppressed activation of AKT and MAPK signaling pathways in PC3 and LNCaP cells by reducing phosphorylation levels of AKT (Yan and Katz 2010). Another study, by Jiang *et al.* (2012b) showed the anticancer efficacy of the dietary supplement ProstaCaid™ (at dose 100, 200, or 400 mg/kg), against invasive prostate cancer. ProstaCaid treatment resulted in the inhibition of tumor volumes in a xenograft model of prostate cancer with human hormone independent PC-3 prostate cancer cells. Moreover, the study demonstrated significant upregulation of p21 and inhibition of IGF2, NR2F2, and PLA2 (uPA) genes expression of by an oral administration of drug in prostate cancer xenografts.

15.12 Conclusion

These days, chemopreventive agents are urgently required as an early interventional move toward preventing cancer growth as well lowering the risk of cancer incidence in human beings. An imperative need for alternate and/or adjuvant therapies is also in demand, since the existing available methods of cancer treatment have significant side-effects. In this way an effective agent is one that will create a magnitude of changes in cancer cells even at minimal nontoxic doses. Cancer prevention strategies making use of combined agents with distinct molecular mechanisms, rather than individual agents, and are considered promising for higher efficacy and lower toxicity. Yet at present, the application of synthetic drugs combinations in cancer therapies are ongoing in clinics but our awareness regarding such combinations of dietary factors is still limited. Given that our diet consists of a number of nutraceuticals, it is likely that nutrients in the diet act synergistically or additively to proffer health benefits. Nutraceuticals are relatively safe and abundantly available in dietary sources, therefore, their protective properties toward cancer prevention and therapeutics can be exploited as alternative medicines. A large database of studies supports the use of nutraceuticals in cancer treatment, albeit a majority of those are from *in-vitro* studies. Regardless of limited *in vivo* studies and clinical trials, nutraceuticals show great promise in cancer treatment considering their safe use. Conceivably, a huge research focus is required to explore such possibilities as avenues of using nutraceuticals in combination for cancer therapies and their translational benefits.

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16

Nutrigenomic Approaches to Understanding the Transcriptional and Metabolic Responses of Phytochemicals to Diet-Induced Obesity and its Complications

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16.1 Introduction

Obesity is an important health problem and a key factor in the development of metabolic syndrome, which is associated with increased cardiometabolic risk. Nutritional factors strongly influence the onset and progression of cardiometabolic syndrome, which include dyslipidemia, glucose intolerance, insulin resistance, inflammation, fatty liver disease, and obesity (Bouchard, 2008). Nutritional excess or imbalanced diets can act as metabolic stressors and play a key role in modulating the cardiometabolic risk phenotype in obesity-related conditions (Phillips *et al.*, 2013). When metabolic stress develops, hormonal and metabolic changes subdue the ability of the immune system to protect the body. This activity is further depressed if nutritional status accompanies the metabolic stress and impaired immunity, which leads to an increased risk of disease. It has been well established that obesity results in a state of chronic low-grade inflammation that contributes to several metabolic disorders (Hotamisligil *et al.*, 1993; Schenk *et al.*, 2008), and specific nutrients can modulate immune and inflammatory responses (Enwonwu and Ritchie, 2007).

Based on the pathology of cardiometabolic syndrome, it is assumed that specific nutrients could modulate obesity and its complications. Diets low in saturated fats and high in fruits, vegetables, and fiber have been recommended for the prevention of a number of inflammatory diseases and conditions, including obesity, fatty liver disease, Type 2 diabetes mellitus, and cardiovascular disease. Antioxidant vitamins (vitamins A, C, and E) and trace elements (selenium, copper, and zinc), which are known to be depleted during periods of inflammation (Semba and Tang, 1999), can counteract reactive oxygen species damage to cellular tissues and modulate the production of cytokines and prostaglandins through the regulation of redox-sensitive transcription factors (Wintergerst *et al.*, 2007). Intake of n-3 polyunsaturated fatty acid increases the tissue concentration of eicosapentaenoic acid and docosahexaenoic acid, which are known to down-regulate inflammation (Vedin *et al.*, 2008). Recently, the potential of natural products to counteract obesity has aroused considerable interest in food research (Krzyszowska *et al.*, 2010; Park and Kim, 2011).

Phytochemicals are the bioactive compounds of plants that do not deliver energy and are not yet classified as an essential nutrient but possess substantial anti-inflammatory, antimutagenic, and anticarcinogenic properties (Krzyanowska *et al.*, 2010; Park and Kim, 2011; Xagorari *et al.*, 2001). Some phytochemicals in diverse plants have other beneficial health effects, such as anti-obesity, by mediating the regulation of various metabolic pathways, including lipid absorption, energy expenditure, increasing lipolysis, lipogenesis, and differentiation and proliferation of preadipocytes. Scientists have identified thousands of phytochemicals, such as polyphenols, terpenoids, organosulfurs, and phytosterols (Figure 16.1). For instance, we have recently focused on luteolin (3',4',5,7-tetrahydroxyflavone) as a food-derived flavonoid, which usually occurs in glycosylated form in celery, green pepper, perilla leaf, and chamomile tea, and exhibits anti-inflammatory, antioxidant, and anti-allergy functions (Choi *et al.*, 2007; Middleton *et al.*, 2000). However, the effects of luteolin on low-grade chronic inflammation of obese adipose tissues remain to be clarified, and precise assessment of the underlying mechanisms of luteolin is necessary. With rapid advances in molecular biology and nutritional genomics in particular, high-density data sets from gene expression profiling technologies can efficiently be used to identify genetic factors that contribute to obesity and cardiometabolic syndrome and to provide functional information on the biological and pathophysiological roles of bioactive compounds. Thus, in this chapter, we partially cover the metabolic action of phytochemicals on diet-induced obesity and its complications from a nutrigenomic perspective.

16.2 Nutrigenomics

Nutrigenomics describes the scientific approach that integrates nutritional sciences and genomics and focuses on investigation of the effects of nutrition on health over the high-throughput “omics” technologies such as genomics, transcriptomics, proteomics, and metabolomics (Figure 16.2). Genomics is the systematic study of an organism’s genome. Genomic analysis with DNA microarray can reveal abnormalities such as chromosomal insertions and deletions or abnormal chromosomal numbers. The most common variations in DNA sequences between people are single nucleotide polymorphisms, which are of particular interest when linked with diseases with a genetic determination and have a role in exploring individual responses to a nutrient or drug (Horgan and Kenny, 2011). Transcriptomics is used to describe the approach in which the total messenger RNA in a cell or organism, and consequently gene expression, is analyzed in a biological sample under certain conditions at any given moment. Proteomics is defined as the complete set of all expressed proteins involved in the biological processes of a certain species. Proteome is a dynamic reflection of both genes and nutrition and can offer a solution when there is a gap between genome sequences and cell behavior due to numerous posttranscriptional/translational modifications. Metabolomics is used to describe quantitative analysis of all metabolites in a biological system. The metabolome is the final downstream product of gene transcription; therefore, changes in the metabolome are amplified relative to changes in the transcriptome and the proteome and are closest to changes in the phenotype (Urbanczyk-Wochniak *et al.*, 2003).

The aim of nutrigenomics is to elucidate how the components of a particular diet (bioactive compound) may affect the expression of genes, which may have increased its potential or which can be suppressed. Nutrigenomics also involves studies of nutritional factors that act to protect the genome and provides the scientific basis for improved public health through dietary means (Liu and Qian, 2011; Ryan *et al.*, 2008). One of the main interests in nutrigenomics research relates to prevention of the most common complex diseases, including obesity and cardiometabolic syndrome, through diet. These disorders are complex and multifactorial in their origin, involving not only genetic factors but also a number of behavioral and environmental factors such as exposure to certain food components (Ordovas *et al.*, 2007). Compounds such as resveratrol, which is present in wine, and soy genistein may indirectly influence the molecular signaling pathways, such as nuclear factor κ B (NF- κ B) (Daimiel *et al.*, 2012; Fialho *et al.*, 2008). The involvement of these factors in the activation and regulation of key molecules is associated with diseases ranging from inflammation to cancer (Fialho *et al.*, 2008; Gilmore, 2006; Hayden *et al.*, 2006; Maeda *et al.*, 2005). As a result, there is a need for molecular biomarkers that allow early detection of the onset of disease or, ideally, the pre-disease state (Gohil and Chakraborty, 2004). The development of nutrigenomic research with advances in microarray technology has created extraordinary opportunities for elucidating the effects of novel functional foods and nutraceuticals on global expression of genetic information and providing new means for discovering biomarkers for efficacy testing of bioactive compounds (Reardon and Cougle, 2002).

16.2.1 Tools for Bioinformatics and Systems Biology

As mentioned previously, nutrigenomics makes use of an integrated analytical approach that includes the latest developments in high-throughput “omics” technologies for the comprehensive study of different aspects of biological complexity. High-throughput technologies in transcriptomics usually generate large lists of differentially expressed genes as the final

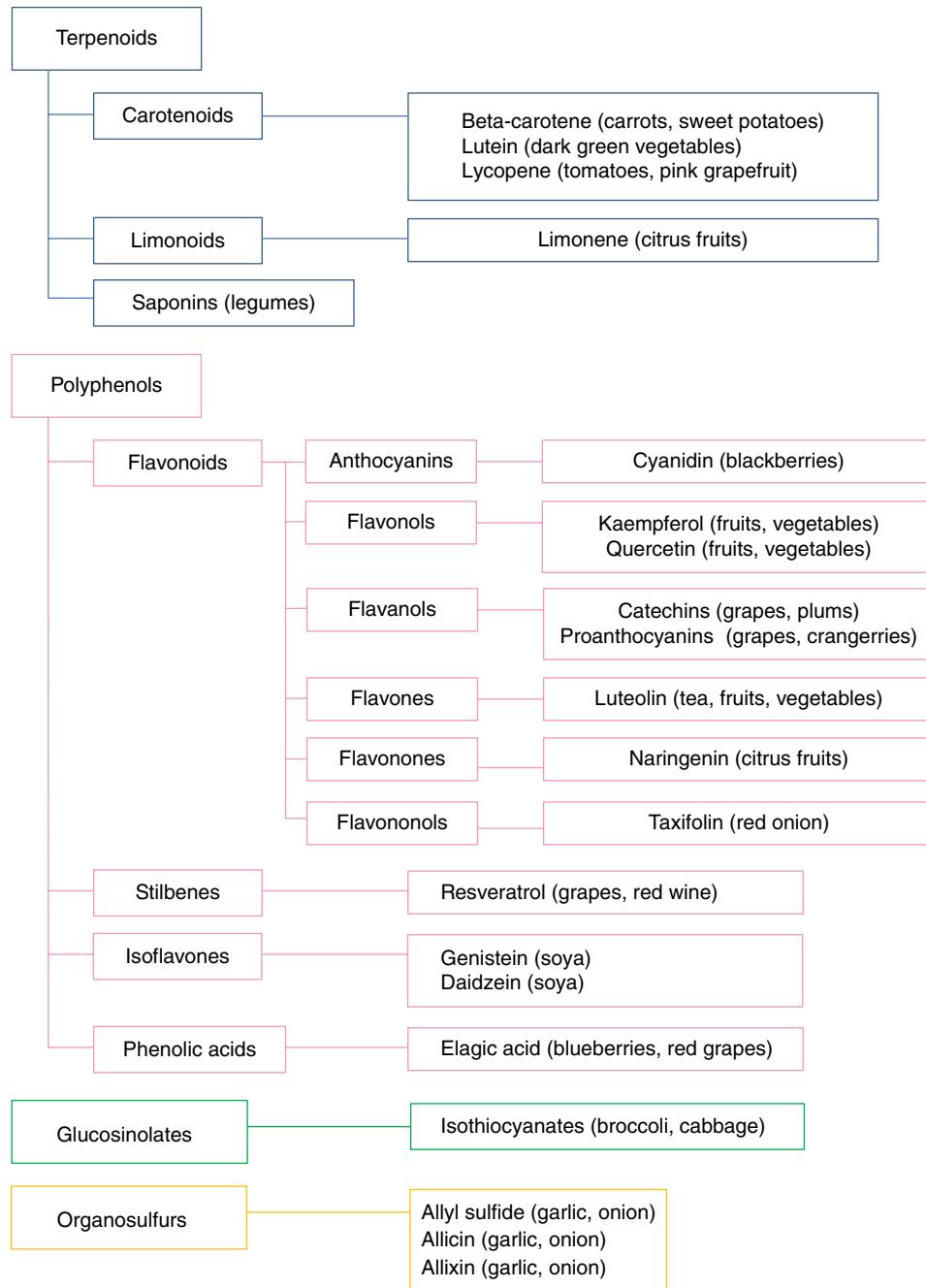


Figure 16.1 Classification of common dietary phytochemicals.

output. However, the biological interpretation of a large gene list is a challenging and daunting task, and therefore an increasing need for high-throughput analysis methods has led to the development of bioinformatics approaches. Such computational tools allow us to systematically analyze large lists of genes in an attempt to summarize the most enriched and significant biological aspects (Waagmeester *et al.*, 2008). The principal foundation of enrichment analysis is that if a

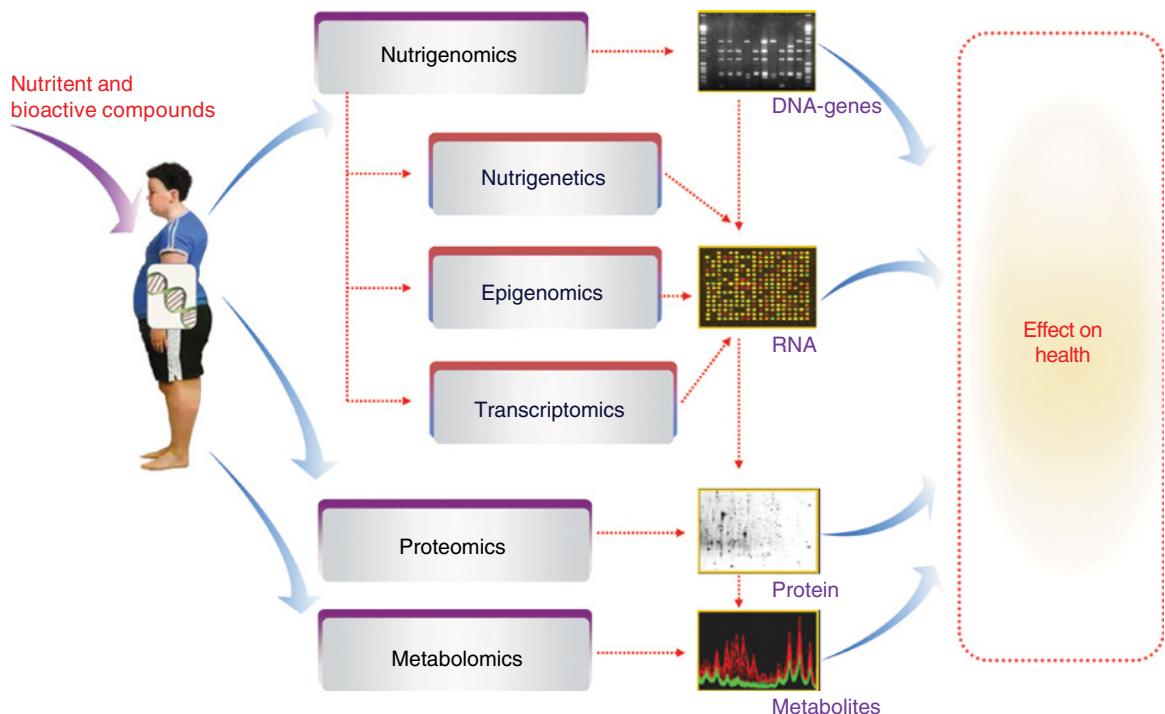


Figure 16.2 “Omics” sciences used in understanding molecular-level interactions between nutrients and other dietary bioactives with the genome.

certain biological process occurs in a given study, such as evaluation of the efficacy of a bioactive compound, the cofunctioning genes should have a higher (enriched) potential to be selected as a relevant group by high-throughput screening technologies (Huang *et al.*, 2009). This approach helps the researcher identify the significance of biological processes and discover a pertinent biological mechanism under study. A number of high-throughput enrichment tools, including DAVID, Onto-Express, FatiGO, GoMiner, and Kyoto Encyclopedia of Genes and Genomes (KEGG) Mapper, have played an important and successful role in contributing to the gene functional analysis of large lists of genes for various high-throughput biological studies (Table 16.1).

Gene Ontology provides an ontology of defined terms representing gene product properties in three key biological domains that are shared by cellular component, molecular function, and biological process (Zheng and Wang, 2008). Thus, Gene Ontology enables the researcher to functionally interpret the experimental data via enrichment analysis. The KEGG pathways (metabolites) are also linked to genomic information with higher-order functional information by computerizing current knowledge. The functional assignment in KEGG is a process of linking a set of genes in the genome with a network of interacting molecules in the cell, such as a pathway complex, representing a higher-order biological function (Kanehisa and Goto, 2000). In addition, recent enrichment tools integrate information extracted from other databases (e.g., Ingenuity Pathway Analysis) to improve the comprehensiveness of this type of study. Ingenuity Pathway Analysis provides interaction information between genes and proteins, related networks, functions and diseases, canonical pathways, and bioactive compound targets and biomarkers.

Nutrigenomics reflects the interaction between an environmental factor (nutrients) and our biological systems (genes). Before evaluating the nutrient-gene interactions, the systemic factors that affect the bioavailability and tissue concentration of the nutrients of interest, including digestion, absorption, and transportation, are considered. For this, “omics” platforms need to be integrated to obtain optimal means to understand the influences of bioactive compounds on the investigated system, giving rise to the growth of a new area of biology called systems biology (Hood and Perlmutter, 2004; Weston and Hood, 2004). In nutrigenomic studies, biological responses to bioactive compounds may be subtle; therefore, the evaluation of nutrigenomic effects requires careful attention and thorough examination

Table 16.1 List of enrichment tools (source: Huang et al., 2009, with modifications).

Name of enrichment tool	Year of release	Key statistical method
Onto-Express	2002	Fisher's exact; hypergeometric; binomial; chi-square
EASE	2003	Fisher's exact (modified as EASE score)
FatiGO	2003	Fisher's exact
GeneMerge	2003	Hypergeometric
GoMiner	2003	Fisher's exact
MAPPFinder	2003	Z-score; hypergeometric
GO	2004	Hypergeometric
GOCluster	2005	Hypergeometric
eGOn/Gene Tools	2006	Fisher's exact
GO Mapper	2004	Gaussian distribution; EQ score
GSEA	2005	Kolmogorov-Smirnov-like statistic
PAGE	2005	Z-score
KEGG* Mapper	2010	Gaussian distribution; EQ score
DAVID	2003	Fisher's exact (modified as EASE score)
GOToolBox	2004	Hypergeometric; Fisher's exact; binomial
Ingenuity Pathway Analysis	2006	Unclear

* KEGG, *Kyoto Encyclopedia of Genes and Genomes*.

of at least 3 interconnected systems: nutrient-nutrient, nutrient-gene, and gene-gene interactions. Because one type of nutrient can significantly affect the metabolism and biological function of another nutrient and one nutrient can affect multiple gene expressions, one gene expression can be modulated by different nutrients. From a practical standpoint, the integrated approach of systems biology can inspire us to create comprehensive strategies for more effective prevention and treatment of diseases. An adequate systems biology approach in nutrigenomics should provide a holistic view of the molecular mechanisms underlying the beneficial or adverse effects of certain bioactive compounds as well as help in the discovery of transcriptional factors that function to regulate metabolic pathways and in which expression is affected by specific bioactive compounds. It is likely that systems biology-based nutrigenomics research will aid in rapidly identifying new biomarkers for nutritional status and disease progression and ultimately provide alternative dietary interventions for complex conditions associated with metabolic disease in addition to comprehensive information for the development of personalized nutrition.

16.3 Obesity and Cardiometabolic Syndrome

Cardiometabolic risk factors frequently occur in obese youth, and these risk factors tend to cluster. Goodman *et al.* (2005) found that obesity has the most substantial influence on cumulative cardiometabolic risk. Obesity, insulin resistance, and chronic low-grade inflammation are hypothesized to be the underlying mechanisms for this clustering of risk factors, known as metabolic syndrome or cardiometabolic syndrome. Central adiposity, atherogenic dyslipidemia, high blood pressure, insulin resistance, glucose intolerance, and a prothrombotic, proinflammatory state all characterize cardiometabolic syndrome.

16.3.1 Obesity

Recent studies have shown that not only environmental factors but also genetic aspects are related to health problems, including obesity and cardiometabolic syndrome (Daimiel *et al.*, 2012; Phillips, 2013). Nutrigenomics is relevant for understanding obesity and how it is linked to associated diseases. An individual genetic background can influence nutrient status, the metabolic response to nutrients/diets, and predisposition to diet-related disease. Not everyone becomes overweight or obese in an obesogenic environment, suggesting that there are genetic or acquired factors that interact with actual environmental factors to predispose some people to obesity.

A number of recent reviews present evidence linking candidate genes to obesity (Perez-Martinez *et al.*, 2010; Ramachandrapuram and Farooqi, 2011). Leptin is a hormone with circulating levels that correlate closely with fat mass. Many studies have shown that leptin stimulates the expression of pro-opiomelanocortin (POMC) in primary neurons located in

the arcuate nucleus of the hypothalamus. POMC is extensively posttranslationally modified to generate the melanocortin peptides, which are agonists at melanocortin 4 receptors (MC4R) and suppress food intake (Cone, 2005; Schwartz *et al.*, 2000). In addition, leptin inhibits orexigenic pathways, which are mediated by neurons expressing neuropeptide Y that can suppress the expression of POMC. Targeted genetic disruption of MC4R in mice leads to increased food intake and increased lean mass and linear growth (Huszar *et al.*, 1997). These hypothalamic pathways coordinate appetite and modulate intermediary metabolism and energy expenditure.

The stress-activated protein kinases JNK1 and IKK β are central signal transducers in innate immunity and stress responses that control the expression of several proinflammatory genes. Both JNK1 and IKK β exert substantial effects on the development of adiposity (Kleinridders *et al.*, 2009; Solinas *et al.*, 2007; Zhang *et al.*, 2008). Many studies have shown that JNK1 affects energy balance and/or lipid metabolism. Mice bearing a conditional *Ikk β* gene disruption in the brain are resistant to high-fat diet-induced obesity and display decreased food intake compared with controls (Zhang *et al.*, 2008). It was proposed that activation of conversion from IKK β to NF- κ B in the hypothalamus promotes food intake and obesity through induction of SOCS3 gene expression, leading to inhibition of leptin signaling (Zhang *et al.*, 2008). Indeed, mice with a targeted disruption of the *MyD88* gene in the central nervous system are protected from diet-induced obesity and leptin resistance (Kleinridders *et al.*, 2009). In the study by Hirosumi *et al.* (2002), *Jnk1*-knockout mice were found to be obesity resistant in both dietary and genetic models; in addition, conditional *Jnk1* gene mutation studies suggest that JNK1 activity in neurons is required for efficient high-fat diet-induced obesity (Sabio *et al.*, 2008, 2010a, b).

Some interplay between toll-like receptors (TLRs) and obesity has been investigated in numerous studies. Activation of TLR signaling (except TLR3) through an MyD88-dependent pathway led to activation of the transcription factor NF- κ B. Increased NF- κ B activity was observed in mice fed a high-fat diet (Zhang *et al.*, 2008). A study by Kwon *et al.* (2012) suggested that adipocytes based on transcriptome *Tlr1*, *Tlr2*, *Tlr6*, *Tlr7*, *Tlr8*, and *Tlr13* expression increase on stimulation with adipogenesis. In addition, diet-induced obesity leads to significant induction of *Cd* antigen genes active in the immune and inflammation system of animals. Synthetically, these investigators proposed that adipose tissue expansion as well as concomitant activation of TLR-mediated inflammatory signaling cascades and induction of CD antigens causes the pathogenesis of obesity and cardiometabolic syndrome (Kwon *et al.*, 2012).

The circadian clock governs a large array of physiological functions that maintain our metabolic flexibility, and current studies suggest that interruption of the circadian system may contribute to obesity and obesity-related complications (Garaulet *et al.*, 2010). The role of this circadian system, including central components such as CLOCK, BMAL1, PER2, and CRY1/CRY2, in human obesity has been demonstrated by clock genes variants in association with obesity and metabolic outcomes (Corbalán-Tutau *et al.*, 2011; Garaulet *et al.*, 2010b, c). In particular, *Per1*-knockout mice showed significant differences in body mass as well as food and water intake and exhibited increased glucose metabolism after intraperitoneal injection of glucose (Dallmann *et al.*, 2006). In this regard, the basal expression of clock genes is significantly associated with abdominal fat content and cardiometabolic risk factors (Gómez-Santos *et al.*, 2009, 2010).

16.3.2 Inflammation and Insulin Resistance in Obesity

Inflammation and insulin resistance is a complex trait characterized by increased proinflammatory cytokine secretion and decreased insulin secretion and insulin action. A number of observational studies have reported an increased prevalence of chronic low-grade inflammation and insulin resistance with obesity, and it is now widely accepted in the field of obesity research that obesity is an important risk factor for cardiometabolic syndrome (such as inflammation and insulin resistance) (Gregoire *et al.*, 1998; Gual *et al.*, 2005; Schenk *et al.*, 2008; Sethi and Vidal-Puig, 2007). Substantial evidence has shown that chronic activation of proinflammatory pathways within insulin target cells can lead to obesity-related insulin resistance. Consistent with this, tumor necrosis factor (TNF)- α levels are elevated in adipose tissue and blood from obese rodents, and neutralization of TNF- α improves insulin sensitivity in these animals (Hotamisligil *et al.*, 1993). It was reported that mice bearing a mutation in the gene encoding *Tlr4*, the sensor for bacterial lipopolysaccharide and other agonists, show improved insulin sensitivity in a model of free fatty acid (FFA)-induced insulin resistance (Shi *et al.*, 2006). The *Irf5* gene is generally involved downstream of the TLR-MyD88 signaling pathway for induction of proinflammatory cytokines, and *Irf5* expression was upregulated in adipose tissue of diet-induced mice (Kwon *et al.*, 2012). As mentioned in the preceding text, adipose tissue expresses nearly all TLR family members and TLR2-knockout mice are protected from high-fat diet-induced obesity and insulin resistance, suggesting a broad role for TLRs in obesity and its associated morbidities (Himes and Smith, 2010; Poulain-Godefroy *et al.*, 2010).

Factors such as fatty acids, microhypoxia in adipose tissue, endoplasmic reticulum stress, and certain cytokines can all initiate a proinflammatory response. In obesity, changes in adipose tissue macrophages (ATMs) and gene expression profile

occur coincident with the development of insulin resistance, so it is possible that ATMs are merely effectors of a coordinated inflammatory response (Strissel *et al.*, 2007). Macrophages are believed to play an important role in obesity-induced insulin resistance by initiating proinflammatory responses through activation of JNK1 and the IKK/NF-κB signaling pathway (Schenk *et al.*, 2008). Furthermore, JNK1 and IKK β contribute to insulin resistance through serine/threonine phosphorylation of insulin receptor substrates 1 and 2, which results in uncoupling of insulin receptor activation from downstream signaling (Aguirre *et al.*, 2002; Tanti *et al.*, 1994; White, 2003). Activation of JNK1 and IKK β occurs downstream of MyD88 and plays a critical role in inflammation in insulin-resistant humans and rodents (Bandyopadhyay *et al.*, 2005; Cai *et al.*, 2005; Hirosumi *et al.*, 2002; Yuan *et al.*, 2001). KO or inhibition of JNK1 or IKK β prevents insulin resistance in cell and mouse models as well as in human models of insulin resistance (Cai *et al.*, 2005; Hirosumi *et al.*, 2002; Solinas *et al.*, 2007; Yuan *et al.*, 2001). The ultimate endpoint for TLR/IKK β and JNK signals is NF-κB-dependent activation of inflammatory gene transcription (Baker *et al.*, 2011). The *Ikke* gene appears to play a role in maintaining body weight and insulin resistance by suppressing thermogenesis, and expression of the *Ikke* gene was induced by a high-fat diet (Chiang *et al.*, 2009).

The transcription factor peroxisome proliferator activated receptor gamma (PPAR γ) has been investigated in numerous studies. This transcription factor is activated by a number of exogenous and endogenous ligands, such as fatty acids and their derivatives. PPAR γ is expressed in monocytic cells, and its modulation by synthetic ligands can influence inflammatory processes. The thiazolidinediones, potent PPAR γ activators (potent full agonists), modulate the expression of several key proteins involved in the metabolism of triglyceride-rich lipoproteins and insulin signaling. Treatment with thiazolidinediones has been shown to reduce the circulatory levels of low-density lipoproteins and triglycerides (Kramer *et al.*, 2001; Martens *et al.*, 2002; Rieusset *et al.*, 1999). Whereas PPAR γ positively regulates the anabolism of lipids in macrophages, activation of PPAR γ acts as a negative regulator of the inflammatory (Pascual *et al.*, 2005). Mice with a conditional ablation of the *Ppary* gene in macrophages show increased susceptibility to obesity-induced inflammation and insulin resistance. This is due to loss of the anti-inflammatory action of PPAR γ (Hevener *et al.*, 2007; Odegaard *et al.*, 2007). Increased numbers of ATMs were also observed in mice bearing a targeted mutation at the *Ppary* gene crossed with *ob/ob* mice (Bento-Abreu *et al.*, 2007). A polymorphism has been identified in the *Ppary* gene in several populations, which appears to affect its transcriptional response. *Ppary* gene variants can promote susceptibility factors of obesity and lipid status, contributing to the development of metabolic syndrome (Bozina *et al.*, 2013).

16.3.3 Obesity and Cardiometabolic Syndrome: A Possible Role for Nutrigenomics

Numerous nutrigenomic studies in which the outcome measure was markers of disease risk, most notably obesity and cardiometabolic syndrome, provide proof of principle. Although producing a more subtle effect than a pharmaceutical agent as proof of concept from a nutraceutical perspective, several nutrients have now emerged as potentially insulin sensitizing, affecting molecular targets by interfering with the (1) TLR4/IKK/JNK/NF-κB axis, (2) target PPAR γ , or (3) target proinflammatory cytokines.

Perhaps the most extensive area of research in relation to their immunomodulating effects is with respect to dietary fatty acids, which are known to interact with several inflammatory pathways. Saturated fatty acids activate TLR4 and increase NF-κB transcriptional activity. In contrast, polyunsaturated fatty acids (PUFAs) have emerged as anti-inflammatory nutrients that exert their effects through a number of biological mechanisms. Recent genome-wide association studies using microarray analysis have identified that the consumption of virgin olive oil is associated with down-regulation of some proinflammatory genes involved in several pathways: the transcription factor NF-κB, cytokines, and mitogen-activated protein kinases in peripheral blood mononuclear cells of patients with metabolic syndrome (Camargo *et al.*, 2010). Similarly, intake of eicosapentaenoic acid and docosahexaenoic acid resulted in decreased expression of genes involved in inflammatory-related pathways by reducing nuclear p65 expression and increasing cytoplasmic I κ B α expression, and docosahexaenoic acid may act as a more potent NF-κB inhibitor than eicosapentaenoic acid (Bouwens *et al.*, 2009; Bradley *et al.*, 2008; Weldon *et al.*, 2007). Furthermore, PUFAs induced a reduction in the expression of some genes related to inflammation, which may constitute the underlying mechanism associated with the decreased adiposity linked to PUFA intake (Kabir *et al.*, 2007). Additionally, PUFAs may exert their anti-inflammatory effect by enhancing adiponectin secretion from human adipocytes, an effect that is elicited at least partially via PPAR γ (Tishinsky *et al.*, 2011; Neschen *et al.*, 2006). Genetic variability between individuals likely influences the responsiveness to an intervention. The common genetic variants of the complement component 3 (C3) locus conferred an increased risk of metabolic syndrome, and PUFA intake may modulate these genetic influences (Phillips *et al.*, 2009). These studies highlight the importance of understanding the interaction between single nucleotide polymorphism genotype and diet in determining the risk of metabolic syndrome, and a personalized nutrition approach may be considered to determine an ideal dietary intervention.

The association between high levels of consumption of fruits and vegetables and a decrease in the risk of obesity and cardiometabolic syndrome is mostly attributable to the high concentration of bioactive compounds such as polyphenols. Several dietary polyphenols modulate the expression of genes related to postprandial hyperglycemia, hyperinsulinemia, and hyperlipidemia, all of which are related to a risk of developing obesity and cardiometabolic syndrome (Murase *et al.*, 2010). Resveratrol has been one of the most studied polyphenols in association with human health in past years. Resveratrol, which naturally occurs in grapes, was shown to inhibit preadipocyte proliferation, adipogenic differentiation, and *de novo* lipogenesis in a SIRT1-dependent manner in adipocytes (Fischer-Posovszky *et al.*, 2010). Moreover, in humans, it has been shown that treatment with resveratrol can improve the metabolic phenotype of healthy obese men by reducing blood glucose and insulin levels and reducing plasma inflammatory markers (Timmers *et al.*, 2011). Another recent study that aimed to identify the nutrigenomic impact of resveratrol revealed the capacity of resveratrol to modulate the expression of numerous genes involved in inflammation and cellular stress response, potentially through the NF- κ B signaling pathway, in peripheral blood mononuclear cells of subjects with obesity and Type 2 diabetes mellitus (de Groot *et al.*, 2012; Tomé-Carneiro *et al.*, 2013). Another polyphenol, hesperidin, modulated the expression of genes such as CCL26, CX3CR1, CXCL17, ITGBL1, and PLA2G7, which are involved in cell adhesion, chemotaxis, chemokines, lipid transport, and/or immune response in circulating leukocytes. This gene expression profile suggests the potential protective effect against foam cell formation that is a hallmark of atherosclerosis (Milenkovic *et al.*, 2011). In addition, curcumin inhibits lipopolysaccharide-induced secretion of TNF- α and interleukin-1 β and completely inhibits TNF- α -induced activation of NF- κ B and the inflammatory response (Chan, 1995; Singh and Aggarwal, 1995). However, few studies have investigated the interaction between polyphenol luteolin and cardiometabolic syndrome, and thus the effect of luteolin on obesity and its complications may be worthwhile to discuss.

16.4 Anti-Obesity Action of Luteolin

Specific food chemicals such as polyphenols have been shown to ameliorate obesity in animals by molecularly defined mechanisms. Among them, luteolin (3',4',5,7-tetrahydroxyflavone) is a food-derived flavonoid that usually occurs in glycosylated form in celery, green pepper, perilla leaf, and chamomile tea. Evidence from previous *in vitro* studies suggests that luteolin is one of the most potent and most efficacious flavonoids that has anti-inflammatory activity. More specifically, luteolin inhibits the lipopolysaccharide-induced production of TNF- α , interleukin-6, and inducible nitric oxide in macrophages (Xagorari *et al.*, 2001; Chen *et al.*, 2007) and suppresses TNF- α -induced NF- κ B pathway activation (Shi *et al.*, 2004). Ando *et al.* (2009) reported that luteolin inhibited the phosphorylation of JNK and suppressed the production of inflammatory mediators in activated macrophages. Furthermore, luteolin exerts antiadipogenic effects by suppressing adipogenic transcription factors and inhibiting the transactivation of PPAR γ (Park *et al.*, 2009).

In contrast to *in vitro* studies, *in vivo* studies associated with evaluation of the effect of luteolin have been overlooked in previous studies; to our knowledge, no systematic empirical research has addressed the impact of luteolin on obesity and its complications. By using nutrigenomic techniques, we have been able to investigate the efficacy of luteolin supplementation in mice with diet-induced obesity. Rodent models provide powerful tools for molecular genetic investigation into the impact of nutritional changes in obesity and cardiometabolic syndrome pathogenesis. High-fat diet feeding is an efficient system promoting obesity, insulin resistance, and fatty liver disease in the majority of rodent models (Almind and Kahn, 2004; Montgomery *et al.*, 2013). By using microarray analysis, we investigated the detailed mechanisms associated with the antioesity action of luteolin based on integration of the transcriptional profile and their phenotype biomarkers in diet-induced obese C57BL/6J mice. Luteolin supplementation was found to be effective for lowering plasma lipid levels in diet-induced obese mice by increasing expression of the chylomicron remnant and LDL receptor gene and decreasing expression of the *Mtp* (microsomal triglyceride transfer protein) gene involved in very-low-density lipoprotein synthesis at the same time. In addition, luteolin supplementation altered hepatic lipid-metabolizing enzyme activities and gene expression and increased fecal lipid levels, thereby helping to lower plasma and hepatic lipid levels. Moreover, luteolin supplementation lowered not only plasma FFA levels but also adipose tissue weight by increasing the expression of genes related to FFA uptake and re-esterification, FFA oxidation, and the tricarboxylic acid cycle in adipose tissue. This important result demonstrates for the first time that luteolin may help ameliorate the deleterious effects of diet-induced obesity and its metabolic complications such as adiposity, dyslipidemia, hepatic steatosis, and insulin resistance (Figure 16.3).

These preliminary data, taken together with the established link between phytochemicals and cardiometabolic syndrome that is focused on the nutrigenomic perspective, lead us to hypothesize that luteolin may help ameliorate the deleterious effects of obesity and its metabolic complications such as adiposity, dyslipidemia, hepatic steatosis, and insulin resistance.

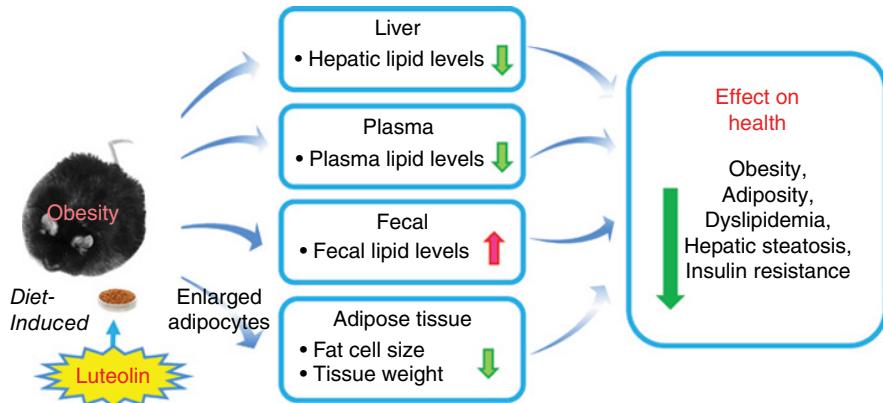


Figure 16.3 Proposed role of luteolin in attenuating obesity and its complications in high-fat fed mice.

16.5 Conclusion

Nutrigenomics investigates the links and interrelationship among diet, genetic makeup, and physiological responses at a genome-wide level and in a systematic manner. Thus, nutrigenomic research in obesity and cardiometabolic syndrome has provided insights into how particular nutrients, and non-nutritive food components such as bioactive compounds, play an important role in detecting and modulating the cellular sensor system that influences gene (genomic) and protein (proteomic) expression and, subsequently, metabolite production (metabolomic). The different “omics” and bioinformatic approaches to realize the vision of nutritional systems biology are in the process of integration and noninvasive techniques to facilitate future studies in humans are in development. Ultimately, nutrigenomics has the potential to provide, at least in part, a proposal of nutritional strategies that is focused on nutraceuticals and the rationale for personalized dietary recommendations based on the individual’s genetic constitution and biochemical individuality to prevent and manage obesity and other diet-related diseases.

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17

Going Beyond the Current Native Nutritional Food Through the Integration of the Omic Data in the Post-Genomic Era: A Study in (Resistant) Starch Systems Biology

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17.1 Introduction

Science and technology have helped improve the quality of human life on the earth dramatically in recent decades. These help make life easy, and speed up the rhythm of daily life with automatic machines and facilities. While people have a more convenient life, there are more concerns on health and demanding a healthy life. Many studies in this era, thus, aim to find good food or functional food for complementing health. Exploring valuable nutrition hidden natively in living organisms has become an equally popular research topic in life sciences, in order to improve product yield. A broad class of organisms has been exploited in research studies, ranging from the small world of microbes (e.g., Romero *et al.* 2007; Yoshida *et al.* 2009) to the green world and exhaustive space of multicellular eukaryotic organisms (e.g., Qi *et al.* 2011; Vigeolas *et al.* 2007; Zhang *et al.* 2008). As autotrophic organisms with large diversity, plants are a highly fascinating source in the search for new nutritional compounds. In addition to primary food and energy sources such as starches and lipids, the most frequently mentioned nutrients include bioactive, antioxidative compounds (e.g., carotenoids, tocopherols, ascorbate acid, Coenzyme Q10, melatonin, lycopene, and flavonoids: Kris-Etherton *et al.* 2002; Zhu *et al.* 2013), pigment (e.g., carotenoid, betacyanins, betaxanthins, chlorophyll-a: Farré *et al.* 2011; Guesmi *et al.* 2013; Zhang *et al.* 2014), and other nutritional supplement compounds (e.g., folate, iron, amino acids, vitamin A, vitamin B (B1, B2, B3, B5, B6, B8, B9, and B12), vitamin C, vitamin D, vitamin E, and vitamin K: Bhullar and Gruissem, 2013; Fitzpatrick *et al.* 2012; Ye *et al.* 2000). These continuously identified compounds have been utilized in diverse applications and most are relevant to supplemental diets; for example, the use of polyunsaturated fatty acids (n-3 and n-6 PUFA) and gamma linolenic acid (GLA) in *Spirulina platensis* as an anti-aging food supplement

(Diraman *et al.* 2009). Recently, resistant starch, of which molecules resist digestion and function in a similar way to fiber, has received great attention as beneficial both in terms of diet food supplement (Bodinham *et al.* 2010; Homayouni *et al.* 2014) and an anticancer substance (Fuentes-Zaragoza *et al.* 2011; Homayouni *et al.* 2014).

To circumvent suffering from the storm of diseases as well as food starvation in the future, advanced technologies in the post-genomic era have been exploited in an effort to increase the capability of dietary supplement identification, and to enhance the yield of the desired products in plants. The advantage of the emerging high-throughput measurement over the early gene-based approach is the capability of measuring the omic data of molecules in living organisms immediately, which allows the global investigation of a system. Here, we demonstrate the contribution of the systems biology as an omic-data-integrative approach to enhance the cellular capacity for phyto-nutrition production through the example of (resistant) starch yield improvement.

17.2 Starch and its Yield Improvement in Plants

Starch is the main carbohydrate storage in plant species that is utilized as food for humans, feed for animals, and recently biofuel feedstock as an energy resource. It is the heteropolymer of amylose (AL) and amylopectin (AP), the linear alpha 1,4-polyglucans and 1,6 branched polyglucans (Sonnewald and Kossmann, 2013; Waterschoot *et al.* 2014). The proportions of the amylose and amylopectin are the crucial characteristics of starch that modulate its physicochemical properties (Homayouni *et al.* 2014; Waterschoot *et al.* 2014) (Figure 17.1). Amylose-rich starches normally have high degree of retrogradability that always results in high crystalline structure. These characteristics enhance resistance of starch molecule to enzyme digestion, so that the amylose-rich starches are more fascinating raw material in resistant starch production over the amylopectin-rich starches. In other words, amylopectin-rich starches, called waxy starch, are more advantageous in de-branched starch production (Charles *et al.* 2005). The diversity of the AL/AP proportion is found among wild plant species (Slavin, 2013; Waterschoot *et al.* 2014) and also among cultivars in a plant species (Charles *et al.* 2005), leading to the spectrum of starch properties available in nature (Figure 17.1). The variety of starch properties is useful for versatile applications, especially in the functional food industry. Nowadays, dietary industries are interested in plant starch not only for primary food, but also for functional nutrition production, a more profitable commodity. Research and development in this area is then directed in two areas. On one side, attempts have been made to explore the desired compounds among plant diversity and to elevate the yield of such compound, while the other has

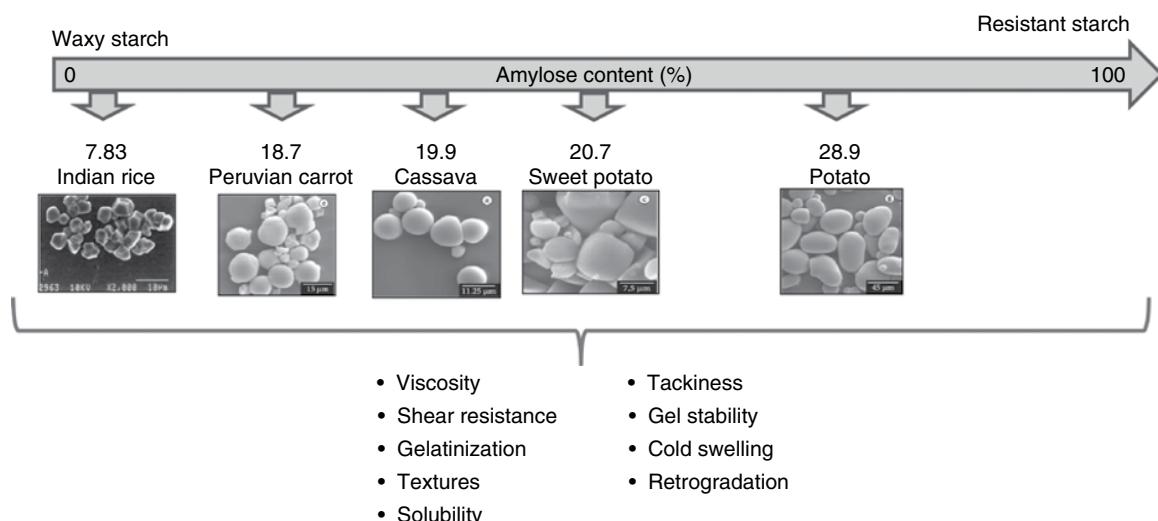


Figure 17.1 A spectrum of amylose contents in plant starches providing the starch varieties with different physicochemical properties. SEM of cassava, sweet potato, peruvian carrot, and potato from Rocha *et al.* (2010), reproduced with permission of Elsevier; Indian rice from Singh, Sodhi and Singh (2003), reproduced with permission of Elsevier.

intended to attain tailor-made products from the native phyto-substances. The progressive science and technologies in this area facilitate going beyond the natural variety of phyto-products for both qualitative and quantitative aspects.

For years, native starches have been modified after harvesting through *in vitro* chemical and bio-enzymatic assays (Jiang *et al.* 2014; Wang and Copeland, 2013). These processing methods have successfully transformed the inherited properties of starches to meet the specific requirements of an application, such as small granule size for cosmetic manufacturing (Sonnewald and Kossmann, 2013). While the chemical-based approaches are considered to be an economical practice for starch modification, they might be rather costly for environmental usage (Waterschoot *et al.* 2014). The developing assays, therefore, aim to alleviate the chemical waste problem in order to be more environmentally friendly. Alternative *in vivo* assays based on genetic and metabolic engineering are believed to be a more green technology, rationally providing an environmentally-safe approach, so they have become popular at the moment. Genetic and metabolic engineering techniques have been introduced to *in vivo* modification for different purposes, which are producing value-added compounds, enhancing the tolerance to biotic and abiotic stresses, and improving crop yield. For more than a decade of applying these techniques for *in vivo* starch modification, it sheds light on the capacity of the green technology to manipulate quantity and quality of starch in plant cells. In this section, the genetic engineering to improve the starch yield and property in plants are briefly reviewed.

At first, genes involved in the starch biosynthesis pathway were subjectively targeted for engineering their metabolic performance. The molecular biological techniques, including mutation, transgene and genetic manipulation, were employed to investigate the effect of a particular gene perturbation on starch biosynthesis (e.g., ADP-glucose pyrophosphorylase: Smidansky *et al.* 2003; Sakulsingharoj *et al.* 2004), and starch synthase (McMaugh *et al.* 2014; Szydlowski *et al.* 2009). Genes related to the four key enzymes in the starch biosynthesis pathway were studied the most: ADP-glucose pyrophosphorylase (AGPase; EC 2.7.7.23), starch synthase (SS; EC 2.4.1.21), starch branching enzyme (SBE; EC 2.4.1.28), and starch debranching enzyme (DBE; EC 2.4.1.41) (more detail in Figure 17.3 later). AGPase starts up the starch formation by converting glucose-1-phosphate (G1P) to ADP-glucose (ADPG) in order to provide the monomer subunit for glycosyl-chain synthesis. Subsequently, SS and SBE function in polymerizing the glycosyl-chain product to amylose and amylopectin, whereby DBE simultaneously decorates the branched-chains of the polyglucans to conform a precise structure for starch granule formation.

As an essential role of AGPase is to supply the ADPG subunits to the starch polymerization, it is the most logical engineered target for yield improvement. Modification of the AGPase in plant is not straightforward because it consists of two small and two large subunits that are encoded from multiple genes (Tuncel *et al.* 2014). Moreover, it was found that plant AGPase has at least two isoforms named according to their localization: cytosolic- and plastidial-isoforms. The activity of the AGPase isoform differs between plant species. In potato tubers and pea embryos, plastidial AGPase is a predominant isoform, whereas in cereals (i.e., maize, barley, and rice), cytosolic AGPase is more dominant. The supporting evidence of the effect of AGPase activity on the starch content has been well described (Ihemere *et al.* 2006; Li *et al.* 2011; Sakulsingharoj *et al.* 2004). Antisense inhibition of AGPase in transgenic potatoes decreased starch formation in tubers (Müller-Röber *et al.* 1992), implying the rationale to increase starch production based on the engineering at AGPase. However, the later works showed contradictory results. Transformation of the mutant *E.coli* AGPase gene into different potato cultivars showed varying results of starch content (Stark *et al.* 1992; Sweetlove *et al.* 1996). In addition to an effect on the starch quantity, perturbation of AGPase activity was reported to affect the properties of starch in potato. Decrease of AGPase level resulted in the reduction of amylose content with high short-chain amylopectin (Lloyd *et al.* 1999b).

To alter the quality of starch, the enzymes downstream of AGPase were considered, including starch synthase (SS), starch branching enzyme (SBE), and starch debranching enzyme (DBE). Starch synthase is categorized into two distinct classes, which are granular-bound starch synthases (GBSSs) and soluble starch synthases (SSs). GBSSs catalyze ADPG polymerization to form amylose, while SSs and mainly respond to amylopectin synthesis. In waxy maize, in which only GBSS was reduced, the starch is mostly composed of amylopectin (Tsai 1974). Recently, different isoforms of SSs have been identified and modified in several plants (Gámez-Arjona *et al.* 2011; McMaugh *et al.* 2014; Roldán *et al.* 2007; Szydlowski *et al.* 2009). Mutations of the different isoforms of SSs in pea, maize, and green algae led to a relative increase in amylose content because of the reduction of the amylopectin synthesis (Craig *et al.* 1998; Fontaine *et al.* 1993; Gao *et al.* 1998). Moreover, antisense inhibition of two starch synthase isoforms (SSII, SSIII) was examined in potato tubers to demonstrate the influence of SSs on amylopectin structure (Edwards *et al.* 1999; Lloyd *et al.* 1999a). Suppression of a single SS isoforms showed that SSII had more effect than SSIII on shifting from longer chains to shorter chains constituent of amylopectin. The double inhibition of the two isoforms exhibited similar results to the single mutants but with more extra-long chains in the amylopectin molecule. For starch branching enzymes (SBE), there

is a huge contribution on amylopectin biosynthesis. Their mutations in pea, maize and rice decrease the total starch content and alter the AL/AP proportion (Bhattacharyya *et al.* 1990; Jiang *et al.* 2013; Mizuno *et al.* 1993; Smith 1988). There are two classes of SBE isoforms, A and B, based on amino acid sequence homology. The two isoforms have distinct substrate affinity to varied chain length of glucans, suggesting the different role in branched-chain formation. SBEs in class A have lower affinity for amylose than class B isoform which prefers relatively longer glucan chains (Guan and Preiss 1993; Martin and Smith 1995; Okamoto *et al.* 2013; Rydberg *et al.* 2001). The last main enzyme in starch biosynthesis pathway, DBEs, hydrolyzes the alpha-1,6 glucan branches of amylopectin. Starch debranching enzymes play an important role in shaping the amylopectin structure, which is crucial for starch granule assembly. Plant DBEs are classified into two groups: pullulanase and isomerase. At least three isoforms of isoamylases have been identified in the cereal genome (Jeon *et al.* 2010; Kubo *et al.* 2005) and located at endosperm. Alteration of the DBE level of either group affects the properties of the starch granule (Fujita *et al.* 2009; Kubo *et al.* 1999). Mutation of isoamylase 1 had dramatic effects on grain morphology, leading to shrunken grains in both rice (Kubo *et al.* 2005) and maize (James *et al.* 1995). Moreover, the proportion of small granules has been increased in barley (Burton *et al.* 2002).

Although genetic modification of enzymes involved in starch biosynthesis pathway demonstrated good progress for starch modification, there might be many unexpected players over the scoped pathway that also influence starch quantity and quality. For instance, a plastidic ATP-ADP transporter in potato also had an effect on starch quality, showing significant increases in amylose content after increasing the protein activity (Bahaji *et al.* 2014; Tjaden *et al.* 1998). Surprisingly, manipulation of several enzymes outside the starch biosynthesis pathway resulted in greater increase in starch content than by modifying the key enzymes in the pathway. The starch content of transgenic potato tubers was increased by 211% of the wild-type level through the manipulation of adenylate kinase (ADK) (Bahaji *et al.* 2014; Reginer *et al.* 2002). These findings have pushed a rational design of the current genetic engineering approach to the margin and have called for a challenge. To resolve the problem of weak target identification, it is vital to exhaustively explore players affecting starch quantity and quality in the overall metabolism rather than focusing on a subjective gene. The complete view of metabolism and regulation as a network would help increase the rate of success in this research.

17.3 An Extension of the (Resistant) Starch Yield Improvement Research on the Systems Biology Regime: Integration of the Omic Data from the Post-Genomic Technology

The reductionist approaches that rely on an investigation of a single or a couple of cellular molecules (i.e., genes, proteins, and metabolites) have established much success in crop yield improvement (e.g., Biemelt *et al.* 2004; Sakamoto 2006; Smidansky *et al.* 2003). However, countless failures have happened throughout the trail of success, mainly due to a lack of information on how the investigated component interacts with the others in the complicated network of cellular regulation. Recently, the emergence of high-throughput technologies has changed the paradigm of the study from the reductionist to a holistic manner, according to the greater capacity of molecular measurement. The power of the technologies to capture the omic data of intracellular molecules provides the overall molecular relationship, a landscape of molecular interaction underlying the physiology of the cell. The stream of the technology development has been shown up at every level of regulation: genome (a whole set of genes in an organism; e.g., Next-Generation sequencing technology; Pareek *et al.* 2011; Shendure and Ji, 2008), transcriptome (the global gene expression at a particular condition; e.g., microarray, RNA-seq; Kogenaru *et al.* 2012; Marguerat and Bähler, 2010), proteome (the global protein abundance at a particular condition; e.g., mass-spectrometry-derived technology such as MS-MS-MaldiTOF; Agrawal *et al.* 2013), and metabolome (the global metabolite abundance at a particular condition; e.g., high-resolution separation technology such as GC-MS and LC-MS; Fernie and Schauer, 2009; Saito and Matsuda, 2010). With the current technology to monitor the intracellular molecules, the coming challenge is the effective exploitation of these omic data to envisage the cellular regulation, especially in the integration point of view. Systems biology is the multidisciplinary science that aims to utilize the overall data to gain more understanding into the systematic governance of the complex system through their integration. The basic regime of the systems biology approach begins with the construction of the molecular network relationship based on the available genome information of the organism, for instance genetic and metabolic networks. Subsequently, the transcriptomic, proteomic, and metabolomic data, which provide a snapshot of cellular dynamics under the measured condition, are integrated into the network to conjecture the cellular regulation (Figure 17.2). The advanced technology, together with the novel rationale of holistic study, is expected to complement the weak points of the reductionist approaches and increase their efficacy, which will push yield-improvement-related research forward.

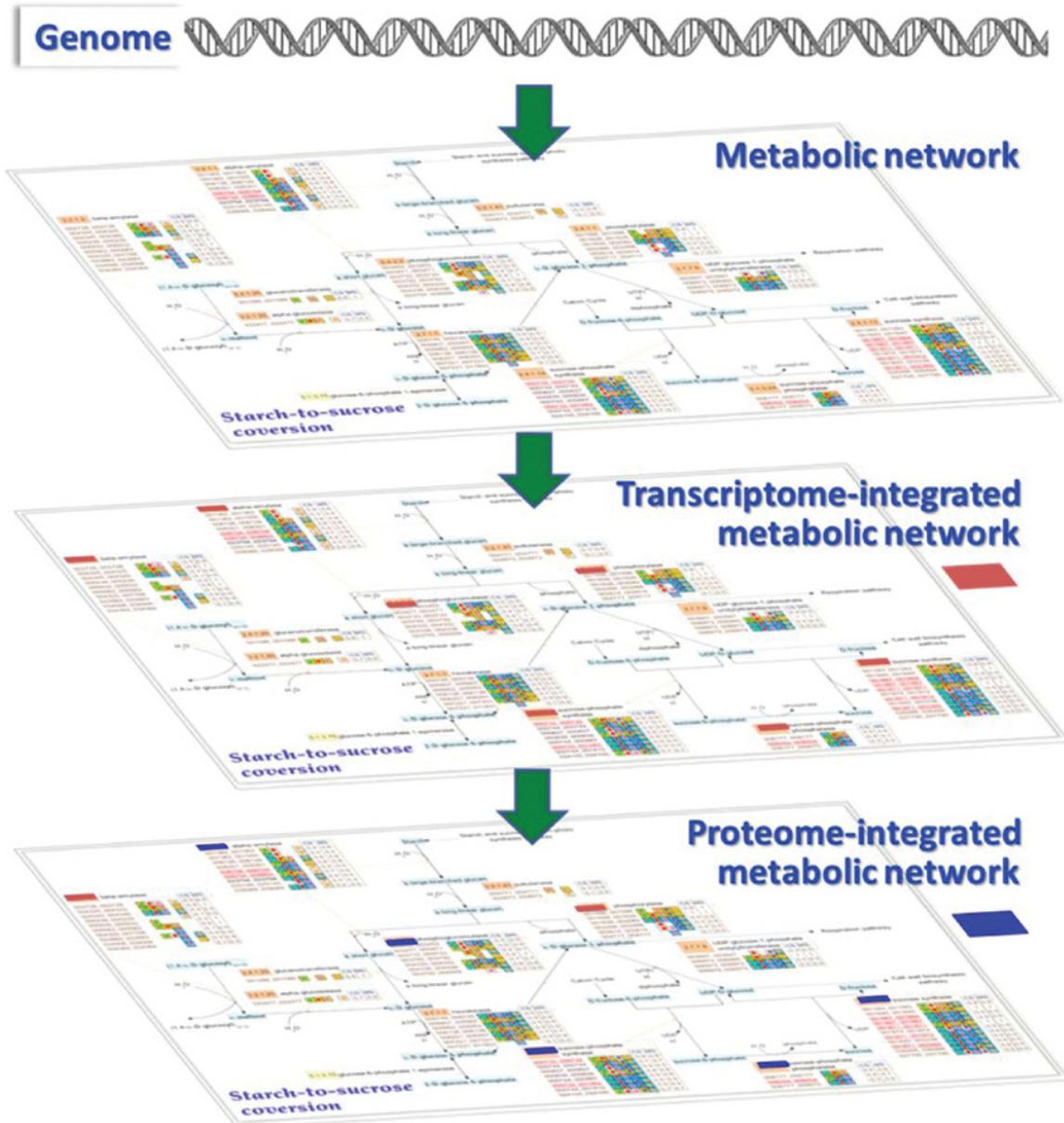


Figure 17.2 Conceptual scheme of omic data integration into the metabolic network. Metabolic network was constructed from the genome sequence of an organism to demonstrate the static regulation of the metabolism, while the subsequent incorporation of the expression data onto the network exhibits the dynamic regulation underlying the metabolic phenotype: shaded boxes denote the active reactions under a particular condition according to gene expression data and protein expression.

Starch and its derivatives are the major desired products from crop plants. The starch quantity and properties in nature are diverse among sources (Figure 17.1), and these features determine the significance of the crops. Cereal crops are important sources of high-nutrition starches, whereas tuber crops are dominant in terms of the quantity of produced starch. Cassava is a starchy root crop that contains starch of up to 80–90% on the dry matter (Zvinavashe *et al.* 2011). Despite the incredible amount of starch content in root, the research on starch yield improvement in cassava is on-going parallel with

the designer starch property. In an effort to manipulate yield and properties of starch in cassava root, the systems biology study to decipher the regulation controlling starch biosynthesis is underway.

Regarding the systems biology regime, the study of cassava starch biosynthesis was begun by the reconstruction of the metabolic network, whereby the series of reactions governing cellular starch production are described on the basis of enzymatic genes and biochemical compounds (metabolites). The cassava starch biosynthesis pathway was first introduced in 2007, based on the full-length cDNA EST microarray data (Sakurai *et al.* 2007). After the release of the cassava genome sequence (Prochnik *et al.* 2012), at least two genome-based pathways were presented to demonstrate the more complete portrait of the starch biosynthesis process in cassava (Saithong *et al.* 2013); CassavaCyc (www.plantcyc.org/). The latest published pathways in addition provided the evolutionary conservation and uniqueness of the cassava starch biosynthesis with respect to those of template plants (i.e., *Arabidopsis*, rice, maize, castor bean, and potato) as exemplified in Figure 17.3 (Saithong *et al.* 2013). The proteins that are allowed across template plants infer their essentiality in starch biosynthesis, whereas the proteins uniquely identified from a single template plant may be highlighted as a niche of starch biosynthesis in cassava root. This information is advantageous in providing the global landscape of choices to be selected for metabolic engineering to attain the desired starch yield and properties.

Metabolic engineering is the promising technique for designer starch research, and the key of achievement greatly lies on effective target selection. The choices of targets for engineering are normally scoped by the background knowledge and experience of the researchers, or confined to the space of the metabolic pathway of interest. However, modification of the target selected in accordance with these criteria is often reported to be failure. Sweetlove *et al.* (2008) showed that starch yield could be improved greatly by manipulating enzyme kinase (adenylate kinase; ADK), located outside the starch biosynthesis pathway, over targeting at the ADP-glucose pyrophosphorylase (AGPase), a key enzyme in starch formation. More examples were summarized in the same publication (Sweetlove *et al.* 2008) (Figure 17.4). The findings obviously suggest that the potential target for metabolic engineering could be anywhere in the metabolism beyond the biosynthesis pathway, and sometimes having vague relationship with the starch based on the reconstructed network. Therefore, the information comprised of the static metabolic network that was reconstructed from all metabolic genes existing in the genome might not be sufficient to select the promising target effectively.

To complement the content of the static metabolic pathway, the expression data of the genes composed of the network at either gene (transcriptomic data) or protein (proteomic data) level are integrated to extrapolate the regulation underlying the metabolism. Figure 17.5 demonstrates an example of incorporating the gene expression data into the starch biosynthesis pathway of cassava (Saithong *et al.* 2013). The cDNA microarray data provided the results of gene expression in the cassava root development condition, which consists of fibrous, developing, and mature roots (Yang *et al.* 2011). The results showed that only a group of genes in the metabolic network were expressed, suggesting their function in the metabolic network at the observed condition. Moreover, the varied expression of a gene across conditions exhibits the dominance of its function in the different types of roots (conditions). Siriwat *et al.* (2012) further demonstrated that the carbon partitioning towards starch formation might be regulated differently throughout the root development. The integrative study of transcriptomic data into the carbon assimilation pathway of cassava inferred the shift in carbon utilization from respiration and cell wall biosynthesis in fibrous roots to the starch biosynthesis in mature stage of storage roots (Siriwat *et al.* 2012). Similar studies are also found in other plant research, for example development of grape berry (Deluc *et al.* 2007) and carbon utilization in *Arabidopsis* (Stitt *et al.* 2007), which assures the advantage of the approach. Overall, the integrated temporal information helps unravel the dynamic regulation behind the static metabolic network (e.g., Deluc *et al.* 2007; Saithong *et al.* 2013; Siriwat *et al.* 2012; Stitt *et al.* 2007) It enables us to refine the reliable set of candidate targets for metabolic engineering, for example by excluding the non-expressed genes.

Besides the temporal omic data integration, comprehensive dynamic regulation could be acquired by conducting mathematical modeling. Constraint-based modeling approaches, such as flux balance analysis (FBA), are the most popular techniques for investigating the metabolic regulation, which would finally bring promising targets. Examples of FBA-aided target identification for the metabolic engineering are well established (Alper *et al.* 2005; Wang *et al.* 2006).

In conclusion, systems biology is a complementary approach to increase the effectiveness of the reductionist approach in phyto-product yield (and property) improvement. The overt contribution of this approach to the metabolic engineering research is on the identification of promising targets. The smart regime to identify the overall candidate target followed by the candidate refinement through the omic data integration would guarantee a shortcut towards the success in phyto-product yield (and property) improvement research.

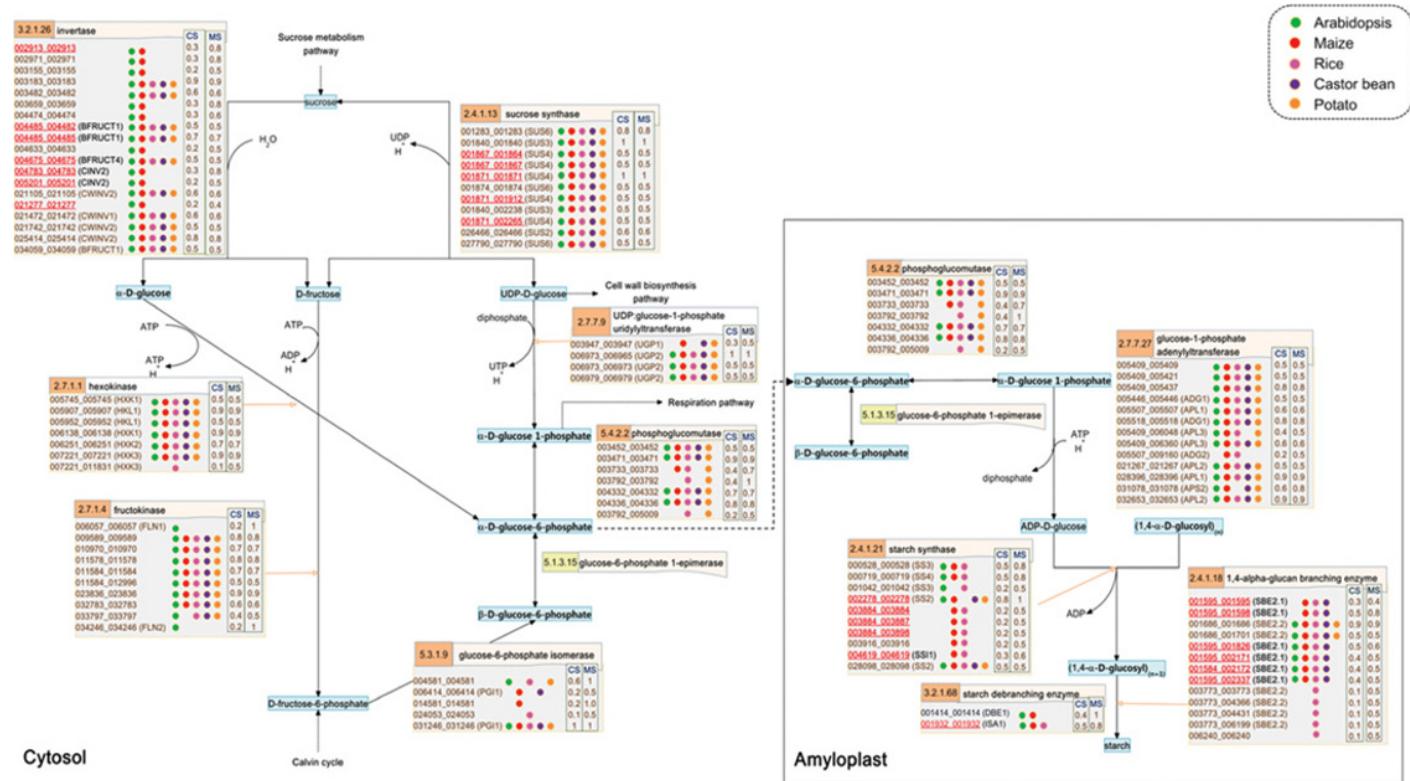


Figure 17.3 Starch biosynthesis pathway of cassava redrawn from Saithong et al. (2013). Cassava proteins responding for a metabolic reaction were denoted by 12-digit gene IDs (the first six digits) and transcript IDs (the latter six digits) of Phytozome database. The shaded circles represent the template plants from which the starch biosynthesis genes in cassava were annotated. The CS (conservation score) and MS (match score) scores indicated the confident level of gene annotation in the reconstructed pathway (see more detail in Saithong et al. 2013).

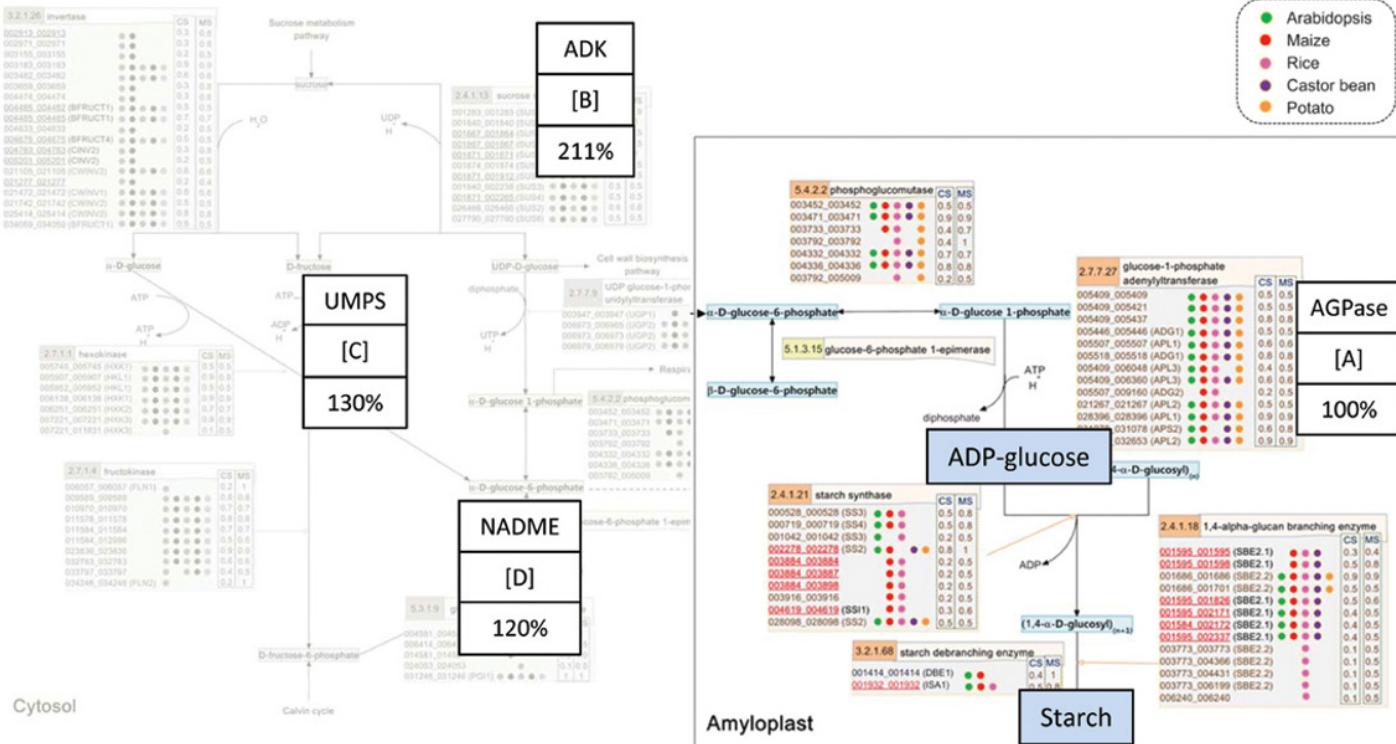
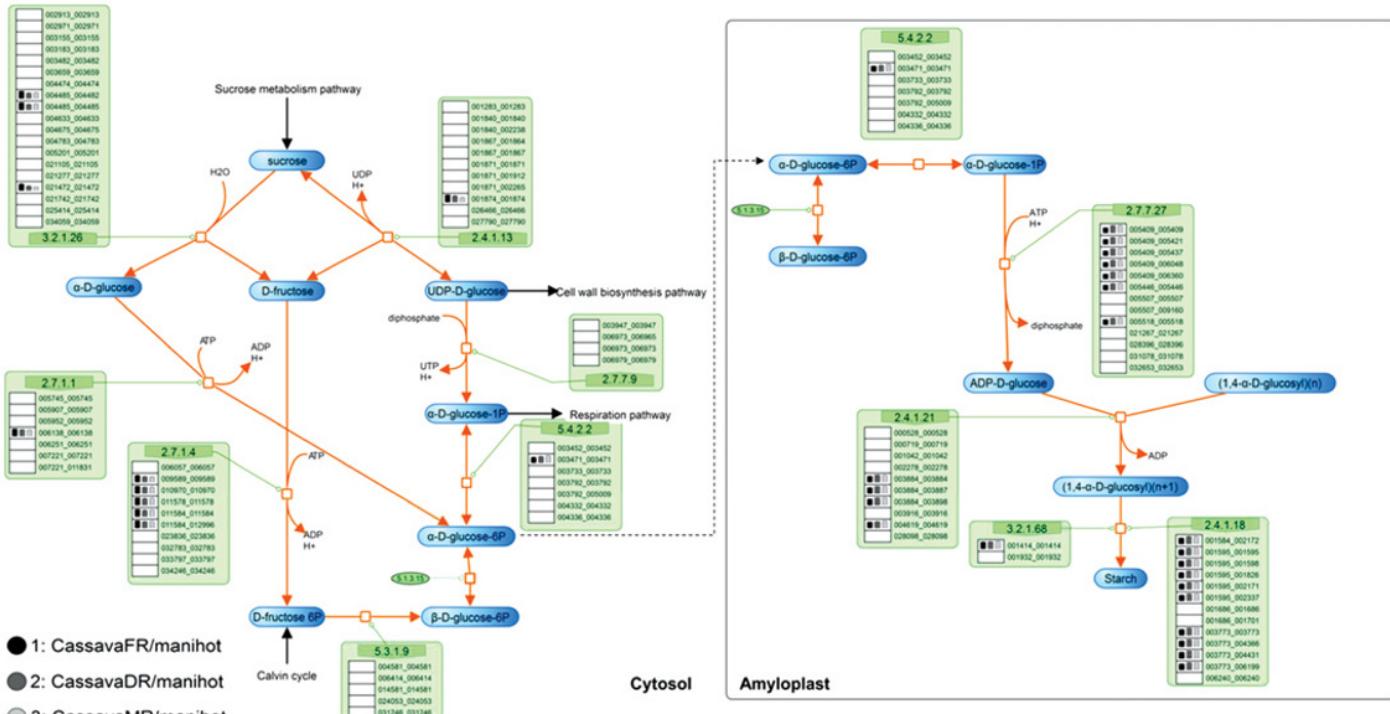


Figure 17.4 Examples of genetic and metabolic engineering at enzymatic genes that influence the starch content in potato. The white boxes contain a name of an enzyme that was perturbed, the corresponding reference and the resulting starch content in the transgenic potato tubers (as a percentage of the wild-type value). The three enzymes in cytosol are distally connected to starch metabolism. AGPase: ADP-glucose pyrophosphorylase, ADK: adenylate kinase, UMPS: uridine monophosphate, NADME: NAD⁺-dependent malic enzyme; [A]: Sweetlove et al. (1996), [B]: Regierer et al. (2002), [C]: Geigenberger et al. (2005), and [D]: Jenner et al. (2001).



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Part III

Proteomics

18

Proteomics and Nutrition Research: An Overview

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18.1 Introduction

Food and a nutritious diet are required as the basic energy source for normal function. However, scientific research into human nutrition and food is essential to maintain good health and also lower the onset of diseases. A well balanced diet will have beneficial effects on human health. Modern technological inventions could lead to new food products, processing methods to enhance shelf life, taste, and texture of food. Biochemical research efforts could be useful to determine physical and chemical composition of food (Capozzi and Bardoni, 2013).

Diet and nutrients in food influence gene expression and ultimately lead to (1) changes in individual proteins/proteome, (2) metabolic alterations at both cellular and molecular levels, and (3) perturbation in interactions between gene and nutrients. The information could be useful to determine the personal diet regimen for an individual.

Current nutrition research is focused on better nourishment through healthy diets, enriched with bio-nutrients like vitamins and other factors. From ancient times, diet and daily consumption of combination of food and beverages have had direct implications on human health and disease. The bioactive components in ingested food have profound effects on gene and protein expression. In recent years the goal of nutrition research has been more toward how to harness the knowledge of micronutrients and bioactive factors available in diet, and use that information for timely intervention to prevent or modulate disease pathophysiology for better health.

To unravel the molecular basis of beneficial effects of bioactive factors of diet, it is critical that a comprehensive and coherent effort be made in tandem through all the available modern “omics” technology. Compared to microarrays, RNA interference, nanotechnologies, genomics, proteomics, and metabolomics provide better options for identification and interactions of bioactive components of diet and their molecular targets associated with disease prevention. Protein profiling approaches have been used to analyze quality, origin, or adulterations of food (Carbonaro, 2004).

18.2 Proteomics

Proteomics represents a comprehensive scientific study of all expressed proteins or entire proteome at any given time in an organism (Liu and Qian, 2011). Proteins are key macromolecules present in all living organisms. Proteomics can provide

details about the changes and comparisons in expression pattern of proteins in a specific physiological or pathological condition (Cozzolino, 2012).

To gain new insight in identification of novel nutritional biomarkers and their effects on individual phenotypes, we need to employ the modern omics technologies. It is important to note that not only will individual response to nutrients depend on individual genome profile, but also on the proteome and its variations. Therefore, with the protein as the target physiologically active molecule, the use of proteomics is the newest addition to nutrition research, and is the main objective of this chapter. Proteomics applications in nutrition research are important for identification and differential quantification of bioactive components and protein biomarkers.

Proteomics provides a unique interactive platform and new insights to unveil the novel mechanisms and critical information on health effects of key micro- and macronutrients present in food. Novel proteomic tools and methodology in conjunction with other omics-based techniques, can be useful to elucidate (1) dietary factors associated with nutrients-related traits, (2) alteration in gene, protein and metabolite levels due to inflammation or changes in metabolism, and (3) to identify key proteins in the diabetes-related pathway that are modified by diet.

In general, the human proteome is a much more complex set of protein molecules when compared to the human genome complex. The complexity in the proteome is often due to a large dynamic range, 6–10 orders of magnitude. In plasma, the range is even greater than 10 (Anderson and Anderson, 2002; Rose *et al.*, 2004) with difficulty in detecting the very-low abundant proteins (Jacobs *et al.* 2005). The wide range and concentrations of proteins differ in cells as per their types, functions and so on, and therefore proteome characteristics can be deciphered by using proteomic tools.

18.2.1 Proteomics Tools and Technologies

In general, various proteomic tools have been used to define the global and sub-proteomics approaches. These tools are applicable to (1) *ex-vivo* samples like biological fluid and tissue samples, and (2) compare disease versus normal or the total proteome and sub-proteome. Proteomic techniques were developed for high-throughput analysis of protein identification (Haynes and Yates 2000). Two-dimensional difference gel electrophoresis (2DE) is a traditional proteomic technique mainly used for a wide dynamic range and differential expression of proteins using cyanine fluorescently-labeled proteins (Tonge *et al.* 2001). For global protein expression study, two approaches were developed; one, the 2D gel and an MS/MS based approach was used to separate, analyze protein abundance, and identify target proteins (Görg 2004). Second, a MudPIT (Multidimensional Protein Identification Technology) was developed by Washburn and Yates (2001) and Wolters *et al.* (2001). In this approach, the protein mixture was digested directly and peptides were separated by strong cation exchange chromatography (SCX). Further sequence and identification was done to overcome large abundance variations from most abundant to least abundant proteins.

Apart from protein profile analysis, another important aspect of proteome complexity, structural, and functional changes, is involvement of post-translational modifications (PTMs) of proteins. Due to notoriously low-abundance of these proteomes in a sample, it is critical to apply a suitable enrichment for further analysis. For example, immobilized metal affinity chromatography (IMAC) technology (Nuhse *et al.* 2003) was used for phosphoprotein analysis, specific enrichment method were used for glycoprotein-like lectins (Kobata and Endo *et al.* 1992). Specific technology was developed to monitor sugar oxonium fragment ions during electrospray mass spec analysis of glycoprotein (Huddleston *et al.* 1993).

Quantitative proteomics has been used as a powerful approach for 2DE, image analysis coupled with mass spectrometric analysis of proteins (Görg *et al.* 2000). Other techniques, like differential isotope-coded tagging (ICT) chromatography with MS, were also useful (Gygi *et al.* 1999; Han *et al.* 2001; Zhang *et al.* 2001). Some of the recent examples for proteomics technology applications to nutrition research have been summarized in Table 18.1. These applications clearly support the importance of proteomics technology in food and diet-related research.

18.3 Nutrition and Proteins

Nutrition serves as major source for essential amino acids, which cannot be synthesized by the body. Diet-based proteins derived from plant and animal sources differ in their composition and nutritional quality. Differences in composition and bioavailability of the food proteins are essential for their nutritional quality.

Table 18.1 Recent examples of proteomics platform applications to nutrition-related research.

References	Study/objective	Proteomics technology	Results/conclusion
Poschmann <i>et al.</i> , 2014	Effects of HFD on weight gain and obesity; mouse model	2DE, LC-MS/MS	Oxidative modification of cysteine in DJ-1 protein, sensor protein isoform for nutrition effects and neurodegenerative diseases
Lim <i>et al.</i> , 2014	Effect of mangiferin (MGF), from mango, on liver proteome, steatosis; mouse model	SDS-PAGE, LC-MS/MS, Quantitative analysis of proteins in plasma and liver by SILAM method	MGF up-regulates mitochondrial bioenergetics proteins, decreases lipogenesis, modulates HFD induced adverse metabolic effects
Nissen <i>et al.</i> , 2012	Identification of unique protein, bioactives in bovine colostrums	Differential fractionation, 2D-LC-MS/MS	Unique subsets of proteins identified for neo-natal nutrition
Li <i>et al.</i> , 2013	Identification of metabolic protein changes during strawberry ripening	Quantitative protein analysis, stable isotope labeling by peptide dimethylation (SMR)	Increase in flavonoid, anthocyanin biosynthetic proteins, antioxidant metabolism, decrease in methionine metabolism
Cole <i>et al.</i> , 2013	Cross-sectional, correlation cohort study for effects of vitamin A, D, E, copper, selenium on plasma proteins in Nepalese children	Quantitative proteomics analysis, iTRAQ mass tags	Protein biomarkers of micronutrient status in undernourished children
Barnett <i>et al.</i> , 2013	Anti-inflammatory effects of green tea extract phenols (GrTP)- enriched diet, Mdr1 a (-/-) mouse model of IBD	2D SDS-PAGE, LC-MS based identification of colon proteins	Reduction in transcript and protein levels of immune and inflammation mediated fibrinogenesis, modulation of intestinal inflammation
Duthie <i>et al.</i> , 2010	Long term effects of folate supplement on human plasma proteins	2DE, LC-MS/MS	A unique set of 62 proteins differentially expressed for modulation of complement fixation, coagulation, and mineral transport pathways
Chakraborty <i>et al.</i> , 2013	To enhance nutritional value of crop, genetic expression of OXDC in tomato	2DE, LC-MS/MS, MALDI-TOF/TOF	Significant decrease in oxalate levels, increase in micronutrients Ca, Mn, Mg, Zn; change in proteins of metabolism, stress response, signaling, redox regulation
Tavakolan <i>et al.</i> , 2013	To improve nutrient quality of genetically modified soybean seeds, decipher soybean protein composition	2D SDS-PAGE, MALDI-TOF, LC-MS/MS	High quality storage proteins like β -conglycinin, glycinin, allergen protein GlymBd60k; helped create web-based soybean protein database (SoyProDB)
Quintela- Baluja <i>et al.</i> , 2013	To early detect and identify Enterococcus strains in food, other strains in food poisoning; improve food safety	MALDI-TOF based genus specific fingerprinting	Total 36 different strains of enterococcus were identified, molecular protein biomarker used for quick and specific ID of different stains and species of pathogens in food
Mora <i>et al.</i> , 2013	To screen GM and other crops for environment and food safety, GM tomato study	Label-free approach, SDS-PAGE, reverse phase chromatography	Helped to link transcriptome to metabolome, deliver nutrient-enriched fruits
Arena <i>et al.</i> , 2011	To monitor and reduce harmful effects of milk processing methods, and enhance quality of dairy-based products for better nutrient enrichment	Phenylboronate chromatography, nano LC-ESI-ETD MS/MS	Significant changes in 35 milk globule fat proteins due to thermal treatment, lactosylation of MGF affects nutritional values of dairy products
Scippa <i>et al.</i> , 2010	Phylogenetic study of lentil for environmental and socioeconomic issues; mature seeds of 7 lentil population	2D-SDS-PAGE, MALDI-TOF protein mass fingerprinting, nano LC-ESI-MS	Protein ID maps compared and 103 differentially expressed proteins between population; multivariate analysis ID to 24 unique marker proteins

Abbreviations: HFD, High fat diet; SILAM, Stable isotope labeling of mammals; iTRAQ, Isobaric tags for relative and absolute quantification; ID, Identification; OXDC, Oxalate decarboxylase; GM Genetically modified.

Mass spectrometry based techniques have been widely used as analytical methods to characterize the quality and quantity bioactive molecules of food. These are also being used to assess the taste and aroma aspects of food using LC-MS/MS and GC-MS, respectively. These techniques also apply to some of the commonly used additives and preservatives.

18.4 Nutritional Biomarkers

Nutritional biomarkers are indicators for nutritional status associated with intake or metabolism of a dietary ingredient, and to indicate both the composition and pattern of diet intake (Potischman and Freudenheim 2003). A major challenge associated with accurate assessment of diet intake and nutrition studies is an error based self-reporting method of food or diet pattern. A robust and novel nutrition based biomarker provides a non-biased option for diet intake or exposure (Hedrick *et al.* 2012). To accurately determine the effects of food consumption on health and disease, biomarkers are needed. Following are few examples of studies related to nutritional biomarkers.

Coffee, a major source of dietary alkaloid caffeine, rich in other phenolic bioactives, affects metabolism. Liquid chromatography and mass spec based methodology were used to determine the better health implications of coffee intake (Rothwell *et al.* 2014).

Mass spectrometry based methods like GC-MS and LC-MS were used to analyze urinary sucrose and fructose as potential biomarkers for sugar intake (Kuhnle *et al.* 2008). Another study has revealed plasma alkyresorcinol (AR) as possible biomarker for wheat/rye intake. The level of AR increases with intake of whole grain and also decreases with consumption of refined bread (Linko-Parvinen *et al.* 2007).

A proteomic study, involving 2D electrophoresis and peptide mass fingerprinting, has revealed the *in vivo* biomarkers for effects of diet intervention with isoflavone-enriched soy extract in postmenopausal women (Fuchs *et al.* 2007b). An increase in the expression levels of anti-inflammatory proteins is an indicative of preventive activities of soy-enriched diet. A total of 29 proteins were significantly expressed in mononuclear blood cells, which include variety of anti-inflammatory, HSP70, and proteins with high fibrinolysis, like alpha-enolase, with a decrease in smooth muscle cell proliferator galectin-1 (Fuchs *et al.* 2007b).

18.5 Nutritional Bioactives

A proteomics study can provide the missing link between the gene expression and the resulting cellular or molecular activity with a final response in an organism. The effects of butyrate, a food derived bioactive molecule, regulates the key proteins involved in cell cycle, apoptosis, and cell differentiation (Costa and Rosa 2011).

Food bioactives were applied for prevention of cancer (Shukla and George 2011). A recent observational study by Valdes *et al.* (2012) has shown effects of rosemary extracts on colon cancer cells using transcriptomics-based protein profiling for factors affecting signaling and metabolic pathways.

A number of proteomic approaches were used to identify natural peptides present in food as bioactives or that are formed later during the digestion process (Gagliaire *et al.* 2009; Gomez-Ruiz *et al.* 2006).

18.5.1 Wheat Proteins

Here is another example for application of proteomics to improve food quality. A 2D-PAGE and MALDI-TOF based multivariate analysis was used to characterize wheat proteins (Gottlieb *et al.* 2002). The wheat protein named “gliadin”, from the wheat gluten protein family and other varieties was differentiated with the help of mass spec data.

18.5.2 Vitamins

Recent studies have shown the roles of Vitamin A, D, and fatty acids affecting gene transcription (Dauncey 2012; Fialho *et al.* 2008; Mahan and Stump 2005). Vitamin C, present in citrus fruits, acts as a potent antioxidant (Frei 1991, 1999). It is transported to mitochondria as an oxidized form dihydroascorbic acid (DHA) through glucose transporter (Glut 1) and protects mitochondria from reactive oxygen species (ROS) induced oxidative stress (Sagun *et al.* 2005). Vitamin C is also a cofactor of enzymes for collagen (Padh 1991) and carnitine biosynthesis (Rebouche 1991). Other studies (Vera, *et al.* 1993, 1994) have also provided evidence to support the transport of vitamin C as a DHA and its intracellular storage as ascorbic acid (AA).

18.5.3 Glucose

A recent study has shown that an enhanced uptake of glucose may lead to vascular complications with an enhanced injury induced pro-inflammatory lesions in a murine model (Adhikari *et al.* 2011).

18.5.4 Wine and Soy Nutrients

The recent studies on the beneficial effects of wine have established a link between the novel ingredients resveratrol and genistein in soy to lipid metabolism and signaling factor kappa b (Dalmiel *et al.* 2012; Fialho *et al.* 2008; Mahan and Stump 2005). Mass-spectrometry (LC-MS/MS) has been used to characterize the protein composition and function in wine (Kwon 2004). Proteomic study related to chemical composition of alcohol has shown an effect of alcohol on cardiac muscle proteins (Fogle *et al.* 2010). A proteomic study has evidently shown that soy extract or mixture of soy isoflavones genistein and daidzein can reverse low density lipoprotein (LDL) induced apoptosis in endothelial cells (Fuchs *et al.* 2007a).

18.6 Diet-Based Proteomics Application to Animal Products (Livestock Applications)

In an experimentally induced weight-loss study in sheep, diet and nutrients influenced wool fiber structure and composition, thereby affecting wool quality (Almeida *et al.* 2014). The non-availability of diet-based nutrients in these experiments led to a decrease in wool fiber diameter with an increase in high sulfur protein (HSP) KAP 13.1 and proteins of the high glycine-tyrosine family KAP 6 family. The 2DE and MALDI-TOF based proteomics methods were used to identify specific markers like B2A family proteins from HSP group for wool quality traits in sheep (Plowman *et al.* 2000).

18.7 Proteomics and Food Safety

Initially, proteomics approaches have been used to study and enhance the production process or quality of plant and animal origin foods including meat, wine, beer, milk, and so on. The food quality and safety and its effect on human health remains a major concern. Recent advancement in sensitive proteomic technology and high throughput capability has improved the screening of food for foodborne pathogens and contaminations at a faster pace with high sensitivity and specificity (D'Alessandro and Zolla 2012).

The use of synthetic growth or performance enhancement agents is being used in the animal meat industry to enhance meat production. However, due to the lack of a sensitive analytical test, these chemicals pose difficulty with their detection. A multivariate MALDI-TOF mass spectrometry based proteomic method using bovine serum samples could detect polypeptide fragment from beta 2-glycoprotein-1 as a potent biomarker for detection of growth promoting agents (Dell Donna *et al.* 2009). An array of omics-driven approaches have been used for optimal detection, prevention, and treatment of foodborne pathogens (Bergholz *et al.* 2014).

18.8 Conclusion

Food and diet affect the outcome of four major “omics” driven high throughput platforms; genomics, transcriptomics, proteomics, and metabolomics. A number of other smaller sub-types of platform, such as lipidomics, interactomics, and so on, could provide complementary information. Proteomics along with other “omics” technologies provides a better platform for understanding of the effects of nutritional factors on health and disease outcomes. It is clearer now that not all individuals respond identically to diet or nutritional intervention, therefore any future nutritional strategy should be based on a personalized approach based on the health-based need and interventions. The success and clinical application of the omics-based nutritional personalized approach will depend on some key factors such as the integration of all the omics driven information, discovery, validation, and quantification of nutritional biomarkers with high degrees of sensitivity and accuracy. Modern systems biology platforms and a personalized medicine approach to characterize proteomic signature profiles could provide more robust applications (Weston and Hood 2004). The most important challenges with respect to the use of proteomics and other omics-driven technology in nutrition research

would consist of two parts. First, the discovery of key bioactive molecules, and their validation, and the second would be the use of bioinformatics towards vast data sets; that is, need and use of specialized bioinformatics algorithm platforms to analyze and manage complex data in an efficient and reliable way.

The major challenge will be to decipher the vast amount of data sets generated from such studies, and provide evidence for their validity, robustness, and physiological significance. The use of innovative new bioinformatics tools and software could provide a better overall opportunity for novel therapeutic intervention.

In conclusion, with fast-paced development and applications of advanced proteomics and other omics technologies, along with bioinformatics, new dimensions and tools have been added to improve diet and nutrition.

18.9 Significance

The new concept and combined approach of all omics-based technology, specifically genomics, transcriptomics, proteomics, and metabolomics, would help connect the missing links of food components and individual diets with implications for health. A comprehensive, high throughput approach could be exploited to improve foodomics outcomes and utilize new information for improvement of human nutrition and health. A personalized nutrition counseling session based upon proteomics data can be used to change diet habits and improve lifestyle.

Conflict of Interests

None.

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19

Proteomics Analysis for the Functionality of *Toona sinensis*

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19.1 Introduction

Proteomics is the study of the “proteome”, which is recognized as the comprehensive analysis of a protein complement in a cell, tissue, or biological fluid at a given time. The word *proteome* was coined in late 1994 at the Siena 2D electrophoresis meeting (Wilkins *et al.*, 1996) and described the entire collection of proteins of an organism, including products arising from events such as the processing of mRNA transcripts (i.e., alternative splicing) and post-translational modifications (e.g., phosphorylation, glycosylation, and oxidation) (Figeys, 2003; Mann *et al.*, 2001). Proteomics-based studies are focused on the interactions of multiple proteins and their role as part of a biological system rather than the structure and function of one single component. Proteins are directly involved in all cellular activities, cell phenotype, and hence the tissue or organ. This phenotype varies under normal physiological conditions (e.g., cell-cycle stage, differentiation, function, and age) or in response to pathophysiological stresses. Environmental changes may alter protein structure and functions, ultimately leading to the progression of diseases. The concept of comparing the global protein profile from tissues between two biological states has given rise to a new era in biological science. Therefore, proteomic studies can be applied to a range of biological systems to answer the desired research question, including human studies, animal models, and/or cell culture systems (Lam *et al.*, 2006). Furthermore, more studies in the food-related products have applied proteomic analysis to elucidate the functionalities of phyto-active and/or bioactive components. In this article, certain functionalities of *Toona sinensis* explored by our laboratory are discussed and used as examples to elucidate the functionalities of *Toona sinensis* by proteomic analysis.

19.2 *Toona sinensis*

Toona sinensis Roem (TS), a widely distributed deciduous arbor in Asia, is a nutritious food in Chinese society and a popular vegetarian cuisine in Asia. Leaves of TS (TSL) have been used as a traditional Chinese medicine (TCM) for treatment of enteritis, dysentery, metabolic disease, infection, and itching.

19.2.1 Functions of *Toona sinensis* Leaf Extracts (TSLs)

Numerous studies showed that *Toona sinensis* leaf extracts (TSLs) provided novel functions including antioxidant properties (Cho *et al.*, 2003; Hsue *et al.*, 2008; Hsieh *et al.*, 2004; Yu *et al.*, 2012a, b, c), increasing the dynamic activity of human sperm (Song *et al.*, 2005; Yu *et al.*, 2012b, c), improving lipolysis of differentiated 3T3-L1 adipocyte and its uptake of glucose (Yang *et al.*, 2003), anticancers (lung cancer, prostate cancer, and ovarian cancer) (Chang *et al.*, 2006; Chen *et al.*, 2009; Yang *et al.*, 2010), anti-low-density lipoprotein (LDL) glycation activity (Hsieh *et al.*, 2005), improvement of learning and memory through the reduction of lipid peroxidation and s-amyloid plaques in the brains of senescence-accelerated mice (Liao *et al.*, 2006), and inhibiting SARS-CoV coronavirus *in vitro* (Chen *et al.*, 2008).

19.2.2 Preparation of TSLs

In our laboratory, leaves of *Toona sinensis* were boiled in reverse osmosis (RO) filtered water for 30 min and extracted by serial different concentrations of ethanol. The filtered concentrates were lyophilized to obtain a crude extract (TSL-CE), which was further extracted serially to obtain TSL-1, TSL-2 (supernatant), TSL-2P (pellet), TSL-3 (ethanol, supernatant), TSL-3P (ethanol, pellet), TSL-4 (ethanol, supernatant), TSL-4P (ethanol, pellet), TSL-5 (H₂O, supernatant), TSL-5P (H₂O, pellet), TSL-6 (H₂O, supernatant), and TSL-7 (H₂O, pellet). Among these, TSL-2, TSL-2P, and TSL-6 were found to be the most active extracts. Therefore, they were utilized for the proteomic analysis for the functionalities of the TSLs (Chang *et al.*, 2013a).

19.3 TSLs Regulate Functions of Testes/Spermatozoa

Proteomic approach based on 2D-gel electrophoresis was carried out to investigate the proteins modulated by TSL extracts in rat testes under oxidative stress (Yu *et al.*, 2012b). Sprague-Dawley (S-D) rats were IP injected with H₂O₂ every other day and fed with normal diet (control), vitamin C, or TSL extracts (TSL-2; 0.053 g/kgBW/day, TSL-2P; 0.94 g/kgBW/day or TSL-6; 0.013 g/kgBW/day) daily for 8 weeks. Testicular proteins were extracted for proteomic analysis. Protein spot determination and comparative analysis of 2-DE among control, vitamin C, and TSL groups showed that 10 protein spots were modulated by TSL-2 at least more than 1.5-fold in the rat testes under oxidative stress, whereas four proteins including glutathione S-transferase (GST), phospholipids hydroperoxide glutathione peroxidase (PHGPx), fatty acid binding protein 9 (FABP9), and thioredoxin were further identified by MS MALDI-TOF and confirmed by western blotting (Yu *et al.*, 2012b). Three protein spots were modulated by TSL-2P more than 1.5-fold in the rat testes under oxidative stress, whereas one protein (phospholipids hydroperoxide glutathione peroxidase, PHGPx) was further identified by MS MALDI-TOF. Eleven protein spots were modulated by TSL-6 more than two-fold in the rat testes under oxidative stress, whereas nine proteins including tumor rejection antigen Gp96, 3-hydroxy-3-methylglutaryl-coenzyme A synthase 2 (HMG CoA synthase 2), glutathione transferase Mu 6 (GST Mu6), cofilin 2, pancreatic trypsin 1, heat shock protein 1- β (HSP 1- β), peptidylprolyl isomerase A, type II keratin Kb1, and heat shock 90kDa protein 1- β were identified by MS MALDI-TOF and confirmed by western blotting (Yu *et al.*, 2012b).

19.3.1 TSL-2 Exhibits Pro-oxidants but Protects Germ Cells from Apoptosis

Four proteins modulated by TSL-2, including GST, PHGPx, FABP9, and thioredoxin, are involved in antistress and sperm functions. GSTs, a family of Phase II detoxification enzymes that catalyze the conjugation of glutathione (GSH) to a wide variety of endogenous and exogenous electrophilic toxic compounds. Aside from a pivotal role in detoxification and defense against oxidative stress, GSTs may play a role in the etiology of diseases, including tumor, neurodegenerative diseases, multiple sclerosis, and asthma (Keppler, 1999; Townsend and Tew, 2003). Previous studies demonstrated that a mix of glucosinolate breakdown products (isothiocyanates, nitriles, and thiocyanates) in cruciferous vegetables synergistically upregulated phase II detoxification enzymes (quinone reductase and GST) (Nho and Jeffery, 2001). Using stannous chloride (SnCl₂) to generate reactive oxygen species (ROS), Yousef and colleagues found SnCl₂ significantly decreased activity of GST in rabbit blood plasma. On the contrary, ascorbic acid alone increased activity of GST and also alleviated SnCl₂-induced harmful effects (Yousef *et al.*, 2007). Consistent with Yousef *et al.*'s results, we found that vitamin C alleviated oxidative damage via upregulation of GST in rat testes. Downregulation of GST by TSL-2 in proteomic analysis also coincided with our previous chemical study that TSL-2 exhibited prooxidant and elevated levels of ROS in human spermatozoa. Glutathione peroxidase

(GPx) converts hydrogen peroxide to H_2O at the expense of oxidizing GSH to its disulfide form (GSSG). GSSG is returned to the GSH form by glutathione reductase (GR) using NADPH. In general, intracellular glutathione peroxidases comprise two distinct proteins, classical GPx (cGPx) and phospholipid hydroperoxide glutathione peroxidase (PHGPx), which exist in the nucleus, mitochondria, and cytosol. PHGPx plays a pivotal role in male fertility through its dual functions (a soluble active enzyme and enzymatically inactive structural protein) during sperm maturation (Ursini *et al.*, 1999). PHGPx was downregulated by TSL-2, known to exhibit prooxidant properties and thus reduced PHGPx expression. Fatty acid binding protein (FABP) is a cytosolic protein that binds unsaturated long-chain fatty acids and acyl-CoA esters (Watanabe *et al.*, 1991). So far, nine different FABPs with tissue-specific distribution have been identified. The primary role of all the FABPs is to regulate fatty acid uptake and intracellular transport. Among these FABPs, FABP9 is predominantly expressed in testis (Chmurzynska, 2006) and involved in spermatogenesis, testicular germ cell apoptosis, and sperm quality preservation (Kido and Namiki, 2000; Kido *et al.*, 2005). FABP9 was upregulated by control and followed by vitamin C and then TSL-2 (Yu *et al.*, 2012b). The elevated H_2O_2 -induced testicular germ cell apoptosis in control may lead to the upregulation of FABP9. TSL-2 and vitamin C protected testicular germ cell from apoptosis and gave rise to the decreased FABP9 expression. Thioredoxin is reported as one of the most important thiol-based systems being involved in many physiological as well as pathophysiological processes. Thioredoxins function as general protein disulfide reductases. Mammalian male germ cells possess a set of three testis-specific thioredoxins (named Sptrx-1, -2, and -3, respectively) that are expressed either in different structures within the sperm cell or at different stages during sperm development. Sptrx-1 is located in the developing tail of elongating spermatids, transiently associated with the longitudinal columns of the fibrous sheath. Sptrx-2 is also associated with the sperm fibrous sheath, but unlike Sptrx-1, Sptrx-2 becomes a structural part of the mature sperm tail and can be detected in ejaculated spermatozoa. Sptrx-3 can be found in Golgi-derived vesicles associated with the developing spermatid acrosome and its function might be related to the posttranslational modification of proteins required for acrosomal biogenesis (Jiminez *et al.*, 2005). Thioredoxin was upregulated by vitamin C and downregulated by TSL-2 (Yu *et al.*, 2012b). However, function of TSL extracts in thioredoxins of rat testes under oxidative stress needs to be further investigated due to the distinct roles of Sptrx-1, Sptrx-2, and Sptrx-3.

19.3.2 TSL-2P Exhibits Prooxidant Properties and Impairs Sperm Maturation

One protein, PHGPx, was modulated by TSL-2P using 2D-gel electrophoresis-based proteomics and confirmed by western blot tests. TSL-2P downregulated PHGPx expression using proteomic and western blotting analyses and exhibited prooxidant properties at high concentration. Therefore, TSL-2P impairs sperm functions via downregulation of PHGPx.

19.3.3 TSL-6 Exhibits Antioxidant Properties and Enhances Sperm Functions

Nine proteins modulated by TSL-6 and identified by MS MALDI-TOF are Gp96, HMG CoA synthase 2, GST Mu6, cofilin 2, pancreatic trypsin 1, HSP 1- β , peptidylprolyl isomerase A, type II keratin Kb1, and heat shock 90kDa protein 1- β .

Gp96, a member of the heat shock protein 90 family, has been implicated in the formation of a functional zona-receptor complex on the surface of mammalian spermatozoa. Gp96 was subsequently found to become co-expressed on the surface of live mouse spermatozoa following capacitation *in vitro* and was lost once these cells had undergone the acrosome reaction, as would be expected of cell surface molecules involved in sperm-egg interaction. GP96 was intimately involved in the mechanisms by which mammalian spermatozoa both acquire and express its ability to recognize the zona pellucida (ZP) (Asquith *et al.*, 2005). Studies also suggested that elevated ROS resulted in decline of sperm-egg interaction (Kodama *et al.*, 1997). Gp96 was highly upregulated by TSL-6 and downregulated by vitamin C and control. The ROS elevated by H_2O_2 in control leading to decreased Gp96 expression implicated a decline of sperm-egg interaction. TSL-6, compared to the control, highly induced Gp96 expression, suggesting that TSL-6 may facilitate capacitation and recognition of zona pellucida in sperm under oxidative stress (Asquith *et al.*, 2005).

HMG-CoA synthase catalyzes the condensation of acetoacetyl-CoA and acetyl-CoA to form HMG-CoA plus free CoA. HMG-CoA synthases are located in two different compartments: the cytosol and the mitochondria. HMG-CoA produced by the cytosolic HMG-CoA synthase is transformed into mevalonate (a precursor of cholesterol) by the action of HMG-CoA reductase. HMG-CoA produced inside the mitochondria by the mitochondrial HMG-CoA synthase is transformed into acetoacetate by the action of HMG-CoA lyase. Acetoacetate is transformed into hydroxybutyrate and acetone; all of these are known as ketone bodies (Aledo *et al.*, 2001; Heggardt, 1999). Testis and ovary express the gene for the ketogenic mitochondrial HMG-CoA synthase, which is involved in *de novo* cholesterologenesis in gonads (Royo *et al.*, 1993). HMG-CoA synthase identified by MALDI-TOF was mitochondrial HMG-CoA synthase, which was upregulated by TSL-6 and downregulated by vitamin C. Therefore, TSL-6 induced HMG-CoA synthase expression to regulate the synthesis of sex hormone in human gonads under oxidative stress.

GST Mu6 is a member of GST family. GST Mu6 is mainly expressed in brain, testis, and lung and functions as a detoxicative enzyme (Eaton and Bammler, 1999). The highest amount of GST Mu6 was upregulated by control and followed by TSL-6 and downregulated by vitamin C. Studies have shown that methyl gallate (MG) from TS prevents intracellular GSH from being depleted following an exposure of H_2O_2 in MDCK cells (Hsieh *et al.*, 2004). Certain phytochemicals in TSL have been isolated and identified, including quercetin, rutin, methyl gallate, gallate, and catechin (Hsieh *et al.*, 2004; Liao *et al.*, 2007). It is found that approximately 7.85 % gallic acid, which is the most abundant and active antioxidant of TSLs, is present in TSL-6. The gallic acid in TSL-6 prevents GSH from being depleted under oxidative stress; therefore, activity of GST Mu6 was not definitely needed and its expression declined when compared to the control. Vitamin C, being an antioxidant possessing a similar mechanism to TSL-6, declined GST Mu6 expression when compared to a control.

Cofilin, an actin-modulating protein of 20 kDa, is widely distributed throughout muscle (cofilin 2) and nonmuscle (cofilin 1) cells (Ono *et al.*, 1994). Nonmuscle cofilin is a component of tubulobulbar complexes with finger-like structures that form at the interface between maturing spermatids and Sertoli cells prior to sperm release and at the interface between two Sertoli cells near the base of the seminiferous epithelium (Guttman *et al.*, 2004). Cofilin 2 was upregulated by vitamin C and downregulated by control and TSL-6, suggesting that vitamin C increased cofilin 2 expression and maintained the contact interface between spermatids and Sertoli cells. Expression of cofilin is also related to survival and differentiation of spermatogenic cells. In a stress condition, phosphorylation of cofilin was severely impaired, and cofilin frequently accumulated in the nucleus, leading to apoptosis of germ cells (Takahashi *et al.*, 2002). Studies from our laboratory have shown that TSL-6 alleviates H_2O_2 -induced apoptosis in human spermatozoa. It is thus speculated that TSL-6 protects against apoptosis through recovery of cofilin 2 phosphorylation.

Pancreatic trypsin 1, a testicular serine proteinases, participates in the later stages of male germ cell maturation and fertilization (Hooper *et al.*, 1999). Acrosin, a serine protease with trypsin-like cleavage specificity, is one of the proteins present in the sperm acrosome. Using acrosin-deficient (*Acr*^{-/-}) mutant mice conclusively showed that sperm did not require acrosin to penetrate the zona pellucida. Further experiments using *Acr*^{-/-} mouse sperm have provided evidence that the major role of acrosin is to accelerate the dispersal of acrosomal components during the acrosome reaction. Acrosomal trypsin-like protease (named pancreatic trypsin 1) other than acrosin is probably essential for the sperm penetration of zona pellucida in mouse (Ohmura *et al.*, 1999). Pancreatic trypsin 1 was upregulated by vitamin C, followed by TSL-6 and control indicating that vitamin C and TSL-6 promoted acrosome reaction of sperm (Yu *et al.*, 2012b).

Exposure of cells to environmental stress including heat shock, oxidative stress, heavy metals, or pathologic conditions results in the inducible expression of heat shock proteins (HSPs) that function as molecular chaperones or proteases. HSPs have been classified into six major families according to their molecular size; HSP100, HSP90, HSP70, HSP60, HSP40, and small heat shock proteins. Heat shock protein 1 belongs to the HSP90 family. The roles of HSP90 have been characterized into: signal transduction (e.g., interaction with steroid hormone receptors, tyrosine kinases, serine/threonine kinases), refolding and maintaining of proteins *in vitro*, autoregulation of the heat shock response, cell cycle, and proliferation (Jolly and Morimoto, 2000). In response to environmental stress such as oxidative stress and heat shock, HSP90 is intensively expressed. HSP90 appears to interact with intermediately folded proteins and to prevent their aggregation but lacks the ability of HSP70 to refold denatured proteins (Nollen and Morimoto, 2002). Accordingly, HSP90 and HSP70 generally are co-expressed and mutually assist in protein refolding. HSP90 was upregulated by vitamin C, followed by control and then TSL-6 (Yu *et al.*, 2012b). Hassen *et al.* (2007) showed that zearalenone (ZEN), a fusarial mycotoxin, was cytotoxic to Hep G2 cells through induction of oxidative DNA damage, depletion of GSH, and induction of HSP70 and HSP90 expression. However, significant reduction of the oxidative DNA damage as well as heat shock protein induction occurred when cells were pretreated with vitamin E prior to exposure to ZEN (Hassen *et al.* 2007). Consistent with Hassen *et al.*'s results, TSL-6, with an antioxidant property, was found to protect rat testes from oxidative damage and concomitantly declined HSP90 expression. In addition, vitamin C recovered oxidative damage in rat testes via refolding of denatured proteins by induction of HSP90 expression when compared to a control (Nollen and Morimoto, 2002). These findings suggest that TSL-6 and vitamin C mediate distinct regulatory mechanism in HSP90 of rat testes under oxidative stress.

Peptidylprolyl isomerase A is highly expressed in spermatogonia and Sertoli cells in adult mouse testes. Using peptidylprolyl isomerase A-depleted mouse model, it has been shown that germ cells in postnatal peptidylprolyl isomerase A^{-/-} testis are able to initiate and complete spermatogenesis to produce mature spermatozoa. However, a progressive and age-dependent degeneration of the spermatogenic cells in peptidylprolyl isomerase A^{-/-} testis leading to the germ cell loss by 14 months of age was observed (Atchison and Means, 2003). Moreover, peptidylprolyl isomerase A was reported to possess nuclease activity. In the event of 2-methoxyethanol induced apoptosis of spermatocyte, peptidylprolyl isomerase A was highly expressed and cleaved substrate DNA into a pattern of DNA fragmentation consisting of around 180–200 base pairs (Wine *et al.*, 1997). Proteomic analysis found that peptidylprolyl isomerase A, which cleaves substrate DNA under oxidative stress, was upregulated by vitamin C, followed by a control and then TSL-6. Vitamin C upregulated peptidylprolyl isomerase

A expression to maintain proper cell cycle progression in testes under oxidative stress. TSL-6, with an antioxidant effect, protected DNA from oxidative damage by reducing peptidylprolyl isomerase A expression. TSL-6 promotes the functions of sperm and testes by regulating multiple testicular proteins in rats under oxidative stress, suggesting that TSL-6 is a valuable functional food supplement to improve functions of sperm and testes under oxidative stress (Yu *et al.*, 2012b).

The 2D-gel electrophoresis-based proteomic and western blotting results showed that TSLs differentially regulate the expression of protein profiles involved in sperm-oocyte interaction, detoxification, spermatogenesis, hormone production, and protein misfolding/refolding in rat testes under oxidative stress. TSL-2P, the upstream extract of TSL-6, exhibits regulatory functions of sperm similar to TSL-6. However, the proteins modulated by TSL-2P are less than that of TSL-6. We speculate that more optimal active components are concentrated and retained in TSL-6, the downstream extract of the TSL-2P, than in TSL-2P. The most important finding, according to the functions of expressed proteins, is that TSL-2 and TSL-6 have been initially discovered to inhibit and promote sperm functions, respectively, which may be beneficial for development of functional products for male contraceptive purpose and male infertility, respectively.

19.4 TSLs Regulate Liver Metabolism

The 2D-gel electrophoresis-based proteomics was used to elucidate a wide spectrum of the molecular mechanisms controlling hepatic metabolism in a condition of high fat diet (HFD) and to characterize protein expression influenced by TSL-CE feeding. HFD is associated with obesity, insulin resistance, and alcoholic and nonalcoholic fatty liver disease (Islam and Loots, 2009). HFD contributes to increase lipogenesis and decrease antioxidation capacity, followed by lipid peroxidation and mitochondrial dysfunction.

19.4.1 TSL-CE Decreases Gluconeogenesis

A high-fat diet (HFD) has been used as a model for obesity, dyslipidemia, and insulin resistance in rodents for many decades (Panchal and Brown, 2011). Numerous enzymes associated with gluconeogenesis in liver were found to be increased in obesity induced by HFD and normalized in HFD+TSL-CE mice including UDP-glucose 6-dehydrogenase (UGDH), electron transfer flavor protein (ETF), and sorbitol dehydrogenase precursor (SDH).

UGDH provides cursors for an array of extracellular matrix glycosaminoglycans (GAGs) such as heparin and hyaluronic acid, and also catalyzes the crucial structural modification of xenobiotic toxins required for their eventual eliminations (Zhou *et al.*, 2011). Disturbed metabolism of GAGs has been proposed to play an important role in the pathogenesis of diabetic complications. Via UDP-glucose, glucose flux from gluconeogenesis was converted to GAGs or glycogen. A HFD increased UGDH, and HFD+TSL-CE restored UGDH to normal level.

SDH, the second enzyme in the polyol pathway, oxidizes sorbitol to fructose in the presence of NAD. Fructose is converted to glucose by way of gluconeogenesis through condensation of triose-phosphate to fructose 1,6-biphosphate. Recent studies have shown that flux via polyol pathway enzymes aldose reductase (AR) and sorbitol dehydrogenase is required to mediate hypertension complications of diabetes (Jang *et al.*, 2010). Oxidation of sorbitol to fructose by SDH results in oxidative stress because its co-factor NAD⁺ is converted to NADH, which is the substrate for NAD(P)H oxidase to generate ROS (Ohmura *et al.*, 2009). Caffeic acid (CA) and ellagic acid (EA) at 5% mediated anti-diabetes effects by decreasing the levels of sorbitol dehydrogenase (Chao *et al.*, 2010). A HFD increased SDH and HFD+TSL-CE restored it to normal levels, suggesting that TSL-CE might inhibit the activation of polyol pathway in HFD condition. The β -oxidation represents an important source of energy for the body during fasting and metabolic stress, providing carbon substrates for gluconeogenesis and contributing electrons to the respiratory chain for energy production. In mitochondria, ETF receives electrons from several mitochondrial flavin-containing dehydrogenases, which are transferred to electron transfer flavoprotein-ubiquinone oxidoreductase (ETF-QO) and subsequently passed to ubiquinone in the respiratory chain. Increased transfer of electrons from reducing equivalents generated by β -oxidation contributes to increased mitochondrial ROS generation (Rocha *et al.*, 2011). HFD mice increased ETF expression that contributed to increase oxidation and gluconeogenesis and HFD+TSL-CE decreased ETF expression in liver (unpublished data).

Therefore, proteomic analysis revealing the downregulation of UGDH, SDH, and ETF by TSL-CE indicates that TSL-CE decreases gluconeogenesis.

HFD decreased transaldolase (TAL), which was increased by HFD+TSL-CE. Expression of TAL in Jurkat and H9 human T-cell lines results in a decrease in glucose-6-phosphatedehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD), two key enzymes in the gluconeogenesis pathway. TAL-deficient mice also exhibit oxidative stress

and mitochondrial dysfunction in hepatocytes that leads to liver disease progressing from non-alcoholic fatty liver disease (NAFLD) to nonalcoholic steatohepatitis (NASH) and cirrhosis (Qian *et al.*, 2008). NAC, an antioxidant compound also prevents or ameliorates liver disease in patients with TAL deficiency (Perl *et al.*, 2011). TSL-CE decreased gluconeogenesis via increasing TAL expression in HFD mice. Expression of phosphoenolpyruvate carboxykinase 2 (PCK2), a protein related to gluconeogenesis and confirmed by western blot, was significantly increased in liver of HFD mice and decreased in HFD+TSL-CE. Hepatic gluconeogenesis contributes to elevation of fasting glucose. Hyperglycemia in Type 2 diabetes is characterized by enhanced glucose production in the liver. In the presence of insulin resistance, enhanced glucose output by liver contributes to hyperglycemia. Inhibition of hepatic glucose production by insulin sensitizers controls glycemic response in diabetic patients. Inhibited or decreased gluconeogenesis has a benefit in patients with Type 2 diabetes and insulin resistance suggesting that TSL-CE can be used as a therapeutic agent for Type 2 diabetes patients.

19.4.2 TSL-CE Enhances Lipolysis

Expression of FABP, which transfers fatty acids between extra- and intracellular membranes, is regulated by dietary long chain fatty acid (LCFA) as well as by stimulating mitochondrial and peroxisomal oxidation of LCFA (Newberry *et al.*, 2008). Proteomic analysis found the increased expression of FABP, which is attributed to the activated expression of peroxisome proliferators-activated receptor γ (PPAR γ) in TSL-CE and comprises 40–75 parts by weight of gallic acid, 15–40 parts by weight of ethyl gallate, and 10–30 parts by weight of methyl gallate (Chang *et al.*, 2013b) in both HFD and HFD+TSL-CE mice. In addition, FABP physically interacts with PPAR α and functionally interacts with both PPAR α and PPAR γ (Atshaves *et al.*, 2010). Short-chain specific acyl-CoA dehydrogenase (SCAD) plays a pivotal role in energy metabolism providing fuel after prolonged starvation or during periods of increased energy requirements (van Maldegem *et al.*, 2006). Although HFD+TSL-CE decreased the protein expression of SCAD compared with HFD, the β -oxidation was upregulated in HFD+TSL-CE feeding mice. Indeed, PPAR α regulates mRNA expression of genes involved in FA oxidation and synthetic PPAR α -agonists decrease circulating lipid levels, so these are commonly used to treat hyperlipidemia and other dyslipidemic states (Baron *et al.*, 2011). The PPAR α expression in HFD+TSL-CE was higher in HFD mice. Similarly, HFD+TSL-CE significantly increased the expression of HMG-CS protein in livers of mice. Activation of PPAR α leads to upregulation of FABP and HMG-CS, which are target genes of PPAR α . FABP enhances cellular LCFA uptake, enhances intracellular transport/diffusion through the cytoplasm, and targets LCFA to peroxisomes for β -oxidation to generate acetyl-CoA. Acetyl-CoA, the product of β -oxidation is passed to the tricarboxylic acid (TCA) cycle for the production of additional adenosine triphosphate (ATP) or is used for the production of ketones under the expression of HMG-CS.

To address the effects of TSL-CE on lipid accumulation, HepG2 and Fl83B cells were treated with 200 μ M oleic acid (OA) to simulate the condition of high plasma levels of free fatty acid (FFA). The combined OA and TSL-CE was either pretreated for 12 h before adding OA or treated for 12 h after exposure of OA. The results showed that TSL-CE decreased lipid accumulation in both the pretreatment and post-treatment of OA, confirming that TSL-CE promoted lipolysis in liver of HFD mice. Insulin resistance in HFD has recorded increasing *de novo* lipogenesis (Oosterveer *et al.*, 2009). However, *de novo* hepatic lipogenesis normally contributed to, in a minor way, total hepatic triglyceride synthesis. Increased plasma FFA levels may result in intracellular accumulation of lipid metabolites in the liver, leading to fatty liver, liver insulin resistance, and Type 2 diabetes (Niu *et al.*, 2012). Therefore, a potential strategy for improving metabolic syndrome is not only to reduce the level of plasma FFA, but also to oxidize the accumulating intracellular TG in the liver. TSL-CE does not decrease lipogenesis but enhances lipolysis in liver of HFD suggesting that TSL-CE may be beneficial for metabolic syndrome.

19.4.3 TSL-CE Decreases Glutamate Metabolism

Liver is the center of amino acid metabolism. Amino acids support numerous processes, including protein synthesis and energy generation. The hepatic glutamate pathway is involved in the metabolism of most amino acids, through transamination or glutamate dehydrogenase (GDH) activity (Fahien and MacDonald, 2011). GDH and glutamine synthase (GS) were up- and downregulated in HFD mice, respectively, and they were restored to normal levels in HFD+TSL-CE mice suggesting an activation of the glutamate shuttle for α -ketoglutarate export in HFD mice and an inhibition of glutamate shuttle flow in HFD+TSL-CE mice. By transamination, glutamate can transfer its amino group to either oxaloacetate or pyruvate, producing α -ketoglutarate and thereby generating new glucose via gluconeogenesis. Therefore, TSL-CE decreased gluconeogenesis via glutamate pathway indirectly in liver. In summary, by suppressing the elevated GDH and PCK2 and increasing the expression of TAL in HFD mice, TSL-CE decreased glucose product generated by gluconeogenesis, and then the low level of hepatic glucose decreased the expression of SDH and UGDH in liver of HFD mice.

19.4.4 TSL-CE Alleviates Oxidative Stress

Under the oxidative stress of HFD, mice increased the expression of antioxidation proteins included Hsp70, catalase (Cat), peroxiredoxin 6 (Prdx6), and Nudix-type motif 9 (NUDT9). Cat is the enzyme that catalyzes the decomposition of hydrogen peroxide to water and oxygen and protects cells. Feeding mice with HFD significantly increased the activity of Cat. High fat diets increase oxidation of fatty acids through the peroxisomal oxidation pathway that is associated with increased generation of hydrogen peroxide. Therefore, the increased activity of Cat suggests a compensatory response of hepatic defense system under condition of enhanced H_2O_2 generation. Hsp70, the central component at the cellular network of molecular chaperones and folding catalysts, assists a wide range of folding processes, including the folding and assembly of newly synthesized proteins, refolding of misfolded and aggregated proteins, membrane translocation of organelle and secretory proteins, and controlling the activity of regulatory proteins (Schiaffonati *et al.*, 1994). Overexpression of Hsp70 in cell lines protects against heat shock and oxidative stress. Elevated levels of Hsp70 in mononuclear cells reflect increased oxidative stress in patients with diabetes (Yabunaka *et al.*, 1995). Hsp70 is thought to be beneficial in defending against oxidative injury. However, continuous over-expression of Hsp70 may impair the ability to respond the subsequent stressful conditions such as aging, exposure to oxidizing chemicals, or reduced antioxidants. In diabetes, sustained hyperglycemia, or attenuated antioxidative capacity may result in chronic overexpression of Hsp70 and subsequent reduction of the ability to cope with stressful conditions. A HFD increased Hsp70 protein expression and HFD+TSL-CE normalized it to that of control groups suggesting that the stress condition in HFD was alleviated by TSL-CE. The peroxiredoxins (Prdx), belong to the rapidly growing family of the thiol-specific antioxidant proteins, are highly conserved from bacteria to mammals. Prdx6 uses GSH and ascorbate as electron donors and is the only member of the Prdx family that has the ability to remove H_2O_2 and phospholipid hydroperoxide and is, therefore, able to reduce the accumulation of phospholipid hydroperoxides in plasma membranes (de Haan *et al.*, 2006). It has been reported that HFD decreases expression of genes associated with antioxidant defense, such as superoxide dismutase-3, metallothionein-1, glutathione peroxidase-5, and Prdx6 (Cui *et al.*, 2012). Consistently, we found that HFD decreased the expression of Prdx6 and HFD+TSL-CE significantly increased Prdx6 expression in liver of HFD mice. Moreover, S-adenosylmethionine (SAM) synthesis is the first step in methionine metabolism in a reaction catalyzed by methionine adenosyl transferase. SAM is the principal biologic methyl donor, the precursor for polyamine bio-synthesis and a precursor of GSH by means of the transulfuration pathway. The increase of SAM leads to an increase in homocysteine; therefore, it increases the generation of GSH. The beneficial effects of SAM in preservation solutions could therefore include direct radical scavenging as well as acting as a precursor for intracellular GSH (Evans *et al.*, 1997). Recently, Fonseca *et al.* (2001) demonstrated that the PPAR γ agonist troglitazone significantly increased hepatic concentrations of S-adenosylhomocysteine (SAH) and S-adenosylmethionine (SAM) plus SAH. Additionally, there was a significant decline in the SAM/SAH ratio (Fonseca *et al.* 2002). TSL-CE treatment increased the SAM level compared with both control and HFD groups indicating that the antioxidative stress activity of TSL-CE might be through the increased level of GSH in liver. Mitochondrial aldehyde dehydrogenase (ALDH) is responsible for metabolizing ethanol and its activity and contributes to the rate of ethanol elimination from the blood (Crabb *et al.*, 2004). Ethanol metabolism also produces reactive species, including acetaldehyde and free radicals, which can directly attack proteins, lipids, and many other cellular components. Diabetic rats exhibited significantly increased ROS, accompanied by decreasing in ALDH2 activity and expression (Wang *et al.*, 2011). A HFD decreased the expression of ALDH2, which was significantly increased and restored to normal levels after feeding with TSL-CE. This result also confirmed the antioxidation effect of TSL-CE in the liver of HFD mice. Nucleoside diphosphate-linked moiety X (Nudix) is likely to be an important auxiliary enzyme in regulating peroxisome acyl-CoA levels, the latter being imperative for β -oxidation to proceed. NUDT9 was reported as having a much greater ADP-ribose hydrolases. Free ADP-ribose is potentially toxic, due to its ability to form non-enzymatic adducts with proteins that can lead to glycation products. NUDT9 is well suited to participate in controlling the levels of ADP-ribose as a second messenger, and also to protect cells from the accumulation of this potentially toxic metabolite. Expression of NUDT9 was decreased in HFD+TSL-CE mice indicating that oxidative stress in HFD have been reduced by TSL-CE treatment. Mitochondria are considered to be the main source of ROS in the cell. ROS-mediated oxidative stress has been associated with lipid peroxidation and loss of mitochondrial membrane potential (MMP) (Raza *et al.*, 2011). To confirm the antioxidation effect of TSL-CE, decreased MMP by ROS resulting from OA in HepG2 using flow cytometry with DiCO6 dye was restored by TSL-CE treatment. Moreover, TSL-CE exhibited antioxidation activity by increasing the expression of Prdx and its GSH supply S-adenosine methionine. Prdx also suppressed the elevated of H_2O_2 generation in HFD mice. Protein oxidation and oxidative damage are associated with diabetes (Johnson *et al.*, 2009). High-fat feeding induced obesity and directed to harmful pathways with intracellular accumulations of toxic metabolites. TSL-CE supplement, while improving dyslipidemia effects, may furnish protection against

HFD-induced liver damage and diabetes by reducing oxidative stress. Biochemical study found that *Toona sinensis* extracts exhibited the antioxidative capacity indicated by increased enzyme activities of Cat, copper/zinc superoxide dismutase (Cu/Zn SOD), GPx, GR, and GST activities in liver (Yu *et al.*, 2012a). Proteomic analysis provides more evidence to confirm the antioxidation activity of TSL-CE. Of particular interest may be the results of Hspd1 (Hsp60), which is a stress protein involved in the oxidative stress response and a potent inductor of pro-inflammatory mediators in innate immune cells. Elevated concentrations of Hspd1 have been measured in the circulation of individuals with Type 2 diabetes. The occurrence of diabetes decreased Hspd1 in the heart and increased Hspd1 and 4-FfNE in the liver, and caused inflammation (Oksala *et al.*, 2006). Proteomic analysis showed that TSL-CE treatment decreased the expression of Hspd1 in livers of HFD mice treated with TSL-CE, suggesting that TSL-CE decreases inflammation in livers of HFD mice.

19.4.5 TSL-CE Increases Protein Kinase C λ

Proteomic analyses indicated that protein kinase C λ (PKC λ) expression in liver was decreased in HFD mice and HFD+TSL-CE mice restored the expression. PKC λ , a Ca^{2+} - and diacylglycerol-insensitive protein, plays a prominent role in the regulation of glucose-induced insulin secretion by modulating the expression of genes required for β -cell function (Hashimoto *et al.*, 2005). Activation of PKC λ is necessary for insulin-mediated glucose transporter type 4 (GLUT4) translocation to the plasma membrane, which enables glucose uptake in skeletal muscle. PKC λ -null mice showed impaired translocation of the insulin-dependent GLUT4 to the plasma membrane. The resultant defect in insulin-mediated glucose transport in their muscles leads to systemic insulin resistance, islet β -cell hyperplasia and impairs glucose tolerance. PKC λ knockout mice also exhibited metabolic syndromes, including abdominal obesity, elevated levels of serum triglycerides, free fatty acids, and low-density lipoprotein cholesterol, and reduced high-density lipoprotein cholesterol levels (Beguinot and Formisano, 2008). The HFD and streptozotocin (STZ)-diabetic rats suppressed PKC λ activation (Kanoh *et al.*, 2003). Thiazolidinediones, chronic insulin treatment, and short-term fasting similarly alleviate defects in insulin-stimulated glucose transport by correcting defects in insulin-induced activation of PKC λ (Kanoh *et al.*, 2001). Proteomic analysis found that HFD mice decreased the PKC λ expression and HFD+TSL-CE mice restored PKC λ expression in liver suggesting that TSL-CE may regulate insulin signaling by activating the expression of PKC λ in HFD mice.

19.4.6 TSL-CE Activates the PPAR α/γ Pathway

We previously found that TSLs activated expression of PPAR α/γ and decreased blood glucose, body weight, and body fat (Chang *et al.*, 2013b). PPAR α regulates genes involved in fatty acid uptake and oxidation, inflammation, and vascular function and plays a central role in fatty acid catabolism in liver and other tissues by upregulating β - and δ -oxidation (Abranches *et al.*, 2011). PPAR γ ligands are particularly interesting for treating Type 2 diabetes mellitus because they restore sensitivity to insulin by increasing glucose uptake into liver and skeletal muscle cells and reducing plasma glucose levels. By promoting β -oxidation via PPAR α activity, TSL-CE decreased gluconeogenesis, which was elevated in HFD mice, and was associated with hypertension and insulin resistance. PPAR α activation also controls hyperlipidemia in HFD mice by decreasing lipogenesis. Moreover, TSL-CE also showed PPAR γ ligand activity, whose expression was increased in livers of mice. PCK2 protein expression was elevated in HFD mice, whereas TSL-CE treatment decreased PCK2. Classic PPAR γ agonists have a variety of side effects, the main one is weight gain due to edema and increased fat mass (Dunn *et al.*, 2011). However, the side effects associated with PPAR γ agonists may be prevented through the combined activation of PPAR α and PPAR γ , which is known to result in a complementary and synergistic increase in lipid metabolism and insulin sensitivity (Huang *et al.*, 2010). Proteomic analysis suggests that TSL-CE may manipulate the lipolysis and energy homeostasis in liver through the regulation of the PPAR α/γ -downstream signaling pathway.

19.4.7 TSL-CE Inhibits the Polyol Pathway

The polyol pathway of glucose metabolism is often linked to the development of various complications of diabetes (Carbone *et al.*, 2009). The increased flux of the polyol pathway induced by hyperglycemia is implicated in the pathogenesis of various complications associated with diabetes, which results in increased oxidative stress. Because oxidative stress results in tissue damage in patients with diabetes, inhibition of oxidative stress is important to prevent diabetic complications. The polyol pathway is a glucose metabolic shunt that is defined by two enzymatic reactions catalyzed by AR and SDH. TSL-CE fed mice decreased SDH and UDGH protein expression, which was significantly increased in HFD mice suggesting that

TSL-CE might inhibit polyol pathway via the antioxidation and lowering gluconeogenesis activity. Hence, TSL-CE inhibits the polyol pathway via the upregulation of PPAR α/γ activity and downregulation of SDH and UDGH proteins.

19.5 TSL as a Novel Antioxidant

SOD, CAT, GPx, GR, and GST are well recognized as antioxidant enzymes in living organisms (Mates, 2000; Wei and Lee, 2002). Certain antioxidant enzymes such as CAT and GPx/GR effectively decreased ROS levels and improved sperm functions (Geva *et al.*, 1996; Patel and Sigman, 2008). Liao and colleagues (2006) found that SOD, CAT, and GPx activities in brain and liver were significantly higher in TSL extracts-treated mice. In an *in vitro* study, using a α,α -diphenyl- β -pricryl-hydrazyl radical-scavenging test, the scavenging activities of TSL extracts were over 80% at a concentration of 0.625 mg/ml (Liao *et al.* 2006). Using biochemical analysis, our results indicated that TSL-2 and TSL-2P significantly reduced activity of GPx as well as GST in testes of rats under oxidative stress. However, TSL-6 did not affect activities of antioxidant enzymes in testes of rats under oxidative stress. Under normal physiological conditions, TSL-2 decreased SOD, CAT, and GR activities and increased GST activity in testes of mice. TSL-2P reduced activity of GR while TSL-6 enhanced GPx activity and declined GR activity in testes of mice under normal physiological condition. Accordingly, different extracts of TSL exhibited specific regulatory function on antioxidant enzyme activities in normal mouse testes. In an *in vivo* study, TSL6 exhibited the best antioxidative effects which increased the enzyme activities of Cat, Cu/Zn SOD, GPx, GR, and GST activities in liver compared to those in TSL-2 and TSL-2P groups, indicating TSLs ameliorate the antioxidant enzymes activity in liver and is beneficial for the hepatic detoxification (Yu *et al.*, 2012a).

Proteomic analysis showed that certain antioxidant enzymes, including GST (downregulated by TSL-2), PHGPx (downregulated by TSL-2 and TSL-2P), and GST Mu6 (upregulated by TSL-6) were modulated by TSL extracts. Biochemical analysis also provided evidence that TSL-6 increased activity of GPx, CAT, Cu/Zn SOD, GPx, and GR. On the contrary, TSL-2, which declines the activities of GPx and GST under oxidative stress and that of SOD, CAT, and GR under normal physiological conditions, is potentially used for contraceptive applications.

19.6 Possible Active Compounds in TSL Extracts

Certain phytochemicals were identified from TS leaves, including quercetin, rutin, methyl gallate, gallic acid, and catechin (Hsieh *et al.*, 2004; Liao *et al.*, 2007), as widely representative of flavonoids that possessed antioxidative properties (Alia *et al.*, 2006; labuda *et al.*, 2003; Kim, 2007). TSL-2, TSL-2P, and TSL-6 were dissolved in methanol and subjected to HPLC for analysis of possible active components, and it was found that 1 mg/ml TSL-2 contained approximately 50.3% gallic acid, 1.4% quercetin, 2.49% rutin, 1.3% catechin, 0.19% vitamin C, and 44.32% unknown compounds. Also, 1 mg/ml TSL-2P contained approximately 22.53% gallic acid, 1.45% quercetin, 2.2% rutin, 0.66% ethyl gallate, 0.2% vitamin C, and 72.96% unknown compounds. Moreover, 1 mg/ml TSL-6 contained approximately 7.85% gallic acid, 0.92% quercetin, 1.17% rutin, 0.36% vitamin C, and 89.7% unknown compounds. Therefore, gallic acid may be the active component contributed to antioxidant/functionality in TSL extracts.

19.7 Conclusion

Proteomic analysis reveals the protein profiles modulated by TSL extracts being involved in sperm-oocyte interaction, detoxification, spermatogenesis, hormonal production, and protein misfolding/refolding in testes, gluconeogenesis, lipolysis, glutamate metabolism, polyol pathway, and PPAR α/γ -pathway in liver under oxidative stress. Biochemical analysis supports and confirms the proteomic analysis that TSL extracts exhibit regulatory functions in testes and liver under oxidative stress condition. TSL-2P, an upstream extract of TSL-6 possessing less optimal active component than that of TSL-6, modulates fewer proteins than that of TSL-6 by 2D-gel electrophoresis and western blot. In addition, according to the functions of expressed proteins, TSL-2 is suggested to inhibit sperm functions and TSL-6 is suggested to promote sperm functions, indicating TSL-2 and TSL-6 may be beneficial for development of functional products for male contraceptive and male infertility, respectively. TSL-CE ameliorates liver dysfunction in HFD mice through controlling blood glucose, enhancing lipolysis, reducing glutamate metabolism, ameliorating oxidative stress, increasing protein kinase C- λ , inhibiting the polyol pathway, activating the PPAR α/γ -pathway, and reducing lipid accumulation, and therefore, is beneficial for alleviating the pathogenesis of lipid metabolic disorder.

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20

Proteomic Approaches to Identify Novel Therapeutics and Nutraceuticals from Filamentous Fungi: Prospects and Challenges

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20.1 Introduction

A wise saying from the Far East translates as “Medicines and foods have a common origin”. Mushrooms are a perfect testimonial to this notion; they serve both as nutritionally functional food and as a natural source of remedies against multiple physiological distresses and ailments. Mushrooms are a kind of macrofungi with a distinctive fruiting body and are large enough to be seen with the naked eye. In culture, they grow by extending multiple filamentous hyphae; this has led to their collective classification under the broad group of filamentous fungi. Most of the macrofungi belong to the class *Basidiomycetes* but there are also others from the class *Ascomycetes*. Mushrooms are valuable resources for food, medicine and nutraceuticals (Lakhanpal and Rana, 2005). The number of existing mushroom species in nature is estimated at around 10,000 from which approximately 10% are likely to be edible (Chye *et al.* 2009). Archaeological evidence indicates that the inhabitants of Chile were probably the first human beings to consume wild mushrooms as food almost 13,000 years ago (Rojas and Mansur, 1995); however, the first authentic report about the consumption of wild fungi came from China, a few hundred years BCE (Aaronson, 2000). The nutritional potential of these wild mushrooms has been extensively reviewed by Ghorai *et al.* (2009). Several exceptional nutritional qualities enable the fungal fruit bodies to stand out from the rest of the vegetarian platter; these are; (1) a balanced protein content (20–30% of dry matter containing all the essential amino acids, thus capable of substituting for meat), (2) a chitinous cell wall that acts as a good source of dietary fiber, (3) high vitamin B content, (4) low LDL, and (5) trace amounts of cholesterol (Agahar-Murugkar and Subbulakshmi, 2005). However, it is not just because of their excellent nutritional status that mushrooms had earned themselves a distinct niche in biological research. The Basidiomycetes also contain a large array of enzymes, conjugated polysaccharides, and phytochemicals loaded with a host of pharmacological properties, such as boosting the immune system, providing anticancer function, as well as controlling blood lipids and glucose levels in humans. The higher Basidiomycetes include about 10,000 species from 550 genera and 80 families. Out of these, approximately 700 species

have been found to be medicinally useful (Mizuno, 1995; Wasser, 1995; Wasser and Weiss, 1999). Historically, hotwater-soluble fractions (decoctions and essences) from medicinal mushrooms were used as healing potions in the Far East, where knowledge and practice of mushroom use primarily originated (Mizuno, 1995). One of the first reports of the medicinal use of the mushroom *Ganoderma lucidum* (Fr.) Karst (commonly known as Lingzhi) dates back to the Ming dynasty and has been documented in the most well-known classical Chinese pharmacopoeia, *Ben Cao Gang Mu*. Mushrooms such as *Ganoderma lucidum* (Reishi), *Lentinus edodes* (Shiitake), *Inonotus obliquus* (Chaga), and many others have been collected and used for hundreds of years in Korea, China, Japan, and eastern Russia (Wasser, 2002). Mushroom metabolites are increasingly being utilized to treat a wide variety of diseases, particularly as they can be added to the diet and used orally, without the need to go through phase-I/II-III trials as in ordinary medicines, and they are considered as a safe and useful approach for disease treatment. Before dwelling on the essence of this chapter, a brief overview has been given in the following section about the developments related to medicinal and therapeutic uses of mushrooms.

20.2 Mushroom Derived Immunomodulators and their Target Cells in the Immune System

20.2.1 Macrophages

Macrophages play a significant part in immunity and immune responses. They assume a defensive role exhibited by their ability to carry on phagocytosis of parasites and microbes. They regulate lymphocyte activation and proliferation and they are essential in the activation process of T- and B-lymphocytes by antigens and allogenic cells. Most, if not all, Basidiomycetes mushrooms have biologically active polysaccharides showing potent antitumor activity with immunomodulating properties. These polysaccharides have various chemical compositions and belong primarily to the β -glucan group. Macrophages are a major distinct population of cells in the nonspecific cellular immune response, and are also intimately involved in specific immunity. They ingest infectious microorganisms and digest them with lysosomal enzymes. Macrophages can be activated by lymphokines and other cell mediators, and can kill tumor cells by producing the tumor necrosis factor and nitric ions. When nonspecific responses fail to check the invasion of pathogenic organisms, specific immune responses are activated whereby the lymphocytes recognize a particular pathogen and generate antibodies to promote destruction of the pathogen. In addition, lymphocytes and macrophages cooperate to mediate the same cellular immune responses. A wide range of immunostimulatory metabolites with different chemical structures have been isolated from Basidiomycetes mushrooms. The majority of immune-active polysaccharides derived from mushrooms are β -glucans, including lentinan from *Lentinus edodes*, schizophyllan from *Schizophyllum commune*, krestin from *Coriolus versicolor*, grifolan from *Grifola frondosa*, and scleroglucan from *Sclerotinia sclerotiorum*. β -Glucans stimulate a wide range of immune responses, such as cytokine release, generation of ROS, generation of NO, release of arachidonic acid metabolites, and activation of NF- κ B (Table 20.1). Quite a number of studies have unraveled so far the mode of action of β glucans on immune cells (Figure 20.1) (Brown *et al.* 2002; Czop and Austen, 1985; Rice *et al.* 2002; Taylor *et al.* 2002; Thornton *et al.* 1996; Zimmerman *et al.* 1998).

20.2.2 Dendritic Cells

Dendritic cells are antigen-presenting cells (APCs), which play a critical role in the regulation of the adaptive immune response. Dendritic cells (DCs) are unique APCs and have been referred to as “professional” APCs, since the principal function of DCs is to present antigens, and because only DCs have the ability to induce a primary immune response in resting naïve T-lymphocytes. To perform this function, DCs are capable of capturing antigens, processing them, and presenting them on the cell surface along with appropriate co-stimulation molecules. DCs also play a role in the maintenance of B-cell function and recall responses. Thus, DCs are critical in the establishment of immunological memory.

The characteristic pattern of high antigen presentation and stimulation of naïve T cells by DC make it a useful tool for vaccine and/or therapeutic design against tumors and infectious diseases. However, the induction of DC maturation is critical for the induction of antigen-specific T-lymphocyte responses and may be essential for the development of human vaccines relying on T-cell immunity. Fully mature DCs show a high surface expression of MHC class II and co-stimulatory molecules (CD40, CD80, and CD86) but a decreased capacity to internalize antigens (Jin *et al.* 2003; Lin *et al.* 2004). Upregulation of CD83, a specific marker for DC maturation, also occurs (Lin *et al.* 2007). Various stimuli, such as proinflammatory cytokines (e.g., TNF-alpha and IL-1), CD40 ligation, bacterial products (e.g., LPS and unmethylated DNA CpG motif), and contact sensitizers, can induce DC maturation *in vivo* and *in vitro* (Pal *et al.* 2007). DC engineered with IL-12, a tumor

Table 20.1 Immunomodulatory activities of mushroom products on macrophages.

Source	Active component	Target cell type	Effects (\uparrow / \downarrow) up-/downregulation	Reference
<i>Coriolus versicolor</i> (Polyporaceae)	Intramycelial and extramycelial PS Polysaccharopeptide Krestin/PSK (protein-bound PS)	Murine peritoneal Macrophage	\uparrow NO \uparrow NO, ROS and TNF- α (oral) \uparrow NO, GSHPx and SOD activity (i.p.)	Wang et al. 1998 Liu et al. 1993 Pang et al. 1998 Hsu et al. 2004
<i>Canoderma lucidum</i> (Ganodermataceae)	Beta-Glucan (1,3)-beta-d-Glucans with (1,6)- β -d-glucosyl branches polysaccharides MD-fraction [beta (1 \rightarrow 6) glucan w/ beta(1 \rightarrow 3) branched chains]	Murine peritoneal and J774.1, human Macrophage RAW 264.7	\uparrow TNF- α	Wang et al. 2014
<i>Grifola frondosa</i> (Schizophyllaceae)	Grifolan, GRN [6-branched beta (1 \rightarrow 3)-d-glucan] beta (1 \rightarrow 3)-d-glucan]	Murine peritoneal and RAW 264.7 macrophage	\uparrow IL-1 (i.p. and oral); \uparrow iNOS message and NO \uparrow IL-12	Sanzen et al. 2001 Kodama et al. 2002
<i>Lentinus edodes</i> (Lentinaceae)	water-soluble homogeneous polysaccharide (GFPBW2) Beta-d-1,3- and beta-d-1,4-linked glucopyranosyl residues, with branches attached to O-6 of beta-d-1,3-linked glucopyranosyl residues Lentinan [beta-(1 \rightarrow 3),, beta(1 \rightarrow 6)-glucan, Mr 500 kDa]	RAW264.7	\uparrow TNF- α , IL-6 activate macrophage	Okazaki et al. 1995. Adachi et al. 1998. Adachi et al. 1994. Ohno et al. 1996. Hashimoto et al. 1997 Ishibashi et al. 2001 Wang et al. 2013 Kodama et al. 2001
<i>Cordyceps sinensis</i> (Clavicipitaceae)	PS	Murine peritoneal and C4M Macrophage, human monocytes	\uparrow NO, ROS, TNF- α , IL-1, phagocytosis and cytotoxicity	Ladányi et al. 1993, Fruehauf et al. 1982 Sipka et al. 1985, Liu et al. 1998,
<i>Lentinus lepideus</i> (Lentinaceae)	PG101 (heteroglycan)	Human U937 Macrophage	\uparrow TNF- α , IL-1h, IL-10,	Mizuno et al. 2000 Chen et al. 1997
<i>Morchella esculenta</i> (Morchellaceae)	Galactomannan (Mr 1000 kDa)	Human THP-1 Macrophage	\uparrow TNF- κ B activation	Jin et al. 2003
<i>Phellinus linteus</i> (Hymenochaetaceae)	Acidic PS	Murine peritoneal Macrophage	\uparrow NO production and cytotoxicity	Duncan et al. 2002
<i>Poria cocos Wolff</i> (Coniolaceae)	Heteromannan (Mr 8 kDa)	Murine RAW 264.7 Macrophage	\uparrow TNF- κ B/Rel, p38 kinase activation \uparrow iNOS and NO	Kim et al. 2003 Lee et al. 2004 Rhee et al. 1999 Lee et al. 2003

(Continued)

Table 20.1 (Continued)

Source	Active component	Target cell type	Effects (↑/↓) up-/downregulation	Reference
<i>Sarcodon aspratus</i> (Thelephoraceae)	PCP (35.6 kDa) is a disulfide-linked heterodimeric glycoprotein consisting of 14.3 and 21.3 kDa subunits with N- and O-glycosylation	RAW 264.7 Murine peritoneal Macrophage	Toll-like receptor 4 (TLR4)-mediated myeloid differentiation factor 88 (MyD88)-dependent signaling	Chang et al. 2009
<i>Schizophyllum commune</i> (Schizophyllaceae)	Fucogalactan	Murine peritoneal Macrophage	↑TNF- α and NO	Mizuno et al. 2000
<i>P. blazei</i>	Schizophyllan/sonifilan/SPG [h(1Y6)-branched h(1Y3)-d-glucan] Water extracts mycelia and fruit bodies Fractions B-4 and B-5 Proteoglycan	Murine peritoneal, human THP-1 and U937 Macrophage	↑TNF- α , IL-6, IL-8, IL-12 and cytotoxicity ↑TNF- α , IL-8, NO ↓IL-2, IFN- γ , and TNF- α production in splenocytes ↓apoptosis of a portion of the activated macrophages and lymphocytes in LPS-treated mice	Hashimoto et al. 1997 Sugawara et al. 1984 Hirata et al. 1998 Pretus et al. 1991 Han et al. 1999 Kim et al. 2003
<i>A. cylindracea</i> <i>T. mongolicum</i> <i>Antrodia camphorata</i>	Ubiquitin-like peptide Lectins (TML-1, TML-2)	TLR2 mutation mice (TLR2 $^{-/-}$), strain B6.129-Tlr2tm1Kir/J, and TLR4 deletion mice (TLR4 $^{-/-}$, strain C57BL/10ScN)	↑NO ↑TNF-alpha, Nitrite ions ↑TLR2-Dependent NF- κ B Activation and M1 Polarization within Murine Macrophages	Ngai et al. 2003 Wang et al. 1997 Sheu et al. 2009
<i>Pleurotus sajor-caju</i> <i>Pleurotus citrinopileatus</i> <i>Hericium erinaceus</i> <i>Cordyceps militaris</i>	(1 \rightarrow 3), (1 \rightarrow 6)-linked beta-d-glucan Nonlectin glycoprotein (PCP-3A) beta-1,3-branched-beta-1,6-glucan 1,6-branched-galactomannan	RAW 264.7 Human mononuclear cells RAW264.7 Mouse macrophage	↑NO, TNF-R, IL-1beta ↑TNF-alpha, IL-2, and IFN-gamma ↑IL-1beta and TNF-beta ↑NO, IL-1beta and TNF-alpha	Elaine et al. 2012 Chen et al. 2010 Lee et al. 2009 Lee et al. 2010

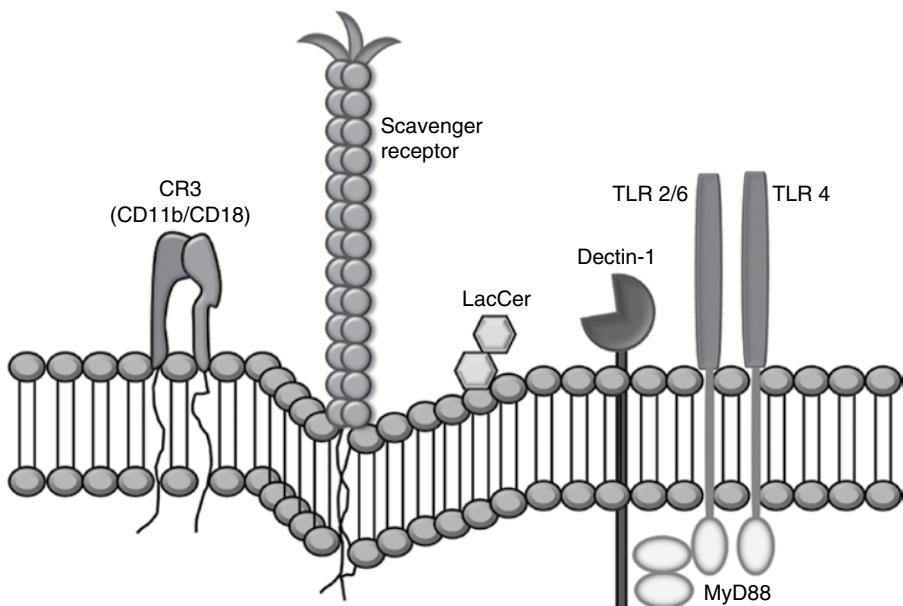


Figure 20.1 The uptake and subsequent actions of β -glucan on immune cells. β -glucans are captured by the macrophages via the Dectin-1 receptor with or without TLR-2/6. The large β -glucan molecules are then internalized and fragmented into smaller sized β -glucan fragments within the macrophages. They are carried to the marrow and endothelial reticular system and subsequently released. These small β -glucan fragments are eventually taken up by the circulating granulocytes, monocytes or macrophages via the complement receptor (CR)-3. CR3 is composed of two subunits: CD11b and CD18. Dectin-1 receptor consists of a carbohydrate recognition domain (CRD), a transmembrane stalk and a immunoreceptor tyrosine-based activation motif (ITAM). It collaborates with the TLR-2. TLR-2 has an adaptor protein, MyD88 leading to NF- κ B activation. Laceramide (LacCer) is composed of hydrophobic ceramide lipid and a hydrophilic sugar moiety. Scavenger receptor consists of three subunits: a-helical coil-coiled, ligand binding collagenase domain and receptor cysteine rich domain.

specific antigen DNA/RNA had been proven an efficient choice for enhancing the efficacy of DC based therapy *in vitro* and *in vivo* (Pal *et al.* 2007). Defect of dendritic cell (DC) maturation in tumor microenvironments is also an important immunological concern, which limits the success of cancer immunotherapy. The fungal molecules are likely to be recognized by a variety of pattern recognition receptors, mushroom polysaccharides, or peptidoglycans may offer the potential to improve existing protocols for the *ex vivo* induction of DC maturation for the purpose of DC-based vaccines and/or therapeutics (Table 20.2).

20.2.3 NK Cells

Within the ecosystem that is our body, NK cells are serious and highly selective predators: in just hours, they kill tumor or infected cells while sparing healthy cells. Natural killer cells (also known as NK cells, K cells, and killer cells) are a type of lymphocyte (a white blood cell) and a component of innate immune system. NK cells play a major role in the host-rejection of both tumors and virally infected cells. NK cells are cytotoxic; small granules in their cytoplasm contain special proteins such as perforin and proteases known as granzymes. Upon release in close proximity to a cell slated for killing, perforin forms pores in the cell membrane of the target cell through which the granzymes and associated molecules can enter, inducing apoptosis. NK cells are activated in response to interferons or macrophage-derived cytokines. They serve to contain viral infections while the adaptive immune response is generating antigen-specific cytotoxic T-cells that can clear the infection. Natural Killer (NK) cells are vital contributors to the innate immune system. Despite lacking conventional antigen-specificity, NK cells can display cytotoxic activity against “altered self” (virus-infected and cancerous) cells by receiving the appropriate signals from complex, NK-specific ligand-receptor interactions. They can also produce potent immunoregulatory cytokines that modulate the adaptive immune response, and thus serve as interesting candidates for cancer or viral immunotherapy. Some mushroom metabolites have been found to exhibit significant stimulating effects on NK cells (Table 20.3).

Table 20.2 Immunomodulatory activities of mushroom products on dendritic cell.

Source	Active component	Target cell type	Effects	Reference
<i>Ganoderma lucidum</i>	Gl-PS	Murine Bone Marrow (BM)-Derived DC	↑ proliferation of one-way MLC induced by DC ↑ phenotypic and functional maturation of DC ↑ membrane molecules, including MHC I, II, CD80, and CD86, and IL-12p70 in DC	Cao et al. 2002
Recombinant LZ-8 (24 kDa. Immunomodulatory protein of <i>Ganoderma lucidum</i>) branched (1→6)-beta-D-glucan moiety branched (1→6)-beta-D-glucan moiety		human monocyte-derived DC human monocyte-derived dendritic cells (DC) human monocyte-derived dendritic cells (DCs)	↑ proliferation of DCs with rLZ-8 resulted in an enhanced, naive T cell-stimulatory capacity and increased, naive T cell secretion of IFN-gamma ↑CD80, CD86, CD83, CD40, CD54, and human leukocyte antigen (HLA)-DR, as well as the enhanced production of interleukin (IL)-12p70, p40 genes associated with proinflammatory chemokines (CCL20, CCL5, and CCL19) and cytokines (IL-27, IL-23A, IL-12A, and IL-12B).	Lin et al. 2009
<i>Phellinus linteus</i>	Proteoglycan	Murine Bone Marrow (BM)-Derived Myeloid DC	↑CD40, CD54, CD80, and CD86). ↑TLR2 and TLR4 ↑ERK, p38, and NF- κ B ↑DC maturation	Kim et al. 2004
<i>C. versicolor</i>	PSK	murine bone marrow (BM)-derived dendritic cells (DC)	↑CD80, CD86, MHC I, MHC II.	Park et al. 2003
Agaricus blazei Murill	Commercially available Agaricus blazei based extract AndoSan™	Human monocyte-derived dendritic cells	↑Promoted both the phenotypic and functional maturation of DC derived from human CD14+ mononuclear cells	Kanazawa et al. 2004
<i>Agaricus bisporus</i>	Water-soluble proteoglycan (WSPG) Extract	murine bone marrow-derived dendritic cells murine bone marrow-derived dendritic cells	↑IL-8, G-CSF, TNF α , IL-1 β , IL-6 and MIP-1 β , ↑CD80, CD86, CD83, and MHC II	Kim et al. 2005
<i>Antrodia camphorata</i>	Extract		↑Extract-pulsed DC presented ovalbumin antigen to T cells in animal model.	Ren et al. 2008
<i>Coriolopsis militaris</i>	Water extract	Mouse bone marrow derived dendritic cells	↑CD80, CD86, CD83, and MHC II and IL-12 phosphorylation of Akt, p38, and JNK/MAPKs in DCs	Lu et al. 2009
<i>Armillaria mellea</i>	Cordylin polysaccharide 6-branched 1,3-beta-D-glucan (sporan)	Mouse bone marrow derived dendritic cells Human CD14+ monocyte-derived dendritic cells	↑CD40, CD54, CD80, CD86, and MHC class II ↑potentiates T cell cytotoxicity during therapy in animal model by DC pulsed with P815 tumor-lysate.	Kim et al. 2010a Kim et al. 2010b Kim et al. 2008a

Table 20.3 Immunomodulatory activities of mushroom products on NK cells.

Source organism	Active component	Target cell	Reference
<i>Ganoderma lucidum</i>	EORP (extract of reishi polysaccharides), a glycoprotein fraction	Enhanced number of CD3-CD16 β CD56 β NK cells in umbilical cord mononuclear cells; increased NK cytotoxicity in highly enriched CD56 β NK cells at an E:Ta ratio of 20:1, but not at 5:1 or 80:1	Chien et al. 2004
<i>G. frondosa</i>	Fractions of the hot water extract of mycelia in submerged culture precipitated with 50% and 67% EtOH, respectively	Stimulated NK activity in human PBMC at an E:T ratio of 50:1	Wu et al. 2006
<i>A. blazei</i>	Aqueous extract (378 C) of fruiting body or ABPC Hot-water extract ABMK (extract of <i>A. blazei</i> Murill Kyowa)	Stimulated NK activity in DC-rich splenocytes; induced IFN γ production by spleen cells NK activity of spleen cells in naïve BALB/c mice NK activity on cancer patients	Yuminamochi et al. 2007 Takimoto et al. 2004 Ahn et al. 2004
<i>Phellinus linteus</i>	73 kDa polysaccharide protein complex	Enhanced NK activity of purified NK cells at an E:T ratio of 50:1, but not 25:1 or 10:1	Kim et al. 2006
<i>Pleurotus ostreatus</i>	Proteoglycan fraction of mycelia	Increased NK activity in non-adherent splenocytes at an E:T ratio of 10:1	Sarangi et al. 2006
<i>Hericium erinaceum</i>	water extracts	cytolytic ability of NK cells via the induction of IL-12 in total splenocytes	Yim et al. 2007
<i>Lentinus edodes</i>	exo-biopolymer from rice bran cultured with <i>Lentinus edodes</i>	Treatment of cancer through NK cell activation	Kim et al. 2007

20.3 Mushroom Derived Metabolites in Treating Cancer

The ancient Egyptians believed that mushrooms were harbingers of long life. While their scientific method was perhaps not entirely sound, modern scientists investigating the medicinal properties of the organism are beginning to produce some fascinating results. There are thousands of species of mushroom growing in the wild, but most studies have focused on three main varieties – reishi, maitake, and shiitake. These mushrooms have complex attributes that cannot be produced synthetically. A lot of useful drugs are originally derived from natural products such as plants – aspirin being the classic example, while the chemotherapy drug taxol comes from the yew tree. The key thing is to find out what the useful chemicals are in these fungi. But until extensive clinical trials are run, the tangible benefits of using medicinal mushrooms to protect against and treat disease remain unknown. Mushrooms are more like a treasure chest that needs to be opened, rather than the cure for cancer. The evidence regarding the use of mushrooms against the cancer has been gathered in Table 20.4.

20.4 Mushroom Derived Metabolites in Infectious Diseases

The increasing occurrence of multi drug-resistant (MDR) pathogenic microbes is a threat to the public health and prompts a call for novel antimicrobial strategies. In Eastern traditional medicine, edible mushrooms have been used for over 3000 years against a range of diseases including infections. β -glucans from yeast and mushrooms and pectin from *Plantago major L.* have anti-infectious properties in rodent models against different microbes, including mycobacteria. The medicinal mushroom *Agaricus blazei* Murill, used traditionally against cancer and hepatitis, has been found to have antitumor effects in mouse models. The mechanisms for development of MDR in microbes under repression of bacteriostatic or bactericidal drugs include lateral transfection of other strains and species of bacteria by plasmids containing genes for drug resistance. One reason is unwise over-use of antibiotics for infections, also most probably viral ones such as those causing otitis media in small children, or too low doses or shortened antibiotics administration. The MDR infection epidemic is of great concern to the public health and new strategies are called for to regain the upper hand in this battle. An *Agaricus* extract, AndosanTM, also containing two related mushrooms has been shown to protect against both Gram-positive and -negative sepsis in mice and has been tested

Table 20.4 Anti-tumor activities associated with mushrooms.

Common name and family	Active component	Function and target cell type	Reference
<i>Auricularia auricular</i>	Linear (1→3)-beta-D-glucan	Immunomodulator; pre-clinical animal models	Zhang <i>et al.</i> 2007
<i>Lyophyllum decastes</i>	Linear (1→3)-beta-D-glucan	Immunomodulator; pre-clinical animal models	Zhang <i>et al.</i> 2007
<i>Lentinus edodes</i>	(1→3)-beta-D-glucan with (1→6)-beta-D branches: Lentinan	Immuno-enhancing activity; clinical trials (gastric, colorectal, prostate and breast cancers)	Zhang <i>et al.</i> 2007 Moradali <i>et al.</i> 2007 Wasser <i>et al.</i> 2002 Zaidman <i>et al.</i> 2005 Lindequist <i>et al.</i> 2005 Poucheret <i>et al.</i> 2006 Ferreira <i>et al.</i> 2010
<i>Schizophyllum commune</i>	Schizophyllan, SPG	Immuno-enhancing activity; clinical trials (gastric, cervical, head and neck cancers)	Zhang <i>et al.</i> 2007 Moradali <i>et al.</i> 2007 Wasser <i>et al.</i> 2002 Zaidman <i>et al.</i> 2005 Lindequist <i>et al.</i> 2005
<i>Grifola frondosa</i>	Grifolan, GRN	Immuno-enhancing activity; clinical trials (gastrointestinal, lung, liver and breast cancers)	Zhang <i>et al.</i> 2007 Zaidman <i>et al.</i> 2005 Lindequist <i>et al.</i> 2005 Poucheret <i>et al.</i> 2006 Moradali <i>et al.</i> 2007 Zaidman <i>et al.</i> 2005
	(1→6)-beta-D-glucan with (1→3)-beta-D branches: D-fraction	Immunomodulator; clinical trials (breast, prostate, lung, liver, and gastric cancers)	Zaidman <i>et al.</i> 2005
	Mannoxyloglucan	Immunomodulator; pre-clinical animal models	Zhang <i>et al.</i> 2007 Moradali <i>et al.</i> 2007 Wasser <i>et al.</i> 2002 Mizuno <i>et al.</i> 2002
	Xyloglucan	Immunomodulator; pre-clinical animal models	Zhang <i>et al.</i> 2007 Moradali <i>et al.</i> 2007 Wasser <i>et al.</i> 2002 Mizuno <i>et al.</i> 2002
	Fucmannogalactan	Immunomodulator; pre-clinical animal models	Zhang <i>et al.</i> 2007 Wasser <i>et al.</i> 2002
<i>Sclerotinia sclerotiorum</i>	Scleroglucan, SSG	Immunomodulator; clinical trials	Moradali <i>et al.</i> 2007
<i>Pleurotus ostreatus</i>	Pleuran	Immunomodulator; pre-clinical animal models	Bobek <i>et al.</i> 2001
<i>Ganoderma lucidum</i>	GLP	Immunomodulator; pre-clinical animal models	Moradali <i>et al.</i> 2007
	(1→3)-beta-glucuronoglucan	Immunomodulator; <i>in vitro</i> cell lines	Zhang <i>et al.</i> 2007 Moradali <i>et al.</i> 2007
<i>Poria cocos</i>	(1→3)-beta-D-glucan with (1→2) or (1→6) branches	Immunomodulator; pre-clinical animal models	Zhang, <i>et al.</i> 2007
<i>Armillariella tabescens</i>	Linear (1→6)-beta-D-glucan	Immunomodulator; pre-clinical animal models	Zhang <i>et al.</i> 2007
<i>Lyophyllum decastes</i>			

Table 20.4 (Continued)

Common name and family	Active component	Function and target cell type	Reference
<i>Agaricus blazei</i>	(1→6)-beta-D-glucan with (1→3)-beta-D branches: D-fraction (1→6)-beta-D-glucan with (1→4)-alpha-branches: (1→3)-alpha-glucan (1→3)-alpha-glucan with (1→6)-beta-branches: (1→6)-alpha-glucan with (1→4)-alpha-branches: Riboglucoman	Immunomodulator; clinical trials (breast, prostate, lung, liver, and gastric cancers) Immunomodulator; pre-clinical animal models Immunomodulator; pre-clinical animal models Immunomodulator; pre-clinical animal models Immunomodulator; <i>in vitro</i> cell lines	Moradali <i>et al.</i> 2007 Zaidman <i>et al.</i> 2005 Zhang <i>et al.</i> 2007 Zhang <i>et al.</i> 2007 Wasser <i>et al.</i> 2002 Zhang <i>et al.</i> 2007 Wasser <i>et al.</i> 2002 Zhang <i>et al.</i> 2007 Moradali <i>et al.</i> 2007 Wasser <i>et al.</i> 2002 Zhang <i>et al.</i> 2007 Moradali <i>et al.</i> 2007 Wasser, <i>et al.</i> 2002 Mizuno <i>et al.</i> 2002
<i>Amanita muscaria</i>	(1→3)-alpha-glucan	Immunomodulator; pre-clinical animal models	Zhang <i>et al.</i> 2007 Wasser <i>et al.</i> 2002
<i>Agrocybe aegerita</i>	(1→3)-alpha-glucan	Immunomodulator; pre-clinical animal models	Zhang <i>et al.</i> 2007 Wasser <i>et al.</i> 2002
<i>Armillariella tabescens</i>	(1→3)-alpha-glucan	Immunomodulator; pre-clinical animal models	Zhang <i>et al.</i> 2007 Wasser <i>et al.</i> 2002
<i>Ganoderma tsugae</i>	Arabinoglucan Glucogalactan	Immunomodulator; pre-clinical animal models Immunomodulator; pre-clinical animal models	Zhang <i>et al.</i> 2007 Moradali <i>et al.</i> 2007 Wasser <i>et al.</i> 2002 Zhang <i>et al.</i> 2007 Moradali <i>et al.</i> 2007 Wasser <i>et al.</i> 2002 Zhang <i>et al.</i> 2007 Moradali <i>et al.</i> 2007 Wasser <i>et al.</i> 2002 Zhang <i>et al.</i> 2007 Moradali <i>et al.</i> 2007 Wasser <i>et al.</i> 2002
<i>Flammulina velutipes</i> , <i>Hohenbuehelia serotina</i> , <i>Leucopaxillus giganteus</i> <i>Polyporus confluens</i> <i>Pleurotus pulmonarius</i> <i>Flammulina velutipes</i>	Galactomannoglucan	Immunomodulator; pre-clinical animal models	Zhang <i>et al.</i> 2007 Moradali <i>et al.</i> 2007 Wasser <i>et al.</i> 2002 Zhang <i>et al.</i> 2007 Moradali <i>et al.</i> 2007 Wasser <i>et al.</i> 2002
<i>Inonotus obliquus</i>	Riboglucoman	Immunomodulator; <i>in vitro</i> cell lines	Zhang <i>et al.</i> 2007 Moradali <i>et al.</i> 2007 Wasser <i>et al.</i> 2002
<i>Pleurotus citrinopileatus</i> <i>Sarcodon aspratus</i>	Xylogalactoglucan Arabinoglactan	Immunomodulator; <i>in vitro</i> cell lines Immunomodulator; pre-clinical animal models	Zhang <i>et al.</i> 2007 Moradali <i>et al.</i> 2007 Wasser <i>et al.</i> 2002 Zhang <i>et al.</i> 2007 Wasser <i>et al.</i> 2002
<i>Dictyophora indusiata</i>	Fucogalactan Fucomannogalactan	Immunomodulator; pre-clinical animal models Immunomodulator; pre-clinical animal models	Zhang <i>et al.</i> 2007 Wasser <i>et al.</i> 2002

(Continued)

Table 20.4 (Continued)

Common name and family	Active component	Function and target cell type	Reference
<i>Pleurotus pulmonarius</i>	Mannogalactan	Immunomodulator; <i>in vitro</i> cell lines	Zhang <i>et al.</i> 2007 Moradali <i>et al.</i> 2007 Wasser <i>et al.</i> 2002
<i>Fomitella fraxinea</i>	Mannofucogalactan	Immunomodulator; <i>in vitro</i> cell lines	Wasser <i>et al.</i> 2002
<i>Hericium erinaceus</i>	Xylan	Immunomodulator; pre-clinical animal models	Zhang <i>et al.</i> 2007 Moradali <i>et al.</i> 2007 Wasser <i>et al.</i> 2002
<i>Hericium erinaceum</i>	Glucoxylan	Immunomodulator; pre-clinical animal models	Zhang <i>et al.</i> 2007
<i>Pleurotus pulmonarius</i>			Moradali <i>et al.</i> 2007 Wasser <i>et al.</i> 2002

against viral infections, as reviewed here (Table 20.5). Thus, in the future, biologically active substances isolated from medicinal mushrooms and plants, may prove useful alternatives in the fight against serious infections by MDR pathogens.

20.5 Fungal Enzymes as Therapeutics and Dietary Supplements

The practice of exploiting fungi as producers of natural bioactive compounds has been in vogue for many decades. The spectrum of fungal components used for alleviation of human health range from antibiotics to dietary enzymes and other food supplements (Ghorai *et al.* 2009; Silveira *et al.* 2008). The use of traditional fungi-borne foods with proven health benefits – such as koji, soyu, tempeh, tofu, and miso – has also been extensively reviewed by Ghorai *et al.* (2009). Apart from the polysaccharide-protein complexes and other uncharacterized molecules of fungal origins, quite a few amongst the plethora of fungal enzymes have been found to be associated with active therapeutic properties such as oncolytics, thrombolytics, or anticoagulants and as replacements for metabolic deficiencies either directly or indirectly (Gurung *et al.* 2013). As compared to the other industrially exploited enzymes, therapeutically useful enzymes are required in much lesser amounts since they are generally characterized by very high substrate affinity and reaction velocity; however, at the same time, the presence of tiny amounts of contaminants can have deleterious consequences in their applicability. Therefore, these should be used in the highest possible level of purity. Digestive enzyme supplements have been used safely for well over a century. Until the late 1800s, animal source derived crude pancreatin was the sole enzyme meant as digestive aid. In 1891, Dr. Jokichi Takamine first formulated the digestive enzyme supplement “Taka kiji” from *Aspergillus oryzae*, a fungus rich in enzymatic activity. In 1894, Dr. Takamine’s formulation was commercialized by Parke, Davis, and Company under the trade name “takadiastase”, on a retail scale that is marketed even today as a digestive aid. Proteases are currently being used extensively to mobilize amino acids from powdered protein drinks. Incompletely digested proteins from protein supplements can be acted upon by the intestinal micro-flora to produce toxins such as urea, phenolics and branch chained fatty acids. Selective addition of highly efficient proteases can ensure complete degradation of the peptides into constituent amino acids and facilitate their absorption in the small intestine. AFP-Peptizyde (developed by Houston enzymes) is a combination of three different proteolytic enzymes isolated from fungal sources, which hydrolyzes protein quickly and efficiently in a concerted manner. Proteases from *Aspergillus niger* have also been found useful as anti-inflammatory agents and research is currently underway to identify other immunostimulatory activities associated with this enzyme (Chanalia *et al.* 2011). Dutch researchers have identified an enzyme from *Aspergillus niger* that can breakdown gluten molecules before they reach the small intestine, thus providing a new effective remedy for the celiac disease. Gout is a painful disorder, characterized by uricemia, recurrent attacks of acute arthritis, deposition of sodium urate in and around the joints, and, in many cases, formation of uric acid calculi (Lee *et al.* 1988). The condition results from defective purine metabolism, which in most cases is associated with a deficient or defective uricase. Administration of fungal uricase in such patients can have promising implications in gout therapy (Ali and Ibrahim 2013). The advent of new generation proteomic tools is facilitating the identification of many such enzymes; Antihypertensive peptides were screened from extracts of fruit bodies of several wild mushrooms, which were later identified as angiotensin I-converting enzyme (ACE) by SELDI TOF MS (Surface Enhanced Laser Desorption Ionization Time-of-Flight Mass Spectrometry) (Lau *et al.* 2012). Very recently, a kappa casein specific milk clotting protease was identified from the culture filtrate of *Termitomyces clypeatus* by LC ESI-MS (Liquid Chromatography Electro Spray Ionization MS) (Majumder *et al.* 2015), which is also in high demand in the dairy industry.

Table 20.5 Anti-infectious agents.

Source organism	Active component	Function and target cell	Reference
<i>Rozites caperata</i>	A protein of 10425 Da	Inhibitor of Herpes simplex Type 1 and Type 2	Piraino <i>et al.</i> 1999
<i>Pleurotus citrinopileatus</i>	A homodimeric 32.4 kDa lectin	Inhibited HIV-1 reverse transcriptase with an IC50 of 0.93 μ M	Li <i>et al.</i> 2008
<i>Agaricus blazei</i> Murill (AbM).	water extract	The extract, protected against systemic <i>S. pneumoniae</i> 6B infection in the mice.	Bernardshaw <i>et al.</i> 2005
<i>Agaricus blazei</i> Murill	Water extract	promastigote and amastigote-like stages of <i>Leishmania amazonensis</i> , <i>L. chagasi</i> , and <i>L. major</i> , with IC50 (50% inhibitory concentration) values of 67.5, 65.8, and 56.8 μ g/ml for promastigotes, and 115.4, 112.3, and 108.4 μ g/ml for amastigotes-like, respectively.	Valadares <i>et al.</i> 2011
		Cures BALB/c mice infected with <i>Leishmania amazonensis</i>	Valadares <i>et al.</i> 2012b
		selected leishmanicidal fraction to treat BALB/c mice infected with <i>Leishmania chagasi</i>	Valadares <i>et al.</i> 2012a
<i>Astraeus hygrometricus</i>	five fractions obtained from <i>Agaricus blazei</i> water extract (AbM), namely, Fab1, Fab2, Fab3, Fab4, and Fab5	<i>Leishmania donovani</i> promastigotes <i>in vitro</i>	Lai <i>et al.</i> 2012
<i>Astraeus hygrometricus</i> and <i>Tricholoma giganteum</i>	lanostane-type triterpenes, Astrakurkuron	significantly inhibited the growth of <i>L. donovani</i> promastigotes	Mallick <i>et al.</i> 2014
Water soluble Fb fractions of <i>A. hygrometricus</i> , <i>Russula laurocerasi</i> ,	80% ethanol extracted	intracellular amastigotes in macrophages	Mallick <i>et al.</i> 2014
<i>Russula albonigra</i> , <i>Termitomyces eurhizus</i> , <i>Russula delica</i> and polyphenolic fraction of <i>R. laurocerasi</i>			
<i>Ganoderma lucidum</i>	Crude aqueous extracts of the fruiting bodies	Swiss albino mice infected with <i>Plasmodium berghei</i>	Oluba <i>et al.</i> 2012

Given that the resource of such potentially beneficial wild mushrooms is far from being optimally explored, there seems to be a gargantuan figure of bioactive fungal enzymes yet to be identified. Currently, the number of whole genome and EST databases of filamentous fungi available for screening is significantly scanty. An integrated platform for a handful of such databases is provided by FungiDB (Stajich *et al.* 2012), which currently caters for the functional genomics studies in this group of organisms. Under the circumstances, the application of new generation high throughput proteomic technologies becomes imperative to enable a robust and comprehensive search of the fungal glycoproteome to fish out novel proteins that have promising health augmentation capabilities.

20.6 Identification and Characterization of Mushroom Derived Bioactive Therapeutics

The unprecedented rise in bioapplicability of mushrooms had witnessed a global initiative to identify the active components responsible for imparting health benefits over the last few decades. Modern technologies have aided the classification of these components as glycoproteins having unconventional glycosylation patterns (Maras *et al.* 1997; Wildt and

Gerngross, 2005) or as complex conjugates of peptides and sugar moieties variously termed *polysaccharide-protein complexes* or *polysaccharopeptide complexes* (Ooi and Liu, 2000), apart from a host of other secondary metabolites and mycelial components. The fungal glycoproteins possess unique high-mannose N-linked glycan motifs (Maras *et al.* 1999). These typically consists of about 100 mannose sugars, including beta-linked mannoses and mannosylphosphates, and differ from mammalian type N-glycans, which are essentially free from mannose and instead are built upon by addition of sugars such as N-acetylglucosamine, galactose, fucose, and sialic acid (Dean 1999; Gemmill and Trimble, 1999; Kukuruzinska and Lennon, 1998). The polysaccharopeptide complexes resemble eukaryotic proteoglycans but belong to a distinctive niche of complex biomolecules unique to the fungal kingdom. They are typically enriched in (1→3),(1→6)- β -glucans, and (1→3) α -glucan sugar moieties (Peng *et al.* 2003), or occur as galactomannan-protein or glucuromannan-protein complexes (Ooi and Liu, 2000). They can be isolated from mycelia by hot water extraction or from culture filtrate itself followed by ethanol precipitation (Figure 20.2). The biological activities of these molecules are influenced by their structure, molecular weight, water solubility, degree of branching, and conformation (Mizuno, 1995). Up until now, very little initiative has been taken on high throughput and robust proteomics based analysis of these molecules. However, a few studies carried out thus far indicate that these tools hold great promise for deciphering these unique biomolecules (Schiarea *et al.* 2013).

20.6.1 Proteomic Methodologies for Characterization of Fungal Complexes

Over the last decade, there has been a momentous advancement in efforts made towards characterizing fungal bioactive components. The conventional tools for structure delineation, such as NMR, FT-IR, GC, and powder diffraction, have played major roles in illuminating the dark niche of these “wonder molecules”. However, in spite of their indispensability in structure analysis, these techniques alone had failed to provide a holistic picture of the complex conjugates of peptides and sugar moieties much required for the development of new-generation drugs and therapeutics. This has mandated the employment of more sophisticated high throughput proteomic approaches to complement conventional structural tools. Proteomics is the name given to a series of concerted methodologies that can potentially reveal the total protein equivalent of either the complete cell or any of its multiple organelles. Thus, proteomics serves as the most important tool in understanding the physiology and behavior of the cell in response to diverse external stimuli. Owing to its competence in yielding proper, authoritative information on protein identity, localization, post-translational modification, and the accuracy of *in silico* gene model prediction in fungi, proteomics has become integral to all “omic” and systems based approaches towards deciphering fungal biology (Doyle, 2011). Fungi or fungus derived components present many intrinsic challenges for proteomic analysis. The comparatively slower development of the fungal omics in relation to other prokaryotes and eukaryotes until recently has been largely attributable to lack of efficient protocols and technology to dissect both the extracellular as well as the intracellular milieu of the organism. As a result, the number of entries of these groups of organisms in public databases corresponding to the genome, transcriptome, and proteome has also been scanty. The lack of satisfactory information about the fungal proteomes has necessitated a surge in the development of methodologies compatible with efficient protein extraction from fungi, and establishing basic data on the types and relative abundances of proteins present in fungi (Lakshman *et al.* 2008). Quite a few works have also been aimed at cataloging the proteomes belonging to different cellular milieu (mycelial, organellar, and secreted proteins) across the fungal kingdom (Bouws *et al.* 2008; Kim *et al.* 2008b).

There are two major approaches in fungal proteome analyses: (1) Fractionation of crude proteins by 2D gel electrophoresis, followed by in-gel tryptic digestion of individual spots, subsequent ionization, and spectra generation by MALDI-TOF (or TOF/TOF for fragmented peptides), and finally algorithm based peak association using intact peptide masses (Peptide Mass Fingerprinting) or fragmented peptide ions (Collision Induced Dissociation spectrum) to identify known, putative, or hypothetical proteins; and then (2) a multidimensional LC approach, where proteins are usually digested into peptides prior to successive separations by cation exchange and C18 reversed-phase column chromatography and subsequent identification after ESI MS/MS analysis (Figure 20.3). The derived sequences corresponding to the fragmented ions can also be submitted to similarity search algorithms such as BLAST and FASTA, which can also fish out homology dependent association and correspondingly identify the query protein/s. The LC-based methods offer flexibility of choosing a wide range of stationary and mobile phases to resolve complex biological samples at the protein or peptide level (Issaq *et al.* 2005; Shi *et al.* 2004). Besides augmentation of the current proteome reference maps, these techniques also enable the researcher a direct comparison between diverse experimental conditions, strains, or mutants. The protein spots successfully analyzed through MS are either categorized as “predicted proteins” or “hypothetical proteins” (de Oliveira *et al.*, 2010) depending upon the match

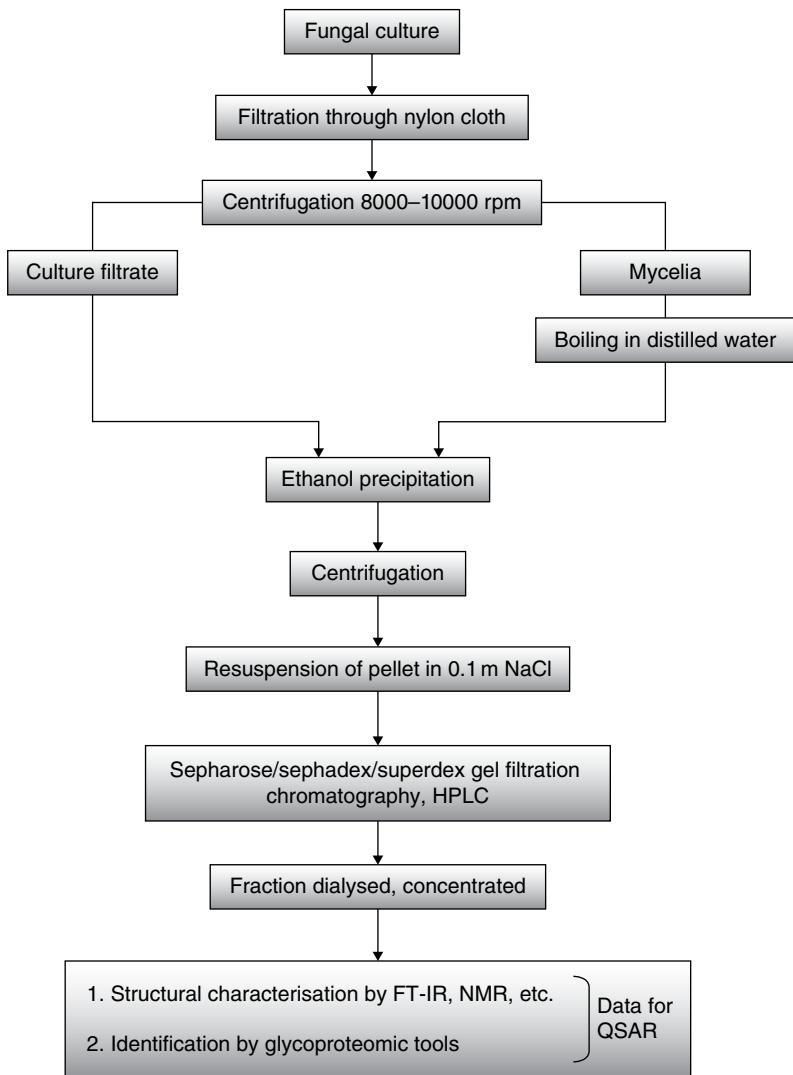


Figure 20.2 Flow sheet for isolation and identification of fungal polysaccharopeptide complexes. Following purification, conventional physicochemical characterization protocols (1) are still in vogue for structure elucidation, however, these can be supplemented by high throughput glycoproteomic approaches (2) to yield more definitive and holistic data for Quantitative Structure Activity Relationship (QSAR) studies of these unique biomolecules.

generated by the MS peaks with the protein databases available over the World Wide Web. The “hypothetical proteins” or “proteins of unknown function”, originating from filamentous fungi presently pose a considerable challenge since (1) the number of annotated entries in fungal databases currently are insufficient for getting successful hits in Peptide Mass Fingerprint (PMF) or other related homology searches, and (2) fungal proteins undergo certain post translational modifications that are unknown elsewhere amongst the eukaryotes thus effectively rendering them “Unknown” or “Putative” during database searches. A database independent search tool is the use of *de novo* sequencing (Bandeira *et al.* 2008) where sequence information is derived from MADI-TOF MS spectra of a nested set of intact N terminal peptides based on Edman chemistry. However, until now it has not been successfully implemented for proteins of fungal origin.

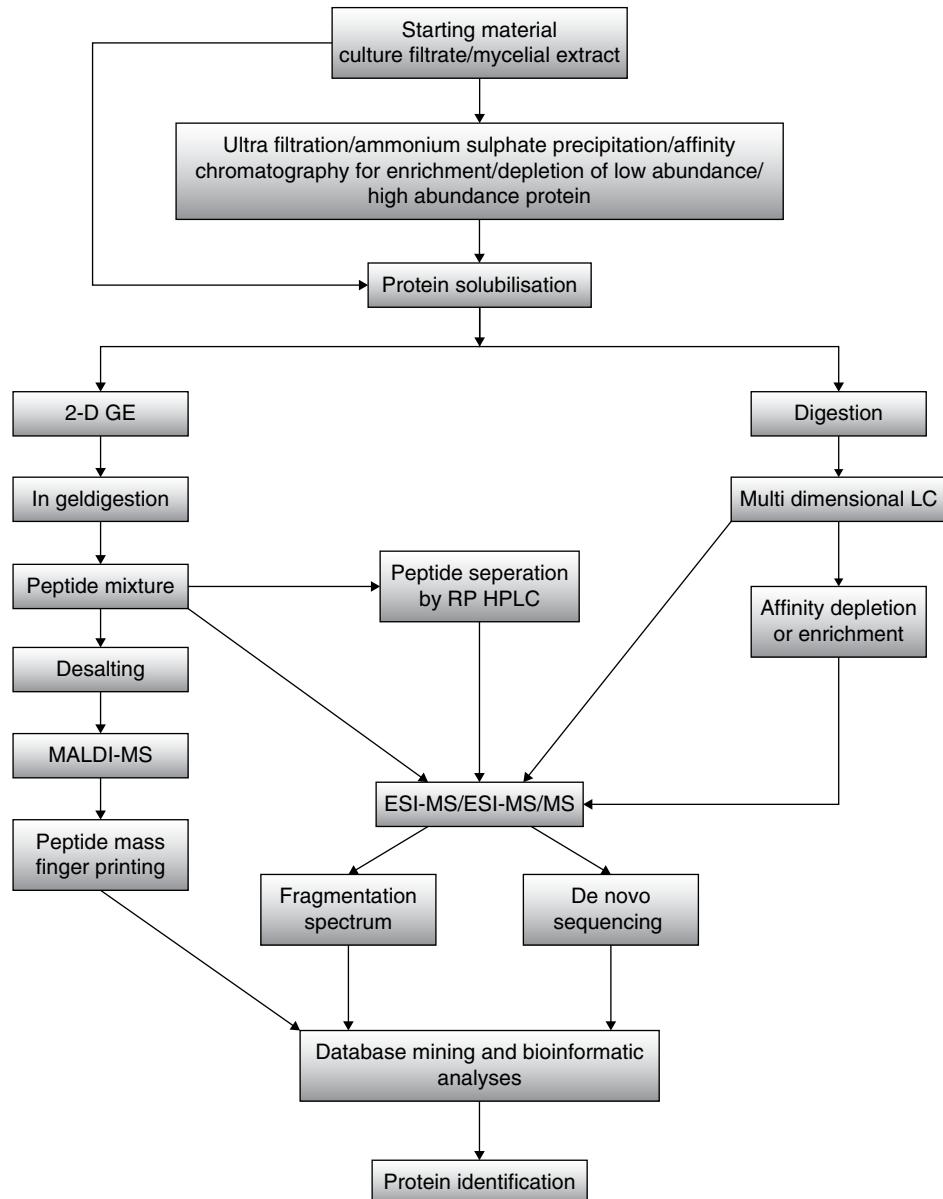


Figure 20.3 Flow sheet for identification of novel therapeutic protein from filamentous fungus. Fractionation of the fungal proteome is carried out either by 2D PAGE or by multidimensional LC; in the former approach, proteins are digested into peptides by electrophoretic separation whereas predigested protein mixture is subjected to chromatographic separation in the latter method. Thereafter, the peptides fragmented are analyzed by MALDI or ESI-MS and the generated spectra are tallied with databases for identification of new proteins having biological activities of therapeutic interest. Although not shown explicitly, the bottom up and top down approaches (as shown in Figure 20.4) also apply for analysis of the fungal proteome.

Analyses of the fungal proteome present a twofold challenge for researchers; filamentous fungi comprise a unique niche amongst the lower eukaryotes. The macromolecular composition of fungal constituents and secretions are distinctly different from the better characterized macromolecules of model lower eukaryotes such as yeast. Additionally, sample preparation for generating faithful proteomes representative of extracellular and various intracellular milieus of the organism is also a major obstacle due to the recalcitrant fungal cell wall and unique chemical nature of the analytes.

20.7 Challenges in Intracellular Proteome Preparation

Since most fungi possess a strong cell wall, the protein extraction protocol is a crucial step for fungal intracellular proteins analysis. In order to live up with the challenge of extracting and solubilising a fungal intracellular or organellar proteome, different optimally and uniquely suited procedures should be adapted for each fungus. However, all of these protocols share some common steps, such as mechanically breaking the chitinous cell wall, carrying out the extraction at low temperatures, use of protease inhibitors to prevent proteolytic digestion of the intracellular proteins, use of reducing agents to solubilize proteins, and so on. The task of disintegrating the fungal cell wall constitutes the first challenge in preparation of the fungal intracellular proteome (Ruiz-Herrera, 1992); accordingly early efforts were dedicated towards achieving this feat; these included use of glass beads for mechanical lysis (Grinyer *et al.* 2004; Melin *et al.* 2002; Mukherjee and Khowala, 2002; Nandakumar *et al.* 2003), which is superior to chemical or enzymatic extraction methods (Nandakumar *et al.* 2002). The problem of efficient cell wall disruption can be bypassed by maintaining the fungal cultures as protoplasts (Shimizu and Wariishi, 2005). Proteomes prepared from protoplasts have a better resolution in two-dimensional electrophoresis (2DE) than proteins obtained from disrupting the fungal cell wall using SDS extraction. However, many physiological conditions cannot be properly probed in the protoplast state; therefore, this technique failed to gain popularity. Since then, grinding in liquid nitrogen using a mortar and pestle has been the most effective and popular method of fungal cell lysis (Kniemeyer *et al.* 2006; Shimizu and Wariishi, 2005; Yajima and Kay, 2006). After cell lysis, the proteins are separated from other cellular components that can interfere with the isoelectric focusing step; use of TCA precipitation is unsuitable for this purpose since TCA precipitated proteins do not solubilize effectively in the buffer for isoelectric focusing. A brief treatment with sodium hydroxide has been shown to improve the solubility of TCA precipitated proteins (Nandakumar *et al.* 2003). Also, a combination of trichloroacetic acid (TCA) and acetone has been proven to be more effective than either TCA or acetone alone (Bhaduria *et al.* 2007). Other notable improvisations include solubilization using zwitterionic detergents (Kniemeyer *et al.* 2006), phosphate buffer solubilization before the precipitation (Fernández-Acero *et al.* 2006), as well as the use of acidic extraction solution to reduce streaking of fungal samples caused by their cell wall (Herbert *et al.* 2006).

20.8 Challenges in Extracellular Proteome Preparation

The extracellular proteome, also called the “secretome” of the filamentous fungi, is one of richest source of glycoproteins, enzymes, antimicrobials, and other metabolites of therapeutic and other industrially lucrative uses. The secretome is also indicative of the physiological and nutritional status of the organism, since these organisms thrive by extracellular digestion of complex organic nutrients found in diverse environmental niche. Under the circumstances, understanding the secretome is of prima-facie importance in understanding the physiology and, hence, productivity of the organism. In the first step of the secretome preparation, culture filtrates are separated from the growing mycelia when desired periods/conditions are reached. This is most commonly achieved by filtration through a Whatman 3MM paper or a nylon cloth filters (Couvans *et al.* 2010; Fernández-Acero *et al.* 2006; Jami *et al.* 2010; Lim *et al.* 2001; Yıldırım *et al.* 2011) followed by subsequent centrifugation of the filtrate. Prior separation of the bulk of the mycelia from culture medium by filtration is necessary since centrifugation of mycelial microorganisms does not result into compact pellets; thus the subsequent washing steps get cumbersome. The filtered mycelia can be washed with sterile distilled water and stored at -20°C for intracellular extraction. A crucial aspect of sample preparation for proteomic analysis of crude fungal samples (culture filtrate/mycelial extract) is the accurate estimation of the protein content. The Bradford method is the most popularly practiced protocol for determination of protein contents in crude samples despite the availability of a number of methodologies for protein estimation because of its ease, sensitivity and non interference from other components. However, significant deviations in estimations might occur if the samples contain a thousand-fold excess of free sugars when compared with that of protein (Banik *et al.* 2009); this is especially the case with fungal culture filtrates. Additionally, most of the enzymes belonging to the fungal secretome are significantly glycosylated (Banik *et al.* 2011), thus rendering conventional methods of TCA precipitation for downstream proteomic analyses inefficient. In order to attain a more reliable representation of the complete subset of proteins present in the culture medium, an initial precipitation with 80% prechilled acetone containing 20 mM dithiothreitol (DTT) at -20°C for 2 h, followed by washing the pellets twice with the same solution, and subsequently with cold 10% trichloroacetic acid, is recommended (Banik *et al.* 2009). Prior to isoelectric focusing, the pellet is dissolved in a lysis buffer and resuspended. In order to visualize the protein spots after second dimensional electrophoretic separation, the MS compatible colloidal Commassie stain, silver stain, or other fluorescence based stains of higher sensitivity are used. High abundance proteins in a fungal secretome may sometimes efficiently mask other proteins of comparatively lower concentrations. In order to reveal these masked proteins on one-dimensional or two-dimensional SDS-PAGE, an initial phase of selection and enrichment (on the basis of molecular weight) is applied on the crude culture medium. This is generally done either by ultrafiltration of the culture

medium through different molecular weight cut off membranes where the proteins having molecular weights equal to or higher than the membrane cut-off are retained above the membrane, whereas those of lower molecular weights pass through and are separated as filtrate. In some cases, increasing percentages of ammonium sulfate solutions are also used to progressively salt out proteins with different solubilities.

20.9 New Generation MS Technologies to Track the Dynamic Proteome

A new workhorse in the field of proteomic research, which has circumvented many such issues of accurately locating and quantifying low abundance proteins, has been Differential Gel Electrophoresis (DIGE) (Lilley, 2003). The technique employs CyDye fluors, a group of chemically N-hydroxysuccinimidylester, which reacts with primary amino groups, typically the terminal amino group of lysine side chains. The fluor:protein ratio is kept such that only 2–5% of the total number of lysine residues are labeled. This ensures that no single protein is tagged with more than one fluor. The three fluors are mass matched, each labeling event adding approximately 500 Da to the mass of the protein. The fluors carry an intrinsic charge of +1, such that the pI of the protein is maintained upon labeling. Labeling with CyDye DIGE fluors results in a detection limit in the order of 150–500 pg for a single protein, depending on the experiment, with a linear response in protein concentration over five orders of magnitude. The sensitivity of silver staining in comparison has a detection limit of 1 ng of protein with a dynamic range of less than two orders of magnitude. The spots detected in DIGE are compatible with downstream MS analysis after tryptic digestion. In spite of the well acclaimed popularity of the DIGE approach, several pitfalls of this technique have been reported over the last few years. Some of the reasons behind this trend include issues related to reproducibility (Lilley *et al.* 2002), poor representation of less abundant proteins (Gygi *et al.* 1999), highly acidic/basic proteins, or proteins with extreme size or hydrophobicity (Ong and Pandey, 2001), as well as difficulties in automation of the gel-based techniques (Hamdan and Righetti, 2002). Many experiments seek methods for relative quantification of proteins/peptides. One such approach has been a prototypical stable isotope labeling methodology termed the “Isotope-Coded Affinity Tags” (ICAT) technology (Gygi *et al.* 1999). ICAT technology fundamentally involves use of biotinylated light and heavy tags to differentially label the cysteine residues of proteins belonging to the two expression states of the organism under study. The labeled proteins are mixed, digested into peptides, and subjected to cation exchange chromatography to remove excess reagents; subsequently, the mixed peptides are affinity purified using immobilized avidin and subjected to MS analyses. Peak doublets in mass spectra (arising out of mass difference between heavy and light isotopes) are identified as the same peptide corresponding to the control and experimental samples. Once the doublets are established for most of the peptides (proteins), peak intensities of the peptides can be directly correlated with the relative abundance of the proteins in the two states. Despite being a powerful technique for ascertaining relative protein levels, ICAT has been confronted with several practical limitations; these include missed identification of proteins with few or no cysteine residue, lost information for post-translational modifications, differential reversed-phase elution of identical peptides labeled with the hydrogen/deuterium isotope pairs, and complicated interpretation of tandem mass spectrometry (MS/MS) peaks due to the addition of the biotin group (Goshe and Smith, 2003; Leitner and Lindner, 2004). In an improvisation of the ICAT technique termed cleavable ICAT(cICAT) that employs ^{13}C isotopes and an acid-cleavable biotin, proteins are uniformly labeled and therefore the absolute requirement of cysteine has been eliminated (Hansen *et al.* 2003; Yu *et al.* 2004). Amongst other newly developed quantitative methods, the iTRAQ (Isobaric Tags for Relative and Absolute Quantization) has been quite popular with the scientific community. In this technology, a 4-plex set of amine reactive isobaric tags is used to derivatize peptides at the N-terminus and the lysine side chains; in this way almost all peptides in a digest are labeled (Ross *et al.* 2004). The peptides labeled with any of the isotopic tags are initially indistinguishable (isobaric). However, upon fragmentation in MS/MS, signature ions (m/z from 114 to 117) are produced, which can yield definitive quantitative information about the proteins upon integration of the peak areas. Very recently, label free LC based quantitative methods have been developed (Chelius *et al.* 2003; Liu *et al.* 2004). These are claimed to yield artifact-free high resolution MS data as compared to the label dependent approaches. Development of next generation MS tools has paved the way for digging deep into more complex realms of life, the intricate interplay between sugars and peptides in the world of filamentous fungi.

20.10 Glycoproteomics: A New Arsenal in the Proteomic Toolbox

Carbohydrate moieties or the glycans are one of the key regulators of a plethora of cellular and physiological functions. However, a systemic study of these molecules has been confronted with many serious challenges chiefly owing to their non-template driven complex sequence and subsequent rich information density. This has given birth to a comparatively

new arena of scientific research, the field of “Glycomics” (Shriver *et al.* 2004). The scope of the term includes all the coordinated scientific attempts to identify and study the carbohydrate molecules directly associated with life, whether in isolation or in conjugation with proteins/peptides and lipid components of that cell, the so called “glycome” of an organism; this encompasses the glycolipids, glycoproteins, lipopolysaccharides, peptidoglycans, and the proteoglycans. However, in the present context, discussion has only been confined to the characterization of glycan-peptide/protein conjugates. “Glycoproteomics”, a sub-discipline of proteomics, thrives to complement the structure analysis tools like X-ray diffraction, NMR, and FT-IR to generate a high throughput holistic picture of the peptides and their attached glycans. The study of glycosylation of protein, one of the many indispensable protein post-translational modifications, has been recognized as crucial field of research because as many as 50% of proteins in the human body are glycosylated (Budnik *et al.* 2006) and a high proportion of therapeutic proteins, including antibodies, growth factors, and cytokines, are derived from endogenous glycoproteins; the peptide part of these exceptional biomolecules serves the functional role whereas the attached glycan moieties modulate their activity, stability, serum half-life, and immunogenicity (Baker *et al.* 2001). Some of the largely marketed glycoprotein-based pharmaceuticals include erythropoietin (Maiese *et al.* 2004), follicle stimulating hormone (Loumaye *et al.* 1998), thyroid stimulating hormone (Szkudlinski *et al.* 1993), and vaccine candidates, such as the heavily glycosylated envelope glycoprotein on the surface of the HIV virus (Go *et al.* 2008). Glycan profiling on these molecules has shown that in cell expression systems, the glycosylation on recombinant therapeutics is different from those found naturally inside human body (Hamilton *et al.* 2006); researchers are currently seeking effective “humanization” or modification of the glycosylation to closely mimic the human system (Gerngross, 2004; Wong, 2005). Accordingly, the importance in the understanding of protein glycosylation has been equivocally acclaimed now. Apart from serving a host of cellular functions, correct glycosylation is also decisive for proper folding and optimal function of the protein. Rapid and ultra-sensitive high-throughput analytical methods employing mass spectrometry (MS) and high-performance liquid chromatography (HPLC) techniques are currently being applied to provide information on the continual process of piling information in the glycan database.

Molecular dissection of the glycoproteome has been confronted with four major obstacles; (1) cellular carbohydrates are generally long molecules with many stereocentres making chemical synthesis and structure determination extremely difficult; (2) unlike DNA and protein, carbohydrate chain synthesis is template independent; (3) synthesis is spread over two cellular organelles, the endoplasmic reticulum and the Golgi body, which adds another dimension to the heterogeneity of the glycans; and (4) the peptide-glycan linkages are more fragile than other substitutions: therefore, predicting the sites of glycosylation is complex.

20.11 Glycoproteomics of Filamentous Fungi

Characterization of the glycan structures in filamentous fungi is all the more challenging since (1) the majority of the fungal glycoproteins are heavily glycosylated (Banik *et al.* 2011), and (2) the glycan structures and linkages are vastly different from those of yeasts and higher eukaryotes (Kainz *et al.* 2008). In spite of the huge promises for therapeutic applications, few inroads have so far been made with respect to development of fungi based therapeutics (Maras *et al.* 1999). This has been largely attributable to two reasons: (1) a unique non-mammalian glycosylation pattern, which is sufficient to elicit adverse immunogenic effects in human hosts, and (2) poor or incompletely characterized glycan spectra, which have pushed back the development of humanized therapeutic trials.

While both fungi and mammals attach a specific oligosaccharide to asparagines in the sequence Asn-X-Ser/Thr/Cys (where X represents any amino acid except proline), the subsequent processing of the transferred glycan differs significantly between mammalian and fungal cells (Wildt and Gerngross, 2005). The biosynthetic glycosylation pathways diverge between fungi and mammals once a glycoprotein leaves the endoplasmic reticulum (ER) and is shuttled through the Golgi apparatus. Filamentous fungi are known to carry small, high-mannose *N*-glycans including beta-linked mannoses and mannosylphosphates (Goto *et al.* 1997; Maras *et al.* 1999), whereas the formation of mammalian glycans generally involves the removal of mannose, followed by the addition of N-acetylglucosamine, galactose, fucose, and sialic acid (Dean, 1999; Gemmill and Trimble, 1999; Moremen *et al.* 1994). Therefore, in order to obtain therapeutic proteins using efficient heterologous production hosts such as the filamentous fungi, the recombinant proteins must be sufficiently “humanized” before they can be safely used for human trials. Recently, glycoengineering in the yeast *Pichia pastoris* and the expression of therapeutic glycoproteins with complex humanized N-glycosylation structures have shown significant progress (Bobrowicz *et al.* 2004; Choi *et al.* 2003; Gerngross, 2004; Wildt and Gerngross, 2005). However, few inroads have been made with respect to glycoengineering of fungal production hosts, such as *Aspergillus*

or *Trichoderma* species, although the N-glycan structures of several secreted glycoproteins have been delineated and scientists have also tried to modify fungal glycan motifs by the insertion of glycan structure modifying enzymes (Harrison *et al.* 1998; Maras, 1999). In this respect, one of the few success stories have been introduction of rabbit N-acetylglucosaminyltransferase I (GnT I) into *A. nidulans* to synthesize an *in vitro* active enzyme, but no evidence for *in vivo* GlcNAc transfer had been detected thus far (Kalsner *et al.* 1995).

20.12 High-Throughput Approaches to Decipher Fungal Glycan Structures

The first step in characterizing the fungal glycan motifs is purification of the representative glycoprotein from intracellular (mycelia extract) or extracellular (culture filtrate) milieu. In general, glycoproteins can be purified by the conventional column chromatography based protein purification strategies including various forms of HPLC (ion exchange, hydrophobic interaction, size exclusion, and affinity chromatography) and electrophoretic separation. The most commonly adopted approaches for glycoprotein/glycopeptides enrichment include hydrophilic interaction chromatography (HILIC) using amide-based columns (Bereman *et al.* 2009), lectin affinity chromatography (Ruiz-May *et al.* 2014), porous graphitized carbon column (Costello *et al.* 2007) covalent capture technology, and immobilized metal ion (usually TiO₂) chromatography (Chen *et al.* 2014). Each such methodology is based on the fundamental chemical and physical principles applicable during the affinity recognition processes: (1) peptide physicochemical properties, such as hydrophilic interactions and chelation/coordination chemistry, and (2) glycan-specific recognition, such as lectin based affinity sorting and covalent bond formation by hydrazide/boronic acid (Chen-Chun *et al.* 2014). Conventional chemical analysis of glycan moieties liberated from purified macromolecules are carried out either by NMR spectroscopy or by HPLC based identification of 2-aminopyridine derivatized fluorescent oligosaccharide moieties (Grass *et al.* 2011). However, applicability of NMR has been limited by the requirement of a relatively higher concentration (0.2 mg/ml) of purified glycans (Grass *et al.* 2011). Apart from 2 AP labeling, other popular methods of HPLC based detection of sugars involve their prior benzoylation for detection (Miyagi *et al.* 2007). In order to achieve a high throughput separation and identification of glycans, scientists have resorted to proteomic approaches. There are two general MS-based strategies for glycoprotein analysis (Figure 20.4). In the “top-down” approach, the intact glycoproteins with minimal sample preparation are directly subjected to MS and tandem MS analysis for *in situ* localization of complex glycans without extensive separation or digestion (Hanisch, 2011); however, the data interpretation is complex and not free from ambiguity if applied for large glycoproteins. The more convenient “bottom-up” or “shotgun proteomics” strategy is more suited for analysis of glycoproteins. In this method, the glycans are first released from the glycoproteins by chemical or enzymatic methods, and then the sugar moieties and peptides are purified and analyzed separately. For N-linked glycoproteins, typical N-glycanase (PNGase) enzymes are used, such as PNGase F. For analysis of N-glycans, glycoproteins are migrated in SDS-PAGE just up to their point of entry into the resolving gel and N-Glycans are isolated directly from these bands by N-glycosidase F digestion (Rendić *et al.* 2007). Analytes are subsequently separated and desalted with a dedicated sugar column in HPLC (Packer *et al.* 1998) before mass spectrometric identification. The constituent monosaccharides of the N-glycans can be determined as anthranilic acid (Anumula, 1994) or 3-methyl-1-phenyl-2-pyrazolin-5-one derivatives (Zhang, 2003) in HPLC following hydrolysis with 4 M trifluoroacetic acid. Alternatively, 2-aminobenzoic acid (2 AA) labeled N-glycans can also be treated with exoglycosidases such as α 1,2-mannosidase and/or β 1, 2-xylosidase before analysis in normal phase HPLC (Park *et al.* 2012). O-Glycans can be obtained from SDS-PAGE bands by reductive β -elimination (Pabst *et al.* 2010; Taylor *et al.* 2006); these are subsequently purified before MS analysis. Alongside structural identification of released glycans, their quantification is also of prime importance for deducing glycan mediated structure-function correlation of therapeutic biomolecules. Typically, quantization can be achieved in either of the two ways: (1) by incorporation of isotopic labels, either chemically or enzymatically, to create a specific mass tag before MS analysis that serves as the basis for relative quantization (Gygi *et al.* 1999; Ong *et al.* 2002; Ross *et al.* 2004), or (2) by either comparing the signal intensity of peptide precursor ions belonging to a particular protein (Bondarenko *et al.* 2002), or counting the number of tandem MS fragmentation spectra identifying the peptides of a given protein (Liu *et al.* 2004). The second approach is essentially label free and although of lesser sensitivity, is able to analyze simultaneously more than two samples. However, since signal intensities can vary between mass spectrometric samples, label-free approaches are not commonly the methods of choice for quantitative analysis. Instead, the popular methods of quantification of labeled glycans involve separation in lectin based affinity columns followed by isotope labeling (Isotope Coded Affinity Tags (ICATs) or isotope coded glycosylation-site-specific tagging (IGOT) before MS based analysis. In all isotope tagging protocols, two samples at most can be analyzed at a time; the control

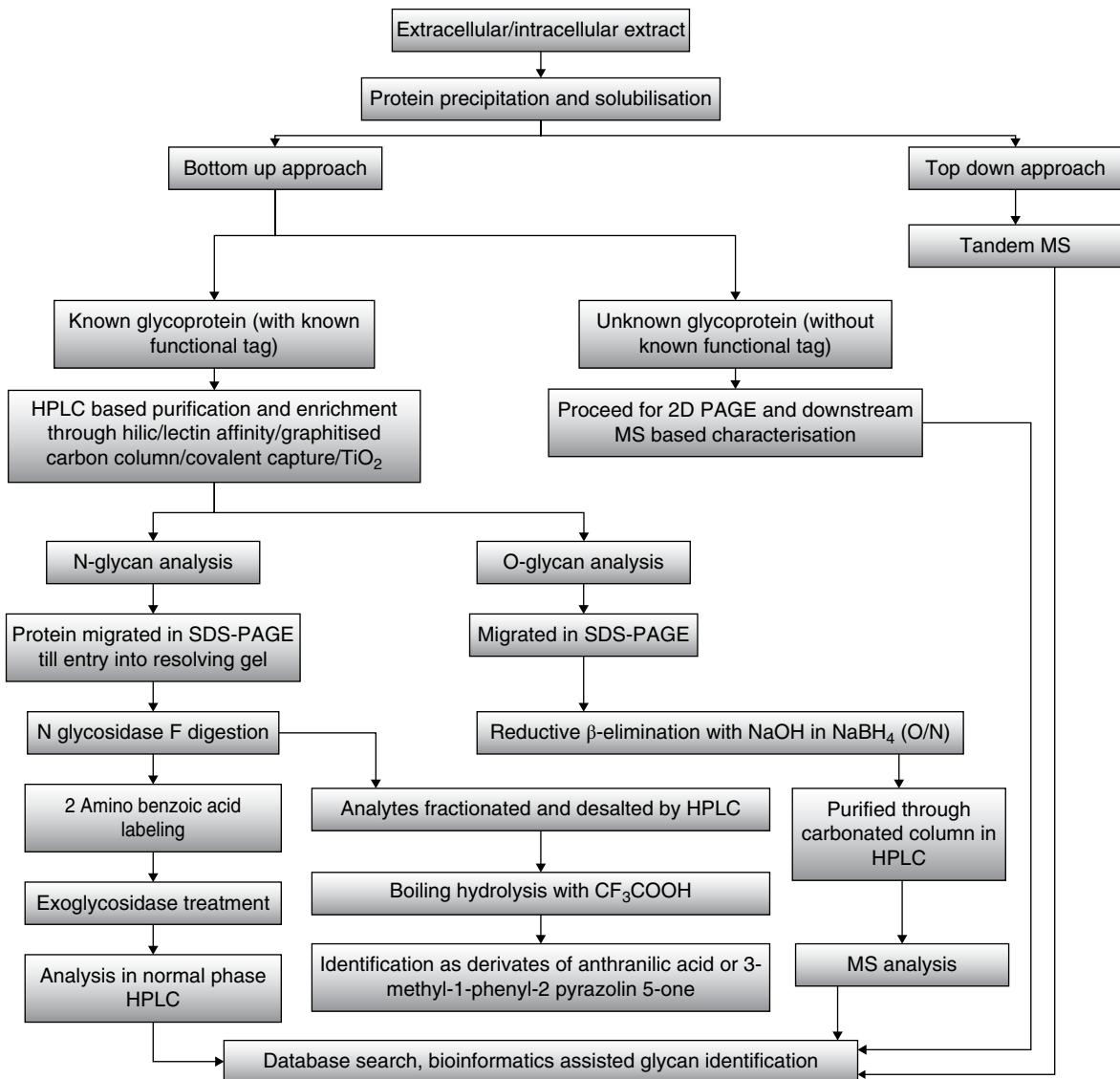


Figure 20.4 Fungal glycoproteome analysis pipeline. Out of the two general approaches for dissection of the fungal glycoproteome, the “bottom-up” approach has been more popular for easy interpretability of MS spectra; on the contrary, the “top-down” approach employs many rounds of MS (tandem MS) making the resultant spectra extremely difficult to analyze and interpret. The scarcity of fungal glycoprotein database is also a major reason accounting for the failure of the top-down approach in most cases.

glycan and its mass shifted pair from the experimental sample. This limitation had been seriously hurting the high throughput analysis of glycans. Very recently, isobaric Aldehyde Reactive Tags (iARTs) for glycan analysis have been developed by Yang (Yang *et al.* 2013), which allows six samples to be analyzed consecutively. The sensitivity of this method is also higher owing to increased hydrophobicity and resultant superior ionization of the fragmented ions. A serious disadvantage of glycan analysis stems from the fact that all information about the site of glycosylation is lost. This introduces many problems in biomarker discovery, such as not being able to decipher whether the increase in glycan’s concentration is attributable to overexpression of the particular protein containing that glycan or if the change

in global glycosylation pattern of the proteome caused the glycan to be more abundant, even though the protein level(s) are not altered (Zhao *et al.* 2007). In order to circumvent this problem, novel label free quantitative approaches have been developed, which compute the relative abundance of a glycopeptide peak with respect to the total glycopeptide peak intensity (Rebecchi *et al.* 2009).

20.13 Challenges in MS Studies of Glycans/Glycopeptides

Over the past decade, mass spectrometry (MS) has become a powerful tool for elucidation of glycan structures in various biomolecules. Despite the advent of cutting edge technologies in MS, the field of glycoproteomics has remained technically challenging; this owes chiefly to glycan structure intricacy, glycan linkage diversity, glycoform heterogeneity, and peptide–glycan complex fragility. A major challenge in glycoprotein analysis is the relatively low abundance and the low signal response of negatively charged glycopeptides during mass spectrometric analyses (Dalpathado and Desaire, 2008). Therefore, for accurate glycoprotein detection or identification with minimum signal suppression, the target glycoproteins are typically enriched through various affinity columns or other chemical strategies. While in the proteomic field one can obtain authentic quantitative data by normalizing the data against the total ion abundance (Callister *et al.* 2006), this method is not very suitable for glycopeptide analysis since they ionize weakly, compared with the nonglycosylated peptides that may also be present. Therefore, subtle changes in the presence of nonglycosylated peptides could introduce large variations in a quantitative assay when the total ion intensity (or pairwise comparison of all the peaks) is used to normalize the ion abundances of the analytes. An effective approach to evade the problem of weak ionization has been permethylation of glycans prior to MS analysis (An *et al.* 2007). Permethylated glycans offer several advantages, including enhanced sensitivity, better interpretation of tandem MS data, and stabilized and efficient ionization of sialic acid residues. Moreover, permethylation of glycan structures allows for the simultaneous detection of both acidic glycans (sialic acids) and neutral glycans in positive MS mode, which enables direct quantitative comparison of both acidic and neutral glycan abundance. An added advantage imparted by permethylation is the substantial increase in hydrophobicity of glycans, which facilitates their separation by reversed phase chromatography (Delaney *et al.* 2001). Besides improving ionization, permethylation of glycans can also stabilize the labile glycosidic bonds. Thus information regarding site of glycosylation is also better preserved.

20.14 Optimized MS Instrumentation for Glycan Analysis

Alongside the development of new extraction and sample preparation protocols, there has been a parallel advance in MS instrumentation for handling persistently complex biomolecules. The following section gives a short introduction of the different components of a typical mass spectrometer. However, a detailed technical discussion on these issues is out of this chapter's scope and therefore has been excluded.

Mass analysers: Five common mass analysers are frequently used for analysis of glycoproteins/glycans in samples: ion trap (IT), quadrupole, time-of-flight (TOF), orbitrap, and Fourier transform ion cyclotron resonance (FT-ICR). The FT-ICR-MS possesses the highest mass accuracy amongst all these; coupled with nano-LC and superior resolution, this technique is ideally suited for detection and identification of low abundance glycoproteins (Li *et al.* 2013). However, at the same time, FT-ICR-MS (MS) and TOF-MS yield complex MS spectrums, which yield authentic information only for smaller glycoproteins. The use of hybrid analysers to identify glycoproteins is also widely practiced since such instruments combine the merits of different mass analysers. For example, the MALDI TOF/TOF MS instrument is extensively applied to identify glycosylation sites and glycan structures and quantifies the global changes in specific glycan motifs (Gomes *et al.* 2013), which may be associated with altered physiology of the cells. Linear ion trap quadrupole (LTQ) instrument and hybrid Q/TOF instruments equipped with online LC separation system are employed in clinical glycoprotein analysis (Zhu *et al.* 2012). The use of Orbitrap analysers fitted with a linear ion trap has been very popular for identification and analysis of glycoproteins with high resolution and mass accuracy.

Ion sources-Electrospray Ionization (ESI) and Matrix Assisted LASER Desorption Ionization (MALDI) are two soft ionization technologies that are widely applied for the analysis of glycoproteins and glycans. Usually, multiply charged ions ($M + nH$) $^{n+}$ of glycoproteins are generated by ESI-MS, which makes it possible to detect the intact glycoproteins but makes interpretation of the generated data challenging. A modification to the conventional ESI is Nano-electrospray ionization (nano-ESI), which works at higher sensitivity owing to smaller initial droplet sizes. Derivatization of glycoproteins, as already discussed, is commonly used to improve ionization efficiency in ESI (Wada *et al.* 2010). The technique is fully

compatible with liquid chromatography (LC) thus enabling on-line LC-MS analysis of multiple glycoproteins (Nicolardi *et al.* 2013). In comparison to ESI, glycoproteins often carry either singly charged species or low-charge ions during vacuum MALDI ionization, which simplifies the subsequent data analysis. Also, vacuum MALDI can handle many samples in a single run and is more tolerant to presence of contaminants as compared to ESI. However, loss of labile sugar monomers during LASER induced ionization is a serious drawback of the technique (Wada *et al.* 2010). A critical factor in achieving efficient ionization of samples in MALDI-TOF-MS analysis is the choice of matrix used for embedding the glycoprotein samples. Several matrices such as 2,5-dihydroxybenzoic acid (2,5-DHB), 2,5-dihydroxyacetophenone (DHAP), 2,4,6-trihydroxyacetophenone (THAP), α -cyano-4-hydroxycinnamic acid (CHCA), and sinapinic acid (SA) have been developed for analysis of glycans/glycoproteins; however, instead of using a single matrix, the binary matrix combinations 2,5-DHB/CHCA and 2,5-DHB/SA have resulted in the best spectra with universal applicability ranging from intact glycoproteins to mixtures of deglycosylated glycoproteins and free underivatized glycans (Laštovičková *et al.* 2009). A variant of MALDI-MS is SELDI (Surface Enhanced LASER Desorption Ionisation) (Tang *et al.* 2004) where the protein mixture is spotted on a surface attached to a particular functional group. Some proteins in the crude sample preferentially bind to the surface, whereas others are removed during a subsequent washing step. The matrix is then applied to the surface and allowed to crystallize with the sample peptides. In this way a subset of proteins (which bind to the SELDI surface) can be analyzed out of a heterogeneous mixture of low abundance and high abundance proteins such as the fungal dynamic proteome. As mentioned already, SELDI-TOF technology has been successfully applied for identification of antihypertensive peptides from wild mushroom extracts (Lau *et al.* 2012).

20.15 Tandem Mass Spectrometry

A powerful tool developed for whole molecule analysis in the “top-down” approach has been tandem mass spectrometry. After an initial fragmentation of the intact glycoprotein, this technique employs additional dimensions of MS analysis where the fragmented ions are further dissociated *in situ* using a variety of technologies such as collision-induced dissociation (CID), electron capture dissociation (ECD), electron transfer dissociation (ETD), higher-energy collision dissociation (HCD, formerly higher-energy C-trap dissociation), infrared multiphoton dissociation (IRMPD), and electron detachment dissociation (EDD) (Alley *et al.* 2013) to yield information about peptide/protein sequences, glycosylation sites and glycan structures (i.e., composition, sequence, and linkage). The oxonium-type ions generated by CID, IRMPD, and HCD are useful to locate the constituent glycopeptides from the cluster of MS peaks. Their complementary structural information can also be obtained if the CID is run in negative ion mode (Ni *et al.*, 2013). One apparent disadvantage of CID stems from the fact that collision energies for the cleavages of glycosidic bonds are much lower than those for required for breaking peptide bonds, therefore, peptide sequence information and glycan attachment site information may not be efficiently recovered. In many cases MS-MS analysis is sufficient to deduce glycan structures; however, for complex linkages, one or two additional MS runs are required to generate new subsets of fragments that can give useful data about the linkage type. Moreover, the dynamic range of glycan concentrations arising out of high abundance motifs to barely detectable moieties in a single sample can lead to saturation of detectors; correspondingly, tandem MS use also gets restricted for such samples. In these cases, bottom-up approaches such as prior chromatographic or electrophoretic separations of the released glycans are more useful for acquisition of reliable MS data.

20.16 Bioinformatics for Glycoproteomics: Hitting Databases with MS Peaks

The final step of any MS based proteomic analysis is identification and interpretation of *m/z* peaks by comparison with available databases. The instrumentation by itself can yield information only about the molecular weight and heterogeneity of the analyte/s without referring to any database. In most cases the reference database is included in the MS analysis software. Therefore, a large part of the success of such applications depends on the availability of bioinformatic tools and glyco-related databases. A resurging interest in this field has resulted in the development of several computational tools for analysis of glycan and glycopeptides (Aoki-Kinoshita 2008; North *et al.* 2009). The UniProt Knowledgebase provides a large number of well-characterized and annotated glycoproteins for association and identification of MS peaks. Many glycopeptide mass spectra are also available in the ever expanding PeptideAtlas library (Deutsch 2008); this database has millions of high-resolution peptide fragment ion mass spectra acquired from a variety of biological and clinical samples for peptide and protein identification.

A major obstacle in comprehensive analysis of glycoproteins is the determination of glycosylation sites. The prevailing technological bottlenecks in this respect as was stated already are fragility of the peptide-glycan bond, low ionization of the sample etc. Therefore, researchers frequently fall back upon *in silico* tools to fish out potential glycosylation sites from databases. Presence of a specific tripeptide sequence Asn-X-Ser/Thr (where X is any amino acid except proline), termed “sequon” in peptide sequence databases, is generally indicative of potential N-glycosylation sites. However, for yet unknown reasons, not all such sequons are glycosylated. Therefore, unambiguous assignment of glycosylation sites from *in silico* tools is not straightforward. Besides other factors, actual and predicted glycosylation sites can also vary due to difference in cellular locations. These issues have resulted in the enlisting of only about 1000 annotated, experimentally verified, glycosylated sequons in the Swiss-Prot; an authentic database of curated protein sequence, which provides annotated sequence information of proteins including a wide range of post translational modifications (Ben-dor *et al.*, 2004). Generally, proteins that are sorted inside the cellular secretory machinery are invariably N-glycosylated. Therefore, an indirect approach of ascertaining which proteins might have N-glycosylation motifs is to search for signal peptide sequences in them. SignalP (Nielsen *et al.*, 1997) is such a prediction tool that sorts out proteins with potential N-glycosylation sites by looking for the signal peptide sequence and its site of cleavage in a protein. There are also other fuzzy logic based tools such as the NetNGlyc, which predicts N-glycosylation sites with some level of confidence. Similarly, O-GLYCOBASE (Gupta *et al.*, 1999) is a database of glycoproteins with O-linked glycosylation sites where only proteins having at least one experimentally verified O-glycosylation site have been included; basic information about the glycan involved such as the species, sequence, a literature reference, and cross-references to other databases are also associated with each entry. Other easy to execute web tools assisting glycan analysis are GlycanMass (Appel *et al.*, 1994), which computes the mass of an oligosaccharide, GlycoMod (Cooper *et al.*, 2001), which generates all possible glycan compositions from the MS molecular weight peak, the GlycoFragment tool (Lohmann and von der Lieth, 2003), which generates all theoretically possible oligosaccharide fragments according the definitions of Domon and Costello (1988).

In spite of the development of advanced tandem mass spectroscopic methodologies, massive expansion in the number of bioinformatic databases, web tools and *in silico* prediction softwares, it has not been possible to integrate all the data generated for a particular glycoform to recreate its structure in entirety. It may be useful to utilize other resources such as the Carbohydrate-Active enZYmes (CAZy) database (Coutinho *et al.* 2003), which is a systematic repertoire of genes from more than 120 organisms including some filamentous fungi coding enzymes that create, degrade, or modify the glycosidic bonds. A knowledge of the function and specificity of these enzymes can also give some essential information about the structure and site of attachment of some specific glycan.

20.17 Predicting Glycan Structures with Computational Tools

A thorough understanding of the dynamic behavior of a given glycan motif under changing cellular conditions mandates the knowledge of its full 3D structure including the various stereochemical centres, anomeric configurations, grooves, and protrusions formed by the array of the constituent sugar moieties and the chemical bondings involved therein both intramolecularly (types of linkages) as well as that with the peptides and the solute interfaces. In order to decipher these aspects, crystal structure of the molecule must be available. However, crystallization of glycans and glycoproteins had been extremely difficult to achieve due to serious technological constraints (Davis and Crispin, 2011). The presence of oligomannoses in glycans characteristic of filamentous fungi makes matters even more complicated. Therefore, scientists often have to resort to computer simulations and molecular modeling tools to build the probable glycan 3D structure by whatever data had been gathered from FT-IR, NMR, and MS experiments. Despite being macromolecules, glycan structures require informatics approaches more similar to those developed for small molecules than for proteins and nucleic acids. Some of the useful *in silico* tools to build 3D glycan structures and conformation maps are SWEET II (www.dkfz.de/spec/sweet2/), Glydicts (www.dkfz.de/spec/glydict/), GlycoMaps DB (www.dkfz.de/spec/glycomaps/) to name a few. Most of these tools work on the basis of Metropolis Monte Carlo approaches and the energy conformation maps are drawn using the relevant set of torsional angles as predicted through Ramachandran plots (Kozar and von der Lieth, 1997).

The success of bioinformatic approaches is hugely dependent on the robustness of the database and the number of annotated entries contained in those. However, as the number of entries rise, the corresponding solution space undergoes an exponential increase thus making prediction and interpretation progressively more difficult. Therefore, development of advanced data mining and management algorithms are the need of the hour to effectively manage and analyse the gigantic data that will be generated by the forthcoming glycomics projects. Since this chapter introduces the technological bottlenecks associated only with glycoproteomic approaches, a comprehensive treatment of these aspects is outside its scope.

20.18 Concluding Remarks: The Road Ahead

The fungal world seems to amuse us every day. There is a continuous tussle between the number of new fungal species discovered every year and the number of novel therapeutic activities associated with or identified from fungal sources. Survey estimates indicate that there are still a huge number of unrecognized and unidentified fungal species (more than 90%), which could be found in diverse ecological niche in association with plants, insects, and animals, as lichen-forming fungi, or in altogether virgin nooks of the ecosystem (Hawksworth, 2001). Apart from the complex protein-glycan conjugates (glycoproteins and PSPs), other fungal metabolites such as the antibacterials also cater for a huge portion of human health requirements. Very recently, Nishino and co-workers (2014) have been able to screen novel and effective *Trypanosoma brucei* kinase inhibitor activity from a previously known fungal compound, hypothemycin, using chemoproteomic approaches. A lot of scientific investigations have been performed to discover possible functional properties, which could be efficient in possible treatments of diseases like allergic asthma, food allergy, atopic dermatitis, inflammation, autoimmune joint inflammation such as rheumatoid arthritis, atherosclerosis, hyperglycemia, thrombosis, human immunodeficiency virus (HIV) infection, listeriosis, tuberculosis, septic shock, and cancer (Lin *et al.* 2004). Considering these data, it can be safely said that sky is the limit for reaping the resources offered by these organisms to mankind (Wasser 2010). However, we need to understand our current limitations before plunging into the quest for a beautiful disease free life. The glycome research has just seen its dawn in the scientific arena compared to the unprecedented advancement in the field of genomics and proteomics. A major reason accountable is undoubtedly the complexity and heterogeneity of glycan structures, making them difficult to study. At present there is no universal approach to comprehensively solve the glycan structure without knowledge of its biosynthetic pathway. Accordingly, cloning the enzymes that are involved in the biosynthesis of complex glycans, and knowing their mechanism of action and substrate specificity might throw some light into the range of complex glycans that are possible (Lowe and Marth 2003; Varki *et al.* 1999). The inception of novel glycoproteomic approaches has surely been a shot in the arm for this field. At present, bottom-up shotgun proteomics is the method of choice for mass spectrometric analysis of glycans and glycoproteins. However, in order to really accelerate the mission of uncovering the fungal paradigm, major breakthroughs in the high throughput top down approach must be realised. With integrated resource usage and concerted collaborative efforts amongst researchers, we may not be far from getting that magic potion, which will transform our lives into a new realm of spiritual wellness.

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21

Proteomics and Metaproteomics for Studying Probiotic Activity

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21.1 Introduction

Probiotics are living microorganisms that confer beneficial effects to the host when supplied in adequate amounts and have a role in the prevention and treatment of several gastrointestinal disorders, as well as food allergies and intolerances (Sanders *et al.* 2013).

Probiotics positively affect human health mainly promoting digestion and uptake of dietary nutrients, strengthening the intestinal barrier function and modulating the immune response. In addition, they enhance the antagonism towards pathogens, either by producing antimicrobial compounds or through competition for mucosal binding sites. Probiotics should be able to withstand the stress conditions of the gastro-intestinal tract (GIT) and adhere to human intestinal mucosa. These are essential features for efficient gut colonization, also used as criteria in the selection of probiotic strains (Figure 21.1) (Marco *et al.* 2006).

The most widely used probiotics include some *Lactobacillus* and *Bifidobacterium* species, indigenous inhabitants of GIT and among the dominant colonists of the small and large intestine, respectively.

The availability of complete genomic sequences of probiotic strains has opened the way to the application of omics technologies (transcriptomics, proteomics, metabolomics, etc.) to elucidate the molecular mechanisms of probiotic functionalities. The integration of complementary information from the different omics platforms in a systems biology perspective, is now contributing to get a holistic vision of the probiotic mode of action. In particular, proteomics, studying proteins directly involved in specific biological activities, holds unique capabilities to investigate cell physiology through the identification of proteins that quantitatively and qualitatively change their expression level due to environmental conditions, and the analysis of post-translational modifications (PTMs; phosphorylation, glycosylation, etc.) and protein cell location. This discipline provide insights into protein biological functions and stress response and adhesion mechanisms in probiotics.

Most of the proteomic studies on probiotics have been performed using a classical approach that integrates two-dimensional electrophoresis (2-DE) for proteome separation and Matrix Assisted Laser Desorption Ionization – Time of Flight Mass Spectrometry (MALDI-TOF-MS) for protein identification (Blackstock and Weir 1999). Tandem mass spectrometry coupled to liquid chromatography (nanoLC-MS/MS) is also used for protein identification and to characterize PTMs. More recently, MS-based advanced approaches and novel computational bioinformatics have been applied to

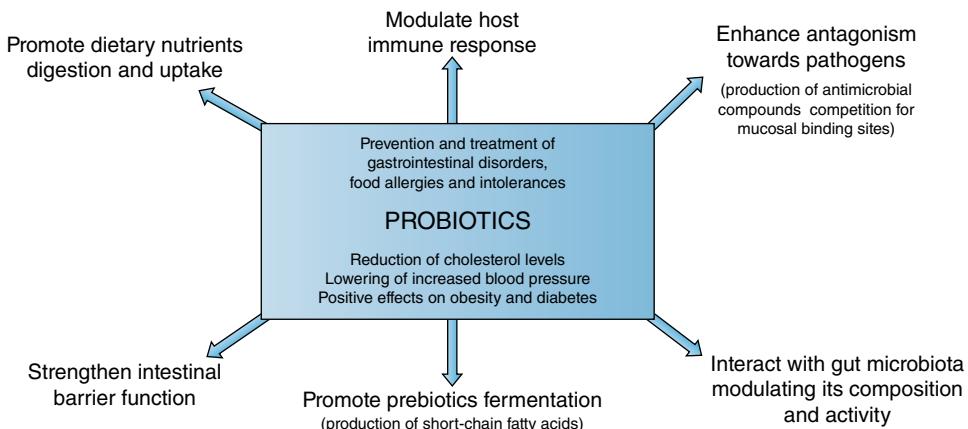


Figure 21.1 Beneficial effects of probiotics on human health.

large-scale protein identification and quantification, thus opening the way to high-throughput proteomics (Aebersold and Mann 2003; Walther and Mann 2010).

21.2 Molecular Mechanisms of Probiotic Action as Studied by Proteomics

21.2.1 Adaptation Mechanisms to GIT Environment

In order to survive the GIT transit and transiently colonize the GIT, probiotics should be able to counteract exposure to acid pH (≈ 2) in the stomach and bile in the small intestine (from 1 to 40 mM). Proteins and DNA are in fact damaged by the lowering of intracellular pH that also changes the transmembrane ΔpH . This alters the proton motive force that acts as energy source for different transmembrane transport processes. Bile, composed of bile acids (weak organic acids), and bile salts (taurine or glycine conjugates), mainly impairs the cell membrane, altering its architecture, and cell wall functionalities. Moreover, bile salts are deconjugated by bile salt hydrolases (BSHs) in the bacterial cytoplasm, thus causing an intracellular acidification. Therefore, response mechanisms to acid and bile exposure share several common features, as clearly assessed by proteomics (Ruiz *et al.* 2013; Sánchez *et al.* 2008a).

Firstly, in the GIT, probiotics activate general stress response mechanisms that involve the activation of the protein folding and degradation machinery, as demonstrated by the accumulation of molecular chaperones and ATP-dependent proteases (GroES, GroEL, DnaK and DnaJ, and Clp family proteins, respectively) to prevent protein misfolding, denaturation, aggregation, and assure protein quality. In addition, stress conditions affect the expression of proteins responsible for maintaining redox balance or involved in transcription and translation (such as RNA polymerases, elongation factors, and ribosomal proteins), nucleotide and amino acid biosynthesis and related to DNA-repair. Harsh GIT conditions also cause a metabolic reorganization, mainly leading to the activation of glycolysis and bifid shunt, a unique hexose metabolism of bifidobacteria (Ruiz *et al.* 2013; Sánchez *et al.* 2008a). In fact, a greater production of energetic intermediates (ATP) is mandatory to sustain ATP depending processes such as folding machinery, transport and detoxifying systems and the activity of proton translocating ATPase ($\text{F}_0\text{-F}_1$ -ATPase). This enzyme, whose expression is induced by both acid and bile exposure, assists proton extrusion thus assuring the cytoplasmic pH homeostasis in anaerobic microorganisms (Figure 21.2) (Koponen *et al.* 2012; Lee *et al.* 2008a,b; Sánchez *et al.* 2007a,b).

pH homeostasis is controlled by lactobacilli also through the activation of the arginine deiminase (ADI) pathway (De Angelis and Gobbetti 2004; Lee *et al.* 2008b; Lee and Pi 2010), while bifidobacteria, that lack this metabolic pathway, produce higher amounts of enzymes involved in the biosynthesis of branched-chain amino acids (BCAA) and glutamine synthetase, that lead to ammonia production (Ruiz *et al.* 2013; Sánchez *et al.* 2007b).

Due to its lipophilic character, bile exposure could modify cell-envelope architecture and affect cell wall lipid composition, in a species- and strain-dependent fashion. In fact, alterations of the expression level of enzymes involved in exopolysaccharides (EPS) biosynthesis or in fatty acids metabolism have been associated to the acquisition of bile tolerance (Alcántara and Zúñiga 2012; Burns *et al.* 2010; Koskeniemi *et al.* 2011; Ruiz *et al.* 2013; Sánchez *et al.* 2007a; Wu *et al.* 2010).

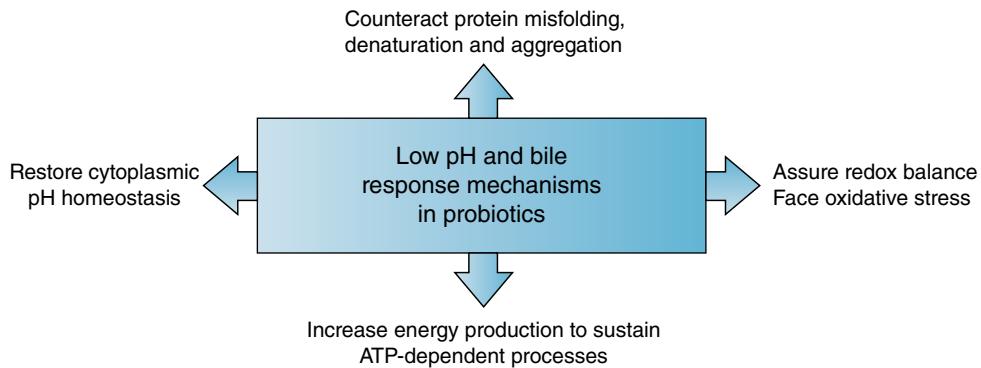


Figure 21.2 Response mechanisms triggered in probiotics by both acid and bile exposure.

Proteomics also highlighted that bile triggered the expression of ABC-type multidrug transporters and the activity of bile efflux pumps for bile salts extrusion (Ctr and BetA in *Bifidobacterium*) (Hamon *et al.* 2011, 2012; Koskenniemi *et al.* 2011; Ruiz *et al.* 2009; Sánchez *et al.* 2008a).

Intriguingly, the role of BSHs in conferring resistance to bile remains unclear: in fact, BSHs accumulated in a bile-adapted *B. animalis* strain, thus suggesting that overexpression of these proteins made this strain more tolerant, while their expression level changed differently in *Lactobacillus* species (Hamon *et al.* 2011; Koskenniemi *et al.* 2011; Lee *et al.* 2013; Ruiz *et al.* 2013; Sánchez *et al.* 2007a; Wu *et al.* 2010).

21.2.2 Adhesion Mechanisms to the Host Mucosa

Probiotics' ability to adhere to epithelial cells and intestinal mucosa is a crucial feature for the transient colonization of GIT and allows them to interact with the host in order to exert beneficial health effects. Colonization strategies seem to be species- and strain-specific, and involve proteinaceous compounds as well as teichoic and lipoteichoic acids, peptidoglycans, and EPS.

Probiotic-host molecular crosstalk and interactions with the host immune system are mainly mediated by extracellular proteins that include cell-envelope and secreted proteins. These proteins represent the first-line interaction with the mucus layer of GIT and/or extracellular matrix proteins (fibronectin, collagen, and laminin) and plasminogen (Sánchez *et al.* 2010).

Proteomics, properly integrated by *in vitro* tests, has been important to definitively assess the presence of cytoplasmic housekeeping proteins in the extracellular proteoma. Such proteins, defined as moonlighting proteins, lack any extracytoplasmic sorting sequence and display different, seemingly unrelated, functions in different cell locations (Jeffery 2003). The large and constantly growing list of moonlighting proteins now includes proteins belonging to the glycolytic pathway (such as enolase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate mutase, and triose-phosphate isomerase), ribosomal proteins, molecular chaperones (such as DnaK and GroEL) and translational factors (such as elongation factor-Tu: EF-Tu) (Beck *et al.* 2011; Sánchez *et al.* 2008b, 2010). These proteins act as adhesion promoting factors in probiotics, as their ability to bind fibronectin, mucin or plasminogen has been reported (Antikainen *et al.* 2007; Bergonzelli *et al.* 2006; Candela *et al.* 2010; Granato *et al.* 2004; Sánchez *et al.* 2010; Vastano *et al.* 2013). As these proteins are also involved in the adhesion of pathogens to GIT, probiotics competitively exclude enteropathogens from binding to intestinal cells and mucus (Lebeer *et al.* 2010).

Proteins binding plasminogen, collagen, fibronectin, or mucin, as well as proteins responsible for fimbriae formation, have been identified in the secretome and membrane proteome of *B. animalis* subsp. *lactis* BB-12 (Gilad *et al.* 2011, 2012).

Proteomics also proved to have a role in deciphering the molecular basis of strain-specificity of probiotic features. The detailed proteome analysis of two *L. rhamnosus* strains (the GG strain and the dairy strain Lc705) led to the identification of a significant number of predicted extracellular proteins, including lipoproteins, integral membrane proteins, proteins anchored to peptidoglycans, and secreted proteins. A large portion of these surface proteins (about 25%) was strain-specific, thus providing molecular evidence of phenotypic and functional differences between these strains (Savijoki *et al.* 2011).

Proteomic studies clearly highlighted that several proteins involved in adhesion mechanisms were also implicated in acid pH and bile adaptation, thus suggesting that GIT stress conditions represent a key signal triggering the colonization process and more, in general, the probiotic-host crosstalk.

21.2.3 Molecular Mechanisms of Probiotic Immunomodulatory Effects

Beneficial effects of probiotic consumption in the prevention and treatment of several gastrointestinal diseases, such as inflammatory bowel diseases, antibiotic associated-diarrhea, neonatal necrotizing enterocolitis, irritable bowel syndrome, *Helicobacter pylori* infection, as well as food allergies and intolerances, have been clearly assessed (Sanders *et al.* 2013). Furthermore, first evidence of probiotic action in reducing cholesterol levels and lowering blood pressure has been reported (Kumar *et al.* 2012). In addition, positive effects of probiotics on obesity and diabetes have been suggested (Panwar *et al.* 2013). However, the molecular basis of the strain-specific probiotic action and the identity of effector molecules (peptidoglycan, teichoic acid, cell surface polysaccharides, and extracellular proteins) still remain to be fully elucidated.

Proteomics integrated by *in vitro* and *in vivo* analyses led to identify extracellular proteins involved in probiotic–host immune system interplay. In *L. johnsonii* NCC533, moonlighting proteins EF-Tu and GroEL elicited a CD14-dependent proinflammatory response stimulating IL-8 release in macrophages and HT29 cells (Bergonzelli *et al.* 2006; Granato *et al.* 2004). Overexpression of GroES and GroEL, induced in *L. acidophilus* by pH decrease in culture media, affected the secretion of cytokines (IL-12, IFN- γ , and IL-10) from splenocytes (Kuwana and Yamamoto 2012). This evidence strongly suggested that immunomodulation could be an additional function of the multitasking moonlighting proteins.

As to surface proteins, the S-layer protein SlpA of *L. acidophilus* has been identified as the first bacterial protein able to bind to the DC-specific ICAM-3-grabbing nonintegrand (DC-SIGN) receptor, thus being functionally involved in the modulation of DCs and T-cell functions (Konstantinov *et al.* 2008). Moreover, adhesion to Caco-2 cells and release of IL-12 from DCs, were related to the amount of SlpA present on the surface of different *L. acidophilus* strains (Ashida *et al.* 2011).

More recently, a global picture of *L. acidophilus* NCFM surface proteins has been depicted by proteomics, leading to the identification of a putative S-layer protein (LBA1029) that contributed to a proinflammatory TNF- α response from murine DCs (Johnson *et al.* 2013). Six proteins with potential immunogenic abilities were identified in the extracellular proteome of *B. animalis* BB12 (Gilad *et al.* 2011).

Interestingly, specific soluble factors produced by probiotics, called postbiotics, could be sufficient to elicit host immunomodulatory response. In fact, a secreted bioactive peptide (STp), enclosed in an extracellular protein of *L. plantarum* and released after protein hydrolysis by intestinal proteases, modulated phenotype, and function of human blood-enriched DCs (Bernardo *et al.* 2012). These findings could prompt the development of novel nutraceuticals or functional foods that could represent a safer alternative for specific clinical applications, such as chronic inflammatory conditions, where probiotic somministration seems to be less effective (Tsilingiri and Rescigno 2013).

21.3 Probiotics and Prebiotics

A prebiotic is defined as “a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora, that confers benefits upon host wellbeing and health”. At present, only fructooligosaccharides (FOS and inulin), galactooligosaccharides (GOS), and lactulose have achieved prebiotic status in the EU (Gibson *et al.* 2004). These molecules exert their positive effects by selectively stimulating specific intestinal bacteria. In fact, these oligosaccharides are neither absorbed by the host nor degraded by bile and digestive enzymes, and move to the small intestine and colon where members of the microbiota, in particular probiotics, have developed the ability to utilize these abundant sources of carbon and energy (Koropatkin *et al.* 2012; Scott *et al.* 2013). This is an important factor modulating the composition of the gut microbial consortium. However, the molecular mechanisms of prebiotic actions still need to be completely documented.

The metabolic endpoint of bacterial carbohydrate fermentation is the production of short-chain fatty acids (SCFAs), predominantly acetate, propionate, and butyrate that host cells use as energy sources (Gibson and Glenn 2004; Topping and Clifton 2001). In addition, prebiotics exert a trophic effect on the intestinal epithelium, increasing cell growth and controlling proliferation and differentiation (Guarner and Malagelada 2003). Recently, synbiotics, that are suitable combinations of prebiotics and probiotics, have been developed to synergically act in order to promote well-being and health (Kolida and Gibson 2011).

In the last decade, omics sciences, in particular transcriptomics and proteomics, have been applied to elucidate oligosaccharide metabolism in lactobacilli and bifidobacteria, showing that bacteria employ several strategies, sometime strain-specific, for utilizing different carbon sources. These strategies involve intra/extracellular or membrane associated enzymes with activity towards oligo- and polysaccharides (such as glycoside hydrolases (GHs)), assisted by complex sugar transport systems (mainly ATP-binding cassette (ABC)-type systems and phosphoenolpyruvate phosphotransferase (PTS) permeases) nested in membranes (Abou-Hachem *et al.*, 2013; Gänzle and Follador 2012). Noteworthy, in bifidobacteria, β -galactosidases also exhibit transglycosylic activity that allows the synthesis of prebiotic substances from lactose (Pokusaeva *et al.* 2011).

Metabolic enzymes responsible for oligosaccharide degradation in lactobacilli so far characterized, are localized in the cytoplasm except amylase and levensucrase. Therefore, transport systems play a pivotal role in prebiotic metabolism. In fact, the expression of the *lac* operon, encoding for lactose permease LacS, enzymes involved in galactose metabolism, two β -galactosidases (GH2 and GH42) and two transcriptional regulators, was induced in *L. acidophilus* NCFM grown on GOS. On the other hand, mutant strains obtained from the deletion of *lacS* were not able to grow on substrates containing GOS, lactose, or lactitol as a carbon source, thus confirming the cytoplasmic localization of these enzymes (Andersen *et al.* 2011). Increased amounts of LacS and GH2 were also observed at proteomic level in the same strain grown on lactitol (Majumder *et al.* 2011). Interestingly, the *lac* operon was overexpressed also in response to bile acids (Pfeiler *et al.* 2007), suggesting a transversal role of proteins encoded by this operon in *L. acidophilus* adaptation to the gut niche.

Proteomic studies highlighted the specificity of hydrolases involved in prebiotics metabolism; in fact, particular GHs were overexpressed in *B. longum* subsp. *infantis* grown on different carbon sources (GOS, FOS, and human milk oligosaccharides), while only four GHs (out of the 16 identified) were induced in all the experimental conditions (Kim *et al.* 2013). Metabolic utilization of emerging prebiotic candidates, such as β -glucans and xylo-oligosaccharides, requires their internalization mediated by transport systems and intracellular degradation by specific GHs (Gilad *et al.* 2010; Zhao and Cheung 2013). Up to now, omics sciences have been barely applied to elucidate the mechanisms of probiosis/prebiosis interplay and future studies in this field will be important for the formulation of synbiotics designed for specific health benefits.

21.4 Investigation on Human Microbiota Dynamics by Proteomics

In recent years, metagenomics, defined as the analysis of complex microbial communities by direct extraction, cloning, and sequencing of DNA from their natural environment (Riesenfeld *et al.* 2004), and metaproteomics, the large-scale characterization of the entire protein complement of microbiota in environmental samples at a given point in time (Wilmes and Bond 2004), have been applied to investigate microbial dynamics of the complex human gut ecosystem and their impact on human health (Aires and Butel 2011; Del Chierico *et al.* 2012; Hettich *et al.* 2012; Siggins *et al.* 2012).

Recent findings highlight that adults have a unique and stable microbial community affected by environmental and genetic factors as well as diet habits (such as the ingestion of functional foods or bioactive peptides) (Qin *et al.* 2010). Gut microbiota participates in the digestion process but, most importantly, it has a critical function in defense processes against pathogens and in development and response of the immune system, while specific alterations in its composition have been associated to different gastrointestinal diseases (Sekirov *et al.* 2010). Probiotics tightly interact with gut microbiota having a role in modulating its composition and/or activity, so they could counteract such pathological alterations (Gerritsen *et al.* 2011; Gueimonde and Collado 2012).

It is worth stressing that metagenomics reveals the potential of microbiota but it does not provide any information on which genes are actually expressed and under which conditions. On the other hand, metaproteomics holds great potential to investigate specific metabolic activities and key biological functions truly exhibited by the gut microbiota and, hopefully, to identify protein markers that may be indicative of a healthy or disease status (Siggins *et al.* 2012).

A pioneering metaproteomic study performed on human fecal samples led to the identification and functional classification of more than 2200 proteins, thus fully demonstrating, for the first time, the feasibility of specifically designed proteomic strategies to analyze such complex samples. Proteins mainly involved in translation, carbohydrate metabolism, and energy production were identified and direct evidence of the dominant microbial populations in the gut ecosystem was provided. In addition, this non-targeted analysis also led to the identification of a significant number of human proteins, some of them related to the host response to microbiota (Verberkmoes *et al.* 2009).

Comparative metaproteomics further assessed the temporal stability of human intestinal microbiota and the expression of a common functional protein core that is mainly involved in carbohydrate transport and degradation. Additionally, a variety of microbial surface proteins was characterized, including potential components of the microbes-host interaction such as flagellins and pili (Kolmeder *et al.* 2012). The metaproteomic analysis of mucosal-luminal interface also revealed significant anatomic region-related (biogeographic) features and established the identity of proteins that demarcated the proximal and distal colon (Li *et al.* 2011).

More recently, the integrated metaproteomics/metagenomics approach was applied to the investigation of dysbiosis of gut microbiota due to gastrointestinal diseases, shedding light on functional differences at gene and protein level and changes in bacterial community related to Ileum Crohn's disease (ICD). Results highlighted that general processes (including carbohydrate transport and metabolism, energy production and conversion, amino acid/lipid/nucleotide transport and metabolism, and defense mechanisms) were deficient in ICD patients. In particular, some enzymes, under-represented in the

ICD patients, were involved in the degradation of complex carbohydrates of plant origin (probably reflecting a functional shift due to dietary habits) and in the production of butyrate and other SCFAs (Erickson *et al.* 2012).

Interestingly, a recent metagenomic and metaproteomic investigation of gut microbial communities in fecal samples from obese and lean adolescents clearly showed a considerable heterogeneity in total and active microbial communities and in the protein expression pattern, and gave first insights into dynamics and mechanisms underlying the intestinal microbiota response to food supply and obesity (Ferrer *et al.* 2013).

These first studies undeniably demonstrate the innovative strength of metagenomics and metaproteomics in the analysis of gut microbiota dynamics and its alterations due to diseases. We can foresee that the near future will witness a boost in such studies, also prompted by the development of more advanced methodologies (Del Chierico *et al.* 2014; Kolmeder and de Vos 2014; Li *et al.* 2011; Rooijers *et al.* 2011). This knowledge could conduct to new criteria for the selection of probiotic strains exhibiting specific ability in maintaining microbiota homeostasis.

21.5 Concluding Remarks and Future Directions

Proteomics has significantly contributed to elucidate the molecular mechanisms underlying probiotic actions, revealing that some proteins actually mediate both adaptation and adhesion to GIT as well as immunomodulation. In addition, proteomics could support the definition of novel probiotic markers, useful for the rapid selection of strains with predictable and improved functionality (Sánchez *et al.* 2012) and prompt the identification and development of new postbiotics (Tsilingiri and Rescigno 2013). Overall, further omics studies will provide the scientific basis for the design of enhanced functional foods tailored for specific health benefits, thus reflecting the need of personalized nutritional advice.

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Proteomics Approach to Assess the Potency of Dietary Grape Seed Proanthocyanidins and Dimeric Procyanidin B2

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This is an exciting time for biological scientists as the “omics” era continues to evolve and shape the way science is understood and conducted. Understanding the interrelationship among genes, gene products and dietary is fundamental to identifying those who will benefit most from, or be placed at risk by, intervention strategies. Unraveling the multitude of nutrigenomic, proteomic, and metabolomic patterns that arise from the ingestion of foods or their bioactive food components will not be simple but is likely to provide insights into a tailored approach to diet and health. Nutritional proteomics or *nutriproteomics* is the application of the proteomics modality to nutrition-related research (Schweigert, 2007; Trujillo *et al.*, 2006). It also represents the interaction of bioactive food ingredients with proteins, whereby the interaction with proteins occurs in basically two specific ways.

We are aware that diet may not only provide an adequate amount of nutrients to meet metabolic requirements, but could also contribute to improving human health. As a consequence of this awareness, plant extracts or single compounds thereof that benefit human health need to be identified and developed for the food market to complement a balance diet. The grape is one of the most widely consumed fruits in the world. Moderate consumption of wine is associated with a reduced risk of cancer. Grape seeds are rich in dimers, trimers, and other oligomers of the flavan-3-ols, named procyanidins. Grape seed extracts are commonly available dietary supplements taken for the antioxidant activity attributed to their proanthocyanidin (oligomers of monomeric polyphenols) content. Grape seed proanthocyanidins (GSPs) have been reported to possess a variety of potent properties including antioxidant, anti-nonenzymatic glycation, anti-inflammation, anti-tumor, and so on (Zhang *et al.*, 2006). Moreover, GSPs have cardiovascular, renal, and cerebellar protective effects (Bagchi *et al.*, 2003; Xu *et al.*, 2008). GSPs exhibit a novel spectrum of biological, pharmacological, therapeutic, as well as chemoprotective properties against oxygen free radicals.

22.1 Chemoprotective Properties of GSPs

22.1.1 Components and Molecules

GSPs exist as oligomers or polymers of flavan-3-ol such as (+)-catechin and (-)-epicatechin that are predicted to form helices in their global minimum-energy conformation. GSPs are formed from the association of several of these monomeric units: 2–5 units for catechin oligomers, over 5 units for catechin polymers. The oligomeric proanthocyanidin (OPC) has the strongest biological activity. Procyanidins differ in the position and configuration of their monomeric linkages. The structures of procyanidin dimers B1, B2, B3, and B4 are the best known. Among various types of procyanidins, dimers distribute the most widely in nature and have received a lot of attention from scientists. Trimers are named C1, C2, and so on, because of the different compositions of monomers and different locations where carbon atoms connected. People are now becoming more and more interested in GSPs because of their beneficial effects on human health.

22.1.2 Antioxidant Effects

Various environmental pollutants have been shown to produce enormous amount of free radicals, resulting in oxidative deterioration of lipids, proteins and DNA, activation of procarcinogens, inhibition of antioxidant defense systems, and changes in gene expression, which contribute significantly to human diseases. The free radical scavenging abilities (RSA) of GSPs, vitamin E, and vitamin C against biochemically generated superoxide anion and hydroxyl radicals were assessed *in vitro* at varying concentrations via cytochrome C reduction and chemiluminescence response. Chemiluminescence is a general assay for the production of reactive oxygen species, while cytochrome C reduction is a specific assay for superoxide anion. At 50 mg/l, GSPs demonstrated 84 and 98% greater RSA against the superoxide anion and hydroxyl radical, respectively, compared to natural vitamin E, and at 100 mg/l, GSPs demonstrated 439 and 575% greater RSA against superoxide anion and hydroxyl radical, respectively, as compared to vitamin C (Bagchi *et al.*, 1997).

GSPs could be useful in the attenuation of ultraviolet (UV) radiation induced oxidative stress-mediated skin diseases in human skin. Treatment of normal human epidermal keratinocytes (NHEK) with GSP inhibits UV-induced hydrogen peroxide (H₂O₂), lipid peroxidation, protein oxidation, and DNA damage in NHEK. It can scavenge hydroxyl radicals and superoxide anions in a cell-free system. GSPs also inhibit UVB-induced depletion of antioxidant defense components, such as glutathione peroxidase, catalase, superoxide dismutase, and glutathione. As UV-induced oxidative stress mediates activation of mitogen-activated protein kinase (MAPK) and nuclear factor- κ B (NF- κ B) signaling pathways, GSPs inhibit UV-induced phosphorylation of ERK1/2, JNK, and p38 proteins. GSPs also inhibit UV-induced activation of NF- κ B, which is mediated through inhibition of degradation and activation of I κ B- α and IKK- α , respectively (Mantena and Katiyar, 2006).

GSP treatment can attenuate tissue MDA level, myeloperoxidase activities and collagen content in bile duct ligated biliary obstruction rats suggesting an anti-fibrosis effect, which is possibly because of its inhibition of neutrophil infiltration and lipid peroxidation; thus, restoration of oxidant and antioxidant status in the tissue.

22.1.3 Anti-Nonenzymatic Glycation and Anti-Inflammation Effects

Using an *in vitro* protein glycosylation system, glucose and bovine serum albumin were co-incubated at 37 and 50°C, respectively, as controls while the experimental groups received additional different doses of GSP or aminoguanidine. Then, the amount of advanced glycation end products (AGEs) was determined using a fluorescence photometer. Results show that GSP in the range of 1.0–2.0 g/l can inhibit the production of AGEs efficiently and the effect of GSP at the concentration of 2.0 g/l was similar to that of aminoguanidine of the same dose. Studies indicate that proanthocyanidins can effectively scavenge the reactive carbonyl species and thus inhibit the formation of AGEs.

Our previous experiments showed that GSP had anti-nonenzymatic glycation and anti-inflammation effects by reducing the receptors of advanced glycation end product (RAGE) protein expression, subsequently leading to decreased expression of high level vascular cell adhesion molecule 1 (VCAM-1) induced by AGEs in human umbilical vein endothelial cells (HUVEC). AGEs activated the expression of RAGE and inhibited peroxisome proliferator-activated receptors (PPAR) gamma expression in HUVEC, whereas GSP inhibited the expression of RAGE through activation of PPAR gamma in HUVEC simultaneously (Ma *et al.*, 2007; Zhang *et al.*, 2006, 2007). These findings indicated that GSP inhibited the cell inflammatory factor expression and protected the function of endothelial cell through activation of PPAR gamma expression and inhibition of RAGE expression.

Although several studies have postulated NF-κB as the molecular site where redox-active substances act to regulate agonist induced ICAM-1 and VCAM-1 gene expression, inhibition of inducible VCAM-1 gene expression by GSP was not via a NF-κB dependent pathway. GSPs modulated inflammatory response in activated macrophages by the inhibition of nitric oxide (NO) and prostaglandin E2 (PGE2) production, suppression of inducible nitric oxide synthase (iNOS) expression, and NF-κB translocation. These results demonstrate an immunomodulatory role of GSP and thus a potential health benefit in inflammatory conditions that exert an overproduction of NO and PGE2.

22.1.4 Protective Effects on the Cardiovascular System

A large body of evidence has shown that GSPs can improve the elasticity of blood vessels, inhibit the activity of Angiotensin II, and lower blood pressure. And GSP has also been proved to decrease plasma low-density lipoprotein (LDL) and total cholesterol (TC), decrease capillary permeability, and prevent thrombosis. In a word, GSPs have been suggested to have a cardioprotective effect.

22.1.4.1 Anti-Atherosclerosis Effects

In previous experiments, GSP was shown to prevent atherosclerosis and this effect may be due to its modulation of lipids and antioxidant properties. Male New Zealand rabbits were divided into three groups and were fed a standard diet, standard +1% cholesterol diet, and standard +1% cholesterol+1% grains feedstuff diet, respectively. At 12 weeks, the animals were sacrificed and the aortas dissected for analysis. Results showed that GSP can reduce plasma TC, low density lipoprotein cholesterol (LDL-C), triglyceride (TG), TG/high density lipoprotein cholesterol (HDL-C), malondialdehyde (MDA), and ox-LDL significantly and elevate HDL-C significantly. Immunohistochemical analysis demonstrated less thickness in the aorta blood vessel wall and fewer foam cells when comparing the GSP group with the high cholesterol group.

Postprandial hyperlipemia is a well-defined risk factor for atherosclerosis. A reasonable contributing mechanism could involve the postprandial increase of plasma lipid hydroperoxides (LPO) affecting the oxidant-antioxidant balance and increasing the susceptibility of LDL to oxidation. The supplementation of a meal with GSP minimizes the postprandial oxidative stress by decreasing the oxidants and increasing antioxidant levels in plasma and, as a consequence, enhancing the resistance to oxidative modification of LDL. It has been shown that a GSP supplement can increase plasma antioxidant capacity in the postprandial phase and the content of LPO in chylomicrons was 1.5-fold higher after the control meal than after the GSP supplemented meal (Natella *et al.*, 2002).

22.1.4.2 Anti-Ischemic Reperfusion Injury of Myocardium

GSPs have cardioprotective effects against ischemic reperfusion induced injury via their ability to reduce or remove, directly or indirectly, free radicals in the myocardium that undergoes reperfusion after ischemia. GSPs reduce the incidence of reperfusion-induced ventricular fibrillation and ventricular tachycardia. In rats treated with 100 mg/kg proanthocyanidins, the recovery of coronary flow, aortic flow, and developed pressure after 60 min of reperfusion was improved (Pataki *et al.*, 2002). Electron spin resonance studies indicated that GSP remove peroxy radical generated by 2, 2'-azobis (2-amidinopropane) dihydrochloride and reduce 7-OH-coumarin-3-carboxylic acid attributing to its hydroxyl radical scavenging activity.

GSP can protect the heart against myocardial injury induced by isoproterenol (ISO) in a rat model. The prior administration of GSP for 6 days a week for 5 weeks maintained the levels of the marker enzymes in all the treatment groups (GSP 50, 100, and 150 mg) when compared to ISO-injected rats. In the ISO-injected group there was a significant rise in TBARS and a significant decrease in glutathione, glutathione peroxidase, glutathione S transferase, superoxide dismutase (SOD), and catalase. The administration of GSP maintained the activities of these enzymes close to normal levels. It also significantly increases the activities of mitochondrial enzymes (isocitrate dehydrogenase, succinate dehydrogenase, malate dehydrogenase, and alpha-ketoglutarate dehydrogenase) and respiratory chain enzymes (NADH dehydrogenase and cytochrome c oxidase), and significantly decreases the activities of lysosomal enzymes in the heart tissues of ISO-induced rats (Karthikeyan *et al.*, 2007). GSP pretreatment significantly maintains the cholesterol, phospholipids, triglycerides, and free fatty acids levels in serum and heart tissue of the ISO-induced myocardial injury in rats, too. All these findings prove the stress stabilizing effect of GSP.

22.1.4.3 Anti-Thrombosis Effects

GSP can inhibit platelet aggregation, improve endothelial function, and reduce oxidative stress. Studies show GSP significantly decreased adenosine diphosphate-stimulated platelet reactivity and epinephrine-stimulated platelet reactivity. Rein *et al.* found that adding GSP to *in vitro* whole blood increased the expression of PAC-1 and P-selectin,

reduced platelet reaction to the agonist, and didn't inhibit platelet activation induced by epinephrine. Vitseva proved that incubation of platelets with GSP could lead to a decrease in platelet aggregation from 50–70% and so lead to a dramatic decrease in superoxide release, as well as a significant increase in radical-scavenging activity, decrease in reactive oxygen species release, and enhance platelet NO release (Vitseva *et al.*, 2005). These findings suggest the potentially beneficial platelet-dependent antithrombotic and anti-inflammatory properties of GSP.

22.1.4.4 Effects of GSP on Blood Pressure

Hypertension is a confirmed risk factor in cardiovascular diseases and one of the mechanisms of hypertension concerns the dysfunction of ACE. GSP can inhibit the activity of ACE *in vitro*. A rabbit experiment showed infusion of GSP (5 mg/kg) lessened responses to Ang I and II, which suggested a therapeutic role of GSP in hypertension (Peng *et al.*, 2005). However, clinic experiments using GSPs to treating individuals with hypertension showed contradictory results, too.

22.1.4.5 Anti-Arrhythmic Effects

GSPs prevented the incidence of reperfusion arrhythmias, and significantly shortened the duration of the episodes of ventricular fibrillation. Simultaneously, the percentage of duration of normal sinus rhythm increased. These results demonstrate antiarrhythmic and cytoprotective effects of oral administration of oligomer procyanidins (Al-Makdessi *et al.*, 2006).

22.1.5 Protective Effects on Diabetes and its Complications

It has been documented that impaired homeostasis in diabetes mellitus is associated with increased production of reactive oxygen species and depletion of antioxidant defense systems. Increased oxidative stress is a widely accepted participant in the development and progression of diabetes and its complications. Application of antioxidants, especially natural antioxidants such as GSPs, to the treatment of diabetes attracts more and more attention.

22.1.5.1 Protective Effect on the Pancreas in Diabetes

In alloxan-induced diabetes rats treated with GSP there is a significant increase in glutathione levels and reduction in lipid peroxidation and total nitrate/nitrite content in pancreatic tissue. The protection effect of GSP on the pancreas contributes to control hyperglycemia (El-Alfy *et al.*, 2005).

22.1.5.2 Protective Effect on the Kidney in Diabetes

According to recent studies, GSP can also protect target organs such as the kidney in diabetes. Diabetic rats induced by alloxan were given GSP intragastrically for 6 weeks, compared with the diabetic group, 24 h urinary protein, levels of blood urea nitrogen and serum creatinine, creatinine clearance rate, and the ratio of kidney weight/body weight were decreased, the SOD activity in kidney rose while MDA content, the NO content, and NOS activity decreased, indicating its nephroprotective effect on diabetic rats (Liu *et al.*, 2006).

22.1.5.3 Protective Effects on Heart in Diabetes

In patients with DM, increased oxidative stress also accelerates the accumulation of AGEs. AGEs are thought to act through receptor independent and dependent mechanisms to promote myocardial damage; fibrosis and inflammation are associated with accelerated diabetic cardiomyopathy. For the cardioprotective effects on streptozocin induced diabetic rats. GSP significantly reduced the plasma AGEs, RAGE, NF- κ B, and transforming growth factor- β 1 (TGF- β 1) gene expression in myocardial tissue of diabetic rats. The structure of the myocardium was improved, too (Cheng *et al.*, 2007). Therefore, GSP can ameliorate glycemia associated cardiac damage.

22.1.5.4 Protective Effects on Hippocampus in Diabetes

Long term chronic hyperglycemia caused the overexpression of AGEs/RAGE and NF- κ B in the CA region of hippocampus in streptozocin (STZ) induced diabetic rats. GSP decreased the expression of RAGE and NF- κ B at a daily oral dosage of 250 mg/kg. This study provides evidence that GSPs can prevent structural changes of the rat brain with diabetes and it suggests that GSP might be a useful remedy in the treatment of diabetic encephalopathy (Xu *et al.*, 2008).

Therefore, these studies may provide some recognition of GSP for the treatment of diabetes and its complications.

22.1.6 Anti-Aging Effects

Aging is the accumulation of diverse deleterious changes in the cells and tissues leading to increased risk of different diseases. Oxidative stress is considered to be a major risk factor and contributes to age related increase in DNA oxidation and DNA protein cross-links in central nervous system during aging. GSP had opposite effects on the accumulation of age-related oxidative DNA damages in spinal cord and in various brain regions such as cerebral cortex, striatum, and hippocampus (Balu *et al.*, 2006). Aging is also characterized by impairment of physicochemical and biological aspects of cellular functions, for example, devastation of normal cell function and membrane integrity. GSPs significantly decrease cell surface charge levels, concomitantly increase protein carbonyls, and decrease glycoprotein as well as antioxidant status in erythrocytes of aged rats. Long term supplementation of GSPs increased erythrocyte surface charge density to near normal levels in aged rats.

22.1.7 Anti-Oncogenesis Effects

Several studies proved that the growth and metastasis of tumor, such as, breast cancer, lung cancer, gastric adenocarcinoma, and epidermoid carcinoma A431 could be inhibited by GSP indicating its anti-oncogenesis effect, which is probably due to the upregulation of tumor suppression genes and/or downregulation of some oncogenes (Singletary and Meline, 2001).

GSPs exhibited cytotoxicity towards some cancer cells, such as MCF-7 human breast cancer cells, A-427 human lung cancer cells, CRL-1739 human gastric adenocarcinoma cells, and K562 chronic myelogenous leukemic cells, while enhancing the growth and viability of normal cell lines, such as normal human gastric mucosal cells and J774A.1 murine macrophage cells. GSPs have been proved to have the ability to upregulate anti-apoptotic gene Bcl-2 and downregulate cell cycle associated and pro-apoptotic genes, such as c-myc and p53 (Joshi *et al.*, 2001).

In vitro treatment of breast cancer cells, 4T1, MCF-7, and MDA-MB-468, with GSP resulted in significant inhibition of cellular proliferation and viability, and induction of apoptosis in 4T1 cells in a time and dose dependent manner. Further analysis indicated an alteration in the ratio of Bax/Bcl-2 proteins in favor of apoptosis, and the knockdown of Bax using Bax siRNA transfection of 4T1 cells resulted in blocking of GSP induced apoptosis. Induction of apoptosis was associated with the release of cytochrome c, increasing expression of Apaf-1 and activation of caspase 3 and poly (ADP-ribose) polymerase. Treatment with the pan-caspase inhibitor (Z-VAD-FMK) resulted in partial but significant inhibition of apoptosis in 4T1 cells suggesting the involvement of both caspase activation dependent and independent pathways in the apoptosis of 4T1 cells induced by GSP. 4T1 cells were implanted subcutaneously in Bal b/c mice. Dietary GSP (0.2 and 0.5%, w/w) significantly inhibited the growth of the implanted 4T1 tumor cells and increased the ratio of Bax: Bcl-2 proteins, cytochrome c release, induction of Apaf-1, and activation of caspase 3 in the tumor micro-environment. These data suggest that GSP possess chemotherapeutic effect against breast cancer including inhibition of metastasis (Mantena *et al.*, 2006).

Cell cycles of cancer cells also get changed when being co-cultured with GSP. *In vitro* treatment of human epidermoid carcinoma A431 cells with GSP inhibited cellular proliferation (13–89%) and induced cell death (1–48%) in a dose and time dependent manner. GSP induced inhibition of cell proliferation was associated with an increase in G1-phase arrest at 24 h, which was mediated through the inhibition of cyclin-dependent kinases (Cdk) Cdk2, Cdk4, Cdk6, and cyclins D1, D2, and E, and simultaneous increase in protein expression of cyclin-dependent kinase inhibitors (Cdki), Cip1/p21, and Kip1/p27, and enhanced binding of Cdki-Cdk. The treatment of A431 cells with GSP (20–80 µg/ml) resulted in a dose-dependent increase in apoptotic cell death (26–58%), which was associated with an increasing protein expression of pro-apoptotic Bax, decreasing expression of anti-apoptotic Bcl-2 and Bcl-xL, loss of mitochondrial membrane potential, and cleavage of caspase-9, caspase-3, and PARP. Pretreatment with the pan-caspase inhibitor (z-VAD-fmk) blocked the GSP induced apoptosis in A431 cells suggesting that GSP induced apoptosis is associated primarily with the caspase-3-dependent pathway (Meeran and Katiyar, 2007). GSP decreased the phosphatidylinositol 3-kinase (PI3K) and the phosphorylation level of Akt at ser473, and the constitutive activation of NF-κB. Treatment with GSP inhibits of the expression of cyclooxygenase 2, iNOS, proliferating cell nuclear antigen, cyclin D1, and matrix metalloproteinase-9 in A431 cells compared with non-GSP treated controls. Treatment of athymic nude mice with GSP by oral gavage (50 or 100 mg/kg body weight) reduces the growth of A431-xenografts in mice, which is associated with the inhibition of tumor cell proliferation in xenografts as indicates the inhibition of mRNA expression of PCNA and cyclin D1, and of NF-κB activity (Meeran and Katiyar, 2008).

In conclusion, GSP is a potential anti-oncogenesis agent, which could be supplement in cancer treatment in the future.

22.1.8 Effect on Wound Healing

Angiogenesis plays a central role in wound healing. The wound site is rich in oxidants, such as hydrogen peroxide, mostly contributed to by neutrophils and macrophages. GSPs influence dermal wound healing *in vivo*. The potential effect of GSP on inducing vascular endothelial growth factor expression is at the transcriptional level. GSP accelerate wound contraction and closure associating with a well-defined hyperproliferative epithelial region, higher cell density, enhances deposition of connective tissue, and improves histological architecture. GSP treatment also increased tenascin expression in the wound edge tissue. Tissue glutathione oxidation and 4-hydroxynonenal immunostaining results supported the idea that GSP application enhanced the oxidizing environment at the wound site (Khanna *et al.*, 2002).

22.1.9 Anti-Osteoporosis

It has been proved that a high-calcium diet combined with GSP supplement is more effective in reversing mandibular condyle bone debility in rats than is low-calcium diet, standard diet, or high-calcium diet alone. GSPs included in a diet mixture with calcium have a beneficial effect on bone formation and bone strength for the treatment of bone debility caused by a low level of calcium (Yahara *et al.*, 2005).

Moreover, GSP treatment can protect brains from reperfusion injuries, too. All these studies show GSP to be a safe, novel, highly potent and bioavailable free radical scavenger, and an antioxidant exhibiting a broad spectrum of pharmacological, therapeutic, and chemoprotective properties. GSPs, functioning at the genetic level and promoting therapeutic efficacy, are potential natural agents in promoting human health.

22.2 Proteomic Platform

Proteomics can be divided into expression proteomics and cell mapping proteomics; expression proteomics focuses on differential display or “discovery” proteomics, global analysis of changes in protein expression during a biological process or in disease, and cell mapping proteomics studies protein interactions, which includes: protein-protein interactions in order to identify components of functional protein complexes, pathway analysis, and the analysis of protein networks; and protein-small molecule interactions such as protein-drug binding.

The extraction, display, and analysis of the individual proteins of tissues and cells together comprise a complex multistage process, involving a variety of biochemical and biophysical principles. This is a rapidly developing field and new or modified techniques continue to emerge. However, two-dimensional (2D) gel electrophoresis, coupled with spot analysis by MS, is still the most widely used technical approach.

22.2.1 Based on Two-Dimensional Gel Electrophoresis (2-DE) Proteomics

22.2.1.1 2-DE

Two-dimensional gel electrophoresis is currently the core technique that can be routinely applied to parallel quantitative expression profiling of large sets of complex protein mixtures, such as whole cell and tissue lists (Gorg *et al.*, 2000). Proteins are separated on the basis of charge in the first dimension and molecular mass in the second. Typically, 1000–3000 proteins per gel can be visualized, for example by staining with silver. Multiple forms of individual proteins can be readily visualized and the particular subset of proteins from the proteome is determined by factors such as initial choice of sample soluble conditions and pH range of the gel strip used for the first dimension.

Analysis of gel with specialized software allows comparisons of multiple gels both within a laboratory and to comprehensive proteome databases on the internet. Proteins of interest can then be identified on the basis of knowledge of the isoelectric point and apparent molecular size determined from the two-dimensional gels, supplemented by a combination of methods, generally applied hierarchically in recent years, and the mass spectrometry technique is now applied widely for high-throughput analysis, which requires smaller amounts of material and has a higher throughput than conventional sequencing methods. Proteins or peptides are ionized by electrospray ionization from liquid state or matrix-assisted laser desorption ionization from solid state and the mass of the ions is measured precisely by various coupled analyzers (Baldwin *et al.*, 2001; Hillenkamp and Karas, 1990). For example, the time-of-flight analyzer measures the time for ions to travel from the source to the detector (MALDI-TOF). If the excised protein spot is first digested by trypsin, which cleaves proteins at specific amino acid (if present), the protein can be broken into a mixture of peptides. The masses of

the peptides can then be measured by mass spectrometry to produce a peptide mass fingerprint. This discriminating signature is compared with the peptide masses predicted from theoretical digestion of protein sequences currently contained within databases, and the protein can be identified. If necessary, the actual sequence can be obtained by tandem mass spectrometry, in which discrete peptide ions can be selected and fragmented and complex algorithms are used to correlate the experimental data with data derived from peptide sequence in protein databases. If the protein or peptide of interest cannot match any known sequence, generally a long enough sequence can be interpreted to design suitable nucleotide-based probes and subsequently to identify new proteins and genes.

22.2.1.2 *Fluorescence 2-D Difference Gel Electrophoresis (DIGE)*

Despite increased reproducibility of the 2-DE process through standardization and automation, alignment of gel images is still an issue. No matter how good the separation, however, some proteins will always conjugate, thereby confounding accurate quantitative analysis. DIGE is a new technology built upon the classical gel approach for quantitative analysis (gel densitometry); it is a difference gel electrophoresis, which separate samples are treated with unique fluorophore tags (binding covalently with lysine ϵ -amino groups); samples are combined and run on the same 2D gel (MW of proteins is negligible); and quantitative analysis is based on relative intensities of fluorescing labels at specific spots (relative quantitation) or to labeled standard (absolute quantitation) (Han *et al.*, 2008). The advantages are obvious: up to three individual samples can be separated on the same 2D gel; Ettan DIGE enables the incorporation of the same internal standard on every 2D gel, thereby negating the problem of gel-to-gel variation; and the Cy dyes afford great sensitivity with detection of 125 pg of a single protein and a linear response in protein concentration over at least five orders of magnitude (105). Meanwhile, the disadvantages are gel based: time, labor, and cost intensive. Also, not all proteins can be run on gels.

22.2.2 “Gel-Free” Proteomics

Based on mass spectrometry technology, “Gel-free” Proteomics can be divided into “shotgun” LC-ESI-MS/MS of total tryptic digest of proteins, and non-gel based quantitative proteomics methods that can be broadly categorized into isotopic and isotope-free methods.

22.2.2.1 *“Shotgun” Proteomics*

2-DE is inadequate for analysis of high molecular weight, hydrophobic, or highly acidic/basic proteins, therefore, so-called “shotgun” proteomics is an emerging concept that has been developed to cope with this problem (Swanson and Washburn, 2005). A protein sample is digested into a large array of small peptide fragments. The protein composition of immunoprecipitates, organelles, cultured cells, and clinical samples has been thoroughly identified by the combination of multidimensional LC and MS/MS.

LC-MS is a proteomic approach coupling of high-performance liquid chromatography and mass spectrometry to separate and to identify proteins or peptides (Elias *et al.*, 2005). In brief, as for sample sources of 1D LC-MS, the sample should be peptide mixture in most time, however, as for 2D LC-MS, the protein or the peptide samples could be loaded onto the first phase of LC, and only peptides are allowed to the second phase of LC; for protein separation if 1D LC-MS the reverse phase column is adopted most of the time, however, as for 2D LC-MS, the first phase column could have multiple choices, ionic exchange resin as priority, and the second phase column is usually reverse phase one; for protein identification ESI-ion trap, ESI-quadrupole TOF or ESI-FT mass spectrometry is often employed for peptide identification, but MALDI-TOF MS is also used for this process; and for annotation: “Sequest” is a major searching engine of proteins adopted by Thermo-Finnigan ESI mass spectrometry, and “MasCot” is a popular searching engine of proteins for number of mass spectrometries.

Electronic spray ionization (ESI) or matrix-assisted laser desorption ionization (MALDI) enables vaporization of non-volatile biological samples from a liquid or a solid-state phase directly into the pseudo-gas phase so that the masses of biological macromolecules could be measured (Vissers *et al.*, 2002).

22.2.2.2 *Quantitation by Stable Isotope Labeling*

Effective and economical methods for quantitative analysis of high throughput mass spectrometry data are essential to meet the goals of directly identifying, characterizing, and quantifying proteins from a particular cell state. Several types of *in vivo* metabolic and *in vitro* chemical and enzymatic isotope labeling methods, including ICAT (isotope-coded affinity tag), SILAC (stable isotope labeling by amino acids in cell culture), and iTRAQ (isobaric tagging for relative and absolute quantitation), have been developed to add a quantitative dimension to MS/MS.

SILAC was developed as a simple and accurate approach for mass spectrometric (MS)-based quantitative proteomics; this method relies on the incorporation of amino acids with substituted stable isotopic nuclei (in this case deuterium 2H, 13C, 15N) (Schmidt *et al.*, 2007). In SILAC, two groups of cells are grown in culture media that are identical except in one respect: the first media contains the “light” and the other a “heavy” form of a particular amino acid. ICAT compares the relative abundance of two tagged proteins, each sample is covalently labeled at cysteine residues with either the heavy tag or the light tag (MW 8Da); cICAT (cleavable isotope-coded affinity tags) then samples are mixed and then digested; the labeled tags are purified by a biotin affinity column (Gygi *et al.*, 1999). iTRAQ uses up to four tag reagents that bind covalently to the N-terminus of the peptide and any Lysine side chains at the anime group (global tagging), each sample set is digested separately and then mixed with the specific iTRAQ tag. In brief, tags were designed to produce fragments in a “quiet” spectral region; the samples are then combined and subjected to LC-MS/MS (Ross *et al.*, 2004).

22.2.2.3 Label-Free Strategies

Recent advances in proteomics approaches developed an integrated platform called label-free quantitative proteomics. This method consists of machinery and software modules that can apply vast amounts of data generated by nanoflow LC-MS to differential protein expression analyses. Label-free methods are based on the relationship between protein abundance and sampling statistics such as peptide count, spectral count, probabilistic peptide identification scores, and sum of peptide Sequest XCorr scores (Σ XCorr).

22.2.3 Protein Chips

This platform includes techniques such as surface-enhanced laser desorption ionization time of flight (SELDI-TOF) (Tang *et al.*, 2004), surface microarray-based systems (protein, tissue, and antibody arrays) (Olle *et al.*, 2005).

22.3 Proteomics Analysis of the Actions of GSPs

Nutritional proteomics or nutriproteomics is not only the application of proteomics methodology to nutrition related research but also the represent of interaction between bioactive food ingredients and the innate proteins, whereby the interaction between bioactive food ingredients and the innate proteins occurs in two basic ways: the influence of bioactive food ingredients on protein synthesis via gene expression; and the interaction between these ingredients and proteins via posttranslational modifications or small-molecule protein interactions (Barnes and Kim, 2004; Schweigert, 2007; Weinreb *et al.*, 2007). The possible target structures, on the cellular and molecular level, as well as the enormous number of bioactive compounds to be tested represent a very heterogenous group of molecular structures.

The method of current nutrition study is to use sophisticated technologies to identify the molecular basis for the activity of various dietary chemicals (Fuchs *et al.*, 2005). Proteomics technologies will benefit a better understanding of the interplay between GSP and diet-related diseases such as cancer, diabetes mellitus, or neurodegenerative diseases. Recently, Kim *et al.* utilized proteomics technology to assess the effects of GSP on proteins in the brains of normal rats that had ingested a defined diet supplemented with 5% GSP over a 6-week period (Deshane *et al.*, 2004; Kim *et al.*, 2006). Moreover, Gao *et al.* studied the mechanisms of action of GSP on kidney in the streptozotocin induced diabetic rats (Li *et al.*, 2008). These studies indicate that the quest to find the interaction between GSP and diseases will cross all scientific disciplines.

22.3.1 Proteomics Analysis of the Actions of GSP in the Brain of Normal Rats

22.3.1.1 Experimental Procedures

Five-week-old female Sprague-Dawley rats were segregated into two groups; one group ($n = 5$) received the AIN-76A (Teklad Industries, Madison, WI) diet supplemented with 5% GSP (Kikkoman Corp., Chiba, Japan) and the second group ($n = 5$) received an unsupplemented AIN-76A diet for 6 weeks (Deshane *et al.*, 2004; Kim, 2005). At the end of the intervention, all rats were sacrificed. Whole brains above the brain stem were dissected out. To perform a differential proteome analysis, the protein samples were extracted in the right hemisphere of brain and separated using 2-DE. The gel spots of interest were identified by MALDI-TOF MS and LC-MS/MS.

22.3.1.2 *Localization Analysis of Differently Expressed Proteins*

The expression of 13 proteins was found to be either up- or downregulated in the brain following ingestion of GSP. The increased proteins were the heat shock protein 60 (HSP 60), heat shock cognate protein 70 (HSC 70), HSC 71, creatine kinase brain β chain (CK-BB), and neurofilament proteins (NF-L and NF-M). Seven proteins were significantly lower in amount in the brains of animals that ingested GSP the ϵ isoform of 14–3–3 protein, glial fibrillary acidic protein (GFAP), actin, vimentin, α and γ subunits of enolase, and polypeptides homologous with the polypeptide sequence predicted by the RIKEN cDNA (NM025994) (Deshane *et al.*, 2004; Kim *et al.*, 2005, 2006). Using the proteomics tool of ExPASy, the subcellular localization of all different expressed proteins consists of cytoplasm, nucleus and mitochondrion. Seventy seven percent of proteins were found to be localized within cytoplasm.

22.3.1.3 *Function Analysis of Differently Expressed Proteins*

Using the dynamically controlled vocabulary of Gene Ontology and a lot of data, the function of all differently expressed proteins consists of response to stress, energy metabolism, cell anchorage, motility, and polarity. Moreover, most proteins are in the cytoskeleton. The cytoskeleton has the functions of anchorage, mobility, information, and polarity.

As chaperones, HSP-60, HSC-70, and HSC-71 have roles in protein folding, assembly, and secretion (Reddy *et al.*, 1999). CK-BB plays an important role in the brain in regenerating ATP from phosphocreatine at discrete cellular sites of high ATP turnover (Friedman and Roberts, 1994). NF-L and NF-M are cytoskeleton components of neurons that are important in brain development as well as in neuronal maintenance (Bajo *et al.*, 2001). These proteins are found at higher levels in the brains of the animals that ingested GSP. Moreover, enolase is the enzyme that converts 2-phosphoglycerate to phosphoenolpyruvate in the glycolytic pathway (Messier and Gagnon, 1996). It was found downregulated in the brain of normal rats with ingestion of GSP.

22.3.2 Proteomics Analysis of the Actions of GSP in Rats with Diabetic Nephropathy

22.3.2.1 *Experimental Procedures*

Ten-week-old male Wistar rats received a single dose of STZ (55 mg/kg, injected into tail veins) freshly dissolved in 0.1 mol/l sodium citrate buffer (pH 4.5) after a 12 h overnight fasting (Li *et al.*, 2008). Only rats with blood glucose higher than 16.7 mmol/l after 5 days were considered as being diabetic in the fasting state.

All rats were divided into three groups: group 1, control rats (C); group 2, untreated diabetic rats (DM); and group 3, treated diabetic rats (GSP, 250 mg/kg body weight/day). GSPs (Lot No: G050412) were provided by Jianfeng Inc (Tianjin, China). GSPs were given in normal saline solution by intragastric administration for 24 weeks. To perform a differential proteome analysis, the left kidney from group C (n = 3), group DM (n = 3) and group GSP (n = 3) were dissected and rinsed thoroughly with ice-cold phosphate-buffered saline to remove blood components. The kidney lysate was labeled with Cy2, Cy3, and Cy5, and separated using 2-DE. The gel spots of interest were identified by MALDI-TOF MS and LTQ-ESI-MS/MS. Line images and 3D images of NADH- biquinone oxidoreductase are shown in Figure 22.1 and Figure 22.2.

2.3.2.2 *Localization Analysis of Differently Expressed Proteins*

In the kidneys of diabetic rats, 25 proteins were found to be significantly changed compared with normal. Nine proteins were found at normal levels among the rats that received GSP treatment. The proteins that were increased in expression were aflatoxin B1 aldehyde reductase (AFAR), phosphotriesterase related protein, and beta actin. Moreover, seven proteins were significantly lower in amount in the kidneys of diabetic rats that were treated by GSP, namely, NADH-ubiquinone oxidoreductase (complex I), glutathione S-transferase mu (GSTM), selenium binding protein 2 (SBP2), F1-ATPase beta subunit, glutamate carboxypeptidase (GCP), and LOC500183 protein (Li *et al.*, 2008). Using the proteomics tool of ExPASy, the subcellular localization of all differently expressed proteins were found to be in the cytoplasm, mitochondrion, nucleus, and cytolemma: 44% of these proteins are localized within cytoplasm and 33% of proteins are localized within mitochondrion.

22.3.2.3 *Function Analysis of Differently Expressed Proteins*

Using the dynamically controlled vocabulary of Gene Ontology and a lot of data, the function of all differently expressed proteins consists of oxidative stress, metabolism, signal transduction, cell proliferation, and apoptosis. Most of proteins are oxidative stress related proteins.

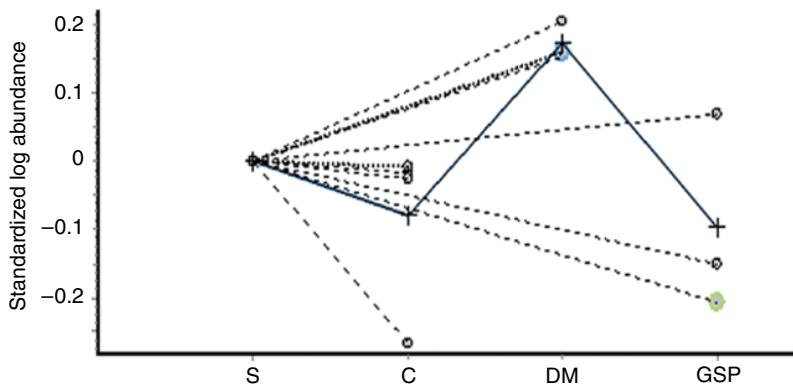


Figure 22.1 Representative line image of NADH-ubiquinone oxidoreductase from C, DM, GSP by proteomics analysis. S: standard; C: untreated control group; DM: untreated diabetic group; GSP: GSP treated diabetic group.

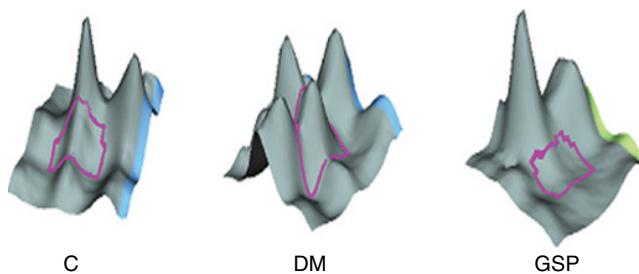


Figure 22.2 Representative 3D images of NADH-ubiquinone oxidoreductase from C, DM, GSP by proteomics analysis. C: untreated control group; DM: untreated diabetic group; GSP: GSP treated diabetic group.

In the present study, complex I was found upregulated in the kidney of diabetic rats and downregulated to normal after GSP therapy. It is well known that increased oxidative stress has been proposed to be implicated in the pathogenesis of diabetes mellitus (DM) and diabetic nephropathy (DN) (Brownlee, 2001). In this way, it was showed that several proteins were involved in the oxidation and reduction changes in DN. Complex I is the point of entry for the major fraction of electrons that traverse the respiratory chain eventually resulting in the reduction of oxygen. Recently, it has been reported that the inhibition of complex I leads to a blockage of more than 95% of activation-induced ROS production (Kaminski *et al.*, 2007). Moreover, complex I is not only the source of mitochondria-derived ROS but also its activity seems to be a prerequisite for subsequent ROS production via the NADPH oxidase. GSPs are a commonly available dietary supplement taken for antioxidant activity that is attributed to its proanthocyanidin (oligomers of monomeric polyphenols) content. It has also been reported that GSPs provide significantly greater protection against ROS and free radical-induced lipid peroxidation and DNA damage than vitamins E, C, and bcarotene, as well as a combination of vitamins E plus C (Bagchi *et al.*, 1997). The results suggested that the inhibition effect of GSP on complex I at least partly contributes to the improvement of DN.

GSTM is a biotransformation enzyme and has functions in the elimination of free radicals, peroxides, electrophilic reagents and heavy metals, the participation of cell protection, and the regulation of cell growth. The expression of GSTM of diabetic rats was significantly higher than those of control rats by Western Blot and 2D DIGE. This might be the mechanism of compensation in diabetes rats. However, it was reported that the presence of the GSTM1 gene was associated with a susceptibility to 1 type DM (Bekris *et al.*, 2005). Therefore, we need do further research on the function of GSTM in diabetes. GCP was found upregulated in the kidney of diabetic rats and back-regulated to normal after GSP therapy. GCP is a membrane peptidase expressed in the prostate, central and peripheral nervous system, kidney, small intestine, and tumor-associated neovasculature. It has been reported that the inhibition of GCP has beneficial effects on hyperalgesia, nerve function, and structural degenerative changes in diabetic polyneuropathy (Zhang *et al.*, 2002).

Moreover, AFAR and beta actin were found downregulated in the kidneys of diabetic rats and back-regulated to normal after GSP therapy. It was reported that AFAR may act as local scavengers of osones and methylglyoxal, and may protect cells and tissues against sugar-mediated damage (O'Connor *et al.*, 1999). The ability of AFAR to reduce the lipid peroxidation product 4-hydroxynonenal suggests that the reductase serves as a protection mechanism against oxidative stress. Previous studies showed that beta actin is a member of the actins family, which maintains the functions of cell structure, intracellular movement, and cell division. Beta actin disassembly is a prominent feature of diabetic nephropathy (Clarkson *et al.*, 2002). It has been suggested that the activation effect of GSP on AFAR at least partly contributed to increasing the activity of scavengers of AGEs in the kidneys tissue. Meanwhile, there was the variation of Glutathione peroxidase 1, SBP2, and phosphotriesterase related protein. The proteins were related to the functions of deintoxication and antioxidant.

22.3.3 Proteomics Analysis of the Actions of GSPB2 in the Aorta of db/db Mice

22.3.3.1 Experimental Procedures

Male C57BLKS/J db/db and db/m mice ($n = 24$, 7 weeks old) were used in this study. C57BLKS/J db/m mice were selected as control group (CC, $n = 8$). The db/db mice were divided into two groups: an untreated diabetic group (DM, $n = 8$) and GSPB2-treated group (30 mg/kg body weight/day, DMT, $n = 8$). At the end of the experiments, all mice were fasted overnight and then sacrificed. The aortas were dissected. Aortic proteins were labeled with iTRAQ reagents (114 for the peptides of group CC, 117 for group DM, and 115 for group DMT), fractionated with Strong Cation Exchange (SCX) chromatography and analyzed on mass spectrometers.

22.3.3.2 Localization Analysis of Differently Expressed Proteins

In total, 557 proteins were shown to have significantly different abundances between the control group and DM group. Of these 557 proteins, the levels of 139 proteins were normalized by GSPB2 treatment. Using AmiGO (Version 1.8), the subcellular localization of the identified proteins in aorta was analyzed. Among these proteins, some are located in one or more subcompartments of the cell: 40% were in cytoplasm, 18.8% in nucleus, 12.1% in plasma membrane, 7.9% in endoplasmic reticulum, 7.3% in mitochondrion, 6.7% extracellular, 6% in centrosome, 3% in ribosome, 2.4% in Golgi, and 1.2% in lysosome.

22.3.3.3 Function Analysis of Differently Expressed Proteins

The differentially expressed proteins that were normalized by GSPB2 were related to many important biological functions including oxidative stress, metabolism, apoptosis, tissue remodeling, and heat shock. In these identified proteins, MFG-E8 was increased 2.4-fold in abundance in db/db mice, which was normalized by GSPB2 treatment. In aorta, MFG-E8 is a protein mainly expressed by adventitial microvessels, medial smooth muscle cells, and luminal endothelial cells (Silvestre *et al.*, 2005). Previous proteomic studies showed that the expression of MFG-E8 in the aorta of type 1 diabetic rats was significantly higher than in control rats and treatment with GSPE significantly inhibited the expression of MFG-E8 in diabetic rats (Li *et al.*, 2010), suggesting that MFG-E8 was involved in oxidative stress and inflammatory process in diabetic aortic disease. MFG-E8 was upregulated with increased oxidation. It has recently been reported that high concentration of glucose upregulated MFG-E8 in the adiposomes, increased ROS production (Aoki *et al.*, 2007); ROS scavenger N-acetyl cysteine decreased MFG-E8 in the adiposomes.

Some proteins contributing to cytoskeletal of the vasculature were found upregulated in the aorta of db/db mice and downregulated in the aorta of GSPB2-treated db/db mice. CSRP1 is expressed primarily in VSMCs and in sensing or responding to pathological vascular stress and maintaining smooth muscle homeostasis (Lilly *et al.*, 2010). It is thought to be critical for smooth muscle differentiation. Our results suggested that the inhibition of CSRP1 partly contributed to the improvement of aortic damage.

Moreover, it is well known that increased oxidative stress has been implicated in the pathogenesis of diabetic vascular complications of type 2 diabetes (King *et al.*, 1996). Our data showed that glutathione S-transferase, theta 1 (GSTT1), an enzyme actively involved in alleviating oxidative stress was downregulated in the db/db mice aorta, which was reversed by GSPB2 treatment. GSTT1 is associated with the regulation of inflammation through modulation of prostaglandin signaling pathways and oxidative stress (Doney *et al.*, 2005). Decreased GSTT1 expression contributes to increased oxidative stress in the db/db mice.

22.3.3.4 Functional Confirmation of Proteins

The global protein changes data in the aorta of db/db mice treatment with GSPB2 were analyzed through the use of Ingenuity Pathways Analysis (Ingenuity Systems, CA). This program uses a knowledge database derived from literature to relate gene products based on their interaction and function. The proteins with their abundance change and corresponding Swiss-Prot accession numbers were imported into the program. Ingenuity software uses the data to navigate the Ingenuity pathways database for interactions between these focus proteins and all the other protein stored in the database to generate biological networks. The aortic IPA analysis shows that the primary pathway involved was cell death, lipid metabolism and small molecule biochemistry. The proteins include Isoform 1 of Apoptosis-inducing factor 2 (AIFM2), Mitochondrial fission 1 protein isoform 2 (FIS1), Isoform 1 of microtubule-actin cross-linking factor 1 (Macf1), Isoform 1 of methylglutaconyl-CoA hydratase (AUH), Platelet isoform of phosphofructokinase (PFKP), and so on. The expression of these proteins was decreased in diabetic aorta, and was normalized by GSPB2. Together with Interleukin 4 (IL-4), Interleukin 5 (IL-5), Interferon- (IFN), Peroxisome proliferator-activated receptor- (PPAR), Leptin (LEP), Heat shock protein-90 (Hsp90), Transforming growth factor- β (TGF β), and other molecules, the differentially expressed proteins normalized by GSPB2 form a network of cell death and lipid metabolism. The pathways would facilitate the understanding of diabetes biomarkers for further study.

22.3.4 Proteomics Analysis of the Actions of GSPB2 in the Kidneys of db/db Mice

22.3.4.1 Experimental Procedures

After adaptation for a week, 7-week-old db/m mice were selected as the control group (CC, n = 8). Sixteen db/db mice were randomly divided into two groups: the vehicle-treated diabetic group (DM, n = 8) administered normal saline solution and the other diabetic group (DMT, n = 8) treated with GSPB2 (30 mg/kg body weight/day) in normal saline solution orally for 10 weeks (Zhang *et al.*, 2013).

Grape seed procyanidin B2 (GSPB2) is a dimeric form of GSP, more powerful than other water-soluble polyphenols in biological activities. GSPB2 (purity >90%, Lot No: 20100915) was provided by Jianfeng Inc (Tianjin, China). To perform a differential proteome analysis, left kidney from group CC (n = 4), group DM (n = 4) and group DMT (n = 4) were dissected and rinsed thoroughly with ice-cold phosphate-buffered saline to remove blood components. Renal proteins were digested, labeled with iTRAQ reagents (114 for the peptides of group CC, 117 for group DM, and 115 for group DMT), fractionated with Strong Cation Exchange (SCX) chromatography, and analyzed on mass spectrometers. The MS/MS spectra were extracted and automatically searched against the non-redundant International Protein Index (IPI) mouse protein database (version 3.72) using the Turbo SEQUEST program in the BioWorksTM 3.1 software suite.

22.3.4.2 Localization and Functional Analysis of Differently Expressed Proteins In the Kidneys of db/db Mice

In total, 2842 proteins were identified to have different abundances between db/db mice and db/m mice. A final set of 113 proteins differentially expressed proteins were identified after GSPB2 treatment, of which, 53 proteins were downregulated and 60 proteins were upregulated in db/db mice after GSPB2 treatment. The differentially expressed proteins were categorized into various location and function classes based on GO analysis according to UniProt protein knowledge database and literature search. In the cellular component of GO analysis, the three highest proportions of differentially expressed proteins were located in nucleus (22%), cytosol (19%), and plasma membranes (14%).

The differently expressed proteins played pivotal roles in a number of cellular pathways, including material metabolism related to glucose/amino acid/lipid/nucleic acid metabolism, apoptosis, oxidative stress, cell cycle and proliferation, signal transduction, and cell adhesion.

22.3.4.3 Protein Pathway Analysis

The ingenuity pathway analysis (IPA) program (www.ingenuity.com) was applied to analyze the pathway of differentially expressed proteins identified by iTRAQ. IPA generated a primary protein network of the differentially expressed proteins, as shown in Figure 22.3. In total, 35 proteins involved in apoptosis, oxidative stress, and metabolism consisted of this network. Among these, 22 proteins were present in our iTRAQ list. In particular, MFG-E8 is directly linked to extracellular signal-regulated kinase 1/2 (ERK1/2) and Akt pathway in this network.

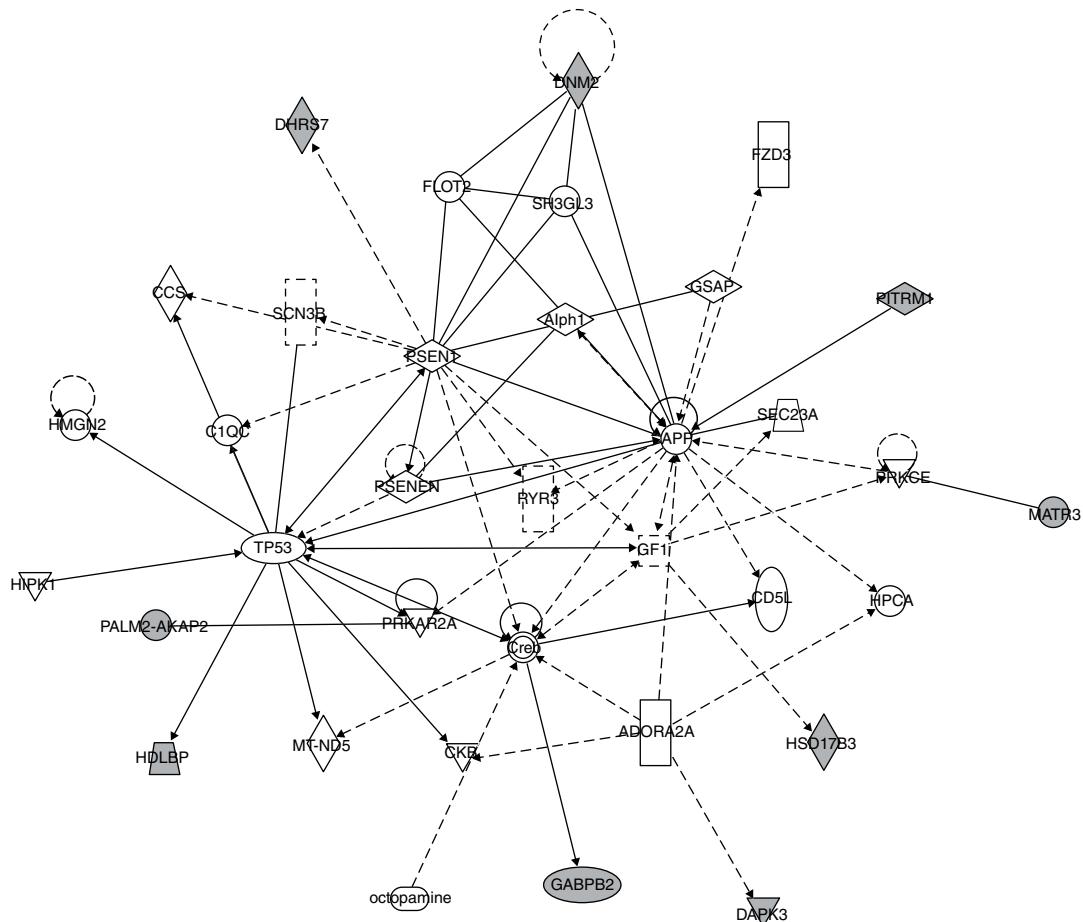


Figure 22.3 Bioinformatic analysis of differentially expressed renal protein after treatment with GSPB2 in *db/db* mice. Interplaying network of proteins with abundance change generated by Ingenuity pathway analysis (IPA).

22.3.4.4 Functional Confirmation of MFG-E8 Protein in Diabetic Nephropathy

A novel molecule MFG-E8 was identified in the kidneys of *db/db* mice, with a 2.23-fold increase in *db/db* mice versus *db/m* mice, and back-regulated by GSPB2 treatment at a ratio of 0.47. MFG-E8 is a membrane associated glycoprotein inducing cell-specific apoptosis and inflammation response, and plays a diverse biorole in various pathophysiologic conditions, including angiogenesis, aging, tissue remodel, and tumor genesis (Aziz *et al.*, 2009; Hanayama *et al.*, 2002; Motegi *et al.*, 2011; Silvestre *et al.*, 2005; Wang *et al.*, 2012). In patients with CKD, elevated serum MFG-E8 level is positively associated with deteriorated renal function (Yin *et al.*, 2010). Knowing down of MFG-E8 gene by transfection of MFG-E8 shRNA improved glomerular hypertrophy, mesangial expansion and thickening basement in *db/db* mice, associating with the decreased phosphorylation of extracellular signal-regulated kinase1/2 (ERK1/2), Akt and glycogen synthase kinase-3beta (GSK-3 β) in kidneys of *db/db* mice. However, exogenous MFG-E8 exacerbated the renal histological lesions with the increased phosphorylation of ERK1/2, Akt and GSK-3 β . GSPB2 treatment significantly decreased protein expression of MFG-E8, inhibiting phosphorylation of ERK1/2, Akt, and GSK-3 β in kidneys of *db/db* mice. These findings indicated that MFG-E8 play a key role in the development of diabetic nephropathy and GSPB2 as a prospective therapy by down-regulation of MFG-E8, along with the ERK1/2, Akt, and GSK-3 β signaling pathways.

22.4 Functional Confirmation of Proteins

The proteins of interest that demonstrated statistic difference could be confirmed with different approaches as an alternative. Consistently, our findings with 2-DE and 2-D DIGE were validated by immunocytochemistry and immunohistochemistry, Western blot analysis, as well as RT-PCR, respectively. In order to further study the functions of differently expressed proteins after treatment with GSP, we will apply the technologies of gene transfection and gene silencing, or gene knockout. Moreover, the interactions between proteins are important for many biological functions. We may study these associations from the perspective of biochemistry, signal transduction, and networks through protein-protein interactions.

22.5 Future Perspectives

We are just entering a new era of postgenomic research and there is no doubt that nutrition science is going to be of central interest, as are nutrients and other food components. A wealth of protein information and novel techniques with high throughput capabilities provide fantastic new tools for nutrition research. As those tools can generate overwhelming data sets there is clearly an emerging need for bioinformatics in nutritional sciences, but also for nutritionists with a good background in cell biology and biochemistry of metabolism. The proteome analysis will be a useful technology with which to solve major nutrition-associated problems in humans and animals (including obesity, diabetes, cardiovascular disease, cancer, aging, and intrauterine fetal retardation).

Moreover, proteomics technologies are powerful tools that will be applied to the dietary GSP in the future to optimize nutritional and health status. This will be accomplished by determining differently expressed proteins and identified molecular targets after treatment with GSP by which “functional” ingredients act to prevent and treat diseases. These methods can improve the velocity and efficiency of drug research, and decrease the cost of new drug research.

Proteomic technologies, including 2-DE, 2-DE DIGE, and MS analyses combined with gene expression determination form a basic research platform for investigating the actions of the GSP. In summary, our recent proteomics analyses and previous studies indicate that GSPs affect mitochondrial function, cell morphology, cell proliferation and apoptosis, oxidative stress, substance metabolism, heat shock, inflammatory pathways, and neuronal survival pathways associated with antioxidant and anti-inflammatory activities. The diverse molecular mechanisms and cell signaling pathways participating in the protection of brain, kidney, retina, and aorta treated with GSP make this multifunctional compound a potential agent for reducing risks of various neurodegenerative diseases and chronic complications of diabetes.

Integrated with other advanced technologies (genomics, transcriptomics, metabolomics, and bioinformatics) and systems biology, proteomics will greatly facilitate the discovery of key proteins that function to regulate metabolic pathways and whose synthesis, degradation, and modifications are affected by GSP. This will aid in rapidly enhancing our knowledge of the complex mechanisms responsible for GSP utilization and designing a contemporary paradigm for dietary prevention and intervention of disease.

Moreover, molecular imaging is an extremely powerful technique that has the potential of analyzing these factors noninvasively, repetitively, and quantitatively in an intact, living whole-body system. In addition, it can be used to track cell survival, monitor endogenous transcriptional regulation, screen transgenic animal phenotypes, study protein-protein interactions, and expedite novel drug discovery. Those who are able to incorporate these technologies in assess the potential of dietary GSP will make meaningful contributions over the next decade.

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23

Genomic and Proteomic Approaches to Lung Transplantation: Identifying Relevant Biomarkers to Improve Surgical Outcome

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23.1 Introduction

In recent years, lung transplantation (LTR) has become increasingly utilized as a therapeutic option in end-stage lung disease (Christie 2005). LTR involves an episode of ischemia-reperfusion (I/R, i.e., stop and restart of blood flow) and the resultant I/R injury (also known as primary graft dysfunction, PGD) has been reported to be directly linked to the outcome of transplant surgery (Christie *et al.* 2005; Diamond *et al.* 2013). Severity of I/R injury correlates with mortality (McGregor *et al.* 1994); indeed PGD (which occurs between 15 and 30% of transplants) accounts for the majority of early mortality after lung transplantation (Christie 2005; Lee *et al.* 2010; Lee and Christie 2011; Suzuki *et al.* 2013). Those who survive PGD have substantial functional impairment 12 months following transplant and increased mortality over the subsequent 4 years (Diamond *et al.* 2013; Kuntz *et al.* 2009; Suzuki *et al.* 2013). In addition, oxidant injury associated with I/R also limits the availability of human lungs for transplant (Punch *et al.* 2007). Transplant patients who develop PGD have an eight-fold higher rate of mortality within 30 days.

With LTR, the lung potentially experiences altered blood flow at several stages (1) the initial ischemic event (removal and storage of the donor lung), (2) the reperfusion during evaluation or reconditioning, (3) a short ischemic episode and (4) reperfusion with transplant into the recipient. These alternating I/R events lead to production of reactive oxygen species (ROS) that cause oxidative damage to tissue. Besides this, ROS also induces inflammation in the transplanted lungs. Immune cells associated with inflammation can attach to endothelial cells and subsequently migrate into lung interstitial spaces and even reach the alveoli. This has an important bearing on lung survival after transplantation.

The pulmonary endothelium and inflammatory cell-specific pathways in lung transplantation have only recently begun to be unraveled and we are beginning to understand the development of the allograft rejection process and the role of ROS and the resultant oxidative damage, either directly or via activation of inflammatory and other pathways

(De Perrot *et al.* 2002; Lafferty *et al.* 2010; Semenza, 2000). Oxidative injury and immunologic rejection are now being considered as the major cause for graft failure; thus it becomes necessary to use our understanding of the signaling factors that underlie rejection to develop relatively noninvasive and reliable means to identify lung donors and transplant recipients who are at risk for rejection. Indeed several studies have shown that patient outcomes vary greatly depending on the specifics of the recipient's pathophysiology and also the donor allograft quality (Christie *et al.* 2005). Biomarkers are cellular, biochemical, molecular (genetic and epigenetic) alterations by which a normal or abnormal process can be recognized or monitored. In this regard, many investigators have attempted to identify diagnostic and predictive biomarkers of acute rejection and chronic rejection. Toward this end, we review the relevant biomarkers associated with lung transplantation. Identification of biomarkers will aid the understanding of underlying mechanisms by indicating damage early on post-transplantation.

23.2 Lung Transplantation

23.2.1 A Case of Ischemia-Reperfusion (I/R)

I/R or stop of blood flow followed by its reinstatement implies lack of oxygen supply followed by its restoration. This process of anoxia-reoxygenation leads to production of ROS in systemic organs. Lung I/R differs from I/R in the systemic organs because oxygen supply to the lung tissue can be maintained with stop and restart of blood flow, as lung alveolar cells also obtain oxygen by gas diffusion from alveolar space. Recognizing the role of anoxia-hypoxia in oxidant production, donor lungs in clinical practice are now ventilated during storage. However, ventilation strategies have not been effective in preventing lung injury (Davis and Pasque 1995; D'Armini *et al.* 1996).

This is because “sensing” of alterations in shear by the pulmonary microvascular endothelium triggers signaling that leads to overproduction of ROS and nitric oxide (NO) that can cause oxidative injury and activation of proteins that participate in processes that drive inflammation and cell death. Past studies from the lab of our mentor have shown that pulmonary endothelium produces ROS with ischemia alone and this is further increased upon reperfusion (Eckenhoff *et al.* 1992; Fisher *et al.* 1994).

Thus, during lung I/R, the pulmonary endothelium senses altered blood (both during lung storage and later surgical attachment into recipient) and initiates a pro-inflammatory cascade leading to oxidative stress; activating pro-inflammatory transcription factors; upregulating pro-inflammatory genes, cytokines, and chemokines; recruiting leukocytes; and losing vascular barrier integrity (Browning *et al.* 2012; De Perrot *et al.* 2002; Lee *et al.* 2013, 2014).

23.2.2 The I/R Signaling Cascade

Based on our past studies, we postulated that the alteration of the mechanical component of pulmonary blood flow triggers an endothelial signaling that leads to ROS production and subsequent lung injury (Chatterjee *et al.* 2006, 2008, 2012; Fisher *et al.* 1991, 1994; Eckenhoff *et al.* 1992). We unraveled the cascade to show that interruption of blood flow caused K_{ATP} channel induced NADPH oxidase 2 (NOX2) activation and ROS generation (Chatterjee *et al.* 2006, 2008, 2012; Chatterjee and Fisher 2014). NADPH oxidase 2 comprises of membrane components (gp91phox and p22phox) and cytosolic components (Rac, p47phox, and p67phox) that, upon assembly on the membrane, catalyze the 1-electron reduction of oxygen into superoxide (Griendling 2000).

The ROS produced by endothelial cells plays a role in the lung response to inflammation. In general inflammation derives from the latin root “inflammo”, meaning “I ignite” is an adaptive response that is prompted by harmful stimuli and conditions, such as infection and tissue injury. Although controlled inflammatory response is beneficial (e.g., providing protection against most pathogens), an excessive response such as septic shock becomes dysfunctional (e.g., septic shock) (Deutschman and Tracey 2014). In the context of lung transplant, the episode of I/R often serves as a trigger for the onset of inflammation.

Endothelial ROS-dependent signals with I/R in the donor lung, facilitate binding and adhesion of polymorphonuclear neutrophils (PMN) of the recipient's circulation into the vascular wall (of the transplanted lungs). Although the exact mechanism by which recruitment and adherence of PMN is unclear, it appears that endothelial ROS regulates the expression of those cell adhesion molecules (CAMs) that control PMN binding to endothelial cell membranes (Gandhirajan *et al.* 2013; Menden *et al.* 2013). The main CAMs found on the surfaces of endothelial cells in injured tissue are the intercellular adhesion molecule (ICAM/CD54) and the vascular cell adhesion molecule (VCAM/CD106) (Granger and Kubes 1994; Jordan *et al.* 1999). The PMN-endothelial cell binding is followed by extravasation into tissue and release of ROS. Lack of CAMs

is reported to prevent PMN-endothelial binding and extravasation into tissue (Basit *et al.* 2006; Yamazaki *et al.* 2012). Thus, the oxidative damage with I/R (or PGD) during transplant is primarily the result of signaling that leads to inflammation as PMN from the recipient's circulation are recruited and adhere to the donor lung (Fiser *et al.* 2002; Granton 2006; Laubach and Kron 2009). Indeed, work from the lab of one of our collaborators shows that ICAM concentrations in circulation are higher for patients with PGD versus no PGD (Covarrubias *et al.* 2007; Shah *et al.* 2012, 2014) (ICAM expressed by lung endothelium is preceded by its shedding into blood and succeeded by its physical association to VCAM: van Buul *et al.* 2010). Besides, CAMs, other binding proteins contributing to PMN sequestration after I/R are the leukocyte adhesion molecule CD18 and P-selectin.

Several inflammation markers associated with lung transplant rejection have been identified; these are IFN- γ (Iacono *et al.* 1997; Moudgil *et al.* 1999; Ross *et al.* 1999), interleukins (IL)-6 IL-4, IL-1, IL-15, and IL-1 β (Magnan *et al.* 1994, 1996; Ross *et al.* 1999; Whitehead *et al.* 1993), tumor necrosis factor-alpha (TNF- α), and monocyte chemoattractant protein (MCP-1). These chemokines and cytokines (signaling proteins secreted by cells that induce chemotaxis or other cellular events) are released either by alveolar macrophages or monocyte like cells that reside in the alveolus of the donor lung or by neutrophils/lymphocytes or endothelium of the recipient. Depletion of alveolar macrophages (by clodronate) or neutrophils significantly reduced pulmonary injury (as assessed by reduced edema and microvascular leak) as well as TNF- α and chemokines (CCL2 and CXCL2) production, indicating that macrophages and neutrophils are critical to the initiation of lung injury with I/R (Zhao 2006). This provides rationale for focus on adhesion molecules, chemokines, and cytokines in assessing the outcome of lung transplant.

23.3 Challenges of Lung Transplantation

Post lung transplant, rejection of the lung results from two distinct but linked, processes: acute rejection, which is an immune response directed against target cells in the transplanted organ, followed by chronic rejection, in which a proliferative response that causes structural changes that impaired lung function. Acute rejection occurs in 35–50% of lung transplant recipients during the first post-transplant year. It is characterized by lymphocytes and mononuclear infiltrates and arises from T-cell mediated immune response by CD4+ and CD8+ T-lymphocytes. Acute lung rejection, although treatable by immunosuppressive therapy has been identified as the strongest known risk factor for chronic rejection (Bando *et al.* 1995; Husain *et al.* 1999; Kroshus *et al.* 1997). Chronic rejection is accompanied by lesions characteristic of severe inflammation. The histology appearance is that of fibrosis of small airways. Chronic rejection is in the form of bronchiolitis obliterans syndrome (BOS) (Khalifah *et al.* 2005) that blocks the airways. Bronchoalveolar lavage (BAL) fluid and cell analyses point to onset of an inflammatory process followed by fibroproliferation in the bronchiolar walls and lumens (Estenne and Hertz 2002; Neuringer *et al.* 2005).

23.3.1 Oxidative Damage and Bronchiolitis Obliterans Syndrome

As mentioned earlier, oxidative injury leads to a cascade of events that activate the recipient inflammatory system against the already damaged vascular endothelium and airway epithelium. Examination of samples of chronic graft rejection shows lesions of lymphocytic inflammation and disruption of the epithelium and ingrowth of fibromyxoid granulation tissue into the airway lumen, resulting in obstruction. This obliterates the lumen of the airways (bronchiolitis obliterans syndrome, or BOS) and is the predominant feature of chronic lung transplant rejection. Although the mechanism by which I/R injury drives BOS is unclear, there is a significant correlation between excessive oxidative stress markers and a lack of glutathione in patients after transplant and BOS (Behr *et al.* 2000; Madill *et al.* 2009; Rahman and MacNee 2000).

23.3.2 Oxidative Damage and Inflammation

The major challenge in lung transplantation arises from oxidative injury, immunologic rejection, and inflammation. Inflammatory mediators such as CAMs, cytokines and chemokines, and induction of matrix metalloproteases (MMPs) is also associated with rejection (Sekine *et al.* 2000; Yano *et al.* 2001; Yasufuku *et al.* 2001). As mentioned earlier, some of these give rise to fibroproliferative responses that cause impaired lung function.

The presence of inflammatory markers and cells in the BAL of patients with chronic rejection has given rise to the demand for biomarkers that can predict or recognize the onset of some of the changes that lead to eventual transplant rejection.

23.4 Inflammatory Biomarkers with Lung Rejection: Markers of Inflammation Signaling such as CAMs, Chemokines, and Cytokines and their Status with Transplants

Biomarkers are cellular, biochemical, molecular (genetic and epigenetic) alterations by which a normal or abnormal process can be recognized or monitored. Due to the difference between acute and chronic allograft rejection (the former is an earlier process that is the most immune driven response, while the latter is a complex multistep cascade where slow changes develop that compromise function) the biomarkers that are monitored for the onset of these, are quite diverse.

Pre-transplant biomarkers are geared toward checking the immune response of both donor and recipient. In general, it has been reported that tolerant recipients showed an expansion of peripheral blood B and natural killer lymphocytes, fewer activated CD4+ T-cells, a lack of donor-specific antibodies. Currently, a match between the human leukocyte antigen (HLA) in the sera of the donor and the recipient is the best pre-transplant biomarker (Terasaki *et al.* 2006).

Post-transplant biomarkers, detected from BAL and cells of transplant recipients, correlate with BOS; indeed several markers of fibroproliferation like platelet-derived growth factor (PDGF), transforming growth factor (TGF) insulin-like growth factor-1, and IL-12 show a significant increase in patients with BOS (Charpin *et al.* 1998, 2000; Hertz *et al.* 1992; Meloni *et al.* 2004).

However, most of these biomarkers require biopsies that are invasive leading to approximately 10–30% of all transplant patients being diagnosed and treated for rejection after the first year post transplantation (Hertz *et al.* 2009) while a large percentage of clinical episodes remain undetected.

Thus non-invasive methods for detection of biomarkers, such as those in blood and exhaled breath, are now gaining increasing importance. Biomarkers in exhaled breath of transplant recipients include markers of oxidative events and sulfur-containing compounds. Patients with undetectable amounts of carbonyl sulfide (COS) were reported to be at low risk for rejection (Studer *et al.* 2001). High levels of nitric oxide (NO) in exhaled breath serve as an indicator for onset of rejection.

Biomarkers from donor and recipient blood can be measured from peripheral blood DNA prior to transplantation; studies have identified single nucleotide polymorphisms (SNPs) that indicate the risk of graft rejection at the pre-transplantation stage. Studies have shown that mutations in immune system proteins like the Toll-like receptor (TLR) and in complement factor C3, both of which control immune response in donor and/or recipient blood have been associated with reduced risk of rejection (Dhillon *et al.* 2010; Ducloux *et al.* 2005; Naesens *et al.* 2009).

23.4.1 Proinflammatory Cytokines and Chemokines

Gene expression patterns of several genes, rather than of only one gene, showed a correlation between the following genes: perforine, TNF α , Fas ligand and the serine protease granzyme B (Choy, 2010; Vasconcellos *et al.* 1998; Strom and Suthanthiran 2000) and the risk of rejection (in renal transplant).

Work elsewhere has shown that the cytokine levels in the first 24 h immediately following transplantation correlates with the severity of PGD following transplantation (Mathur *et al.* 2006). Others have also shown that elevation in IL-6, 8, and 10 following transplantation (De Perrot *et al.* 2002; Mal *et al.* 1998; Pham *et al.* 1992) correlate with rejection. Markers of fibroproliferation have also been found in BAL cells of lung recipients with bronchiolitis obliterans syndrome. The chemokines CXCL9 and CXCL10 and the chemokine receptor CXCR3 have been identified as potential biomarkers to predict acute rejection; all these can be assessed in transplant patient serum, peripheral blood, urine, and bronchoalveolar fluid.

23.4.2 Cellular Adhesion Molecules

ICAM-1 and leukocyte CD11/CD18 integrins (including CD11a, CD11b, and CD11c) were reported to be significantly increased during episodes of rejection in patients after lung transplantation as compared to those without complication. Soluble ICAM-1 was quantified in serum and BAL while CD11/CD18 was detected in macrophages by immunocytochemistry (Bewig *et al.* 1999).

23.5 Microarray Technology to Identify Transplant Rejection Biomarkers

Global gene expression profiling has been used to identify expression patterns in organ transplant biopsies and predict outcomes post-transplant such as acute rejection, acute dysfunction without rejection, and functioning transplants (Gimino *et al.* 2003). Microarray analysis of BAL cells could identify genes and gene expression patterns indicative of acute rejection

via single-nucleotide polymorphisms (SNPs), epigenetic studies and analyses of mRNA, and microRNA profiling. Studies 364 genes were differentially expressed between lung transplant patients and normal controls showed an array of upregulated (72 genes) and downregulated (292) genes. The downregulated genes were associated with pathways that participate in blood coagulation and homeostasis while the upregulated genes were associated with biological processes that dealt with immune (such as CCR4, encoding chemokine receptor 4) and inflammatory response [(IL8, IL1 β , and CD86 lipopolysaccharide (LPS) CD86) (encoding CD86 molecule)], were downregulated in patients in response to wounding and bacteria (Zhang, 2013).

Thus, microarray technology is being used by organ transplant researchers to identify specific patterns of gene expression that predict and characterize acute and chronic rejection.

23.6 Challenges and Future Directions

Current screening for lung donor suitability is based on a number of empirical criteria, such as donor age, smoking history, arterial blood gas concentrations, chest radiograph findings, bronchoscopy findings, and physical examination of the lung at the time of organ retrieval.

However, genetic predisposition to inflammation, exaggerated immune response, and oxidative stress response to I/R all play a role in determining transplant outcome. Gene expression profiling of samples (BAL, cells, and biopsy) from transplant recipients, with and without rejection, provides a somewhat comprehensive information on the expression of proteins that are associated with process that promote the onset of rejection.

Important future directions for this research involve more extensive studies using larger cohort of patients and patient samples. Microarrays of cells (lung or BAL) that describe the chain of events between donor and recipient genotypes, need to be combined with information on lung and “onset of rejection” phenotypes. Finally, although biomarkers of acute rejection have been identified, patterns of gene expression that predict onset of inflammation and oxidative stress pathways need to be discovered.

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Proteomics in Understanding the Molecular Basis of Phytochemicals for Health

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24.1 Introduction

Functional food compounds are dietary constituents that provide health benefits or medicinal effects beyond their nutritional value. Accumulating epidemiological evidence suggests a link between a diet rich in fruits and vegetables and a reduced risk of chronic diseases such as cancer, cardiovascular disease, and obesity (Boeing *et al.* 2012; Liu 2013). Plant-based natural dietary compounds, known as phytochemicals, have been receiving increasing attention as key molecules responsible for the health benefits of fruits and vegetables (Gonzalez-Vallinas *et al.* 2013). Phytochemicals are comprised of a variety of natural chemical compounds present in plants. Among these, the most studied major group is the polyphenols, which include flavonoids, phenolic acids, and stilbenes. Multiple lines of evidence indicate that phytochemicals improve health and exert protective effects against the development of certain types of disease including cancer, heart disease, and neurodegenerative diseases by exerting antioxidant, anti-inflammatory, and various molecular regulatory functions (Lee *et al.* 2013; Rasool *et al.* 2014). In an effort to understand the actions of phytochemicals for health and their potential to be utilized as novel medicines, vigorous research efforts have focused on uncovering their mode of action, and their molecular targets and subsequent mechanisms regulated are gradually being discovered (Bode and Dong 2013; Chung *et al.* 2013). Recent lines of evidence provide insight into the molecular basis of phytochemical functions, which act by regulating cellular signaling pathways by directly targeting key signal transduction molecules (Lee *et al.* 2011).

As phytochemicals are becoming increasingly recognized for their molecular regulatory effects, efforts to identify the molecular targets responsible have been growing with the development of new technical tools to aid this process. Recent omics-based approaches including genomics, proteomics, and metabolomics are being rapidly developed and utilized in phytochemical research and are fueling the identification of new molecular targets responsible for phytochemical-dependent health (Ovesna *et al.* 2008; Sales *et al.* 2014).

The large scale analysis of changes in the genome and proteomes in the presence of phytochemicals allows for the discovery of interactions between phytochemicals and the genome or proteome by detecting alterations in the expression of specific

genes or proteins, providing evidence for the direct molecular target of the phytochemical in question. Combined with data concerning a phytochemical's effects and function, this analytical approach provides key insights for crucial biomarkers in health and disease. Metabolomics provides valuable tools to study a broad range of phytochemical metabolites generated after consumption, which will further aid in uncovering the wider physiological impact of phytochemicals and to assess their value as potential nutraceuticals and functional foods.

Proteomics can be considered as more complex than genomics because proteome changes are extensively dynamic due to post-translational modifications and protein-protein interactions. Genomics approaches have been in focus for the study of the effects of phytochemicals on numerous health conditions (Shankar *et al.* 2013). Recently, an increasing number of studies have been utilizing proteomics to study phytochemical action and the resultant effects (Joo *et al.* 2010; Madden *et al.* 2009). With recent investigations showing a direct interaction between phytochemicals and cellular signaling pathway proteins, the need for complete analysis from a proteomic perspective has been increasing (Lee *et al.* 2011). In this chapter, we focus on the advance of proteomics and its implication for phytochemical research for health and chronic diseases, including cancer, metabolic disease, and neurodegenerative diseases.

24.2 Proteomics in Phytochemical Research in Cancer Prevention

Cancer is a growing health problem around the world, and is one of the major causes of deaths in many countries. Cancer being a disease generally requiring a long time to develop and progress, dietary factors can play a major role in promotion as well as the progression phase of cancer. Currently, many researchers are focusing on identifying dietary components that have preventive and therapeutic effects on the development of cancer, and examining how and why these dietary factors are effective in cancer prevention. Also, numerous reports have been published regarding natural and synthetic chemicals, which show chemopreventive activity by inhibiting certain proteins and subsequently disrupting carcinogenic cell signaling (Kang *et al.* 2011).

Cancer is linked to disruption of cell signaling and abnormal regulation of protein expression. For many years people have thought that phytochemicals in food possess anticancer activity mainly because of their antioxidant activity. However, recent research suggests that phytochemicals can act like small molecule inhibitory drugs similar to Gleevec in leukemia and Iressa and Tarceva in lung cancer. Compounds that are capable of specifically targeting certain enzymatic activities or protein expression could act like a drug to prevent cancer-related cell signaling (Lee *et al.* 2011). Proteomics can aid in holistically understanding how the genome is differentially expressed by consumption of food components. Hence, utilization of proteomics in phytochemical research and cancer can not only lead to identifying the mechanism of phytochemicals but also contribute to understanding the relationship between food components and health outcomes. In the current chapter we will focus on reports using proteomics technology to analyze the mechanism of phytochemicals.

24.2.1 Genistein

Multiple lines of studies have proved that soy isoflavones possess strong chemopreventive effects against multiple cancer types including breast, ovary, prostate, colon, and gastric cancers (El Touny and Banerjee 2009; Nagaraju *et al.* 2013; Yan *et al.* 2012). Especially, genistein, the primary isoflavone component of soy beans, have been shown to induce cell cycle arrest and apoptosis *in vitro* and *in vivo* (Li *et al.* 2012). Several studies utilized proteomics approaches to assess the overall pattern of change in protein levels to understand the molecular mechanism of genistein. Mammary glands of rats injected with genistein were subjected to two-dimensional gel electrophoresis (2-DE) to identify alterations in protein levels by genistein treatment. The study concluded that genistein caused upregulation of GTP-cyclohydrolase1, subsequently leading to increased tyrosine hydroxylase and downregulation of vascular endothelial growth factor receptor 2 (VEGFR2) rendering the mature mammary gland less proliferative and less susceptible to cancer (Rowell *et al.* 2005). In addition, another study investigating the mammary gland after administration of genistein found a decrease in Annexin A2 from proteome analysis as well as reduction in VEGFR2 (Betancourt *et al.* 2012). These studies suggest that suppressed expression of VEGFR2 and Annexin A2 in genistein treated groups might explain the preventive effects in mammary carcinogenesis. However, it is of noteworthy that neither of the studies found VEGFR2 expression alteration directly from the proteome analysis. Additional experiments based on the proteome results led to subsequent examination of VEGFR2 levels and found reduction of VEGFR2 in genistein exposed animals. In a separate study, stable isotope labeling by amino acids in cell culture (SILAC)-based proteomics was used to examine the estrogen receptor α (ER α) and β (ER β)-mediated effects on protein expression in T47D breast cancer cells treated with genistein. Since, genistein was known to bind to ER, this study

investigated the consequences of the ER α /ER β ratio for the effects induced by genistein. Through comparing the proteomic profiles in different ER α and ER β expression status, the study shows that the effect of genistein is dependent on receptor phenotype (ER α /ER β ratio) in the cells to exert its estrogenic effects (Sotoca *et al.* 2011). This study shows that comparison of the overall proteome in different conditions of a specific protein can lead to a more comprehensive understanding of how the phytochemical works in cells.

In a gastric cancer model, using SILAC-based quantitative proteomics, a study demonstrated that genistein induced simultaneous downregulation of five kinesin family proteins including KIF11, KIF20A, KIF22, KIF23, and CENPF. And they additionally prove that knockdown of KIF20A inhibited cell viability and induced G2/M arrest, whereas overexpression of KIF20A significantly attenuated genistein-induced cell viability inhibition and G2/M arrest (Yan *et al.* 2012). This particular study exhibits the benefit of proteomics to identify the “functional target” of phytochemicals. Analysis of the proteome provides crucial information in altered protein levels of which might contain key factors for the compound to exert its bioactivity, thus aiding in identifying functional targets of the molecule. Using a gastric cancer cell SGC-7901, a study attempted to identify the tyrosine-phosphorylated proteins and sites regulated by genistein by high-resolution MS in combination with tyrosine-phosphoprotein immunoaffinity enrichment and SILAC technologies. This led to identification of a number of kinases that was affected by genistein including, EGFR, PDGFR, insulin receptor, Abl, Fgr, Itk, Fyn, and Src, providing a clue of how a genistein might regulate the carcinogenic signaling pathway (Yan *et al.* 2010).

24.2.2 Curcumin

Curcumin is a diarylheptanoid found in turmeric well known for its anticancer effect in a variety of cancer types (Johnson and Mukhtar 2007; LoTempio *et al.* 2005; Lu *et al.* 1994). Analysis of 2-D electrophoresis in LOVO colorectal cancer cell protein extract showed 54 proteins to be differentially expressed after curcumin treatment. The study found that curcumin reduced proteins involved in the removal of reactive oxygen species (e.g., GSTM5, PRDX4) as well as altered the expression of proteins involved in formation and destruction of disulfide bonds and suggested these changes as the primary reason for inducing apoptosis in LOVO cells (Zhu *et al.* 2014). In another study, the proteome was analyzed in HeLa cervical cancer cells after curcumin treatment and found several proteins associated with cancer initiation and progression which seemed to be responsible for its anticancer effects (Madden *et al.* 2009). However, though both studies used similar methods to separate proteins, the list of altered proteins in HeLa cells did not overlap with the list of proteins changed in the LOVO cells after curcumin administration, suggesting that the overall proteomic results can vary significantly depending on the cell type and condition. A shotgun proteomic approach to find differentially expressed proteins in response to curcumin treatment in neuroblastoma cell line reported that curcumin reduced expression of proteins involved in biosynthesis and glycolytic activity and caused accumulation of polyubiquitinated proteins in association to cell cycle arrest (D’Aguanno *et al.* 2012). Based on this result and observation of increased amount of polyubiquitinated proteins after curcumin exposure, they proposed that inhibition of the ubiquitin–proteasome system may be the primary mechanism of action of which curcumin exerts its multi-target activity.

24.2.3 Sulforaphane and β -Phenylethyl Isothiocyanate

Various reports showed that isothiocyanates are potent chemopreventive and chemotherapeutic agents and sulforaphane (SFN) and β -phenylethyl isothiocyanate (PEITC) are among the most extensively studied (Brown and Hampton 2011; Holst and Williamson 2004). Several studies have used proteomics approaches to understand the mechanism and target of SFN and PEITC. SFN exerted cytotoxicity in LNCaP cells and proteomics study revealed that levels of nine proteins including tubulin, phosphoglucomutase-3 (PGM3), and activin A type I receptor precursor were changed in SFN treated LNCaP cells compared to untreated control. In this study, they focus on PGM3 and suggest that PGM3 plays a critical role in SFN-induced cell death. Other studies utilized radiolabeling on SFN and PEITC to identify target proteins (Mi and Chung 2008; Mi *et al.* 2011). A549 non-small cell lung cancer cells were treated with ^{14}C -PEITC and ^{14}C -SFN, and the cell lysates were subjected to 2D gel electrophoresis and mass spectrometry. Examination of cellular proteins containing radioactivity, resulting from selective binding with PEITC or SFN led to identification of multiple binding proteins. These studies demonstrate an advanced method to identify direct targets of small molecules using proteomics which is a critical step in understanding the mechanism of phytochemicals. Using this method the study suggests several group of proteins (e.g., cytoskeleton proteins, redox regulating proteins, heat shock proteins, and mitochondrial proteins) that might be responsible for the chemopreventive effects of PEITC and SFN (Mi and Chung 2008; Mi *et al.* 2011). In another study, 2D gel electrophoresis was used in human hepatoma

HepG2 cell line after PEITC treatment for 3 and 6 h to assess the proteomic alteration. Based on protein expression pattern modification, they suggested PEITC-induced apoptosis is connect with PEITC's effect on heterogeneous nuclear ribonucleoprotein K, which was also reported to be a direct target of PEITC in the data found in A549 cell line (Mi *et al.* 2011; Neo *et al.* 2005). The separate results from these studies are interesting in that the binding protein of PEITC is also found as a protein to be reduced in PEITC treated cells. It is likely that sometimes binding of a compound to a protein may affect the stability, which in turn might cause a change in expression and hence can be a concept to be used in certain situations to understand the mode of action of a phytochemical.

24.2.4 Apigenin 7-Glucoside

Apigenin is a flavone that is abundant in various fruits and vegetables with anti-inflammatory, antioxidant and anti-carcinogenic properties (Byun *et al.* 2013; Shukla and Gupta 2010; Tong and Pelling 2013). Apigenin is more commonly present as a glucoside conjugated form in foods, apigenin 7-glucoside, which is more stable. Analysis of 2D gel electrophoresis with leukemia K562 cells treated with apigenin 7-glucoside showed several proteins affected, including ran-binding protein 1, elongation factor Tu, 14–3–3 ζ/δ , and multifunctional protein ADE2 (Tsolmon *et al.* 2011). In a different study examining the proteomic alterations that may be important in HL-60 cell differentiation, Nakazaki and colleagues found a number of proteins to be affected including elongation factor 1-beta, 14–3–3 α/β , ϵ , γ , and proliferating cell nuclear antigen (Nakazaki *et al.* 2013). It is interesting to point out that both studies found similar proteins such as elongation factor and isotypes of 14–3–3. Comparing the common hits from different conditions in proteomic analysis might help to narrow down to a more accurate target or alteration that is responsible for a phytochemical's effect.

24.2.5 Quercetin

Quercetin is a naturally occurring dietary flavonol that has been recognized in many studies to possess cancer chemopreventive as well as chemotherapeutic activities (Lee *et al.* 2008; Russo *et al.* 2014; Vijayababu *et al.* 2005). A study utilized 2D gel electrophoresis and mass spectrometry to identify proteins altered in expression level after quercetin exposure to colon cancer cells. The study found various proteins with altered expression associated with the regulation of heat-shock proteins and annexins as well as cytoskeletal caspase substrates (Wenzel *et al.* 2004). While this study focused on consequential protein expression changes made by quercetin, a recent study sought to find the direct target of quercetin using immobilized quercetin beads and proteomics. By employing affinity chromatography and mass spectrometry, the study identifies that quercetin directly targets heterogeneous nuclear ribonucleoprotein (hnRNP) A1 (Ko *et al.* 2014). This study shows that target identification can be conducted by conjugating the phytochemical to a bead and using proteomics.

24.3 Perspectives

A notable finding from various proteomic studies that analyzed expression profile or targets of phytochemicals is that most of the results often share a list of several common of proteins. As shown in Table 24.1, several proteins show up multiple times as hits in different studies, even though these results are generated using different compounds, cell lines or tissues, incubation time, and method of analysis. There could be various reasons for these phenomena. First, there could be common target protein(s) that are susceptible to be influenced by phytochemicals leading to such outcomes. Second, since most of the studies were performed in order to investigate the anticancer activity of a compound, there might be certain group of proteins that are more vulnerable in cancer cell survival and their stability or expression could change more easily when exposed to compounds with anticancer effects. However, considering the differences in structure, chemical properties, and concentration of phytochemicals analyzed, these could be partially responsible but, it is unlikely that this would be the explanation for all of the occasions. It is estimated that the whole proteomes are composed of 1.5×10^6 proteins (Melton 2004). Given the number of proteins in a cell it could be reasonable to be able to detect more variety of proteins with less common hits. In some cases it could be the case that certain proteins are more often detected compared to other proteins due to higher expression leading to easier detection or their biochemical properties are more suitable to survive the experimental conditions causing more common detection. The current methods used to analyze the proteome for expression profile or target identification generally relies on 2D gel separation or SILAC. In the future, there could be enhanced methods to increase the accuracy to overcome such problems.

Table 24.1 Commonly detected proteins from various proteomic analysis results.

Identified protein	Phytochemical	Analyzed cell or tissue	Reference
Peroxiredoxins	Genistein, green tea, piperlongumine, curcumin, lycopene	Mammary gland, HPAF-II, U2OS, EJ, LOVO, SH-SY5Y, LNCaP	Betancourt <i>et al.</i> 2012; Rowell <i>et al.</i> 2005; Zhang <i>et al.</i> 2011; Raj <i>et al.</i> 2011, Zhu <i>et al.</i> 2014; D'Aguanno <i>et al.</i> 2012; Goo <i>et al.</i> 2007
Annexins	Apigenin 7-glucoside, genistein, quercetin, piperlongumine, curcumin, 6-shogaol, lycopene	HL-60, T47D, HT-29, U2OS, EJ, LOVO, HeLa, LNCaP	Nakazaki <i>et al.</i> 2013; Sotoca <i>et al.</i> 2011; Wenzel <i>et al.</i> 2004; Raj <i>et al.</i> 2011, Zhu <i>et al.</i> 2014; Liu <i>et al.</i> 2012; Goo <i>et al.</i> 2007
Heat shock protein 70	Dibenzoylmethane, pomegranate juice, quercetin, curcumin, PEITC, SFN	LNCaP, DU145, HT-29, SH-SY5Y, A549,	Frazier <i>et al.</i> 2004, Lee <i>et al.</i> 2012; Wenzel <i>et al.</i> 2004; D'Aguanno <i>et al.</i> 2012; Mi <i>et al.</i> 2011
Heat shock proteins (except HSP70)	Dibenzoylmethane, genistein, EGCG, green tea, PEITC	LNCaP, mammary gland, AZ521, HPAF-II, HepG2,	Frazier <i>et al.</i> 2004; Betancourt <i>et al.</i> 2012; Tanaka <i>et al.</i> 2011; Zhang <i>et al.</i> 2011; Neo <i>et al.</i> 2005
actin	Dibenzoylmethane, pomegranate juice, PEITC, SFN, curcumin, 6-shogaol,	LNCaP, DU145, A549, HepG2, LOVO, HeLa,	Frazier <i>et al.</i> 2004, Lee <i>et al.</i> 2012; Mi <i>et al.</i> 2011; Neo <i>et al.</i> 2005, Zhu <i>et al.</i> 2014; Liu <i>et al.</i> 2012
Heterogeneous nuclear ribonucleoprotein	Dibenzoylmethane, PEITC, SFN, genistein, EGCG, green tea, 6-shogaol, quercetin, lycopene	LNCaP, DU145, A549, mammary gland, AZ521, HPAF-II, HeLa, PC-3, LNCaP	Frazier <i>et al.</i> 2004, Lee <i>et al.</i> 2012; Mi <i>et al.</i> 2011; Betancourt <i>et al.</i> 2012; Tanaka <i>et al.</i> 2011; Zhang <i>et al.</i> 2011; Liu <i>et al.</i> 2012, Ko <i>et al.</i> 2014; Goo <i>et al.</i> 2007
Elongation factor	curcumin, green tea, PEITC, 6-shogaol, quercetin, lycopene	SH-SY5Y, HPAF-II, HepG2, HeLa, PC-3, LNCaP	D'Aguanno <i>et al.</i> 2012, Zhang <i>et al.</i> 2011; Neo <i>et al.</i> 2005; Liu <i>et al.</i> 2012, Ko <i>et al.</i> 2014; Goo <i>et al.</i> 2007
Enolase	pomegranate juice, curcumin, green tea, 6-shogaol,	DU145, SH-SY5Y, HPAF-II, HeLa,	Lee <i>et al.</i> 2012, D'Aguanno <i>et al.</i> 2012; Zhang <i>et al.</i> 2011; Liu <i>et al.</i> 2012
14-3-3s	curcumin, PEITC, SFN, green tea, 6-shogaol,	SH-SY5Y, A549, HPAF-II, HeLa,	D'Aguanno <i>et al.</i> 2012, Mi <i>et al.</i> 2011; Zhang <i>et al.</i> 2011; Liu <i>et al.</i> 2012
Eukaryotic translation initiation factor 40S ribosomal proteins	curcumin, EGCG, green tea, lycopene	SH-SY5Y, AZ521, HPAF-II, LNCaP	D'Aguanno <i>et al.</i> 2012, Tanaka <i>et al.</i> 2011; Zhang <i>et al.</i> 2011; Goo <i>et al.</i> 2007
Tubulin	curcumin, PEITC, SFN, green tea, 6-shogaol, lycopene	SH-SY5Y, A549, HPAF-II, HeLa, LNCaP	D'Aguanno <i>et al.</i> 2012, Mi <i>et al.</i> 2011; Liu <i>et al.</i> 2012; Sotoca <i>et al.</i> 2011; Raj <i>et al.</i> 2011
Vimentin	PEITC, SFN, EGCG, 6-shogaol, piperlongumine	A549, AZ521, HeLa, U2OS, EJ	Mi <i>et al.</i> 2011, Tanaka <i>et al.</i> 2011; Liu <i>et al.</i> 2012; Raj <i>et al.</i> 2011
Glutathione S-transferases	quercetin, curcumin, lycopene, piperlongumine	HT-29, LOVO, LNCaP, U2OS, EJ	Wenzel <i>et al.</i> 2004, Zhu <i>et al.</i> 2014; Goo <i>et al.</i> 2007; Raj <i>et al.</i> 2011

NB: Different isoforms of proteins are omitted and are grouped in the same category.

24.4 Proteomics in Phytochemical Research for Metabolic Diseases

In addition to cancer, recent studies have utilized proteomic analysis methods in various metabolic disease models, including obesity and cardiovascular disease, to discover novel molecular targets of phytochemicals. Obesity is a growing health concern worldwide and is a major contributing factor in the development of numerous metabolic complications, including diabetes. A number of phytochemicals have been shown to exhibit anti-obesity effects *in vitro* and *in vivo*, and recent studies have provided evidence for direct targeting of protein kinase signal pathways in adipose tissue development (Kwon *et al.* 2012; Wang *et al.* 2014). Comparative proteomics analyses have been adopted in a number of studies to screen and compare a wide range of proteome changes to identify the most affected molecules and signaling pathways involved (Beattie *et al.* 2011; Joo *et al.* 2010).

Capsaicin is a phytochemical found in red pepper and has been shown to suppress the development of obesity by activating thermogenesis. Joo *et al.* have shown evidence for the effects of capsaicin on obesity in rats fed on a high-fat diet, using proteomic analysis (Joo *et al.* 2010). Analysis by 2-DE together with MALDI-TOF on the white adipose tissue of capsaicin-fed mice compared to control mice revealed that heat shock protein 27 and Steap3 protein were significantly downregulated, while the olfactory receptor is up-regulated, indicating that thermogenesis and lipid metabolism pathways are regulatory targets of capsaicin in attenuating obesity. In a study by Beattie *et al.*, ginger phytochemicals including 6-gingerol and goryangkang suppressed high fat diet-induced obesity in mice, and by using proteomic analysis and mass spectrometry, discovered that their effects were elicited mainly by targeting cholesterol metabolism and fatty acid oxidation (Beattie *et al.* 2011). They showed that acetyl-coenzyme A acyl transferase 1 and enoyl CoA hydrates were significantly increased by ginger phytochemical supplementation.

Cardiovascular disease is one of the leading causes of mortality worldwide. It is another chronic disease that has been the subject of research using various phytochemicals and their protective effects, since a diet rich in polyphenols has been linked to a decreased risk of the disease (Kishimoto *et al.* 2013). A study by Mullen *et al.* showed that polyphenol-rich drink consumption altered the level of urinary biomarkers of coronary artery disease in humans (Mullen *et al.* 2011). From urine proteome analysis, 93 polypeptides were identified to have significant differences in expression levels between the polyphenol-rich drink consumption group and the control group, 27 of them having a more than four-fold difference. Notably, seven of the peptides have been previously identified as urinary biomarkers for coronary artery disease, indicating the potential impact of polyphenols for improving coronary health. Alperujo extract, which is a waste product from the olive oil extraction process, holds the majority of polyphenols present in olive fruits and has been shown to exhibit anti-platelet effects, which thereby may contribute to protection against the formation of atherosclerotic plaques (de Roos *et al.* 2011). From isolated human platelets treated with alperujo extract, the platelet proteome was analyzed and nine proteins were identified to be regulated by alperujo extract upon platelet aggregation induced by ADP, which may explain its effects.

The root of *Salvia miltiorrhiza* has been used as a traditional Chinese medicine and its aqueous extract containing numerous polyphenolic acids has been shown to exert anti-atherosclerotic effects by reducing oxidative stress in rat aortic smooth muscle cells. 2-DE coupled with MALDI-TOF was used to evaluate the changes in protein content in rat aortic smooth muscle cells and revealed that protein kinase C beta-1 (PKC beta-1) and phosphorylated mitogen-activated protein kinase (p-MAPK) are suppressed, suggesting the involvement of PKC/p44/42 MAPK-dependent pathways in *Salvia miltiorrhiza* root extract action (Hung *et al.* 2009). Resveratrol has been linked to a reduced risk of cardiovascular disease and has been shown to induce changes in nine proteins including elongation factor 2 (EEF2) and acetyl-EIF5A in human umbilical vein endothelial cells, suggesting a molecular mechanism for resveratrol in promoting coronary health (Shao *et al.* 2012). The cardioprotective effects of salvianolic acids (SA) and notoginsenosides (NG) were investigated in a cardiac ischemia-reperfusion (IR) injury rat model and the analysis of protein profiles of heart tissues revealed that 18 proteins were involved in IR injury and both SA and NG compounds exerted regulatory effects on a number of proteins, including those involved in energy metabolism (Yue *et al.* 2012).

24.5 Proteomics for Neuroprotective Phytochemicals

With an increasing proportion of the aging population and extended life expectancies, the prevalence of neurodegenerative disease is becoming a serious modern health issue. Evidence suggests that a number of phytochemicals counteract a number of processes involved in the development of neurodegenerative diseases (Rasool *et al.* 2014). Proteomics analyses suggest evidence for an underlying molecular mechanism responsible for the effects of epigallocatechin gallate (EGCG),

a green tea catechin (Weinreb *et al.* 2007, 2008). In human neuroblastoma cells, EGCG induces changes in the expression levels of various proteins, including factors associated with cytoskeletal regulation, cell survival and differentiation, heat shock, and protein binding (Weinreb *et al.* 2007). Grape seed extract (GSE), which is rich in proanthocyanidins, was shown to induce changes in a number of brain proteins which may explain the neuroprotective effects of GSE (Deshane *et al.* 2004, Kim *et al.* 2006). *Rosmarinus officinalis* has been suggested to protect against neurodegenerative disease and improve memory and cognition (El Omri *et al.* 2010). *R. officinalis* polyphenols including luteolin, carnosic acid, and rosmarinic acid, have been shown to induce changes in stress-associated proteins such as heat shock protein 90, transitional endoplasmic reticulum ATPase, nucleoside diphosphate kinase, and hypoxia upregulated protein 1 (A *et al.* 2012). *R. officinalis* polyphenols have also been shown to upregulate tyrosine hydroxylase and pyruvate carboxylase in rat neuronal cells, which may explain the antidepressant effects of *R. officinalis* (Sasaki *et al.* 2013).

Sung *et al.* demonstrated that ferulic acid, a phenolic compound with neuroprotective effects, prevents cerebral ischemic injury by regulating peroxiredoxin-2 and thioredoxin (Sung *et al.* 2014). MALDI-TOF analysis revealed that decreases in peroxiredoxin-2 and thioredoxin levels during cerebral ischemic injury were counteracted by ferulic acid. In addition, red wine polyphenol compounds have been shown to exhibit neuroprotective effects by regulating proteins associated with neuronal caliber and axon formation related to protection against oxidative stress and energy metabolism in a rat ischemic cerebral stroke model (Ritz *et al.* 2008).

24.6 Proteomics for Phytochemicals with Other Functions for Health Benefits

The protective effects of phytochemicals against harmful environmental chemicals which exhibit neurotoxicity and carcinogenic effects have been reported (Kita *et al.* 2014). Genistein, a soy isoflavone, has been shown to exhibit numerous health benefits including anticancer and anti-obesity effects. The rat serum proteome was shown to be altered by the carcinogenic action of bisphenol A and a protective effect of genistein was seen via Tandem Mass Tags quantitative mass spectrometry combined with MudPIT technology (Betancourt *et al.* 2014). The protective effect of acai berry extract against paraquat dichloride (a non-selective herbicide) toxicity has been shown in rat lung and its regulatory target has been evaluated by proteomics (Kim *et al.* 2013). Calcium signaling-related proteins including calcium binding protein 1 were shown to be induced by paraquat poisoning, and this was prevented by acai berry extract treatment in rat lung.

The anti-inflammatory effects of phytochemicals have been linked to their various health benefits including anticancer effects. Pycnogenol, a type of procyanidin, has been suggested to exert regulatory effects on inflammatory signaling pathways and to play a role in immune modulation. In RAW 264.7 macrophage cells, pycnogenol increased the expression levels of 4 proteins including cathepsin D, keratinocyte lipid-binding protein, proteasome subunit alpha type 1, and annexin IV, as evidenced by proteomic analysis (Wu *et al.* 2007). The result indicates that pycnogenol may play a role in the regulation of the phagocytic function of macrophages. Green tea extract, with high levels of polyphenols, was shown to reduce colonic inflammation in *Mdr1a*–/– mice by regulating a range of colon proteins linked to immune and inflammatory responses, as well as xenobiotic metabolism pathways (Barnett *et al.* 2013). Olive oil, rich in polyphenols, was shown to induce changes in levels of hepatic antioxidant enzymes in *APOE*–/– mice, underlining its potential for reducing oxidative stress (Arbones-Mainar *et al.* 2007).

24.7 Conclusions

Bioactive food components with health benefits have emerged as potential novel medicines of the future with fewer side effects than conventional drugs. Dietary phytochemicals, which can be consumed for extended periods of time without serious side effects, can provide a long term solution in contrast to synthetic drugs with unpredictable results. To fuel the development of beneficial phytochemicals as functional foods, continued research is needed to determine their exact modes of action.

Proteomics is an emerging technique in the future of functional food research. The development and sustenance of chronic diseases requires a large number of proteins involved in an intricate system, with multiple complicated signaling pathways involved. Phytochemicals with disease preventive properties are known to attack these pathways, resulting in preventive and therapeutic effects. Proteomics approaches in phytochemical research have greatly improved the understanding of how phytochemicals work at a molecular level. The correct utilization of proteomics tools can produce reliable predictions of clinical outcomes, since proteins represent and primarily determine the final phenotype. RNAs have a short

life span and do not accurately reflect the end product of translation. In addition, proteomics analysis can also be used to identify target proteins of compounds in addition to investigating post-translational modifications such as phosphorylation status. Several studies have relied on proteomics methods to analyze a broad spectrum of phosphorylation statuses for multiple proteins (Ramljak *et al.* 2005; Volate *et al.* 2010; Yan *et al.* 2010).

A limitation present in a number of current studies utilizing proteomics techniques is that they use plant extracts containing mixtures of phytochemicals instead of identifying a single compound in their studies. To further identify the direct target molecules in the function of phytochemicals and assess their potential application as bioactive molecules with disease preventive and therapeutic effects, continued research focusing on single compounds combined with proteomics analysis will be needed.

Proteomics-driven approaches can aid in understanding alterations in the proteome as a whole, and also help to identify direct molecular targets. Proteomics therefore represents a promising strategy to further develop and better understand the molecular mechanisms of phytochemicals, for the benefit of human health.

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Genomics/Proteomics of NEXT-II®, a Novel Water-Soluble, Undenatured Type II Collagen in Joint Health Care

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25.1 Introduction

Arthritis is a form of joint disorder accompanied with pain and inflammation. There are wide varieties of arthritis. The most common form of arthritis receiving more attention in recent years is osteoarthritis (OA). OA is characterized as degenerative joint disease, frequently occurring in knee joints. Risk factors related to the etiology of OA include age, gender, race, genetics, nutrition, congenital abnormalities, obesity/excess body weight, infection, metabolic disorders, and others (Bagchi *et al.*, 2010). While the other common form of arthritis is rheumatoid arthritis (RA), which is known as an inflammatory autoimmune disorder, representing 0.5–1.0 % of the global population (Feldmann, 2001).

To alleviate the symptoms such as pain and inflammation in joints, particularly in the knee, various drugs such as nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin (Hochberg *et al.*, 2003), disease-modifying anti-rheumatic drugs (DMARDs) such as methotrexate and sulfasalazine (Saag *et al.*, 2008), and biologicals, for example, the TNF- α blocker (Finckh *et al.*, 2006) have been provided to patients or subjects who suffer from RA and OA. Drugs like non-steroidal anti-inflammatory drugs (NSAIDs) and disease modifying anti-rheumatic drugs (DMARDs) are commonly associated with serious side effects, and hence are not appropriate for long term use (Bagchi *et al.*, 2011, Day, 2002; Singh *et al.*, 1996; Wofe *et al.*, 1999). These drugs also cause nausea and fatigue as adverse reactions (Bagchi *et al.*, 2011, Day, 2002; Singh *et al.*, 1996; Wofe *et al.*, 1999). On the other hand, dietary supplements such as fish oils containing n-3/n-6 fatty acids (Dawczynski *et al.*, 2009; Rosenbaum *et al.*, 2010), botanicals including *Boswellia serrata* (Sengupta *et al.*, 2008), and the bark of *Pinus pinaster Ait.* ssp. *attantica* extract (Farid *et al.*, 2007), and animal-derived supplements from chicken sternum cartilage or type II collagen (Trentham *et al.*, 1993), and others such as glucosamine/chondroitins (Bagchi *et al.*, 2011) have been demonstrated to have potential efficacy in ameliorating both inflammation and pain in RA and OA subjects.

The mechanistic actions of the nutraceuticals, which are emerging as dietary supplements for RA and OA, are still not clear; further investigations and human trials are required for appropriate use of the dietary supplements. However, attempts to elucidate the mechanistic actions underlying the effectiveness of nutraceuticals have become apparent with the emergence of “omics” which are proven to be useful techniques. The representative “omics” including genomics, proteomics, and metabolomics are frequently employed in molecular and cellular studies to understand mechanistic pathophysiology (Bagchi *et al.*, 2010).

The dietary supplement NEXT-II, which is water-soluble and a undenatured type II collagen from chicken sternum cartilage developed in our laboratories, seems to be very effective in alleviating the inflammation of joints as demonstrated in our earlier studies conducted in animals. Earlier studies in RA (Trentham *et al.*, 1993) demonstrated significant efficacy of type II collagen in RA subjects. Although the associated mechanisms were not elucidated, however, many studies were conducted to elaborate the mechanism of action for type II collagen at molecular levels (Yoshinari *et al.*, 2015). In this chapter, we aim to unveil the mechanism of RA and OA. We also conducted a series of safety studies such as acute oral toxicity, acute dermal toxicity, primary skin irritation, primary eye irritation, mutagenicity tests, and long-term safety studies in dogs (Yoshinari *et al.*, 2013a). The efficacy of NEXT-II was assessed using genomics and proteomics in the pathogenesis of inflammatory response and RA.

25.2 Mechanism of RA

RA is an autoimmune disorder, which involves several inflammatory biomarkers working in complicated pathways. Events triggering such auto-immune mechanisms are thought to be the result of a combination of genetic predisposition and certain environmental factors. The pathophysiology of inflammation is complex and is also the outcome of an overlapping, reverberating process that involves both adaptive and innate arms of the immune system. In recent years, a remarkable number of studies have been reported on the pathogenesis of RA. Genetic predisposition and exogenous environmental factors are believed to contribute to T-cell-mediated autoimmune disorder for joint pain and arthritis. This chapter summarizes recent progress in understanding the cellular and molecular mechanisms of arthritis by focusing on helper T-cells, Th17 cells, and Treg cells.

T-helper (Th) cells with diverse cellular functions differentiate from native CD4+T cells upon stimulation by antigen in the presence of different cytokines produced by cells in the immune system. Until recently, two major cell subsets, Th1 and Th2, were known to provide effective responses to intracellular and extracellular pathogenesis, respectively, through the production of specific cytokines. Th1 cells produce interferon (IFN)- γ and lymphotxin- α , while Th2 cells produce interleukin (IL)-4, IL-5, IL-13, and other cytokines (Mosmann and Coffman, 1989). However, recent studies have demonstrated that T-helper lymphocytes require IL-12 and IL-23 to differentiate and secrete pro-inflammatory cytokines, rather than Th1 cells, which are major mediators of inflammatory responses in most of these Th1-mediated autoimmune diseases (Hue *et al.*, 2006; Murphy *et al.*, 2003). These cells produce IL-6, IL-17, IL-22, and TNF- α , and are also recognized to belong to a distinct effector cell subset, the Th17 cells (Bettelli *et al.*, 2007), and contribute to a variety of autoimmune diseases such as RA (Romagnani, 2008) (Figure 25.1). Th17 cells are present in the T cell-rich area of RA synovium

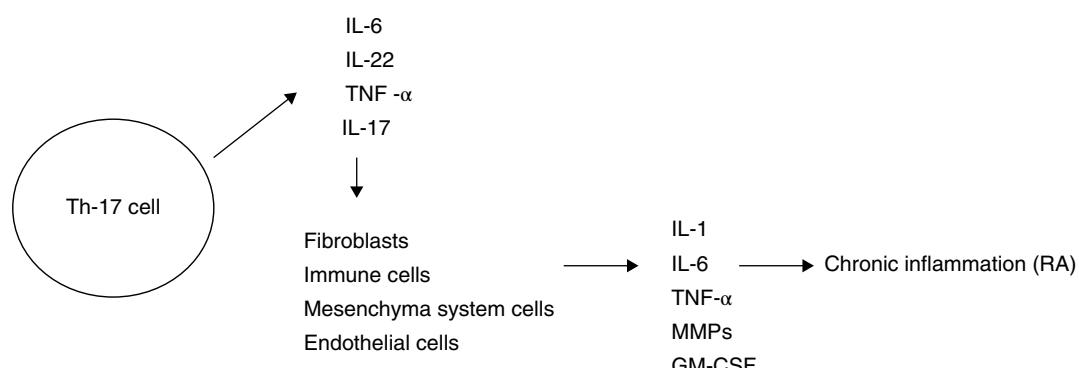


Figure 25.1 Cytokines involved with Th-17 cell in RA.

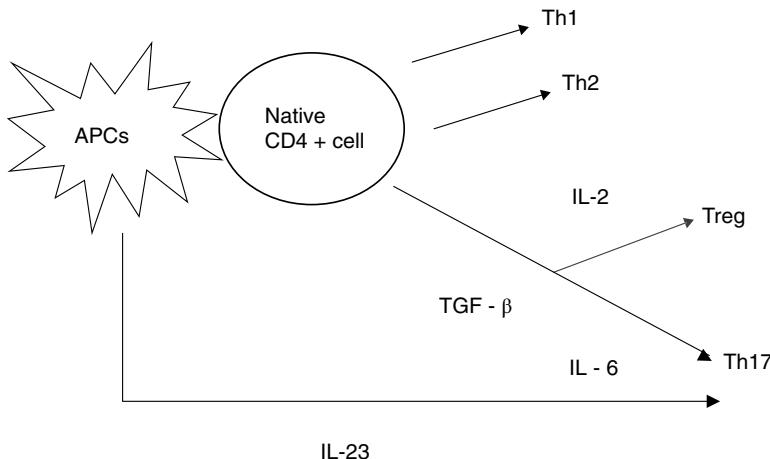


Figure 25.2 Differentiation of native CD4+ cell.

(Fossiez *et al.*, 1996). Th17 cell migrates to the inflammation site expressing CCR6 (Hirota *et al.*, 2007), this gene has been identified in RA pathogenesis (Kochi *et al.*, 2010). Thus, RA patients have higher concentrations of IL-17 in their serum and synovial fluid compared to control subjects (Kotake *et al.*, 1999; Ziolkowska *et al.*, 2000). Furthermore, IL-17 is responsible for chronic inflammation and joint destruction. It also promotes the production of inflammatory cytokines (IL-1, IL-6, and TNF- α) mediated through macrophages and fibroblasts (Yao *et al.*, 1995). Moreover, IL-17 acts on a mesenchyma system cell, aggravating the expression of matrix metalloproteinase (MMPs) and promoting bone fusion (Fossiez *et al.*, 1996), which also causes inflammation in cells and increases osteoclast cells by increasing receptor activator of nuclear factor- κ B (RANK) (Sato *et al.*, 2006).

CD4+CD25+ Treg cells constitute 5–10% of the peripheral CD4+ T cells (Baecher-Allen *et al.*, 2005), which play a major role in the maintenance of immune tolerance and in the control of autoimmunity (Kohm *et al.*, 2002; Morgan *et al.*, 2005). It is important to mention that CD4+CD25+ Treg cells are primarily responsible for the maintenance of self-tolerance and prevention of destructive autoimmunity. Recent research demonstrates their natural function extending to the regulation of tumorigenesis and pathogen immunity (Belkaid *et al.*, 2002). These CD4+ T cells are activated with IL-2 and TGF- β and become Treg cells (Davidson *et al.*, 2007; Zheng *et al.*, 2007). Also, the identification of specific markers such as the IL-2 receptor α -chain, CD25, has facilitated the experimental manipulation and characterization of Treg cells (Sakaguchi *et al.*, 1995). On the other hand, activation with IL-6 and TGF- β results in the enhanced production of Th17 cells (Park *et al.*, 2005; Veldhoen *et al.*, 2006). TGF- β thus plays a dual role required for Th17 cell differentiation and promotion of Treg cell development. Therefore, it plays emerging roles in the differentiation of both inducible Foxp3 Treg cells and ROR γ t cells from CD4+ T cells (Bettelli *et al.*, 2006; Ivanov *et al.*, 2006), along with IL-2 and IL-6 acting as switch factors between Treg and Th17 cells, respectively (Bettelli *et al.*, 2006; Laurence *et al.*, 2007) (Figure 25.2). IL-6 inhibits the function of Treg cells (Pasare and Medzhitov, 2003), while Th17 cells reinforce immune function. Alternatively, IL-2 acts through inhibiting STAT5, the key transcription factor of Th17, ROR γ t upregulation and Treg cell modulation (Ivanov *et al.*, 2007). Thus, the IL-2 and IL-6 milieu influences the balance of differentiating inducible Treg and Th17 cells.

25.3 About NEXT-II®

Oral administration of type II collagen (C II) has been demonstrated to improve signs and symptoms in collagen-induced arthritis in animals and humans suffering from RA and OA (Crowley *et al.*, 2009; Nagler-Anderson *et al.*, 1986; Park *et al.*, 2009; Thorbecke *et al.*, 1999). However, the effect is more pronounced in undenatured type II collagen, because of the maintenance of the structural integrity of the triple helical polypeptide structure with active epitope. Previous studies revealed recognition of the epitope located within a polypeptide fragment of C II 256–273, which might have played an important role (Michaëlsson *et al.*, 1992). Moreover, it was found that lysine in the 264th position (Lys264) in native C II could be post-translationally hydroxylated and later glycosylated. As a result, the processing of this hydroxylsine (Hyl264)

and its glycosylated derivatives are important features of the epitope for T-cells (Corthay *et al.*, 1998). It was previously reported that denatured collagen did not induce immunological hyporesponsivity, implying the importance of intact and active epitopes (Nagler-Anderson *et al.*, 1986). It is important to mention that the structure of collagen is very delicate and can be ruptured by heat and acid easily. Even if the C II is denatured immunological tolerance may only occur by retaining the structure of the epitopes. However, the integrity of denatured structures is easily lost in presence of protease. Therefore, we measured undenatured C II levels by using a commercial ELISA kit and also screened the source of type II collagen, which is useful in human arthritis.

Induction of oral tolerance using an antigen ingested orally has been considered a promising approach to the treatment of chronic autoimmune diseases, including RA. The antigen characteristics, administration route, and primary contact with the immune system regulate the induction of oral tolerance (Weiner, 2000). Agents which enhance anti-inflammatory cytokine profiles from immune cells have been shown to the induction of oral tolerance. Oral administration of undenatured C II has been proven to improve signs and symptoms in CIA animal and human studies for RA (Nagler-Anderson *et al.*, 1986; Thorbecke *et al.*, 1999).

We examined undenatured C II from the cartilages of chicken, salmon, and shark since C II from swine or bone are challenged by causing disease such as Bovine Spongiform Encephalopathy (BSE). On the other hand, cartilages of chicken are comparatively safe for use in critical diseases of humans and animals. These are commonly utilized in health food industry. Type II collagens derived from aquatic animals may not be efficacious, because of the absence of the epitope. Hence, we developed undenatured C II from chicken cartilage. In our previous study, we have reported the broad spectrum safety in animals and humans (Yoshinari *et al.*, 2013a). Bagchi *et al.* have reported that undenatured type II collagen is safe and able to reduce joint pain and swelling, and enhance mobility in human (Bagchi *et al.*, 2001).

25.3.1 Preparation of NEXT-II®

We developed a novel, water-soluble, undenatured type II collagen (NEXT-II®) and evaluated the safety and efficacy.

Briefly, sternum cartilages of commercially available chickens were pulverized. The ground cartilage was extracted using 0.05 M acetic acid and reacted by adding 1.0% pepsin at 4°C. The mixture was filtered to remove undissolved particles, evaporated under vacuum and dried using a lyophilizer, and stored at -20°C.

25.3.2 Safety of NEXT-II®

Acute oral and dermal toxicity, primary skin irritation, primary eye irritation, and Ames' bacterial reverse mutation assay were conducted, which demonstrated its broad-spectrum safety (Yoshinari *et al.*, 2013a). The acute oral LD50 of NEXT-II® was found to be greater than 5000 mg/kg b.w. in female rats. The acute dermal LD50 of NEXT-II® was found to be greater than 2000 mg/kg b.w. in both male and female rats. Primary skin irritation was investigated in female rabbits to evaluate irritation after a single topical application. The mean primary dermal irritation index (PDII) for NEXT-II® was calculated to be 1.8 using Draize primary dermal irritation scoring criteria, thus classifying NEXT-II® to be slightly irritating to the skin (Draize *et al.*, 1944). Primary eye irritation was conducted in rabbits from a single instillation via the ocular route in accordance with the Draize scale for scoring eye lesions and the Kay and Calandra scheme for classifying eye irritants (Draize *et al.*, 1944; Kay and Calandra, 1962). The maximum mean total score of NEXT-II® was determined to be 7.3, classifying NEXT-II® to be minimally irritating to the eye (Yoshinari *et al.*, 2013a).

The mutagenicity of NEXT-II® was evaluated in the five tester strains of *Salmonella typhimurium*, such as TA 1535, TA 97a, TA 98, TA 100, and TA 102 in the presence and absence of a metabolic activation system (S9) (Ames *et al.*, 1977) and NEXT-II® was used at the doses of 5000, 1500, 500, 150, and 50 µg/plate. There were no significant increases in five of the colony tested strains following NEXT-II® treatment. The results demonstrate that NEXT-II® is non-mutagenic and non-cytotoxic (Yoshinari *et al.*, 2013a). NEXT-II® was also assessed by *in vitro* mammalian cell gene mutation assay for its potential to induce mutation at the mouse lymphoma thymidine kinase locus (Thymidine Kinase Locus/TK+/-) using the mouse (*Mus musculus*) lymphoma cell line L5178Y. No biologically relevant increases of mutants were found after treatment with NEXT-II® (with or without metabolic activation). Thus, NEXT-II® is considered to be non-mutagenic in the mouse lymphoma thymidine kinase locus using the cell line L5178Y (Yoshinari *et al.*, 2013a).

Long-term safety studies were carried out in twenty large breed dogs over a period of 150 days (Yoshinari *et al.*, 2015). In brief, 20 adult dogs (body weight range: 18–34 kg, age between 7 and 12 years) was divided two groups, placebo and NEXT-II® (10 mg/day as active undenatured C II level). Compared to the control group all safety parameters were evaluated from blood samples collected on days 0, 30, 60, 90, 120, and 150 of treatment to evaluate alanine aminotransferase

(ALT), blood urea nitrogen (BUN) and creatine kinase (CK) to demonstrate that NEXT-II doesn't induce hepatotoxicity, nephrotoxicity, or cardiotoxicity. No significant changes were observed. Furthermore, body weight, heart rate, and respiration rate were also evaluated in both groups on days 0, 30, 60, 90, 120, and 150. There were no significant differences between the control and treated groups, or between the treated groups at each time point over a period of 150 days. Thus, NEXT-II® was well tolerated and no adverse events were reported (Yoshinari *et al.*, 2015).

25.3.3 Efficacy of NEXT-II® in Collagen-Induced Arthritic Mice

Efficacy of NEXT-II® (1 mg/kg as undenatured type II collagen) was assessed in collagen-induced arthritis (CIA) in mice (Yoshinari *et al.*, 2013b). Mice (DBA1/J, 8-week-old) were divided into two groups; control group and collagen-induced arthritis (CIA) group. C II-Freund's complete adjuvant was injected intradermally into the dorsal root of the tail of the mice to induce CIA.

This was regarded as day 0 of treatment. On day 21, the same booster injection was administered to the CIA group. On day 39, the CIA group was divided into the following groups. Groups include the (1) CIA group control and (2) NEXT-II-treated CIA mice group, and administered with saline only (for CIA group or NEXT-II®) up to day 48. Control group of mice was treated with saline only as the CIA group from day 39 to 48.

The arthritis index in the NEXT-II group was significantly lower compared to the CIA group. Serum IL-1, IL-6 and TNF- α levels in the NEXT-II group were significantly lower compared with the CIA group, while serum IL-2 was higher. Furthermore, NEXT-II® enhanced the proportion of CD4+CD25+ T-cells, and gene expressions of stimulated dendritic cells-induced markers for regulatory T-cells such as Foxp3, TGF- β 1 and CD25 (Yoshinari *et al.*, 2013b). Furthermore, the proportion of CD4+CD25+ T-cells in CD4+ T-cells, and CD4+IL-10+ T-cells in the NEXT-II group increased compared to the CIA group as demonstrated by flow cytometry. A reduction in the frequency of CD4+IL-10+ T-cells in inflamed synovium and peripheral blood of patients with RA contributes to the loss of tolerance (Yudoh *et al.*, 2000). There is a greater induction of IL-10 producing CD4+CD25+ cells among spleens in mice fed C II before CIA induction occurs (Min *et al.*, 2004). In this study, it may be possible that the production of IL-10 is from CD4+CD25+ T cells. Phenotypically, these CD4+CD25+ T-cells resemble Treg cells, based on the expression of their intracellular and surface markers (Foxp3 and CD25) by measuring their gene expression. Foxp3 has been identified as a specific molecular marker for Treg cells and its expression is essential for programming Treg cell development and function (Fontenot *et al.*, 2003). Recently active suppression of autoreactive T lymphocytes by Treg cells has emerged as an important pathway in the maintenance of self-tolerance. Frey *et al.* (2005) have reported a significant role for Treg cells in the control of chronic arthritis. Wang *et al.* (2010) have also demonstrated a significantly higher frequency of CD4+CD25+ T-cells in spleens of control rats as compared to the CIA rats. With respect to Foxp3, TGF- β 1 has been reported to require Foxp3 expression for maintenance and suppressive activity of Treg cells (Marie *et al.*, 2005; Zheng *et al.*, 2008). IL-2 is also required for the maintenance of Foxp3 expression (Fontenot *et al.*, 2005). In this study, high level of serum IL-2 in the NEXT-II® group regulates to the upregulation of CD25 (IL-2 receptor α -chain) expression. Conversely, IL-6 inhibits Treg function (Thorbecke *et al.*, 1999) and expansion (Wan *et al.*, 2007), as well as TGF- β activity (Dominitzki *et al.*, 2007). These results demonstrate that administration of NEXT-II® may induce higher proportions of Treg cells when compared with the CIA group.

In conclusion, oral administration of NEXT-II® resulted in the reduction of arthritis in CIA mice and was also associated with decreased production of anti-C II IgG, IL-1, IL-6, and TNF- α , and enhanced secretion of IL-2. Proportion of CD4+CD25+ T cells in the NEXT-II® group increased compared to CIA group. Furthermore, gene expressions of Foxp3, TGF- β 1 and CD25 in the NEXT-II® group were significantly increased. From these results, oral NEXT-II® treatment is thought to induce CD4+CD25+ Treg cells. The present study demonstrates big advantage of using NEXT-II in human suffering from arthritis without any adverse events.

25.4 Hypothesized Mechanism of NEXT-II®

Although a wide range of studies both *in vitro* and *in vivo* are required to understanding of the mechanism of NEXT-II®, we hypothesize that NEXT-II® may have some kind of activity of antigen presenting cells (APCs) such as dendritic cells (DCs) and macrophages. A native CD4+T cell receives antigen-specific signals (signal 1) through its clonally derived T cell receptor (TCR), which interacts with antigenic peptides presented by MHC class molecules expressed on the surface of APCs. A second pathway of signals may also be present, which includes signals delivered by secreted cytokines that are produced by either the antigen presenting cells on the activated CD4+T cell itself, and signals delivered by co-stimulatory

molecules expressed by activated APCs, such as CD80/86, which may further interact with the CD28 co-receptor that can presumably be expressed by CD4+T cells. Furthermore, Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) in Treg cells have been reported to suppress CD80/86 expression in DCs (Fehérvári *et al.*, 2004; Onishi *et al.*, 2008). The DC, which downregulates the expression of CD80/86, cannot activate effector T-cells. As a result of induction of Treg cells by NEXT-II®, it may be possible that immune response is controlled and downregulated. On the other hand, Zheng *et al.* (2010) have reported that CD80/86-silenced DCs generated more Treg cells. It has been demonstrated that DMARDs attenuate effector T-cell activation by regulating the activation of T cells and inhibiting CD80/86-CD28 pathway (Korhonen *et al.*, 2009). It may also be possible that NEXT-II® potentially acts on DCs, resulting in inducing Treg cells. Moreover, immune response may be adjusted through interacting Treg cell with CD80/86 of the DCs. Additional studies are warranted to understand the complex mechanism of action of NEXT-II® with DCs.

25.5 Future Perspectives

We are interested in using NEXT-II® in OA. Our dog study demonstrated the efficacy of NEXT-II® in controlling pain and inflammation of arthritic dogs (Yoshinari *et al.*, 2015). Currently, a pilot clinical trial with moderately OA subjects have shown that NEXT-II® improves joint knee mobility and flexibility as demonstrated by WOMAC and VAS scores. The results demonstrate that rapid improvement for some volunteers after administering NEXT-II® for 4 weeks, and apparent improvements are observed in most of the volunteers (unpublished).

Furthermore, we will use dendritic cells and CD4+T cells incubated with NEXT-II® at different doses and time points to measure the production of Treg cells and immune response.

25.6 Conclusion

NEXT-II® is a novel, safe, water-soluble, and effective ingredient for both RA and OA. Our dog and pilot human studies have shown the efficacy of NEXT-II® in OA. Further studies are in progress to unveil the complete mechanism of action of NEXT-II® in OA and RA.

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Part IV

Metabolomics

26

Harnessing Metabolic Diversity for Nutraceutical Plant Breeding

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26.1 What is Metabolomics?

A metabolome is defined as the entire set of metabolites produced by any organism at any point in time during its lifecycle. The study of the metabolome, known as “Metabolomics”, describes biochemical processes and underlying biological roles of metabolites in an organism (Fiehn 2002; Hegeman 2010; Patti *et al.* 2012). Unlike genes and proteins, metabolites cannot be assessed in a linear form with computers as the structure of metabolites is complicated due to the order and stereochemical orientations of atoms (Fiehn 2002). Moreover, plant based plant metabolites (levels and types) can change due to both genotypic and environmental interactions. Although genes contain information for metabolite synthesis, production of metabolites can be triggered by growth stages and environmental factors, such as, light, temperature, humidity, soil type, fertilizers, pests and pesticides, and so on (Hounsome *et al.* 2008). At present, it is not possible to map the entire metabolome of any plant; however, “metabolic profiling” can analyze a subset of metabolites at any given time or stage of plant growth (Nanda *et al.* 2011; Shyur and Yang 2008).

Metabolomic studies has been used for discriminating metabolites, obtaining new information (informative) to discover bio-compounds, and for predicting variables that are difficult to estimate otherwise (Cevallos-Cevallos *et al.* 2009). The analyses can be “targeted” towards a specific group of metabolites in order to achieve focused results, or “untargeted” analyses to comprehend and detect a vast amount of metabolites at a given state (Cevallos-Cevallos *et al.* 2009). Additionally, an integrated analysis of the transcriptome, metabolome, and metabolic pathways can be used to understand regulation from gene to metabolite networks through modulation of primary and secondary metabolites, as demonstrated by Putri *et al.* (2013).

Our current knowledge about the end products of genes, their interactions, and regulation is limited, but increasing rapidly (Chen *et al.* 2012; Wishart 2008). Transcriptomics, proteomics, next generation sequencing, and bioinformatics are revolutionizing our understanding of genes and their functions. For example, Fiehn (2002) was able to assign putative functions for several new sequences of nucleotides and amino acids based on homology. Hirai *et al.* (2004) applied metabolomics for identifying the functions of putative genes and their associated proteins involved in metabolism. As plants produce thousands of metabolites at any given time, which cannot be distinguished into a phenotype, Sumner *et al.* (2003) used unbiased differentiation of genotypes based upon their metabolic profile with the help of metabolomics (Sumner *et al.* 2003). In certain cases, where measurable

phenotypic changes could be observed among genotypes, the study of biochemical causes and the consequences of metabolic pathways in different phenotypes were achieved via metabolomics (Sumner *et al.* 2003). Specific to agriculture, it is thus expected that metabolomics could be used to increase select metabolic fluxes as a means to enhance the nutritive and pharmaceutical value in plant based food, while decreasing the need for pesticide sprays (Fiehn 2002). Still, the ultimate goal of metabolomics is to understand and predict the functions of metabolism along with its cellular networks. As metabolomics also has the power to monitor cellular systems, it can also provide information on various functions of genes (Putri *et al.* 2013). The metabolome must therefore be considered the definitive response to genetic and environmental variations.

An upward trend in assessing and enhancing food quality has occurred recently with the help of metabolomics. Pre and post-harvest diseases are most common causes attributed to the poor food quality. Food safety is another of major concern in most societies. Pathogens, such as *Escherichia coli* that contaminate several types of leafy vegetables on a regular basis pose significant health hazard to a great number of people. The metabolomic technique known as neutral desorption extractive electrospray-ionization was able to rapidly discriminate food samples containing *Escherichia coli* (Chen *et al.* 2007). Additionally, many developed nations are skeptical about the health hazards of genetically modified foods in their diets and this is a major growing concern worldwide. Metabolomics offer numerous solutions to all these problems as well.

Many of the metabolomics techniques were discovered four decades ago, but its importance has only been understood in the last decade of the post-genomic era. Genomics, proteomics, and metabolomics have provided new tools to the plant breeder for breeding nutraceutical crops. This review will provide insight on metabolomics, human nutritional deficiencies, nutraceuticals, secondary metabolites, and the latest tools for plant breeders regarding nutraceutical breeding that utilizes a system biology approach.

26.2 Nutraceuticals

The world is facing a dichotomous problem of food malnutrition, that is, over- and under-nutrition of human beings in developed and developing nations, respectively (Hall *et al.* 2008). The majority of people in developed nations are suffering from obesity, diabetes, and coronary heart diseases due to the excessive intake of calories (Everitt *et al.* 2010). Deficiency of vitamins, minerals, and sometimes absence of food is the primary concern in developing nations (Everitt *et al.* 2010). A close inspection reveals that both of these circumstances are related to an imperfect diet.

Nutraceuticals (or dietary bioactives, bio-protective foods) are classified as any safe components of food sources that deliver health benefits beyond our daily nutritional requirements, whereas functional foods are foods that contain dietary bioactives. Such products can take many forms, ranging from whole foods to processed foods to dietary extracts from their original source to isolated nutraceuticals delivered as a supplement (Elliott and Ong 2002; Hall *et al.* 2008; Milner 2000). As a result, nutraceuticals overlap the food, pharmaceutical, and agricultural area, significantly influencing each sector similar to biotechnology industry during the past century. Several nutraceutical supplemented foods containing increased amounts of vitamins (A, B, C, E), bioflavonoids, isoflavones, antioxidants, lycopene, folic acid, pigments, healthy plant oils, decreased toxins, and edible vaccines are either currently available, or will be soon (Davies 2007; Raskin *et al.* 2002; Stewart *et al.* 2011). However, very little is known about the activity of bioactives within the product as a whole and the interaction/activities of such components when delivered to another transport matrix. Bioactivity, levels and types of nutraceuticals are further complicated by cultivar, farming practices, extraction, and other downstream processing/formulating operations. Therefore, there is a critical need not only to discover novel bioactive components but to also standardize their types and levels throughout the field to fork continuum. Moreover, it is expected that personalized nutrition will be achieved by understanding interactions between human genes and diet (Brown and Ouderaa 2007). Although nutritional genomics uses high throughput genomic technologies to elucidate the complexities between the human genome and diet (Brown and Ouderaa 2007; Elliott and Ong 2002; Hall *et al.* 2008), the current science does not provide definitive answers on the impact of secondary metabolites on human health. However, new emerging techniques, such as single cell metabolomics (Heinemann and Zenobi 2011), can aid the scientific community in gaining a deeper understanding.

26.3 Importance of Secondary Metabolites

The basic needs of plant survival, growth, and development depend upon the synthesis of primary metabolites, such as, amino acids, organic acids, phyto-sterols, acyl-lipids, and so on. However, the ecological needs of a plant depend upon the production of secondary metabolites, such as pigments for attracting pollinators and seed dispersal agents, protection

against pests, survival in specialized ecological niches, and so on (Croteau *et al.* 2000; Fiehn, 2002; Gibney *et al.* 2005; Rhodes, 1996; Sumner 2010). Plant secondary metabolites are highly diversified and are present in more than a quarter million forms (Arbona *et al.* 2013; Carreno-Qunintero *et al.* 2013; Croteau *et al.* 2000; Davies *et al.* 2010). In a single plant species, a few thousand secondary metabolites are easily detected using metabolic profiling approaches. Four major groups of secondary metabolites are: terpenoids, alkaloids, phenylpropanoids, and sulfur containing metabolites (Croteau *et al.* 2000; Hounsome *et al.* 2008).

Terpenoids, compounds comprised of five carbon molecules, are the most common secondary metabolites in plants. There are several types of terpenoids present in the plants, such as, monoterpenes (C_{10} – volatile essence and essential herbal oils), sesquiterpenes (C_{15} – phytoalexins, antibiotics), diterpenes (C_{20} – phytol, gibberelic acid), triterpenes (C_{30} – toxins, waxes, brassinosteroids), tetraterpenes (C_{40} – carotenoids), and polyterpenes (plastoquinone, ubiquinone, latex). Terpenoids are usually associated with specialized structures, such as, glandular trichomes, secretory cavities, petal epidermis, resin ducts, and so on. Overall, terpenoids contribute to plant aroma, flavor, color, and defense mechanisms against pests.

Alkaloids are another important group of secondary metabolites, which are nitrogen-containing basic compounds, such as codeine, chloroquine, and tropicamide. These metabolites are useful as purgatives, sedatives, and several other medicinal purposes. Alkaloids also build a plant's constitutive defense mechanism against insects by synthesizing products, such as morphine, caffeine, nicotine, and so on (Croteau *et al.* 2000).

Phenylpropanoids are a third group of secondary metabolites in plants that use the shikimic or malonate/acetate pathway for synthesis, and are responsible for defense against pests, color, flavor, taste, wood, and bark production for durability, and so on. Lignins, suberized tissues, flavanoids, furanocoumarins, stilbenes, and so on, are examples of various types of secondary metabolites synthesized from phenylpropanoid pathways. Lignans, lignins, and suberized tissue provide woody strength to plants and help the plant to acclimatize to the cold (Hounsome *et al.* 2008). Flavanoids are present mostly in the plant's vacuoles and provide pigments (anthocyanins), protect against ultraviolet rays (kaempferol), act as signal molecules between different metabolic pathways, and defend the plant against pests and diseases. Flavanols, such as quercitin, coumarins, and stilbenes, are mainly associated with antimicrobial, antifeedant, and nematocide properties (Croteau *et al.* 2000). Lastly, the sulfur-containing secondary metabolites (glucosinolates) act as natural insecticides during tissue disruption by converting into different forms of thiocyanates (Croteau *et al.* 2000; Hirai and Saito, 2008).

Our diet contains thousands of secondary metabolites depending upon the amount and variety of plant origin food consumed. Secondary metabolites may provide significant human health benefits (Rist *et al.* 2006), but studies designed to measure their efficacy are challenging and are in need of more work. Nonetheless, potential health benefits of secondary metabolites include, but are not limited to, immune system modulation, anti-inflammation, anticancer, anti-viral, anti-bacterial, anti-toxic, hepato-protective, antioxidant, anti-estrogenic, anti-atherosclerosis, and cholesterol reduction (Croteau *et al.* 2000; Hounsome *et al.* 2008; Raskin *et al.* 2002).

A wide range of plant originated vegetables and fruits are abundant sources of secondary metabolites (Crozier 2007). Traditional breeding efforts have improved the horticultural and agronomic traits of several crops tremendously. Despite these advances, breeding for nutraceuticals requires metabolic screening thereby necessitating the integration of metabolomics with traditional breeding for releasing new cultivars with nutraceutical properties. For example, de Vos *et al.* (2011) used the tomato as a model vegetable crop to understand the link between nutritional value and plant physiology through the application of extensive metabolomics tools and system biology. It is thus entirely targeted crop improvement that produces higher quality products can be achieved by modifying breeding programs to include metabolomics based approaches (Hall *et al.* 2008).

26.4 Complementing Plant Breeding with “Omics”

The current paradigm of plant breeders across the globe is to develop new cultivars and hybrids to meet the food demands of fast growing world population. Yet the present food demand has not only increased in terms of quantity over the years, but also in terms of food quality, such as nutritional values, organic food production, and overall food safety. Changing climates, need for low inputs, organic food production, and presence or absence of genetically modified foods are all factors that must now be considered when developing new plant cultivars and hybrids. Genetic background, environments where the crops are grown, cultivation practices of growers also contribute to the allelic variability in newly developed germplasm, and hence new diversity in metabolites (or biochemical phenotype) (Davies *et al.* 2010).

The biochemical phenotype of any individual is the accumulated response of genotype as expressed in a given environmental condition. Developmental stages of an individual also play critical roles in expressing different sets of genes and therefore adding complexity to the phenotype. Spatial and environmental effects are also pronounced – different part of the leaf, different part of the day (temperature, sunlight) – changes in metabolite are constant, and effectively represent the impact of the surroundings (Khakimov *et al.* 2013). The genetic composition of nucleic acids of cells throughout the organism are more or less similar, however, metabolites are highly diverse in an organism based on parts (such as, roots, foliage, flowers, seeds), and its growth stages (Hegeman 2010). Therefore, metabolomic analysis has been considered as an effective approach for capturing phenotypic biomarkers. Understanding changing patterns during various development phases would assist plant breeders to strategize based on metabolic profiling (Harrigan *et al.* 2007).

An added advantage of metabolomics is that it not only integrates the information of proteomics and transcriptomics, but also captures the impact of environmental and developmental stages to present the complete picture (Arbona *et al.* 2013). Current metabolomics initially focuses on proper identification of unique metabolites relative to changes in the environment, and progresses into quantifying those metabolites. Therefore, metabolomics offers immense potential to plant scientists to comprehend phenotype of crops in response to their ever changing natural surroundings.

The ability to predict the phenotype based on genotypic and environmental data has been a critical problem for plant biologists and breeders. However, a recent study successfully combined various “omics” with quantitative genetic analysis on tomatoes and corn to predict the phenotype based on the presence of metabolite variation (Carreno Qunintero *et al.* 2013). Advancements in bioinformatics and molecular marker technologies have also facilitated the ability to generate faster and more accurate genotypic data. In additionally, historical climate dataset is readily available. Still, predicting plant phenotype is unattainable without the information provided by metabolomics. The amount of information generated in any given metabolomic study is rather vast, and often poses a challenge to deduce meaningful information. The real potential of metabolomics can only be exploited through the use of statistical analysis, where hundreds and thousands of metabolites are identified, quantified and then correlated to a specific phenotypic characteristic (Thissen *et al.* 2011). As a result, metabolomics provides multiple challenges and opportunities at the same time.

A plant breeder requires new tools for enhancing crop quality, particularly for nutraceuticals. The use of molecular markers in traditional plant breeding is increasing rapidly to drive this area. Marker assisted selection has been used as many genetic markers were developed. Genomic selection was developed during early 2000, and was of particular interest due to its ability to account for all available DNA markers. Because phenotypic evaluation is often time-consuming and cost-prohibitive, combining genomics and metabolomics is a potential alternative to improve the quality of crops provided they predict phenotypic performances in different environments and non-additive effects (Jonas and de Koning 2013). Techniques, including mass spectrometry, nuclear magnetic resonance spectroscopy, chromatography, and so on, have been utilized to study the impact of time, stress, and environmental conditions and to analyze hundreds of metabolites simultaneously (Dixon *et al.* 2006). Correlation of metabolic composition to yield and biomass traits is paving a way for metabolomics assisted breeding (Sumner 2010). It is thus expected that integrating such metabolomic based techniques with genomics will provide new molecular markers of quality traits for marker assisted selection in existing breeding programs (Hall *et al.* 2008). Recent estimates of metabolite heritability of 25–35% are also facilitating future breeding efforts with metabolomics (Fernie and Schauer 2008).

26.5 Nutraceutical Breeding

Addition of a desirable trait, and/or removal of a deleterious trait from a plant are the most important aspects of nutraceutical breeding as several new secondary effects in the organism arises. Therefore, characterizing the dynamics of those secondary effects is important to understand the full impact of new trait (Harrigan *et al.* 2007). Metabolomics is especially useful with respect to elucidating secondary metabolite profiles when developing nutritionally enhanced crops. The presence of gene co-suppression and inherent feedback metabolic loops for maintaining homeostatic nutrient levels in plants pose problems in increasing expression levels of anabolic biosynthetic genes (Tang *et al.* 2007). New techniques for decreasing catabolic enzymes and anti-nutritional compounds are thus required for nutraceutical breeding. RNAi techniques show such potential for developing nutraceuticals by shutting down specific pathways related to the production of toxins and other undesirable substrates (Harrigan *et al.* 2007; Newell-McGloughlin, 2008). Association mapping is another relatively new approach for linking metabolic compounds to a final phenotype (Carreno-Qunintero *et al.* 2013). For example, Tohge and Fernie (2010) provided an elaborated protocol of combining co-response gene analysis to identify target unknown compounds using metabolomics as an extension of association mapping.

26.6 Crop Quality

Crop quality is mainly comprises sensory properties, shelf life, health, safety, and yield, as well as the absence of plant diseases and other pests (Thissen *et al.* 2011). With our broadened information on role of secondary metabolites, many crop quality traits could be assessed in the future by their composition, that is, or the plant's metabolome. Conventional breeding practices face challenges to produce cultivars that possess both higher quality and increased productivity (Hall *et al.* 2008). While wild progenitors are rich in several metabolites, existing cultivars contain lower quantities. The poor nutritional value of existing cultivars is a result of continuous breeding for higher yield, easier processing, and pest resistance (Morris and Sands, 2006). Perez-Fons and coauthors (2014) described this phenomenon of plant breeding as "domestication syndrome". One example of improving nutritional value of current crops using wild relatives is the study conducted with tomatoes (*Solanum lycopersicum*) from (*Solanum pennellii*) by identifying QTLs for health related carotenoids and tocopherols (Perez-Fons 2014).

The identification of new molecular markers for quality traits requires a thorough understanding of the biochemical basis of intricate pathways for metabolites. Quality-enhancing metabolites are often governed by quantitative trait loci, and epistatic interactions make a major contribution to these phenotypic variations (Fernie and Schauer 2008). The diversity in quality-enhancing metabolites in plants, not only originates from genes, but also from divergent, substrate-specific enzymes and sub-cellular, non-enzymatic activities (Hall *et al.* 2002). Many transcription factors and regulatory proteins are known for impacting yield, agronomic, and quality traits (Harrigan *et al.* 2007). Metabolic engineering using in conjunction with information of biochemical pathways, modern biotechnology, molecular tools, and conventional breeding is gaining momentum to deliver the needs. Transcription factors play a major role in secondary metabolite biosynthesis and offer new a potential alternative for nutraceutical breeding through their integration into marker assisted breeding (Davies, 2007; Newell-McGloughlin 2008).

Several plant biochemical pathways cross-talk for effective regulation of cellular processes in an organism. Nevertheless, cross-talk between several biochemical pathways presents several challenges to plant scientists. Considering that a single precursor synthesizes multiple metabolites in a typical plant biochemical pathway, the presence of a precursor does not assure the synthesis of any specific metabolite (Dixon *et al.* 2006). An end product of one pathway becomes the precursor or intermediate substrate for another pathway. Furthermore, a single enzyme affects several biochemical pathways. Therefore, a single point mutation can lead to significant changes in a metabolome (Newell-McGloughlin, 2008). Alteration in one pathway could also potentially change the flux in non-target metabolites (Davies 2007). Forward genetic approaches that utilize pathway based solutions to identify metabolic traits, such as nutritional status, relied on metabolomic composition (Tohge and Fernie 2010). A combination of various plant science disciplines with metabolomics offers other approaches to manage these complex problems and provide pragmatic solutions to develop higher quality crops. An understanding of the plant's metabolome is thus expected to bridge the gap between genotype and phenotype (Harrigan *et al.* 2007).

Allwood and coauthors (2011) described several mainstream and specialized techniques of metabolomic plant analyses. Moreover, Cevallos-Cevallos (2009) provided a list of most common metabolomics approaches used for food analysis; while Carreno-Qunintero *et al.* (2013) summarized the metabolic studies for several crops. It is expected that this enormous amount of information can be utilized to bio-refine current cultivars (by applying selection pressure) and to optimize nutraceutical breeding. Instead of monitoring a single putative bioactive metabolite, hundreds of biosynthetically-related metabolites and enzymes could be analyzed by metabolic profiling; which in turn could be used to develop stable genotypes for nutraceutical cultivars (Dixon *et al.* 2006).

26.7 Metabolomics and Plant Stresses

With global climate changes and rapid urbanization, the majority of our current crops are dealing with abiotic and biotic stresses on an increased level. Higher plants have evolved a series of responses to fight and avoid sundry stresses, such as drought, salinity, low and high temperature, flooding, and so on. (Zhuang *et al.* 2014). However, with increased stress levels the quantity and quality of food products for any crop suffers drastically. The same cultivar of a given crop can exhibit different phenotype responses. These effects are even greater if growing conditions are significantly different (Khakimov *et al.* 2013). It is thus important to develop cultivars that are adaptable to those changes but still maintain their quality attributes, that is, high producers, nutrient rich, safe, tolerant to stress, and resistant to disease. Molecular markers, high throughput genome sequencing, and several omics techniques are being used in combination as a means to develop crops with higher tolerances to stress. Metabolomics as a tool for both abiotic and biotic stresses can elucidate the metabolic network in

response to various stress levels from a comprehensive perspective (Tohge and Fernie 2010; Zhuang *et al.* 2014). Khakimov *et al.* (2013) and Zhuang *et al.* (2014) have provided thorough reviews on recent metabolomics based studies as applied to cereal crops and to vegetable crops, respectively, toward this end.

26.8 Food Safety

Worldwide concern with respect to transgenically-enhanced foods has been attributed to the potential and perceived risks of epistatic transgenes (Schubert 2008). The use of intragenic and famigenic techniques, in conjunction with metabolomics, offers immense potential for nutraceutical breeding, which in turn may garner greater acceptability in the market (Davies 2007; Rommens *et al.* 2007). Several studies conducted during the last decade showed no major changes in food quality or food safety of genetically modified food (Cevallos-Cevallos *et al.* 2009; Putri *et al.* 2013). As an example, transgenes in barley were comparable to no-transgenic barley isolines – as the differences observed between different cultivars were much greater compared to transgenes (Kogel *et al.* 2010).

26.9 Future

Among the many omics-based sciences, metabolomics has a unique distinction for improving the quality of crops, due to its ability to elucidate the cellular phenomenon, and to characterize primary and large number of secondary metabolites, as well as their changes in magnitude in response to physiological, ecological, and developmental stages (Moco *et al.* 2007). Langridge and Fleury (2011) summarized the application of omics technologies to 20 of the most important agricultural crops. With these rapid advancements, developing healthy crops for our growing populations is quickly becoming a necessity. It would be especially beneficial for all those crops, where extensive genomic sequencing and molecular information is not available or inadequate (Hegeaman 2010).

Although basic research on the link between human health and plant based secondary metabolites remains limited; recent scientific discoveries provide evidence of the on the benefits of nutraceutical-rich crop consumption. It is expected that plant breeders will benefit from a system biology approach, with an emphasis on metabolomics, to develop cultivars intended not only to feed the world, but to benefit human health.

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27

Metabolomics and Fetal-Neonatal Nutrition: An Overview

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27.1 Introduction

Human and animal epidemiological studies have provided evidence that nutritional imbalance and metabolic disturbances, during critical time windows of developmental programming, may have a persistent effect on the health of the children and later in adults (Junien 2006). The literature suggests that fetal malnutrition as in the case of intrauterine growth restriction (IUGR) or large for gestational age (LGA), carries an increased risk of developing chronic diseases in adulthood as metabolic syndrome (Dessì *et al.* 2013). Medical research in the last few years has laid the foundations for future developments of personalized treatments aimed at remedying pathological conditions on the basis of subjective characteristics. In the cases of IUGR or LGA, integration of breast feeding and formulas is suggested to be specifically adapted to individual needs, taking into account the long-term consequences, such as the accumulation of inappropriate fat. The development of customized therapeutic approaches for each individual calls for the application of diagnostic systems capable of providing as detailed and complete as possible information on the metabolic state of an individual. In this context, increasing attention has turned to the definition, at the biochemical and molecular levels, of the different pathophysiological states that characterize human beings in the period going from embryonic development to aging and death. To this aim, metabolomics, one of the youngest “omics” sciences, appears a powerful tool capable of describing the chemical profile of a biological system in terms of low-molecular-weight metabolites present in cells, tissues, organs, and biological fluids, and thus to provide in real time the photograph of the metabolic response of the system to pathophysiological stimuli and genetic modifications (Nicholson *et al.* 2002). Recently introduced in the nutrition field, “nutrimetabonomics” has already delivered interesting insights for the understanding of metabolic responses of humans or animals due to dietary interventions and for the definition of metabolic phenotypes (Llorach *et al.* 2013). Therefore, to date, the effects of nutrients on the metabolites generated from endogenous metabolic processes or exogenous dietary nutrients can be studied and the relationship between metabolism and diet can be established. The new holistic metabolomic approach may lead to an early diagnosis of disease, identification of new predictive markers, development of a better personalized medicine and save large amounts of economic resources (Iacovidou *et al.* 2014).

27.2 IUGR and LGA: Fetal Programming

The most widely used definition of IUGR is a fetus that fails to reach its potential growth, whose estimated weight is below the tenth centile for its gestational age and abdominal circumference is below the 2.5th centile. Approximately 70% of fetuses with a birth weight below the tenth percentile for gestational age are constitutionally small; in the remaining 30%, the cause of IUGR is pathologic. Intrauterine growth retardation is associated with a number of endocrine and metabolic changes. Metabolic disturbances in IUGR include hypoglycemia (decreased glycogen stores/gluconeogenesis) and hypocalcemia (idiopathic or glucagon stimulated calcitonin) (Committee on Practice 2001). It defines LGA or macrosoma as a neonate with a birth weight above the ninetieth centile for gestational age. The major risk factors for LGA include maternal diabetes, obesity, and excessive nutrition/weight gain during pregnancy (Ju *et al.* 2009).

Fetal growth retardation is generally thought to be a consequence of inadequate provision of nutritional substrates across the placenta. Fetal pre-exposure to adverse intra-uterine conditions plays a causal role in perinatal mortality and negative metabolic consequences in adulthood. When it comes to fetal nutrition, one refers to the contribution of nutrients needed for proper intrauterine development. Fetal growth may be influenced by several factors, including maternal diet. Under conditions of non-optimal supply of oxygen and nutrients, it can alter the balance between anabolic and catabolic pathways and triggers an effect of programming to the metabolic syndrome. The first to support this hypothesis was Barker according to which, during pregnancy, exposure to factors such as nutrient deficiency or reduction in placental blood flow can have permanent effects on *fetal programming* (Barker 1995; Hales *et al.* 2003); that is, on the structure or function of the organism. The fetus in this adverse condition puts in place a series of adaptive mechanisms to increase the immediate chances of survival such as saving glucose by reducing insulin secretion. This state of insulin resistance is maintained even in the post-natal life, exposing the subject to the risk of developing metabolic diseases in adulthood (Dessì *et al.* 2012). In fact, these adaptations, although useful in the short term, are in contrast with the environmental conditions of extrauterine life, where there is a sufficient supply of nutrients. In the post-natal period, indeed, impaired insulin function is associated with accelerated and excessive growth: precisely the neonates with low birth weight with rapid post-natal growth, are the ones most at risk of developing diabetes, obesity and coronary heart disease in adulthood (Eriksson *et al.* 1999, Morrison *et al.* 2010, Claris *et al.* 2010). The “Barker theory” was later also sustained by other researchers, who found an association between low birth weight (LBW) and the risk of presenting a chronic metabolic pathologies in adulthood (Table 27.1). At first, Barker *et al.* (1989) noted an association between LBW and mortality from coronary heart disease in adults. Later, the small for gestational age (SGA) has been also associated by other authors to metabolic diseases such as obesity, Type 2 diabetes (T2D) and metabolic syndrome. It is interesting to note that, in 1998, Fall *et al.* (1998) hypothesized that the high birth weight (HBW) could also be associated with the risk of developing T2D in adulthood. Subsequently, other studies have supported this hypothesis, claiming that factors such as maternal obesity and diabetes, which leads to glucose intolerance during pregnancy and changes the macrosomic fetus, can result in an impaired glucose tolerance in adult life. Harder *et al.* performed a meta-analysis on the relationship between low/high birth weight and the onset of T2D (Harder *et al.* 2007). They found a nonlinear, U-shaped association between birth weight and T2D, which led to an augmented risk both for high birth weight and low birth weight neonates with respect to their gestational ages.

27.3 Metabolomics in Nutritional Research

The terms *metabolomics* and *metabonomics* are often used interchangeably to indicate a scientific area aimed at identifying and quantifying the *metabolome*; the dynamic set of low molecular weight (typically <1500 Da) metabolites produced by an organism, which are the end products of gene expression (Nicholson and Lindon 2008). Since the metabolome can be viewed as a mirror that reflects the physiological, evolutionary, and pathological state of a biological system, metabolomics allows us to photograph the genome in its interaction with the environment and, thus, to investigate the metabolic status of an organism in determined physiological conditions, as a consequence of drug treatment, environmental influences, nutrition, lifestyle, genetic effects, and so on. The choice of the analytical approach to be used depends on the nature of the project. Basically, three different approaches can be employed: *target methods*, *metabolic fingerprinting*, and *profiling methodologies*. *Target methods* are usually developed for quantification of a single class of compounds, which are isolated with dedicated and optimized methods. *Metabolic fingerprinting* focuses on the total profile, or fingerprint, as a unique pattern characterizing a snapshot of the metabolism in a particular cell line or tissue, ignoring the identification or precise quantification of all the metabolites in the sample. *Metabolic profiling* is used when one is interested in the identification and quantification of a broad class of metabolites.

Table 27.1 Epidemiological studies evidencing a correlation between high and low birth weight and chronic diseases in adulthood. SGA: small for gestational age; LGA: large for gestational age; T2D: type 2 diabetes.

Study	N° Patients (sex)	Type of patients at birth	Country	Associated diseases in adult
Barker <i>et al.</i> (1989)	5654 (M)	SGA	England	Death from ischemic heart disease
Hales <i>et al.</i> (1991)	468 (M)	SGA	England	T2D, hypertension
Barker <i>et al.</i> (1993)	1586 (M)	SGA	England	Death from cardiovascular disease
Phipps <i>et al.</i> (1993)	140 (M)	SGA	England	T2D
	126(F)			
Osmond <i>et al.</i> (1993)	10141 (M)	SGA	England	Cardiovascular disease
	5585 (F)			
McCance <i>et al.</i> (1994)	1179 (M)	SGA	United States	T2D
Martyn <i>et al.</i> (1995)	337 (M&F)	SGA	England	Hypertension
Curhan <i>et al.</i> (1996)	22846 (M)	SGA	United States	T2D, hypertension
Frankel <i>et al.</i> (1996)	1258 (M)	SGA	England	Coronary heart disease
Lithell <i>et al.</i> (1996)	2322 (M)	SGA	Sweden	T2D
Stein <i>et al.</i> (1996)	517 (M&F)	SGA	South India	Coronary heart disease
Rich-Edwards <i>et al.</i> (1997)	121700 (F)	SGA	United States	Non fatal cardiovascular disease
Leon <i>et al.</i> (1998)	14611 (M&F)	SGA	Sweden	Ischaemic heart disease
Fall <i>et al.</i> (1998)	506 (M&F)	LGA	India	T2D
Ravelli <i>et al.</i> (1998)	702 (M&F)	SGA	Amsterdam	Glucose intolerance, obesity
Forsén <i>et al.</i> (1999)	3447 (F)	SGA	Finland	Coronary heart disease
Carlsson <i>et al.</i> (1999)	2237 (M)	SGA	Sweden	Glucose intolerance, T2D
Levitt <i>et al.</i> (2000)	137 (M&F)	SGA	South Africa	Glucose intolerance, hypertension
Dyck <i>et al.</i> (2001)	1366 (M&F)	SGA and LGA	Canada	T2D
Eriksson <i>et al.</i> (2003)	8760 (M&F)	SGA	Finland	T2D
Wei <i>et al.</i> (2003)	1966 (M&F)	SGA and LGA	Taiwan	T2D
Xiao <i>et al.</i> (2010)	2019 (M&F)	SGA	China	Metabolic syndrome
Meas T (2010)	45(M)	SGA	France	Glucose intolerance, metabolic syndrome
	55(F)			

Among many different technological platforms, Nuclear Magnetic Resonance (NMR) spectroscopy and Mass Spectrometry (MS) are the analytical tools mostly used in metabolomics (Pan and Raftery 2007). Both techniques usually generate multiple signals, which is an advantage for metabolite identification and a disadvantage in terms of spectral complexity. NMR can provide uniquely identification and simultaneously quantification of a wide range of organic compounds with relatively simple sample preparation. Furthermore, it is a highly reproducible and non-destructive technique. However, compared with MS, NMR yields relatively low-sensitivity measurements, with limits of detection on the order of 10 μ M or a few nmol at high fields using new cryoprobes. Analysis of samples by MS usually requires the separation of compounds by chromatographic techniques. The one largely employed include gas chromatography (GC), high-performance liquid chromatography (HPLC), and capillary electrophoresis (CE). Two are the most important limitations of MS-based methods: the inability to quantify or identify components that do not ionize and the inherent variation and reproducibility problems associated with any chromatography technique. On the contrary, compared to NMR, MS offers high selectivity and good sensitivity (limits of detection of picomole to femtomole) (Scalbert *et al.* 2009).

In fingerprint analysis, multivariate statistical methods, when combined with either MS or NMR, create enormous opportunities for metabolomic research where they are used as data reduction methods (Trygg *et al.* 2007). Basically, there are three categories of analysis: exploratory, discriminative, and/or predictive. Exploratory analysis, such as principal component analysis (PCA), provides an overview of the metabolite composition of samples, pointing out trends, patterns, or clusters. Discriminant analysis, with *a priori* classification hypothesis, is used to identify the metabolites responsible for the classification of samples. This includes partial least squares discriminant analysis (PLS-DA) and soft independent modeling of class analogies (SIMCA). Predictive models are used to find a quantitative relationship between two data matrices, X and Y. These models are usually produced by PLS regression.

Over the past decade, metabolomics has emerged as a promising approach mainly in the field of human diseases. More recently, due to the interactions between metabolome with commensals and symbiotic partners such as gut microbiota, and environmental and behavioral factors including food preference, metabolomics has proven to be an useful tool also in the nutrition research, taking the name of *nutrimetabolomics* or *nutritional metabolomics*, that is the study of the human or animal metabolome as a function of nutritional status or as a function of a nutritional challenge (Rezzi *et al.* 2007). Nutrimetabolomics, together with nutrigenomics, is the foundation on which personalized diets are shaped and planned: through the study of the metabolome, it is possible to assess changes induced by diet in gene expression and thus, by modifying the nutriments and biomolecules assumed in the diet, we can intervene in the interaction between nutrients and the human metabolism to reach and maintain the best state of health. Thanks to metabolomics, it appears to be possible to assess an individual's state of nutrition to understand how single nutrients influence metabolic regulation and, thus, to formulate personalized diets which, if followed at an early age, may prevent the onset of certain chronic diseases such as diabetes, inflammatory diseases and obesity. Nutrimetabolomics appears a promising technique also in pediatric and neonatal research studies. Here, comprehension of changes in metabolic profiles during one's lifespan, starting from the earliest stages, may represent an important point of reference in arriving at an understanding of their fundamental mechanisms and their consequent metabolic alterations.

27.4 Nutrimetabolomics in Animal Models

In recent years, rodent and non-litter bearing animal species, such as rats, mice, sheep, monkeys, and pigs, have become the most frequently used animal models for SGA and fetal programming of adult disease (Vuguin 2007). Indeed, since these animals are mammalian systems with embryology, anatomy, and physiology similar to humans, they can mimic the human IUGR condition. The use of these animal models has, so far, provided observations comparable to that observed in humans born small for gestational age, leading to a greater understanding of the pathophysiology and consequences of intrauterine growth retardation.

Notably, the pig exhibits the most severe naturally occurring IUGR among domestic mammals due to placental insufficient. Metabolomic studies on pigs with IUGR have provided important contributions to the use of novel biomarkers for fetal programming. Twenty-four plasma samples from two experimental groups, LBW and HBW piglets, were analyzed by Nissen *et al.* using NMR and GC-MS (Nissen *et al.* 2011). Piglets were taken from the uterus at day 110 of gestation in order to study piglets close to birth, but at the same time ensuring that they did not suckle and thereby ingest food. PCA, performed on the ¹H NMR spectra, evidenced a clear separation between the metabolic profiles of plasma from the two groups. In addition, the PLS model performed on the same data set evidenced a clear positive relationship between glucose concentration in plasma and birth weight and a negative relationship of myo-inositol plasma concentration both with birth weight and the naturally occurring pig model of IUGR. The semi-quantitative plasma analysis by GC-MS confirmed that LBW piglets have a significant higher concentration of both myoinositol and D-chiro-inositol than HBW piglets. Since myoinositol and D-chiro-inositol are coupled with glucose intolerance and insulin resistance in adults, the present results suggested that IUGR is related to impaired glucose metabolism during fetal development, which may cause T2D in adulthood.

By using a pig model and MS technique, Lin *et al.* showed that disorder of nutrients (such as glucose, lipids, and amino acids) and energy metabolism as well as endocrine imbalance may contribute to fetal growth restriction (Lin *et al.* 2012). A comparison between metabolomes of umbilical vein plasma between normal birth weight (NBW) and IUGR fetal pigs at different gestational stages (90 and 110 days) was performed by the authors. Complementary information was also gained from the analyses of hormones, amino acids, and related metabolites using assay kits and chromatographic methods. The PC analysis of the MS data set showed excellent separations between IUGR and NBW piglets. Concentrations of many metabolites were markedly different between the two groups. For instance, compared to the NBW group, IUGR fetuses displayed lower levels of arginine, glutamine, and dodecanoylecarnitine but higher levels of pyroglutamic acid both at day 90 and 110 of gestation. On day 90, concentrations of phenylalanine and tyrosine, valine, leucine, isoleucine, and proline were lower in IUGR umbilical vein plasma than in the NBW group. In contrast, increases in carnitine and creatinine and a decrease in tryptophan were noted in IUGR fetuses compared with the NBW group on day 110 of gestation.

Maternal low protein (MLP) models of fetal programming are extensively used to study the mechanisms that link maternal nutrition with impaired fetal growth and later cardiovascular disease and diabetes. A direct comparison between a maternal adequate protein (MAP) diet and isocaloric MLP diet in relation to their influence on the metabolism in early life of rat (until weaning) and once they reached adulthood (8 months) has been recently performed by a metabolomic

approach (Gouabau *et al.* 2011). To this aim, Liquid Chromatography Tandem Mass Spectrometer (LC-HRMS) was used to analyze the plasma metabolomic fingerprints of male offspring of rat dams exposed to MAP diet during pregnancy and lactation (CC) or an isocaloric MLP diet during pregnancy only (IUGR with rapid catch-up growth, RC) or through pregnancy and lactation (IUGR with slow postnatal growth, RR). A total number of 126 pups were recruited at six postnatal ages (0, 5, 12, 16, 22, and 260 days of life). The findings evidenced a dependence of the long-term deregulation in feeding behavior and fatty acid metabolism in IUGR rats on postnatal growth velocity. Indeed, compared to CC rats, RR pups had clear-cut alterations in plasma metabolome during suckling, but none at adulthood; interestingly, in RC pups, alterations in metabolome were minimal in early life but more pronounced in the long run. In particular, alterations in proline, arginine, and histidine in RR rats were observed, compared to CC rats, and persistent differences in tyrosine and carnitine, compared to RC rats at adulthood. A link between these results and the expression of proteins collected from the same plasma samples was later investigated by the same authors using different statistical strategies (Moyon *et al.* 2011), pointing out the importance of combining metabolomics and proteomics data to understand the complex relationship between nutrition and metabolism.

The impact of effects of fetal undernutrition, alone or in combination with postnatal hypernutrition, on metabolic pathways was investigated by Nyberg *et al.* (2010) in a sheep model (Nyberg *et al.* 2010). During the prenatal period, one control group of animal was treated according to normal nutritional requirements, while another group was exposed to feed restriction during the last 8 weeks of gestation. Urine was sampled from 2, 6, 19, and 24-month-old lambs and analyzed by ¹H NMR spectroscopy. During the first 6 months of the post-natal period, half of the lambs in each group received differential dietary treatments: one subgroup followed a conventional diet, while the other received a high carbohydrate high fat (HCHF) diet. Later, all animals followed the same normal diet. The PCA model of ¹H NMR spectra provided useful information about the nutritional status of the animals and rumen development. A clear V-shaped metabolic trajectory was observed as a function of age and diet. In particular, the separation between groups fed with different diets in early postnatal life (HCHF vs Conv) was clear at the two first sampling periods (2 and 6 months of age). At the later stages of life, when the animals had received the same diet for 6–18 months, there was no observable carryover of effects from different dietary exposures earlier in life. Urines at the beginning of the metabolic trajectory were characterized by higher levels of glucose, indicative of animal with a monogastric, non-ruminant like digestion, while, at the other edge of the V-curve, hippuric acid provided the most prominent contribution to the metabolic profile. These differences were interpreted in terms of development of the ruminant mode of digestion depending on microbial flora, and the effect of HCHF diet bypassing the rumen. Furthermore, urines from 2-month-old animals exposed to prenatal undernutrition followed by normal postnatal diet showed metabolic patterns that were ahead in time on the metabolic trajectory relative to the prenatal control group. No long-term effects of fetal undernutrition, alone or in combination with postnatal hypernutrition were observed.

In rabbit, as well as in humans, the temporal pattern of oligodendrocyte maturation, myelination and functional changes occur most rapidly during the perinatal period, starting several days before birth and continuing during the postnatal period. Therefore, rabbits provide a relevant and useful model to study perinatal processes of brain development. To investigate if IUGR leads to metabolic differences in the fetal rabbit brain, a targeted metabolomic analysis was performed on control and IUGR fetal brain tissues of rabbits by using liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) (Van Vliet *et al.* 2013). Seventy-eight metabolites were identified, among which 18 were found to be significantly different between the two groups. In particular, comparing to the control group, IUGR fetal brain tissue samples were characterized by lower levels of neurotransmitters/peptides, amino acids, fatty acids, energy metabolism intermediates and oxidative stress related metabolites, and higher levels of fatty acid. Overall, from a clinical perspective, this study provided preliminary evidence supporting further research on the development of metabolic biomarkers associated with growth restriction and its neurodevelopment. In particular, these results supported the role of N-acetylaspartylglutamic acid (NAAG) and N-acetylaspartate (NAA) on an abnormal neurodevelopment in IUGR, thus revealing the potential of these biomarkers for the perinatal diagnosis of IUGR. Regarding the clinically relevance of the composition of lipid and oxidative stress related metabolites in the fetal brain, further studies should be programmed to have a more comprehensive understanding of their role.

27.5 Nutrimetabolomics in Human Models

Nutrimetabolomics in pediatric models has been so far applied to two types of biological fluid: cord blood and urines. On one side, analysis of umbilical cord blood soon after birth allowed the exploration of the mother–fetus interaction, while, on the other, urine metabolomics provided novel insights into metabolic abnormalities experienced by IUGR infants.

A few metabolomic analysis of cord blood has been carried out on samples collected from LBW newborn aimed at identifying deregulated metabolites as biomarkers of IUGR. Horgan and coworkers (Horgan *et al.* 2011) examined metabolomic profiles using ultra performance liquid chromatography-mass spectrometry (UPLC-MS) in three independent studies: (1) venous cord plasma from normal and SGA babies, (2) plasma from a rat model of placental insufficiency and controls, and (3) early pregnancy peripheral plasma samples from women who subsequently delivered a SGA baby and controls. The authors observed that 19 metabolites (including sphingolipids, phospholipids, and carnitines) differentiate SGA from controls, providing a base for a valid screening test for presymptomatic SGA or fetal growth restriction. Later, Ivorra *et al.* analyzed the NMR metabolomic profile of plasma from umbilical cord of LBW and NBW newborns (Ivorra *et al.* 2012). For the sake of comparison, the metabolomic profiles of their mothers were also analyzed. Seven metabolites were able to discriminate a specific LBW metabolome: LBW newborns had lower levels of choline, proline, glutamine, alanine, and glucose than did the control newborns, while plasma levels of phenylalanine and citrulline were higher in LBW newborns. No significant differences were found between the two groups of mothers. Tea and coworkers (2012), analyzing the cord blood from very-low birth weight (VLBW) infants, demonstrated that a number of metabolites (glucose, acetate, lipids, pyruvate, glutamine, valine, and threonine) vary depending on gestational age at delivery. In the study by Gouabau *et al.* (2013), samples of umbilical venous, umbilical arterial, and maternal blood from mothers delivering VLBW premature or full-term (FT) neonates were analyzed by LC-HRMS. The findings revealed alterations in energy and polyamine metabolism, and oxidative stress in VLBW infant. In particular, a significant elevation in the levels and maternal-fetal gradients of butyryl-, isovaleryl-, hexanoyl-, and octanoyl-carnitines was observed, suggesting enhanced short- and medium chain fatty acid β -oxidation in human preterm feto-placental unit. The significant decrease in glutamine-glutamate in preterm arterial cord blood, together with lower levels of amino acid precursors of Krebs cycle, suggested increased glutamine utilization in the fast growing tissues of preterm fetus with a deregulation in placental glutamate-glutamine shuttling. An increase in both the circulating levels and maternal-fetal gradients of several polyamines in their acetylated form (diacetylspermine and acetylputrescine) suggested an enhanced polyamine metabolic cycling in extreme prematurity.

Evident modifications between the metabolic profile of serum from cord blood of IUGR and appropriate for gestational age (AGA) fetuses were also reported in the study by Favretto and coworkers (2012). By a non-targeted LC-HRMS analysis, the authors observed changes in 22 metabolites with the most evident modifications in the following aminoacids composition: phenylalanine, tryptophan, and glutamate. Cut-off values for phenylalanine and tryptophan were identified.

Dessì *et al.* have analyzed the ^1H -NMR urine metabolic profiles of neonates with IUGR and compared them with controls to define the metabolic patterns associated with this pathology (Dessì *et al.* 2011). Twenty-six neonates with IUGR diagnosed in the neonatal period and with weight at birth below the tenth percentile were recruited for the study together with 30 neonates of proper gestational weight at birth (controls). Urine samples were taken in the first 24 h (prior to feeding) and about 4 days after birth. The PLS and PLS-DA model performed on the NMR data highlighted significant differences between the two groups. Among the metabolites responsible for these differences, myo-inositol was found to be present in higher level in IUGR neonates. This result was in good agreement with the study carried out on piglets (Nissen *et al.* 2011) as well as with the data in the literature (Kennington *et al.* 1990; Ostlund *et al.* 1993). Furthermore, also the urine concentrations of creatine, creatinine, and sarcosine in IUGR neonates was found to be significantly higher compared to controls.

Recently, Moltu *et al.* have explored the urinary metabolite profiles of 48 premature infants randomized to an enhanced or a standard diet during neonatal hospitalization (Moltu *et al.* 2014). Substantial changes in the urinary NMR profiles of all infants were observed during the early postnatal period that correlated with gestational age at birth and with chronological age. Furthermore, the analysis of the metabolic differences between SGA and AGA newborns at birth and over time, evidenced glycine and threonine as potential biomarkers of an altered metabolic phenotype. Differently, any significant difference between the metabolic trajectories with regard to the two different diets was observed.

27.6 Conclusions

Even though the impact of poor fetal growth on the development of adult disease is well documented, the mechanisms underlying this physical predisposition have not been well characterized yet. Undoubtedly, more knowledge of the perinatal and neonatal maturational processes and their metabolic background would help the management of IUGR and LGA neonates as well as the prevention of the long-term adverse effects of fetal malnutrition. Metabolomics is predicted to provide significant contributions to nutrition science in neonatology, particularly promising in the monitoring of metabolic postnatal maturation and the identification of new biomarkers as early predictors of outcome, diagnosis and monitoring of pathological conditions such as IUGR and LGA.

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28

Metabolomics, Bioactives, and Cancer

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28.1 Introduction

While metabolite identification and monitoring is not a new concept, untargeted, comprehensive metabolite analysis, commonly designated metabolomics, has only become possible in recent years (Fiehn 2002). For decades, biochemists have evaluated metabolites found in blood, urine, and tissue samples to understand pathway regulation, diagnostics of disease, and cellular response to treatments (Fernie *et al.* 2004; Tiziani *et al.* 2008). In fact, with the proper tools, metabolites can provide insight into processes occurring at the subcellular, cellular, tissue, organ, and organism level (Fiehn 2002; Forcisi *et al.* 2013). Historically, the instrumentation and computational technologies available have meant that it was only possible to measure a small number of metabolites or classes of metabolites that were determined *a priori* (Gibney *et al.* 2005; Kell, 2004). As a consequence, metabolite identification gained little attention as scientists pursued greater understanding of biological processes through identification of genetic mutations and later protein activity (Joyce and Palsson 2006). The complete sequencing of the human genome initiated a systems biology approach to research and “omics” disciplines were born. Metabolomics is the newest of the omics (Beger 2013; Joyce and Palsson 2006; Schuhmacher *et al.* 2013). In 1999, Nicholson and colleagues proposed the term *metabonomics*, which they defined as “the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification” (Nicholson *et al.* 1999). Shortly after, Oliver Fiehn presented a simpler and broader alternative, describing *metabolomics* as the “...comprehensive analysis in which all the metabolites of a biological system are identified and quantified” (Fiehn 2002). Metabolomics has become the more universally accepted term when addressing the complexity of comprehensively evaluating metabolites (Gibney *et al.* 2005). Great efforts have been made to create libraries of complete genomes, proteomes, transcriptomes, and metabolomes in order to provide standards for comparison (Jones *et al.* 2012; Ludwig *et al.* 2012; Madhavan *et al.* 2014). Furthermore, future integration of libraries will elucidate biological perturbations across all levels of expression (Bino *et al.* 2004). Much work still remains, for the metabolome in particular, as it is by far the most complex. Moreover, consensus regarding reporting standards and analytical methods are still under development (Kell 2004; Kind and Fiehn, 2006; Madhavan *et al.* 2014; Patti

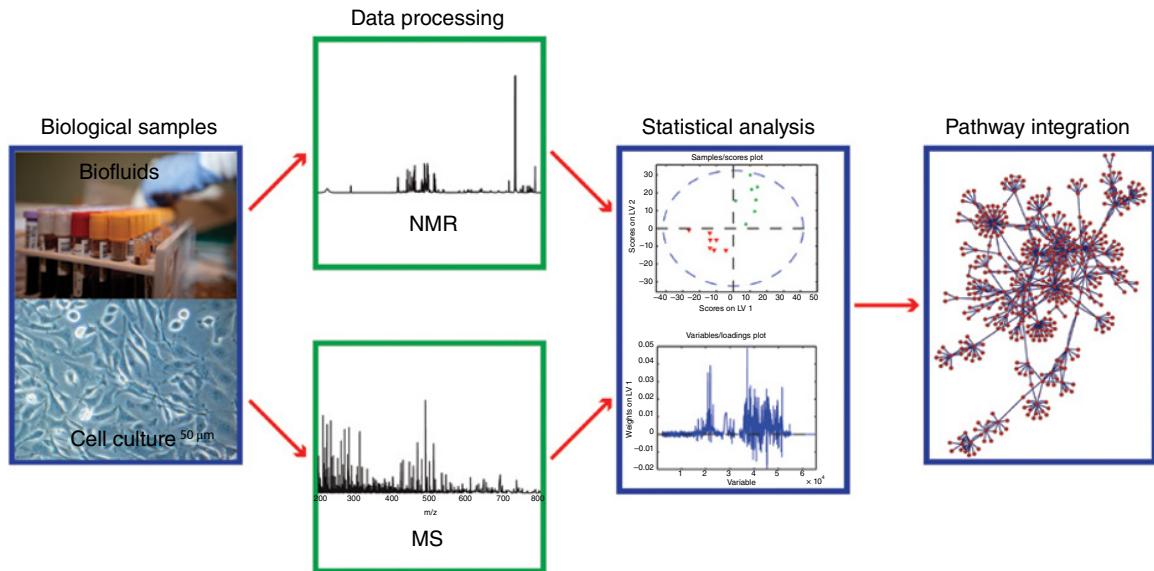


Figure 28.1 Metabolomics workflow schematic highlighting the parallel application of both NMR and MS to capture a more comprehensive picture of the metabolome.

et al. 2012). The number of metabolites in mammalian samples is enormous and mostly unknown, with estimates ranging from a few thousand to hundreds of thousands of small molecules, depending on the source (Fernie *et al.* 2004). Combining several platforms, 4229 and 2651 metabolites have been identified in the human serum and urine metabolomes, respectively (Bouatra *et al.* 2013; Psychogios *et al.* 2011). Even more metabolites are detectable in both serum and urine, but currently remain unidentified (Kell, 2004; Patti *et al.* 2012). Furthermore, the chemical diversity of the metabolome makes it virtually impossible to comprehensively measure every metabolite with a single platform (Brennan, 2013; Fernie *et al.* 2004; Kell, 2004; Schuhmacher *et al.* 2013; Saito and Matsuda, 2010). Instead, platforms should be applied in concert to provide complementary data sets (Beckonert *et al.* 2007). Advancements in nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) instrumentation have contributed most significantly to the development of metabolomics (Beckonert *et al.* 2007; Gibney *et al.* 2005; McNiven *et al.* 2011). In addition, because these instruments generate large amounts of data, in some cases tens of thousands of data points in a single scan, advanced computation and statistical methods have made it possible to address the complexity and size of the information generated (Saccetti *et al.* 2014; Patti *et al.* 2012; Zhang *et al.* 2010). Figure 28.1 provides a visual representation of a typical metabolomics workflow integrating NMR and MS data processing, statistical tools, and biological modeling.

28.2 Nuclear Magnetic Resonance Spectroscopy

High-resolution NMR spectroscopy is probably the most commonly used analytical technique for metabolomics research (Tiziani *et al.* 2011; Whitfield *et al.* 2004; Wishart, 2008). NMR, also known as MRS (magnetic resonance spectroscopy) (Tiziani *et al.* 2013), takes advantage of the inherent magnetic properties of certain nuclei (Emsley and Feeney 1995). In particular, nuclei that have a nuclear spin, I , equal to $\frac{1}{2}$ are easily detected with NMR spectroscopy. Common spin $\frac{1}{2}$ nuclei include ^1H , ^{13}C , ^{15}N , and ^{31}P ; the most abundant and widely used in metabolomics is $^1\text{H-NMR}$. Under normal conditions nuclear spins are completely random and, therefore, do not lead to bulk magnetic properties. However, when subjected to a strong, static magnetic field, typically denoted B_0 , the nuclear spins will align and precess around the magnetic field at a characteristic frequency, known as their Larmor frequency (Levitt 1997). Application of a much weaker, transient oscillating radio frequency (RF) pulse results in the excitation of the nuclei (Kovacs *et al.* 2005). Pulses are typically applied perpendicular to the static magnetic field, B_0 , which changes the orientation of the magnetization. The removal of the RF pulse causes the excited nuclear spins to relax according to their T_1 , spin-lattice, and T_2 , spin-spin relaxation times. T_1 is a

measure of the time it takes for the spins to return to their lower energy states and T_2 is a measure of the loss of coherence of the spins that occurs after the removal of the RF pulse (Fullerton *et al.* 1982). This relaxation is observed as a free induction decay (FID). After collection, the FID can be Fourier transformed to convert the signal from the time domain into the frequency domain, resulting in a classic NMR spectrum, typically given in ppm.

The NMR spectrum contains a wealth of information including the structure and abundance of the molecules present. The chemical shift of peaks in an NMR spectrum depends on the local environment the nucleus experiences (Emsley and Feeney 1995). Nuclei that have greater electron densities will be found at lower ppm, whereas electron deficient nuclei will be shifted to higher ppm (Arnold *et al.* 1951). Furthermore, sensitive nuclei can couple with neighboring nuclei to create a multiplet instead of a single peak (Emsley and Feeney 1995). This phenomenon, known as J-coupling, is due to overlapping electron orbitals and can be used for structural identification (Sattler *et al.* 1999; Wilkens *et al.* 2001). Due to these characteristic properties, NMR was initially utilized as a powerful analytical chemistry tool to elucidate molecular structure (Emsley and Feeney 1995, 2007). In fact, it is still used extensively for protein structural and functional analysis (Grzesiek *et al.* 1995). In addition, NMR peak areas are directly proportional to the number of sensitive nuclei making it an inherently quantitative technique. Spiking with a single standard compound, usually TSP (Trimethylsilyl propanoic acid) or DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid), at a known concentration is sufficient to correct chemical shift and extrapolate the concentrations of all the identifiable metabolites (Wishart 2008).

NMR spectroscopy is one of the most extensively used analytical techniques for metabolite identification because it is so well suited for molecular analysis. The application of NMR to biological samples was first explored by Nicholson *et al.* in the late 1990s (Lindon *et al.* 1999). It is particularly well suited for an omics approach because it is naturally untargeted and thus, completely unbiased (Wishart, 2008). If a metabolite is present at an adequate concentration, it will be detected. Furthermore, relatively minimal sample preparation is required (Whitfield *et al.* 2004; Wishart, 2008). This results in highly efficient and reproducible sample preparation (Beckonert *et al.* 2007). As a result, NMR is extremely reliable and ideal for absolute quantification of metabolites (Whitfield *et al.* 2004; Zhang *et al.* 2010). Because NMR spectroscopy is inherently untargeted, it allows for the identification of metabolites of interest and those outside hypothesized outcomes. As our knowledge of pathway redundancy and interconnections continues to expand, these types of analyses are becoming more and more critical for understanding biological data.

Sensitivity is the only major limitation of NMR spectroscopy (Kovacs *et al.* 2005; Wishart, 2008). Advances in superconducting material, magnet strength and quality, cryogenic probe technology, and pulse sequences have vastly improved sensitivity (Emsley and Feeney, 2007; Kovacs *et al.* 2005; Wishart, 2008). Superconducting materials and improvements in magnetic field homogeneity have created extremely powerful instruments. There are now magnets that can generate a magnetic field of 23.5 Tesla, which gives a resonance frequency of one gigahertz (GHz) (Bhattacharya 2010). Improved pulse sequences and pulsed-field gradients, used to select for desired signal have significantly enhanced signal quality (Emsley and Feeney 2007; Hurd 1990; Price 1997). Progress in water suppression techniques, such as excitation sculpting, have also improved NMR spectra (Hwang and Shaka 1995). Furthermore, advances in probe quality and size have improved excitation profiles and sensitivity, and decreased sample volumes (Kovacs *et al.* 2005). Typically, NMR requires rather large sample volumes, in the order of 200 μ l, but newer probes can obtain high quality signal with as little as a 30 μ l or less (Molinski 2010). While these volumes may appear small, they are relatively large compared to other commonly used metabolomics instruments. Also, because NMR is a nondestructive process, there is the potential for sample recovery and use in additional analyses. Nonetheless, spectra are still dominated by higher abundance metabolites and complex samples often require some degree of deconvolution to identify less abundant molecules (Wishart 2008). Additionally, because no separation techniques are employed, lower abundance metabolites may be undetectable due to overlap with more abundant molecules.

28.3 Mass Spectrometry

In the simplest terms, all mass spectrometers work by manipulating electromagnetic fields to apply a force to an ionized particle. Because the force being applied is dependent on both the mass and the charge of the molecule, mass analyzers are designed to separate ions by their mass-to-charge ratio (m/z). While NMR spectrometers primarily vary in the magnetic field strength and the properties of the probe, there is a great deal of diversity in mass analyzers. Because each have their strengths and weaknesses, they can be application specific depending on the desired properties. Common mass analyzers utilized in metabolomics research include: Triple Quadrupole (QqQ-MS), Time of Flight (TOF-MS), Quadrupole Time of Flight (QTOF-MS), Fourier Transform Ion Cyclotron Resonance (FT-ICR/MS), and Orbitrap (Forcisi *et al.* 2013).

Quadrupole mass analyzers function similar to a filter, only stabilizing a selected mass-to-charge ratio that will reach the detector. On the other hand, time of flight mass analyzers separate ions based on the time it takes to reach the detector. FT-ICR/MS and Orbitrap mass analyzers trap and manipulate ions, which provides the highest mass accuracies, whereas others are preferred because of their rapid scanning rates (Forcisi *et al.* 2013).

Although direct injection methods, often called “shotgun” techniques, are used in metabolomics, more often mass spectrometers are coupled with either gas (GC) or liquid chromatography (LC), especially ultra-high performance liquid chromatography (UPLC or UHPLC), to enhance chemical separation of complex biological matrices (Forcisi *et al.* 2013; Oresic 2009). This adds an additional layer of diversity to developing and applying methods to MS. Although GC-MS was developed and gained popularity before LC-MS, the latter has been shown to be effective over a greater number of metabolites and can separate a broader diversity of compounds, whereas GC-MS is primarily limited to volatile molecules or requires derivatization of non-volatile compounds (Kell 2004). LC-MS requires an interface between the chromatography column and the mass analyzer because the eluted metabolite must be ionized and converted into the gas phase before detection. There are three common interfaces: atmospheric pressure photo ionization (APPI), atmospheric pressure chemical ionization (APCI), and pneumatically assisted electrospray ionization (ESI). Of these, ESI is by far the most commonly used in metabolomics analyses and across other disciplines (Forcisi *et al.* 2013; Oresic 2009).

To add another layer of complexity, mass spectrometers can be used in tandem (MS/MS) to positively identify ions by their fragmentation pattern. The first MS isolates ions and the second MS fragments the ions in order to positively identify metabolites by their characteristic fragmentation pattern. However, if the sensitivity of the first MS is high enough, a compound can be identified by its accurate mass alone and tandem MS/MS experiments are unnecessary.

One advantage of many mass spectrometers is sensitivity. In fact, the impressive sensitivity of MS makes it an ideal complement to NMR. In addition, lipid analysis, which is difficult with NMR, is possible with MS, making it an extremely valuable technique for lipidomics. However, MS has its own set of challenges. Aside from the great diversity in mass analyzer types and coupled techniques, sample preparation is often tedious and can result in significant metabolite loss (Whitfield *et al.* 2004). Furthermore, chromatography column selection, elution solvents, and analysis time all introduce limitations for untargeted analysis and can lead to undesired matrix effects (Kell 2004). While it is challenging for single MS experiment to capture all the detectable metabolites in any given sample, its remarkable sensitivity and ability to separate overlapping metabolites makes it a valuable technique. Finally, mass spectrometry is not well suited for absolute quantification due to variable ionization efficiencies (Whitfield *et al.* 2004). For this reason, internal standards labeled with stable isotopes are required to accurately quantify metabolites. Although robust, internal standards introduce additional expenses in the form of both financial cost and time for sample preparation (Whitfield *et al.* 2004).

28.4 Application of Scientific Computing and Data Analysis

Advances in computer programming and processing speeds have made untargeted metabolite analysis possible (Gibney *et al.* 2005; Schuhmacher *et al.* 2013). For example, a single NMR spectrum can contain more than 64,000 complex data points (Ludwig *et al.* 2012; Tiziani *et al.* 2006). Similarly, LC-MS is able to separate and detect thousands of metabolite peaks from a single complex sample (Beckonert *et al.* 2007; Kell 2004; Zeisel 2007). Without computers and software capable of processing this information, it would be impossible to advance the field of metabolomics.

Multivariate analysis can be applied to multiple spectra for pattern recognition (Gibney *et al.* 2005). These methods fall under two broad categories, unsupervised and supervised, the most common of which are principal component analysis (PCA) and partial least squares discriminant analysis (PLS), respectively (Whitfield *et al.* 2004; Zhang *et al.* 2010). Both are powerful statistical tools that can be used to observe global differences between groups (Bylesjo *et al.* 2006; Whitfield *et al.* 2004). Supervised and unsupervised methods analyze entire spectra to evaluate statistical trends without identification of individual metabolites. PCA is typically considered more powerful than PLS because it is completely unbiased and is usually applied first to determine if inherent grouping exists within the data (Brennan 2013). On the other hand, with PLS, the groups are predetermined and a model is developed based on the key characteristics of those groups to create a predictive model (Brennan 2013, Mahadevan *et al.* 2008). A related supervised method, orthogonal PLS (O-PLS), is also common in metabolomics research (Brennan 2013; Bylesjo *et al.* 2006; Westerhuis *et al.* 2010). In order to avoid bias from higher abundance metabolites, it is important to apply mean center and autoscaling functions prior to evaluation with either supervised or unsupervised methods (Jones *et al.* 2012). Furthermore, care must be taken to avoid overfitting the data when applying supervised methods to prevent false positive results (Manach *et al.* 2009).

Metabolite identification is one of the biggest challenges in metabolomics (Patti *et al.* 2012; Zeisel, 2007). Progress in the development of metabolome databases and libraries has significantly improved identification of metabolites detected by either NMR or MS (Kind and Fiehn 2006; Madhavan *et al.* 2014). The three most comprehensive mammalian databases are the Human Metabolome Database (HMDB), LIPID MAPS, and HUMANCYC (Madhavan *et al.* 2014). There are also several smaller databases freely available online specific to different biological fluids or techniques (Bouatra *et al.* 2013; Ludwig *et al.* 2012; Psychogios *et al.* 2011). Frequently, the majority of compounds detected are not present in any database (Kell 2004; Patti *et al.* 2012). To alleviate this deficiency, the Metabolomics Standards Initiative (MSI) has been formed and proposes thorough minimum reporting standards to maintain consistency between laboratories employing different instruments and methods (Sumner *et al.* 2007).

28.5 Metabolomics, Bioactive Food Components, and Cancer

The application of metabolomics to nutrition offers a unique advantage, specifically by globally monitoring metabolism across all levels (Whitfield *et al.* 2004). Nevertheless, it should be done with great discretion and forethought, to ensure useful and meaningful results (Gibney *et al.* 2005). Furthermore, many factors, including age, gender, lifecycle stage, metabolite turnover, and microbiome metabolism can contribute to the complexity and challenge of generating meaningful nutritional metabolomics data (Jones *et al.* 2012; Gibney *et al.* 2005; Manach *et al.* 2009; Medina *et al.* 2013; Whitfield *et al.* 2004). Despite the many sources of variation and sometimes subtle effects of dietary intake, nutritional metabolomics studies have already yielded promising results. Identification of exogenous metabolite biomarkers have been successfully correlated with dietary intake of a number of foods including vegetables, fruit, tea, coffee, red meat, fish, and whole grains (Brennan 2013; Medina *et al.* 2013). These markers provide useful information about dietary consumption and are more reliable than currently used self-reporting methods (Medina *et al.* 2013). However, metabolites are more than indicators of dietary intake. Various metabolites have broad and complex influences on multiple cellular processes. These bioactive food components are large groups of metabolites that can be derived from a number of exogenous and endogenous sources (Manach *et al.* 2009). Bioactive food components can be categorized as nutrients, including, carbohydrates, lipids, and proteins/amino acids. They can also be classified as non-nutrients, including, polyphenols, terpenoids, alkaloids, and other nitrogen containing compounds (Manach *et al.* 2009). Non-nutrient compounds are frequently termed phytochemicals because of their abundance in plants (Surh 2003).

Understanding the relationship between diet, health, and disease is of critical importance for nutrition research. Many diseases are the result of poor diet and/or metabolic dysregulation (McNiven *et al.* 2011; Medina *et al.* 2013). Obesity is associated with diabetes, cardiovascular disease, and increased cancer risk and malignancy (Ford *et al.* 2002; Hursting *et al.* 2007, 2012). Cancer is, in part, a disease of altered cellular metabolism (Hanahan and Weinberg 2011), which makes metabolomics perfect for early detection, biomarker identification, and treatment success. Ease and speed of analysis also make it an ideal tool for these applications (Beger 2013). Many successful metabolomics studies have already identified biomarkers and potential targets for disease treatments. For example, acetylcarnitine has been identified as a novel serum marker for active multiple myeloma (Lodi *et al.* 2013). Metabolic signatures have also successfully predicted: malignancy in breast, ovarian, and prostate tissues, differentiated brain tumor type, and stage of oral cancer progression (Spratlin *et al.* 2009; Tiziani *et al.* 2009). Similarly, unique saliva metabolite profiles were used to accurately predict the probability of oral, breast, and pancreatic cancers (Sugimoto *et al.* 2010). In addition to tissues, serum, and saliva, potential cancer biomarkers have been identified in urine and breath via metabolomics analysis (Beger 2013). These biomarkers are not only indicators of disease, but may also provide insight into the aberrant processes occurring in malignant cells. Consequently, identification of dietary components that influence these pathways can provide novel targets for disease interventions.

While obesity and poor diet are associated with worse outcomes, calorie restriction is linked to increased longevity, reduced risk of disease, and tumor inhibition (Hursting *et al.* 2003). Many phytochemicals display calorie restriction mimetic properties and are also associated with longevity and decreased incidence of age-related chronic diseases, including obesity, diabetes, Alzheimer's disease, and cancer (Chiba *et al.* 2010; Si and Liu, 2014; Timmers *et al.* 2011). Moreover, epidemiological studies have correlated both vegetarian and Mediterranean diets with decreased risk of many diseases, including cancer and cardiovascular disease, and all-cause mortality (de Lorgeril *et al.* 1999; Dwyer 1988; Key *et al.* 1999; Knoops *et al.* 2004). Additionally, diets rich in fruits and vegetables are associated with overall improved health (Manach *et al.* 2009). One reason may be the abundance of phytochemicals present in these foods and their ability to inhibit metabolic dysregulation.

The application of metabolomics to the field of nutrition is still relatively new and research is ongoing to identify the metabolic relationship between nutritional components and health status (Gibney *et al.* 2005; Trujillo *et al.* 2006). Although many studies have successfully employed nutritional therapies for disease prevention and treatment, these relationships have not yet been explored at the metabolite level (Beger, 2013; Wang and Chen, 2013). Here we discuss a few bioactive food components that have notable potential as chemopreventive and cancer therapeutic agents.

28.5.1 Resveratrol

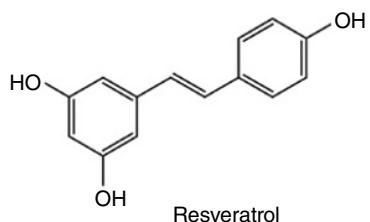
In recent years, resveratrol has received a lot of research attention as a potential explanation for the “French Paradox” (Boocock *et al.* 2007; Kammerer *et al.* 2004; Vitaglione *et al.* 2005). Resveratrol, shown in Figure 28.2(a), is a phytoalexin present in the skin of grapes that is found in especially high concentrations in wine (Jang *et al.* 1997; Jeandet *et al.* 1995; Kammerer *et al.* 2004). It has long been thought to be cardioprotective, but recent evidence suggests anticancer activity as well (Baur and Sinclair 2006; Surh 2003). Cardioprotective properties are likely from a combination of decreases in platelet aggregation, atherosclerosis, lipid peroxidation, serum triglycerides, and cholesterol and an increase in vasorelaxation (Baur and Sinclair 2006). Additionally, resveratrol appears to be able to affect cancer cells at three stages; initiation, promotion, and progression; though an overall mechanism is still unclear (Baur and Sinclair 2006; Espin and Wickers 2000; Jang *et al.* 1997; Surh *et al.* 2001). What is clear, however, is that resveratrol prevents activation of NF- κ B via inhibition of the protein kinase C (PKC) signaling cascade and suppression of I κ B phosphorylation and degradation, which both keep NF- κ B sequestered in the cytoplasm (Surh 2003). The effect of NF- κ B inhibition is the downregulation of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), which attenuates inflammatory signaling and slows tumor development (Jang *et al.* 1997; Surh 2003; Surh *et al.* 2001). Resveratrol increases the expression of the sirtuin gene SIRT1, which is involved in cell survival, cell cycle regulation, and DNA repair (Boocock *et al.* 2007). Sirtuin 1 also negatively regulates the cellular energy sensor, mammalian target of rapamycin (mTOR), which controls growth and proliferation (Ghosh *et al.* 2010).

Despite these findings, the vast majority of metabolomics data involving resveratrol is completely unrelated to health and disease. Spectroscopy has been used to characterize resveratrol and other polyphenols present in different types of wine (Flamini 2003; Jeandet *et al.* 1995; Kammerer *et al.* 2004; Lee *et al.* 2009; Mattivi *et al.* 1995). For example, during wine-making, maceration of grapes is required to release polyphenols from the skins (Jeandet *et al.* 1995). Proton NMR based metabolomics data has been correlated with meteorological records to evaluate the role of environmental factors in quality and polyphenol content. Annual changes in harvesting time, production techniques, and aging also influence the chemical composition of different vintages (Lee *et al.* 2009). Studies involving humans and animals have centered almost exclusively on bioavailability and metabolism in an effort to better understand resveratrol’s true biological effects and potential as a therapeutic (Boocock *et al.* 2007; Burkon and Somoza 2008; Jiang 2008; Vitaglione *et al.* 2005; Walle *et al.* 2004; Yu *et al.* 2002). Resveratrol has been found to be readily absorbed, with as much as 70% of a dose being detected in plasma, but it is also quickly metabolized into sulfate and gluconuric acid conjugates, which limits bioavailability and biological activity (Walle *et al.* 2004). However, these studies have been limited to healthy individuals and there is a need to expand metabolomics analysis to the application of phytochemicals in the prevention and treatment of disease (Boocock *et al.* 2007; Burkon and Somoza 2008; Vitaglione *et al.* 2005; Walle *et al.* 2004). One study, with colorectal patients, found that treatment with resveratrol for one week prior to surgery resulted in detectable metabolites and reduced cell proliferation in the colorectal tissue. Although the reduction was small, this observation suggests a potentially beneficial effect of resveratrol in colorectal tissue (Patel *et al.* 2010). Similar studies are necessary to better understand the beneficial role of resveratrol in the treatment of cancer and other diseases.

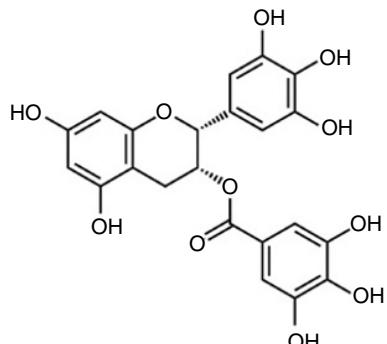
28.5.2 Epigallocatechin Gallate

Tea is one of the most popular beverages worldwide, second only to water (Dalluge and Nelson 2000; Le Gall *et al.* 2004). It is also a rich source of dietary polyphenols, particularly flavonoids (Davis *et al.* 1996). The most abundant flavonoids in tea are catechins, of which six have been well characterized; catechin (C), epicatechin (EC), epigallocatechin (EGC), gallocatechin gallate (GCG), epigallocatechin gallate (EGCG), and epicatechin gallate (ECG) (Dalluge *et al.* 1997). Because of the potential health benefits of dietary polyphenols, a great deal of effort has gone toward characterizing the molecules present in the three major types of tea, green (unfermented), oolong (partially fermented), and black (fermented) (Le Gall *et al.* 2004; Lee and Ong 2000). In particular, metabolite content has been shown to change with region of origin (Le Gall *et al.* 2004). In addition, metabolic fingerprinting has been successfully used to correlate metabolite content with sensory

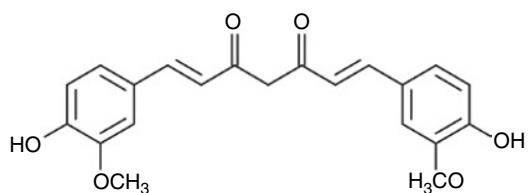
(a)



(b)



(c)



(d)

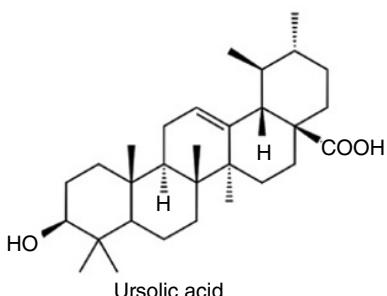


Figure 28.2 Select chemopreventive phytochemicals and their dietary sources. (a) Resveratrol, which is abundant in grapes and red wine, (b) epigallocatechin gallate (EGCG), found in high amounts in green tea, (c) curcumin originating from the Indian spice turmeric, and (d) ursolic acid, found in many fruits and herbs including rosemary, apples, and cranberries.

evaluation to identify objective measures of product quality in both Chinese and Japanese teas (Le Gall *et al.* 2004; Pongsuwan *et al.* 2008). Specifically, catechin concentrations correlated with tea quality (Pongsuwan *et al.* 2008). Spectroscopic techniques have also been popular for elucidation of molecular structure of parent compounds and conjugates formed *in vivo* (Davis *et al.* 1996; Li *et al.* 2000; Lin *et al.* 2008). These studies have improved understanding of the molecular structure of catechins, their differential antioxidant properties, and identified EGCG, shown in Figure 28.2(b), as the most potent antioxidant present in green tea (Stewart *et al.* 2005). In addition to quality assessment and molecular characterization, many metabolomics studies have focused on the bioavailability and metabolism of catechins (Lin *et al.* 2008; Meng *et al.* 2002; Roowi *et al.* 2010; Sang *et al.* 2011; Stalmach *et al.* 2009; Van Dorsten *et al.* 2006). Absorption of catechins is much greater than most other phytochemicals, in some cases as high as ten times greater than known polyphenols in some fruits and vegetable (Stalmach *et al.* 2009). While unconjugated catechins are detectable in blood, the most common metabolic fates involve modification via methylation, glucuronidation, and sulfation (Meng *et al.* 2002). Current data suggests that some of these conjugates can be converted back to the parent compound in select tissues prior to excretion. Despite the wealth of *in vitro* data demonstrating antioxidant, anti-inflammatory, anti-mutagenic, and anti-carcinogenic properties, the human *in vivo* data is mixed (Roowi *et al.* 2010; Sang *et al.* 2011; Stalmach *et al.* 2009). Similar to resveratrol, EGCG blocks activation of NF- κ B through several mechanisms including suppression of I κ B degradation and inhibition of the phosphoinositide 3-kinase (PI3K) activation of Akt/protein kinase B (PKB). Furthermore, EGCG inhibits constitutive activation of signal transducer and activator of transcription 3 (STAT3), a transcription factor that prevents apoptosis and promotes proliferation (Surh 2003). Current EGCG and tea consumption metabolomics studies have involved predominantly healthy individuals (Li *et al.* 2000; Roowi *et al.* 2010; Stalmach *et al.* 2009; Van Dorsten *et al.* 2006). There is an opportunity to learn more about the beneficial health effects of EGCG and other catechins using metabolomics analyses.

28.5.3 Curcumin

The Indian spice turmeric has long been used as a medicinal herb in Asia. It was traditionally used to treat inflammation, dermatological problems, infections, parasites, and tumors (Ireson *et al.* 2002; Peret-Almeida *et al.* 2005). Curcuminoids are pigments responsible for turmeric's distinctive yellow color (He *et al.* 1998). Curcumin, shown in Figure 28.2(c), is the most well studied and abundant of the curcuminoids (Peret-Almeida *et al.* 2005). Experiments with curcumin *in vitro* have shown promising results as an antioxidant, anticancer, anti-mutagenic, antibiotic, antiviral, antifungal, antiangiogenic, antidiabetic, and anti-inflammatory, but curcumin is characterized by extremely low bioavailability (Barry *et al.* 2009). In rats, at much as 75% of curcumin is never absorbed and is excreted in feces (Pan *et al.* 1999). Interestingly, curcumin formulations with phosphatidylcholine show a five-fold increase in plasma concentrations in humans when compared to curcumin alone (Marczylo *et al.* 2007). Curcumin and its gluconuride and sulfate conjugates are all detectable in plasma, urine, and several organs. However, the biological activity of curcumin metabolites is not well understood (Pan *et al.* 1999; Sharma *et al.* 2004). Furthermore, a general mechanism for curcumin's physiological effects is also unknown. Using NMR spectroscopy, curcumin has been observed to insert itself into the phospholipid bilayer altering the membrane structure in a manner similar to cholesterol (Barry *et al.* 2009). This activity may be responsible for some of curcumin's biological effects. Short term curcumin supplementation resulted in detectable levels of circulating curcumin and its metabolites in human malignant colorectal tissue. Treatment also decreased M₁G, a marker of DNA damage, in the target tissue (Garcea *et al.* 2005). In non-malignant human hepatocytes, curcumin decreased COX-2 expression and prostaglandin E₂ (PGE₂) synthesis, both markers of inflammation that are often elevated in cancer cells (Ireson *et al.* 2001; Surh *et al.* 2001). Curcumin can also inhibit NF- κ B activation by several mechanisms, including signaling through PKC, I κ B, and tumor necrosis factor alpha (TNF α) (Surh 2003). Constitutive activation of STAT3 is common in certain cancer types. Curcumin has been shown to inhibit both constitutively active and inducible STAT3 in cancer cells *in vitro* (Aggarwal and Shishodia, 2006; Bharti *et al.* 2003; Rajasingh *et al.* 2006). Despite this promising data, more metabolomics studies are necessary to better characterize the systemic and targeted activity of curcumin in unhealthy individuals.

28.5.4 Ursolic Acid

Ursolic acid, shown in Figure 28.2(d), is a pentacyclic triterpene that is commonly found in fruits, vegetables, and herbs. It is abundant in rosemary, basil, apples, and cranberries (Aggarwal and Shishodia 2006; Pathak *et al.* 2007). In the United States, apples represent the primary dietary source of phenolic compounds, including ursolic acid (He and Liu 2007).

Ursolic acid displays potent antioxidant, anti-inflammatory, and anticancer activities (He and Liu 2007, Subbaramaiah *et al.* 2000). In human liver, breast, and colon cancer cell lines, micromolar concentrations of triterpenoids isolated from apple peels have been shown to be effective at blocking proliferation (He and Liu 2007). In non-small cell lung cancer cells, ursolic acid attenuated proliferation, caused cell cycle arrest, and triggered apoptosis (Hsu *et al.* 2004). It also inhibits *in vitro* growth of leukemia, prostate, and cervical cancer cell lines (Neto 2007). In the multi-stage chemical carcinogenesis model in mouse skin, ursolic acid reduced inflammation, inhibited tumor initiation and promotion, and decreased tumor multiplicity (Banno *et al.* 2004; Huang *et al.* 1994; Tokuda *et al.* 1986). Mechanistically, ursolic acid has been shown to suppress the carcinogen induced activation of NF- κ B through the PKC signaling pathway and degradation of I κ B (Shishodia *et al.* 2003; Subbaramaiah *et al.* 2000). This results in decreased expression of COX-2 and other targets downstream of NF- κ B, suggesting a potential mechanism for ursolic acid's anti-cancer activity (Subbaramaiah *et al.* 2000). In addition, ursolic acid inhibits the PI3K-Akt and mitogen-activated protein kinase (MAPK) pathways (Achiwa *et al.* 2007). The PI3K-Akt pathway is critical for growth, proliferation, and cell survival and is activated in many cancers (Aggarwal and Shishodia 2006). Similarly, the MAPK pathway modulates gene expression involved in cell cycle regulation, differentiation, proliferation, survival, and apoptosis (Achiwa *et al.* 2007). Although, ursolic acid is a promising anti-cancer bioactive compound, very little metabolic data exists. A metabolomics study evaluating the metabolic changes that occur prior to apple scalding reported detectable amounts of ursolic acid, but was interested in predicting fruit quality, not nutritional properties (Rudell *et al.* 2009). NMR has also been used to measure quality and purity of natural products. Several ursolic acid products were found to contain more contaminants than reported by the standard method, HPLC-UV. However, no analyses were performed with respect to product efficacy or biological activity (Pauli *et al.* 2008). Clearly there is a need to combine metabolomics based analysis with biologically relevant ursolic acid research to better understand the health promoting activity of this bioactive compound.

28.5.5 Omega-3 Fatty Acids

Polyunsaturated fatty acids (PUFAs), especially the ω -3 fatty acids, eicosapentanoic acid (EPA) and docosahexaenoic acid (DHA), are compounds of research interest for their potential anti-cancer properties (Yee *et al.* 2005, 2010). Both EPA and DHA are ligands for the transcription factor, peroxisome proliferator-activated receptor gamma (PPAR γ), and are associated with reduced proliferation and metastasis in cancer (Yee *et al.* 2005). PPAR γ ligands may induce cell cycle arrest, sensitizing cancer cells to apoptosis. In mice, dietary ω -3 PUFAs were associated with reduced tumor incidence, multiplicity, and histopathology (Yee *et al.* 2005). PUFAs are also incorporated into lipid bilayers and can significantly impact membrane properties, especially cellular signaling (Shaikh *et al.* 2004; Stillwell and Wassall 2003). Furthermore, PUFAs are substrates for the cyclooxygenase (COX) and lipoxygenase (LOX) enzymes that are responsible for the synthesis of eicosanoids (Zhou and Nilsson 2001). EPA treatment of two lung cancer cell lines revealed differential prostaglandin concentrations, measured by mass spectrometry, despite similar COX-2 expression. Instead, phospholipase A₂ (PLA₂) was found to correlate with differences in prostaglandin levels (Pirman *et al.* 2013). However, this mechanism of action is still not well understood. In a clinical trial involving women at high risk for breast cancer, a dose dependent response to ω -3 supplementation in serum and breast tissue was established, but further research is needed to identify the effects of this treatment (Yee *et al.* 2010). One study identified changes in metabolic profiles with supplementation of ω -3 PUFAs and found them to be consistent with cardioprotective effects, but no chemopreventive or therapeutic analyses were performed (Rudkowska *et al.* 2013). There is an evident need to expand metabolomics investigations into the relationship between ω -3 fatty acid consumption and cancer.

28.6 Future Perspectives

There is a significant insufficiency of metabolomics data connecting nutrition and disease, especially diseases of metabolic dysregulation, such as cancer. As the newest omics science, it is still a young discipline; consequently it comes as no great surprise that there remain many areas yet to be explored. Metabolomics has great potential in the application of nutritional prevention strategies and treatments for a wide range of disease. A great deal of foundational knowledge has been laid regarding the chemistry and biochemistry of many nutritional components, but their application in disease is still limited. However, the currently available findings are promising and greater efforts should be made to expand this field.

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29

NMR-Based Metabolomics of Foods

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29.1 Introduction

A better understanding of food quality is increasingly being recognized as an essential process for assuring food safety and developing functional food products that can contribute to improvements in human health. The metabolites of food are affected by diverse factors such as cultivars, botanical or geographic origins, climates, storage conditions, physicochemical or biological processing, and more. Therefore, the comprehensive and quantitative analysis of metabolites, namely metabolomics, is useful for describing the detailed features of foods. An effective metabolomics approach aims to optimize the quality of food and aid the discovery of novel biomarkers to measure changes in the sensory or biological function of foods.

Nuclear magnetic resonance (NMR) spectroscopy is an analytical technique used to analyze natural organic compounds, synthetic polymers, inorganic materials such as those used in semiconductors, biological macromolecules, cells, tissues, and human bodies in various research and development fields. The excellent advantages of NMR are its nondestructive and noninvasive nature and its ability to detect a broad range of substances with different chemical properties such as sugars, lipids, and organic acids, quantitatively and reproducibly. These benefits in food metabolomics were initially demonstrated in the nondestructive analysis of milk (Hu *et al.* 2004, 2007), and they have helped evaluate the quality of various foods based on the comprehensive information of their metabolites, which are the most important factors determining tastes and nutritive values.

This chapter focuses on the comprehensive analysis of food metabolites using NMR. Recent techniques and applications of NMR have been developed as NMR profiling, which can determine various features of food products such as the botanical origins, states of food processing, sensory functions, and bioactivities. NMR pulse techniques allow us to observe minor food metabolites in a nondestructive way without any separation from major metabolites such as water, sugars, and lipids. Multivariate analyses not only define the differences among food products based on their NMR spectra but also suggest candidate metabolites contributing to the features of foods as biomarkers. Recent NMR profiling studies will be also reviewed herein.

29.2 Principal Aspects of NMR in Food Analyses

To describe the NMR-based metabolomics of foods, we briefly outline the measurement principle related to the nondestructive and comprehensive analysis of foods afforded by NMR. Food metabolites are small organic compounds comprised mainly of hydrogens and carbons. Hydrogen nuclei (protons or ^1H) are positively charged particles that possess an angular momentum, which induces a magnetic moment. The protons thus behave as tiny bar magnets called “nuclear spins”. Nuclear spins degenerate in energy because the magnetic moment is randomly oriented due to thermal motion. When protons are put into a magnetic field, there is an interaction between the magnetic moment and the applied field. The lowest energy levels of nuclear spin are when the magnetic moment is parallel to the magnetic field, and the lowest energy levels are when the magnetic moment is opposed to the magnetic field. This splitting of the energy levels referred to as the “Zeeman effect”.

The term “nuclear magnetic resonance” indicates the phenomenon in which protons in an applied magnetic field absorb a microwave with the resonant frequency corresponding to the difference between two energy levels (ΔE) split by the Zeeman effect. In this case, parallel nuclear spins are excited into opposed spins with a higher energy level. The excitation of nuclear spins can be induced with a low-energy microwave such as a radio wave because of the small energy difference, which assures the nondestructive property of NMR. However, the low sensitivity of NMR is also due to the small energy difference.

Nuclear spins are also affected by the local magnetic field, which is modified by the electrons surrounding the protons. Therefore, the resonant frequency of protons, which is observed as signals in ^1H NMR spectra, depends on the type of metabolites and the attached positions of hydrogens. Although food products are complex mixtures of various metabolites, NMR can simultaneously observe the metabolites as individual signals separated by the unique resonant frequencies of the protons. The ^1H NMR signals of low-molecular-weight compounds (<1000 Da) are generally sensitive in the NMR instruments with a high magnetic field; therefore, most of the identified food metabolites have been comprehensively detected in ^1H NMR spectra. Although proton (^1H) is the most frequently used as an observation nucleus because of its sensitivity, other nuclear isotopes constituting food metabolites (e.g., ^{13}C , ^{15}N , and ^{31}P) are also observed in NMR measurements.

As noted previously, NMR measurements easily detect most of the food metabolites regardless of their types, which means that an optimization of the measurement conditions is not required to observe individual metabolites. ^1H NMR spectra are completed in a relatively short time (within a quarter-hour per sample), and the sample preparation for NMR measurements is quite simple; most foods can be applied to NMR measurements after solvent extraction with deuterium reagents, and no processing is necessary for liquid samples such as beverages, fruit juices and seasonings. The derivatization process, which improves the ionization efficiency in liquid chromatography-mass spectrometry (LC-MS) and gas chromatography (GC)-MS, is not required for NMR measurements. Thus, in NMR no chemical reaction suppresses any sample loss and/or composition changes of metabolites.

29.3 NMR Techniques Applied to Food Metabolomics

In the application of NMR to the assessment of food quality, including the identification of botanical origins or states of food processing, it is necessary to assign the signals detected in ^1H NMR spectra to individual metabolites in foods. The metabolites detected with nondestructive NMR are often composed of many known compounds, which can be assigned by the use of assignment information in the databases for NMR signals such as the Biological Magnetic Resonance Data Bank (BMRB) and the Spectral Database for Organic Compounds (SDBS). However, in the NMR spectra of foods or food extracts, the chemical shifts of metabolites are often different from the values deposited in the databases, caused by the interaction between the metabolites in complex mixtures. For example, chlorogenic acids in coffee bean extracts interacted with caffeine by the stacking between their aromatic rings (Wei *et al.* 2012b). The chemical shift values varied depending on the concentration ratio of chlorogenic acids to caffeine. On the other hand, organic acids such as citric acid and malic acid often coordinate with metal ions (e.g., copper, iron, and manganese) in foods (Wei *et al.* 2010, 2011). Such paramagnetic metal ions have relaxation effects that cause line broadening in the ^1H NMR signals of the coordination compounds to the metals. These findings indicate that NMR is a useful tool for detecting the existing states of metabolites in foods, and that NMR has the potential for analyzing synergistic actions or the masking effects of food metabolites that may affect sensory function.

If chemical shift values are significantly affected by the existing states of metabolites, accurate assignments should be assured by two-dimensional NMR spectra such as those obtained by heteronuclear single quantum coherence spectrometry (HSQC), heteronuclear multiple bond correlation (HMBC), correlated spectroscopy (COSY), and total correlated spectroscopy (TOCSY). For the extensive signal overlaps on the ^1H NMR spectra, two-dimensional NMR spectra are also useful for detecting the signals of interest that are hidden under the other signals. Diffusion-ordered spectroscopy (DOSY) was also applied to separate the metabolites related to the antibacterial activity of manuka honey (Gresley *et al.* 2012). This

NMR technique can separate the ^1H NMR signals based on the difference in the diffusion coefficient of each metabolite and the approximate molecular mass is estimated with the diffusion coefficient. In that study, the signals in the DOSY spectra were assigned to 12 metabolites including 4-methoxyphenyllactic acid, methylglyoxal hydrate, methyl syringate, *proto*-quercitol, and 3,4,5-trimethoxybenzoic acid. There was a positive linear correlation between the antibacterial activity and the amount of 3,4,5-trimethoxybenzoic acid ($r^2 = 0.83$).

Foods contain various metabolites, including the major metabolites (e.g., ethanol in wine and sake, and acetic acid in vinegar) in a wide range of concentrations. Generally, fruit juices are more than 10% sugars, and the content of all other metabolites is only 1%. The minor metabolites are frequently important in the characterization of foods. However, the relatively weak signals of the metabolites are often not detected because of a limitation of the dynamic range, which contains much stronger signals of the major metabolites. The dynamic range is defined as the ratio of the largest to the smallest detectable signal intensity; therefore, the weak signals from minor metabolites are lost because most of the dynamic range is used to digitize the strong signals from major metabolites. This problem can be solved by pretreatment such as freeze-drying or solid-phase extraction to physicochemically remove the major metabolites, but changes in the chemical properties of foods are not avoidable. NMR can detect minor metabolites by selectively suppressing the strong signals from major metabolites without any physicochemical separation (Furihata *et al.* 2014; Rastrelli *et al.* 2009), which does not change the food quality (including the composition of metabolites).

As an effective method for detecting the NMR signals of minor metabolites, the band-selective excitation method was first developed for the analysis of olive oil and honey (Rastrelli *et al.* 2009). The band-selective excitation method was also applied to detect the NMR signals of 30 minor metabolites (e.g., adenosine, 11 amino acids including γ -aminobutyric acid, choline, citric acid, glutathione, myo-inositol, nicotinamide mononucleotide, quinic acid, shikimic acid, trigonelline, and uridine) in mango juice, which contains a large amount of sugars (Koda *et al.* 2012a). Five cultivars of mango juice (Carabao, Irwin, Keats, Kent, and Namudokumai) are effectively distinguished by using the ^1H NMR spectra of the minor metabolites, which indicated that the use of signal patterns from minor metabolites was effective in the evaluation of foods.

The band-selective excitation method has been used together with the F_2 -selective excitation method (Koda *et al.* 2011). This method can sensitively detect the cross-peaks of minor metabolites in two-dimensional NMR spectra such as those provided by TOCSY, double-quantum filtered (DQF)-COSY, and nuclear Overhauser effect spectroscopy (NOESY) without the excitation of major metabolites, which is useful for the assignment of the minor metabolites. The F_2 -selective excitation method was also applied to alcoholic beverages, and the results showed that the signal patterns of minor metabolites such as several amino acids and peptides were good indicators of the classification of alcoholic beverages (Koda *et al.* 2012b). More recently, we reported the “broadband WET (water suppression enhanced through T_1 effects)” method which selectively suppressed the signals of major metabolites and quantitatively detected a wide range of ^1H NMR spectra (Furihata *et al.* 2014). This improved method for detecting minor metabolites can be applied to the evaluation of food quality using the comprehensive pattern of minor metabolites.

29.4 Monitoring of Metabolic Changes in Food Processing Using Quantitative NMR

Since NMR observes the resonances of nuclei, the integral value of each signal in the NMR spectra is proportional to the number of nuclei in the same magnetic environment, which assures the quantitative property in principle regardless of the type of compounds. Therefore, food metabolites can be simultaneously quantified from their peak area observed in the NMR spectra when a peak area corresponds to a known concentration. In other words, one-dimensional NMR spectra depict the comprehensive and quantitative information of metabolites, which is the advantage of NMR compared to other analytical methods.

Quantitative NMR (qNMR) was applied to monitor the metabolic changes of coffee beans during roasting processes (Wei *et al.* 2012a). The signals of 16 metabolites were observed in the extracts of arabica coffee beans before roasting (Wei *et al.* 2010). The medium roasted coffee beans showed ^1H NMR spectra composed of 24 metabolites (Wei *et al.* 2011). The qNMR study of coffee beans by Wei *et al.* (2012a) revealed how a total of 29 metabolites changed during roasting processes; for example, three isomers of chlorogenic acids (3-CQA, 4-CQA, and 5-CQA), which function in hepatic glucose and lipid metabolism (Wu *et al.* 2014), were degraded during the roasting process (Figure 29.1). After roasting for 2 min, the three isomers were decreased and then disappeared after dark roasting for 9 min. In contrast, quinic acid was increased along with the roasting time for 4.5 min, decreased from 4.5 to 5.5 min, and increased again along with the further roasting time. The quinic acid derivatives γ -quinide and *syllo*-quinic acid were continuously increased during the roasting process. The production of quinic acid was considered to be a pyrolysate of chlorogenic acids, which are abundant in green coffee beans. In addition, γ -quinide and *syllo*-quinic acid were isomerized from quinic acid after roasting for 2 min. The generation of

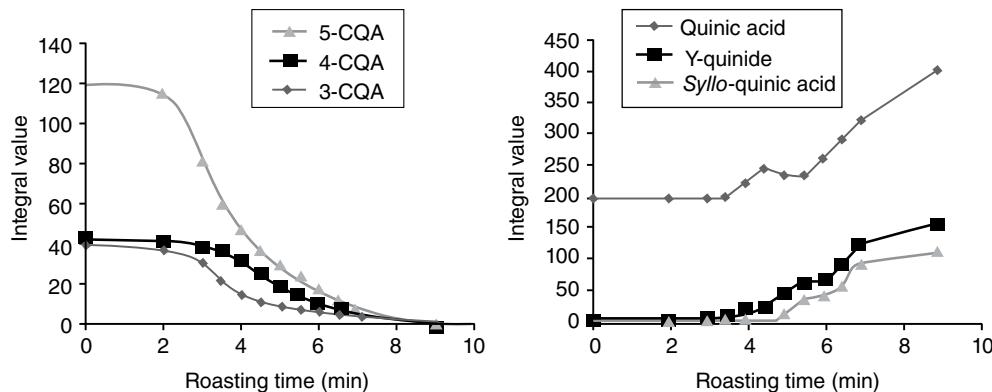


Figure 29.1 Monitoring of coffee metabolites during the roasting process.

quinic acids and the decomposition of chlorogenic acids are closely related to the alteration of the bitterness taste from the acidity achieved by the roasting of coffee beans.

Changes in oligosaccharides during roasting were considerably complicated compared to other metabolites. Although mannooligosaccharide continued to be increased during the roasting process, other oligosaccharides were increased by 5-min roasting and were then decreased along with the further roasting time. Mannoooligosaccharide stimulates the proliferation of “good” enteric bacteria. The increase in mannoooligosaccharide during roasting explains well the fact that coffee regulates the functions of the intestines. These findings indicate that qNMR can be applied to the simultaneous monitoring of multiple key metabolites related to food quality.

In other studies, qNMR was used to monitor the metabolic changes in the fermentation or aging processes of food products such as red wine (López-Rituer *et al.* 2009) and soy sauce (Ko *et al.* 2009). Wine consists of several hundred components. qNMR quantified several major metabolites including ethanol, acetic acid, lactic acid, malic acid, succinic acid, alanine, arginine, and proline, in the alcoholic and malolactic fermentation processes of 28 commercial red wines over a 207-day period (López-Rituer *et al.* 2009). A wine’s strength is expressed in terms of ethanol concentration. qNMR data of the time-course evolution of ethanol were very similar to those obtained by the winery using infrared spectroscopy (López-Rituer *et al.* 2009). The contents of malic and lactic acids are measured in order to obtain a quality wine. Low levels of malic acid are considered to be a prerequisite for the commercial production of red wines. Malic acid is reduced by the spontaneous growth of lactic acid bacteria, which in turn provides malolactic fermentation. The time-course evolution showed the formation of lactic acid from malic acid in the wine a 20–40-day period. This malolactic fermentation finished after 40 days. The proline/arginine ratio was also monitored using qNMR during the fermentation processes. The ratio provides a useful indication of the favorable nutritional value of the grape-to-yeast growth because arginine is an important nitrogen source for yeasts and the ratio reflects the proportion of nonassimilable (proline) to assimilable nitrogen (arginine).

The aging processes of soy sauce were analyzed up to 12 years using qNMR (Ko *et al.* 2009). The ¹H NMR spectra of soy sauces were dominated by a total of 37 metabolites including acetate, betaine, choline, ethanol, formate, fucose, glucose, glycerol, hypoxanthine, lactate, malonate, oligosaccharides, phosphocholine, succinate, trimethylamine, tyramine, uracil, and 13 types of amino acids. Aging for 12 years led to elevated levels of amino acids and organic acids and the consumption of carbohydrates, which were associated with a continuous involvement of microflora. Metabolic changes of soy sauces were also observed with high-pressure treatment (Ko *et al.* 2010). Acetate, choline, glycine, and tyrosine were increased by the high-pressure treatment. These results indicate that NMR-based metabolomics is also useful for gaining a better understanding of the influence of physical treatments in food processing.

29.5 NMR Profiling Based on Multivariate Analyses

NMR reveals the signals from various metabolites in foods simultaneously. The spectra provide comprehensive and quantitative information about metabolites, and this information can be used as a metabolic profile to characterize the food in a nondestructive way. For evaluations of food quality based on the NMR spectra, a multivariate analysis can be used to statistically analyze the differences in the spectra. Figure 29.2 shows the flow of NMR profiling combined with multivariate

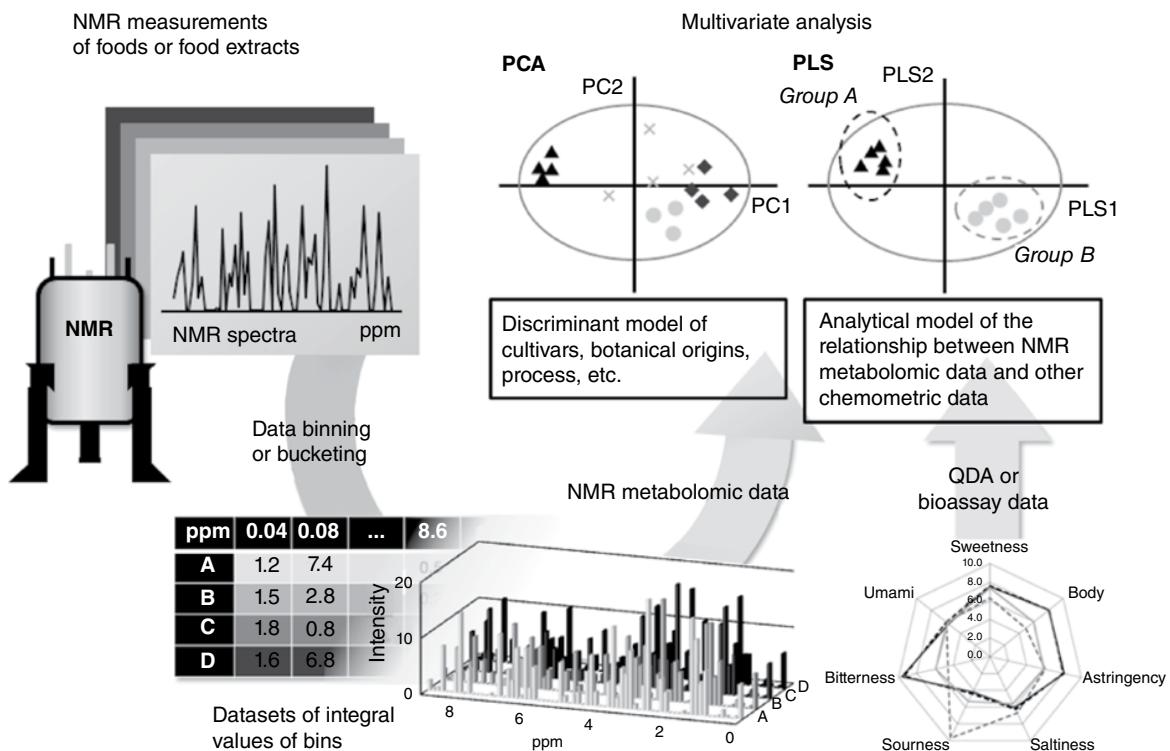


Figure 29.2 Overview of NMR profiling using a multivariate analysis.

analysis. NMR spectra are divided into bins of fixed spectral width. Data binning or bucketing is used to quantify the spectra as datasets of integral values of individual bins.

For example, when ^1H NMR spectra recorded with the spectral width of 10 ppm are divided into the bins of 0.04 ppm, the datasets are composed of 250 variables. The datasets are applied to a multivariate analysis such as a principal components analysis (PCA) or a partial least squares (PLS) regression analysis. A PCA reduces the number of dimensions (variables) without loss of information by compressing the datasets, and it highlights the similarities and differences of patterns between multiple datasets as score plots. In addition, loading plots can describe the variables that contribute to the differences of patterns. PLS is a statistical method for modeling relations between datasets of observed variables (bins of the NMR spectra) by a small number of latent (not directly observed or measured) variables. PLS provides models to predict the type of latent variables from the datasets of observed variables. The methods derived from PLS, for example, the orthogonal projections to latent structures (OPLS), PLS-discriminant analysis (DA), and OPLS-DA methods, are powerful tools for the discovery of metabolites that function as biomarkers. The next section, Section 29.5.1 describes the recent applications of NMR profiling combined with multivariate analysis.

29.5.1 Food Quality and Safety

NMR profiling has been increasingly used for the quality assessment of a variety of foods, such as beans (Choze *et al.* 2013), cheese (Piras *et al.* 2013), cherry (Longobardi *et al.* 2013), garlic (Ritota *et al.* 2012), milk (Sundekilde *et al.* 2013), olive oil (Agiomyrgianaki *et al.* 2012), potato (Pacifico *et al.* 2013), salmon (Wagner *et al.* 2014), tomato (Iglesias *et al.* 2014), white wine (Mazzei *et al.* 2013), and more. The usefulness of NMR profiling has been recognized especially for the discrimination of the cultivars or growing district of foods, which are the starting points of food traceability.

The previously described cultivar discrimination of mango juice with the band-selective excitation method is an example of NMR profiling based on a multivariate analysis (Koda *et al.* 2012a). In that study, the PCA score plot provided a good discriminant model of the five mango cultivars (Awin, Carabao, Keitt, Kent, and Nam Dok Mai) by using the low-field region (6.3–10.0 ppm) of the band-selective excitation NMR spectra. Carabao and Nam Dok Mai overlapped in a

two-dimensional score plot with PC1 and PC2, whereas the use of a third dimension (PC3) separated these two cultivars. The loading plots showed that the cultivar Keitt contained higher amounts of acetaldehyde, arginine and histidine than the other cultivars. Phenylalanine was a characteristic metabolite of the cultivar Awin, and the amounts of glutamine and shikimic acid were high in the cultivars Carabao and Nam Dok Mai. The amount of trigonelline was higher in the cultivars Carabao and Kent compared to the other cultivars, a finding that contributed to the separation of Carabao from Nam Dok Mai.

Metabolic profiling using ^{13}C NMR was also applied to arabica coffee beans from four geographic origins (Brazil, Colombia, Guatemala, and Tanzania) and robusta coffee beans from two origins (Indonesia, Vietnam) to distinguish the species and origins of green coffee beans (Wei *et al.* 2012b). Since ^{13}C NMR spectra are relatively insensitive to the interactions between metabolites such as caffeine and chlorogenic acids, the spectral patterns were compared between different samples without the significant chemical-shift changes caused by the metabolite interactions. PCA score plots clearly discriminated between arabica and robusta coffee beans. The arabica beans contained higher amounts of citrate, malate, sucrose, and trigonelline compared to the robusta beans, and the robusta beans were characterized by high amounts of caffeine, choline, and CQAs. In addition, the four geographic origins of arabica beans were distinguished from one another using a three-dimensional PCA score plot. The arabica beans from Tanzania contained higher amounts of acetic acid, sucrose and trigonelline. The loading scatter plots also showed the relatively high amounts of citrate, CQAs and sucrose in the beans from Columbia. Guatemala and Brazil beans were characterized by caffeine and amino acids, respectively. The S-plot generated from the OPLS-DA model, which helps identify statistically significant metabolites as biomarkers, captured 4-CQA and 5-CQA as the characteristic metabolites in the robusta beans from Indonesia and showed a relatively high amount of 3-CQA in the Vietnam beans. Thus, three isomers of caffeoylquinic acids can be used to distinguish the geographic origins of robusta coffee beans based on their NMR profiling.

The NMR profiling of honey is an interesting example of the construction of a quality evaluation model. Metabolites of honey depend on the types of source flowers. The ^1H NMR spectra of chloroform extracts of 118 honey samples with four different botanical origins (chestnut, acacia, linden, and polyfloral) were used to discriminate the different botanical origins of honey and identify characteristic metabolites for each honey type (Schievano *et al.* 2010). The PLS-DA model was constructed using 85 honey samples, which are randomly chosen as a training set. When 33 honey samples were estimated using the constructed PLS-DA model, 30 samples were classified into the correct botanical origins. The botanical markers of linden honey were directly identified using two-dimensional NMR spectra as two terpene acids, 4-(1-hydroxy-1-methylethyl)cyclohexa-1,3-dienecarboxylic acid and 4-(1-methylethenyl)cyclohexa-1,3-dienecarboxylic acid. The markers of acacia and chestnut honeys were considered to be crysin and 3-(2'-pyrrolidinyl)-kynurenic acid, respectively. That study illustrated a promising approach to discriminate several honey samples using a single ^1H NMR measurement.

29.5.2 Sensory Assessment for Food Development

The evaluation of sensory function is an important analytical task for the development of food products in the food industry. However, it has been difficult to identify a universal evaluation method because such evaluations depend on the discriminating abilities of trained assessors, and a quick, simple and more objective method for sensory assessment is needed. NMR metabolomics is a quantitative descriptive analysis method that has been shown to be useful to describe sensory features of foods. To date, NMR profiling has been applied to the sensory evaluation of food products such as canned tomato (Malmendal *et al.* 2011) and extra virgin olive oil (Lauri *et al.* 2013). These pacesetting analyses provided a general way to construct predicted models for the different sensory descriptors using the OPLS of quantitative descriptive analysis (QDA) and NMR data. The model of canned tomato showed good predictions for bitterness, redness, density, and metal and tomato tastes. All possible correlations between the NMR signals and the sensory descriptors were also analyzed using an OPLS model to determine the metabolites responsible for each sensory descriptor. For example, bitter taste was positively correlated with bitter amino acids including isoleucine, phenylalanine, tryptophan, tyrosine, and valine. Glutamate was also positively correlated with bitter taste, although it does not possess a bitter taste itself. This result suggests the bitter taste-enhancing effect of glutamate.

The OPLS model of extra virgin olive oil also showed good predictions for artichoke, bitter, pungent, rosemary, and tomato tastes. The correlation between sensory descriptors was also analyzed using the OPLS model. For example, artichoke, bitter, and pungent tastes showed highly correlations and similar metabolic profiles. However, no metabolites were associated with any sensory descriptors, due to the lack of convincing assignments of NMR signals.

The OPLS model was recently proposed for evaluations of the sensory function of coffee and for analyses of the relationship between NMR signals and sensory descriptors (Wei *et al.* 2014). ^1H NMR measurements and a QDA were carried out using four types of coffee beans, arabica and robusta beans with light (L value 25.9) and dark (L value 18.2) roasting degrees. Among the sensory descriptors selected for QDA, bitterness, body and sourness showed significant differences among the four types of

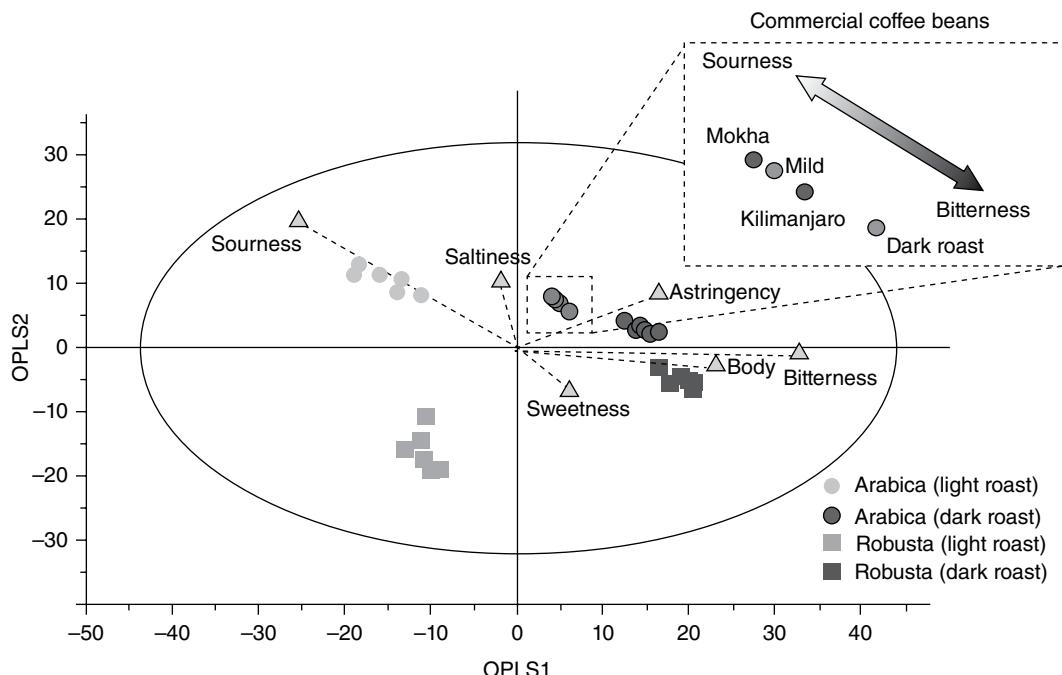


Figure 29.3 OPLS model for the sensory assessment of coffee.

roasted coffee beans. Sweet taste was characterized by all of the tested coffee beans except for light roasted arabica beans. Astringency varied significantly according to the roasting degrees. The score plot of the OPLS model using NMR data discriminated all types of roasted coffee beans even though the dark roasted coffee of arabica and robusta beans showed quite similar QDA patterns. The correlations between NMR signals and the analyzed sensory descriptors were highlighted by the S-plots for each sensory descriptor. Chlorogenic acid was negatively correlated with the bitterness of the coffee beans. Although chlorogenic acids have been described to have a mild bitter taste, the OPLS models suggest that the degradation products of chlorogenic acids (i.e., quinic acid, quinide, and sylo-quinic acid) have strong bitter tastes and may contribute to the bitterness of coffee.

In that study (Wei *et al.* 2014), the predicted OPLS model was also used for evaluating the sensory features of all four types of commercial coffee beans, named Mokha, Mild, Kilimanjaro, and Dark roast tastes (Figure 29.3). Mokha taste had the highest level of sourness. The Dark roast taste showed the highest level of bitterness. These results were consistent with the marked sensory features of the commercial coffee beans, demonstrating that NMR profiling was useful as a sensory evaluation method of coffee as a “magnetic tongue”.

29.5.3 Food Functionality and Identification of Bioactive Metabolites

Food development for the benefit of human health requires effective methods for exploring functional metabolites in food products. The conventional methods include the isolation of each metabolite. This process may waste some of the bioactive metabolites that play significant roles in the functionality of foods because the metabolites often act in concert rather than independently (Seeram 2008). Statistical analysis methods for multivariate datasets take advantage of the large datasets of metabolites. The NMR profiling approach is considered effective for understanding the functionality of foods and has been applied to identify the bioactive metabolites in combination with different chemometric data.

Based on the antiproliferative activities against HT-29 colon cancer cells, bioactive metabolites of black raspberry (BR) fruit extracts were identified using NMR profiling (Paudel *et al.* 2014). Although the antiproliferative metabolites of BR have been identified as anthocyanins, nonanthocyanin phenols, flavonols, and their derivatives, these metabolites did not account for the total efficacy of BR treatments. In that study, the antiproliferative assays and ^1H NMR measurements were carried out using 73 BR samples of three cultivars (Bristol, Jewel, and MacBlack) with varying degrees of ripeness. A variety of BR juice types inhibited the growth of HT-29 cells and showed dose dependency in the antiproliferative activities. A PLS

model was constructed for the antiproliferative activities using chemometric data and the ^1H NMR data of the BR juice. The model revealed diverse antiproliferative metabolites, benzoic acid glucose ester, citric acid derivatives, epicatechin, flavonol glycosides (i.e., quercetin 3-glucoside and quercetin 3-rutinoside), methyl ellagic acid derivatives (i.e., methyl ellagic acid acetyl pentose), *p*-coumaric acid, and salicylic acid derivatives (i.e., salicylic acid glucosyl ester) in addition to anthocyanins such as cyanidin 3-rutinoside and cyanidin 3-xylosylrutinoside. Among these metabolites, epicatechin, quercetin derivatives, and salicylic acid derivatives are known to be powerful phenolic antioxidants that have chemopreventive activities against several types of cancers including colon cancer. In addition, a benzoic acid, glucose ester, was reported to show antiproliferative potential against cancer cells for the first time.

A similar approach was applied to a search for the bioactive metabolites of red wines (Ali *et al.* 2013). Wine phenolics possess several health benefits, which are often associated with their antioxidant and radical scavenging activities. The Ali *et al.* (2013) study focused on the suppressive effects of red wines on inflammatory diseases. Tumor necrosis factor alpha (TNF α) is secreted during the early phase of the inflammatory response, and the overproduction of TNF α leads to systemic toxicity. NMR measurements and anti-TNF α production assays were carried out using the water, methanol–water (1:1), and methanol fractions of 11 wine samples from solid-phase extraction (SPE) resin. A PLS-DA model was constructed to evaluate the metabolites associated with anti-TNF α activities, using NMR profiling. The evaluation model revealed several phenolics such as caftaric acid, catechin, and quercetin as the most influential metabolites in suppressing TNF α production. These results demonstrated that the NMR profiling based on a PLS model is a useful approach for seeking bioactive metabolites in food products. In addition, the SPE approach is useful to detect the metabolites at trace amounts and helps to develop chemometric data such as anti-TNF α production activity and NMR spectra.

29.6 Conclusion

NMR is an analytical technique for food metabolomics with nondestructive and quantitative properties and reproducibility, based on the principle of NMR measurements. NMR signals can be detected with minimal changes in the composition of food products, and the overall patterns (i.e., NMR spectra) can be compared among different food products as comprehensive chemical profiles. As described in this chapter, NMR profiling has been used for the traceability and quality control of foods. Recent studies raise the possibility that NMR profiling can also be useful in evaluations of the sensory functions and biological regulation functions of food products. With further applications of NMR profiling to various food samples showing different sensory functions and degrees of nutrition, models for the assessment of sensory evaluation and nutrition will be developed for use in the food industry. NMR profiling may also be an effective method for the identification of bioactive components and functional assessments of foods, which will contribute to a better understanding of food nutrition and the development of supplements, pharmaceutical products and functional foods based on the bioactive metabolites in foods.

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Cancer Chemopreventive Effect of Curcumin through Suppressing Metabolic Crosstalk between Components in the Tumor Microenvironment

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30.1 Introduction

Despite the development of various novel molecular targeted agents as well as conventional chemotherapy, cancer remains the leading cause of death in the USA. This may indicate that most current anticancer strategies based on the modulation of a single target are not likely to prevent or treat cancer effectively. This previous failure of mono-targeted therapies in the combat against cancers is driving many researchers to have interest in developing multi-targeted therapies, which incorporate not only the combination of several mono-targeted drugs but also new agents with multi-targeting properties, such as *nutraceuticals*. A “nutraceutical”, a term coined by Stephen DeFelice in 1989 (Borwer, 1998), is simply any substance considered to be a food or part of food that provides medical and health benefits (Gupta *et al.*, 2010a). Nutraceuticals are known to target various steps of metabolic alterations in not only cancer cells but also the tumor microenvironment and in-between interaction, directly and indirectly preventing cancer hallmarks; such as limitless replicative potential, tissue invasion, sustained angiogenesis, and metastasis (Gupta *et al.*, 2010a). Moreover, these products are generally less expensive, safer, and more readily available than are synthetic agents, and therefore, attract enormous attention as emerging promising anticancer agents. Among nutraceuticals, non-nutritive components in the plant-based diet that possess substantial anti-carcinogenic and anti-mutagenic properties are specifically called phytochemicals (Surh, 2003), which include allicin, capsaicin, carotene, curcumin, epigallocatechin gallate (EGCG), genistein, lycopene, quercetin, and resveratrol.

Curcumin (diferuloyl methane) is a naturally occurring yellow polyphenol derived from the rhizomes of the plant *curcuma longa*, a perennial herb belonging to the ginger family that is broadly cultivated in south and south-east Asia. Curcumin has been used for thousands of years in traditional oriental medicine as a healing agent for a variety of illnesses, such as biliary disorders, anorexia, cough, diabetic wounds, hepatic disorders, rheumatism, and sinusitis

(Shishodia *et al.*, 2007). Curcumin has been considered safe and tolerable, based on the fact that phase I trials with daily doses as high as 8 g were safely conducted (Cheng *et al.*, 2001). Epidemiologic data suggested that there was a correlation between the widespread use of dietary curcumin and the low incidence of gastrointestinal mucosal cancer in south-east Asia (Bisht *et al.*, 2010). Curcumin mediates its anticancer effects by modulation of several important molecular targets including transcription factors (e.g., nuclear factor-kappaB [NF- κ B], activator protein-1 [AP-1], β -catenin), enzymes (e.g., cyclooxygenase-2 [Cox-2], matrix metalloproteinases [MMPs]), pro-inflammatory cytokines (e.g., tumor necrosis factor- α [TNF- α], interleukin-1 β [IL-1 β], and IL-6), and cell surface adhesion molecules (e.g., Cadherins and Integrins) (Buhrmann *et al.*, 2014).

This chapter deals with the scientific evidence currently available for cancer chemopreventive mechanisms of action and molecular targets of curcumin, with special focus on metabolic modulation within the tumor microenvironment.

30.2 Cancer Metabolism

30.2.1 The Warburg and Reverse Warburg Effect

In the 1920s, Otto Warburg demonstrated that tumor cells show an increased dependence on glycolysis to meet their energy needs, even in the presence of oxygen (the Warburg effect). High rates of glucose uptake, fueling glycolysis, are now used clinically to identify cancer cells. Using an isotope-tagged glucose analog, F-18 fluorodeoxyglucose ([18]FDG), [18]FDG-positron emission tomography ([18]FDG-PET) is currently one of the most sensitive imaging technique clinically localizing and diagnosing cancer (Chung *et al.*, 2012). However, the Warburg effect does not account for the metabolic diversity that has been observed amongst cancer cells nor the influences that might direct such diversity. Despite the accelerated glycolysis in several tumors, its total contribution to the cellular ATP supply only reaches 10% in breast cancer, melanoma, uterine cervix cancer, and lung cancer cells (Moreno-Sanchez *et al.*, 2009). In contrast, in other cell lines, such as glioblastoma multiforme, hepatoma, and sarcoma, glycolysis supports 50–70% of the ATP demand. The development of anticancer agents that target the enzymes involved in glycolysis are hereto not as effective as single agents against cancer (Suh *et al.*, 2011). One possible explanation of the disappointing efficacy of the anti-glycolytic agents is the *metabolic symbiosis*, that is, “reverse Warburg effect”, which is the dominant aerobic glycolysis of the stromal cells adjacent to the cancer cells. In contrast to the Warburg effect, which is the dominant aerobic glycolysis of the cancer cells, the reverse Warburg effect emphasized the importance of tumor stromal cells supplying energy metabolites and chemical building blocks to the rapidly proliferating cancer cells (Suh *et al.*, 2014). Briefly, activated catabolic fibroblasts in the tumor stroma, which are called cancer-associated fibroblasts (CAFs), fuel high-energy metabolite such as lactate, ketones, glutamine, other amino acids, and fatty acids, to anabolic adjacent cancer cells, to metabolize via tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS). Catabolic metabolism and glycolytic reprogramming in the tumor microenvironment, rather than in the cancer cells, are known to be orchestrated by oncogene activation and inflammation, which originates in cancer cells. Oncogenes drive the onset of the CAF phenotype in adjacent normal fibroblasts via paracrine oxidative stress (Lisanti *et al.*, 2013). Two divergent oncogenes such as *RAS* and *NF- κ B* use reactive oxygen species (ROS) production and inflammatory cytokines to induce oxidative stress and glycolysis in adjacent CAFs. Thus, oncogenes act at a distance, to metabolically reprogram the tumor microenvironment (Lisanti *et al.*, 2013). Clinically supporting evidence of the reverse Warburg effect in ovarian cancer was reported by Labiche *et al.* (2010). They evaluated the stroma proportion of the surgical tumor specimens of 194 patients with advanced ovarian cancer (whole stromal area/whole tumor area) using histochemical staining and fully automatic virtual slide processing (PixCyt; Tribvn, Chatillon, France). Women with a high stroma proportion were shown to have a significantly worse overall survival rate regardless of the histologic subtype.

30.2.2 Paradigm Shift from Cancer Cells to Cancer Microenvironment

Currently, the concept of cancer developing not simply due to mutations in a single cell but rather as a complex process creating a permissive environment within the host has now taken hold (Bruno *et al.*, 2014). The tumor microenvironment can be roughly defined as all of the non-transformed components residing in the vicinity of the tumor, including blood vessel cells, mesenchymal stem cells (MSCs), adipocytes, fibroblasts, immune cells, and the extracellular matrix (ECM) (Madar *et al.*, 2013). The tumor microenvironment in solid tumors is very distinct from that in normal tissues. Due to deregulated cancer cell metabolism, highly heterogeneous vasculature and defective blood perfusion, the tumor

microenvironment is characterized by hypoxia and acidosis (Cairns *et al.*, 2006). The acidic tumor microenvironment is the place where a crosstalk between different cell types takes place, thereby helping tumor cells be fueled for proliferation and invade to metastasize. In terms of the interplay between tumor microenvironment elements, disorganized inflammation, neovascularization, and immune modulation are the central mechanisms of cancer progression.

30.2.3 Cancer-Associated Cells in the Tumor Microenvironment

Cancer cells corrupt their microenvironment to be a primary active factor in determining whether dysfunctional epithelial cancer cells will continue to grow and invade or merely become a indolent micro-hyperplasia or even be eliminated (Albini and Sporn, 2007). The “corrupted cells” in the tumor microenvironment, such as, CAF, tumor-associated endothelial cells (TECs), carcinoma-associated MSC, and tumor-associated macrophages (TAM), were shown to play important roles in this paracrine control (Suh *et al.*, 2014).

30.2.3.1 Cancer-Associated Fibroblasts

CAFs are the most abundant subpopulation of cells residing within the tumor microenvironment with the ability to promote tumorigenic features, such as cell proliferation, angiogenesis, inflammation, and metastasis (Madar *et al.*, 2013). Tumors have been described as “wounds that do not heal” (Dvorak, 1986). Given that fibroblasts play critical roles in tissue remodeling during the wound healing process, it is reasonable that CAFs may be associated with chaotic disorganization of inflammation and repair. Inflammation is now recognized as a driving force in carcinogenesis and many environmental causes of cancer and risk factors are associated with some form of chronic inflammation (Bruno *et al.*, 2014). CAFs promote tumor initiation and progression by stimulating angiogenesis and tumor cell growth and invasion (Cirri and Chiarugi, 2012). CAFs secrete increased level of stromal cell-derived factor 1 (SDF-1; also called CXCL12) that facilitates angiogenesis by recruiting endothelial progenitor cells into the tumor (Orimo *et al.*, 2005). CAFs are the sources of transforming growth factor- β (TGF- β), a critical mediator of the epithelial-mesenchymal transition (EMT). Moreover, CAFs facilitate cancer cells to invade ECM and metastasize by releasing ECM-degrading enzymes, MMPs (Boire *et al.*, 2005). Therefore, CAFs might contribute to EMT in nearby cancer cells and promote their invasiveness (Shimoda *et al.*, 2010).

30.2.3.2 Tumor-Associated Endothelial Cells

TECs are the major player in the tumor angiogenesis through sprouting from pre-existing blood vessels. TECs are different from normal counterparts. It has been reported that human hepatocellular carcinoma-derived endothelial cells, when compared the ones from adjacent normal liver tissue, show increased apoptosis resistance, enhanced angiogenic activity and acquire more resistance to the combination of anti-angiogenic and chemotherapeutic agents (Xiong *et al.*, 2009).

30.2.3.3 Carcinoma-Associated MSCs

Carcinoma-associated MSCs, which were isolated in ovarian cancer, highly express bone morphogenetic protein (BMP) 2, BMP 4, and BMP 6 compared with other MSCs, and promote tumor growth in three ways: development of chemoresistance, enhancement of angiogenesis, and immune modulation.

30.2.3.4 Tumor-Associated Macrophages

TAM is characterized by pro-tumoral and immunosuppressive functions (Suh *et al.*, 2014). Ovarian cancer cells were also shown to secrete TGF- β and chemokine (C-C motif) ligand 2 (CCL2), stimulating normal peritoneal macrophages to acquire the features of TAMs (24). TAMs were shown to produce cytokines (e.g., TNF- α and IL-1), chemokines (e.g., CCL2 and CXCL12), angiogenic factors (e.g., vascular endothelial growth factor [VEGF], platelet-derived growth factor [PDGF], fibroblast growth factor [FGF], and IL-8), and matrix-degrading enzymes (e.g., MMPs, cathepsin proteases, and heparanase) (Grivennikov *et al.*, 2010).

The new insights on the roles of cancer microenvironments in cancer metabolism have moved the target of the development of anticancer agents mainly from intra-cellular signaling pathways associated with glycolysis and apoptosis to multiple components in the tumor microenvironments and inter-component interactions (Junttila and de Sauvage, 2013). Considering the well-known roles of phytochemicals in inhibiting tumorigenesis through modulating inflammation and detoxifying oxidative stress, metabolic interaction between tumor microenvironment elements is likely one of the main metabolic onco-targets of phytochemicals.

30.3 Metabolic Onco-Targets of Curcumin in the Tumor Microenvironment

30.3.1 Xenohormetic Inhibition of NF-κB

NF-κB is a ubiquitously expressed pro-inflammatory transcription factor that regulates the expression of over 500 genes involved in cellular transformation, survival, proliferation, invasion, angiogenesis, metastasis, and inflammation (Gupta *et al.*, 2010b). Extensive *in vitro* and *in vivo* studies have indicated that NF-κB activation, one of the masters of inflammation, has a promoting role in most cancers (Zlotogorski *et al.*, 2013). Many of important activities of NF-κB are exerted through components of the tumor microenvironment. Activation of NF-κB has been implicated in cellular transformation, tumor promotion, angiogenesis, and tumor invasion and metastasis. One of the mechanisms that may a role in the anticancer properties of curcumin may be related to the down-regulation of NF-κB (Zlotogorski *et al.*, 2013). Curcumin inhibited IκB degradation through downregulation of NF-κB-inducing kinase and IKK (Gupta *et al.*, 2010a). Curcumin has also been reported to suppress the TNF-α-induced nuclear translocation and DNA binding of NF-κB in a human myeloid leukemia cell line through suppression of IκBα phosphorylation and subsequent degradation (Singh and Aggarwal, 1995). Curcumin has been shown to inhibit IκBα phosphorylation in human multiple myeloma cells and murine melanoma cells through suppression of IKK activity, which contributed to its proliferative, pro-apoptotic, and anti-metastatic activities (Gupta *et al.*, 2010a).

However, NF-κB activation is considered to be a “double-edged sword”. Although NF-κB has therapeutic effects when inhibited in a tumor-specific manner, it could have devastating effects if it is inhibited non-specifically in the host (Aggarwal and Sung, 2011). The tumor-specific inhibition of NF-κB seems too complicated to achieve biochemically in the near future. Natural products such as curcumin that are known to suppress NF-κB activation could be alternatives because curcumin is pharmacologically safe, highly affordable, and can chemosensitize a variety of tumors (Gupta *et al.*, 2010b). The underlying molecular mechanisms of tumor-specificity of curcumin are still obscure. However, its tumor-specific toxic effects probably originate from xenohormesis.

The dietary phytochemicals that are synthesized as secondary metabolites function as toxins, that is, “phytoalexins”, and hence protect plants against insects and other damaging organisms and stresses. However, at the relatively low doses consumed by humans and other mammals, these same toxic plant-derived chemicals, as mild stressors, activate adaptive cellular response signaling, conferring stress resistance and other health benefits. This phenomenon has been referred to as *xenohormesis* (Surh, 2011). The xenohormesis mechanisms underlying chemopreventive effects of some dietary chemopreventive phytochemicals were so far mainly focused on the nuclear transcription factor erythroid 2p45 (NF-E2)-related factor 2 (Nrf2)-mediated induction of cellular defense detoxifying/antioxidant enzymes, which protect against reactive oxygen species/reactive nitrogen species (ROS/RNS) and reactive metabolites of carcinogens (Surh, 2011). In addition, these compounds would kill transformed cancer cells *in vitro* and *in vivo* xenografts via diverse anticancer mechanisms including anticancer effects by inhibiting the IκB kinase (IKK)/NF-κB pathway, inhibiting signal-transducer-and-activator-of-transcription-3 (STAT-3), and causing cell cycle arrest. All of the anticancer effects of curcumin might be exerted through multiple pathways, which are involved in cancer cell-tumor microenvironment crosstalk related to anti-apoptosis, cell proliferation, angiogenesis, and invasion in oral carcinogenesis (Figure 30.1).

30.3.1.1 Cell Survival and Proliferation

Anti-apoptotic gene products regulated by NF-κB include Bcl-2, Bcl-x_L, survivin, cyclin D1, and cellular inhibitor of apoptosis (cIAP)-1 (Bharti and Aggarwal, 2002), the expression of which curcumin was shown to downregulate. This led to the suppression of proliferation and arrest of cells at the G(1)/S phase of the cell cycle. Suppression of NF-κB complex by IKK γ / NF-κB essential modulator-binding domain peptide also suppressed the proliferation of multiple myeloma cells. Curcumin also activated caspase-7 and caspase-9 and induced polyadenosine-5'-diphosphate-ribose polymerase (PARP) cleavage (Bharti *et al.*, 2003). With use of an orthotopic murine model of ovarian cancer, curcumin was shown to inhibit tumor growth that correlated with inhibition of NF-κB and a STAT-3 activation pathway (Lin *et al.*, 2007).

30.3.1.2 Angiogenesis

Curcumin was found to completely prevent induction of VEGF synthesis in microvascular endothelial cells stimulated with glycation end products, which was mediated by down-regulation of NF-κB and AP-1 activity (Okamoto *et al.*, 2002). Although the precise molecular mechanism is not fully understood, hypoxia was shown to increase expression of VEGF, IL-6, and cancer stem cell signature genes such as Nanog, Oct4, and EZH2 consistent with increased cell

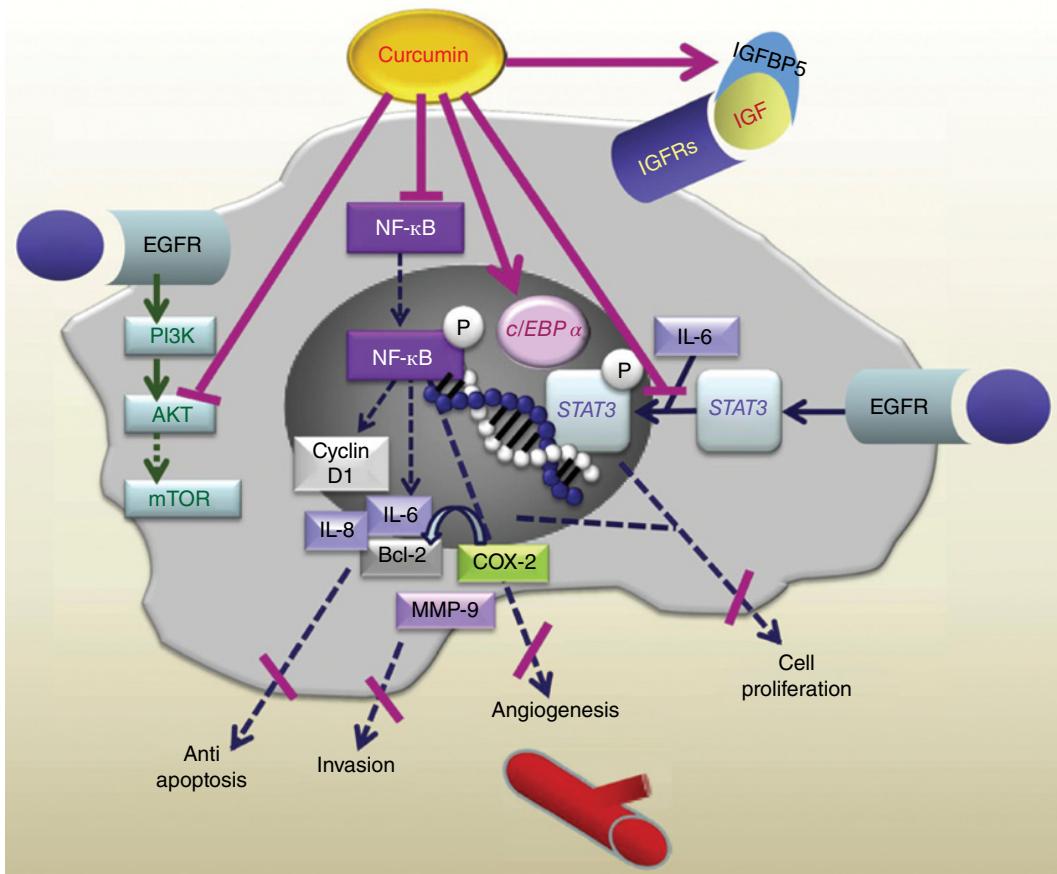


Figure 30.1 Curcumin acts on oral squamous cell carcinoma cells through multiple pathways. Curcumin's anticancer effect consists of inhibiting the NF-κB pathway, thereby holding back the downstream NF-κB-related factors (e.g., cyclin D1, Bcl-2, IL-8, MMP-9, and COX2). Curcumin also acts through epithelial growth factor receptors (EGFRs) to inhibit two downstream pathways, STAT3 and AKT-mTOR. These pathways participate in cancer cell proliferation, have an anti-apoptotic effect, and are involved in cancer cell-tumor microenvironment crosstalk related to extra-cellular matrix degradation and angiogenesis. Unlike these pathways, curcumin activates the C/EBP α transactivator by interacting with binding elements in the IGFBP-5 promoter. The resultant upregulation of C/EBP α and IGFBP-5 by curcumin is crucial to the suppression of oral carcinogenesis. Adapted from Zlotogorski et al. (2013), with permission from Elsevier.

migration/invasion and angiogenesis in human pancreatic cancer cells (Bao *et al.*, 2012a). The treatment of pancreatic cancer cells with a novel synthetic derivative of curcumin (CDF) inhibited the production of VEGF and IL-6, and downregulated the expression of Nanog, Oct4, EZH2 mRNAs under hypoxia. CDF also led to decreased cell migration/invasion, and angiogenesis under hypoxia. The same study group also demonstrated that hypoxia-induced aggressiveness of prostate cancer cells might be linked with deregulated expression of VEGF, IL-6, and miRNAs that were attenuated by CDF (Bao *et al.*, 2012b).

30.3.1.3 Invasion and Metastasis

In a recent experimental study with 3D-co-culture model that mimics *in vivo* tumor microenvironment using colon cancer cells HCT116 and MRC-5 fibroblasts in a monolayer or high density tumor microenvironment (Buhrmann *et al.*, 2014), high density tumor microenvironment co-cultures synergistically increased tumor-promoting factors (NF-κB, MMP-13), TGF- β , and EMT-factors (increased vimentin and Slug, decreased E-cadherin) in HCT116 compared with high density

HCT116 mono-cultures. Curcumin dramatically reduced this synergistic crosstalk between HCT116 and MRC-5 cells, and induced mesenchymal-epithelial transition (MET). Thus, curcumin could suppress metastasis of colon cancer cells through modulating the synergistic crosstalk between cancer cells and stromal fibroblasts. Curcumin was also shown to inhibit prostate cancer bone metastasis by upregulating BMP-7 and modulating TGF- β signaling *in vivo* (Dorai *et al.*, 2014). In addition, the reversal of TAMs to tumor growth inhibiting phenotype M1 by curcumin was reported in breast cancer (Zhang *et al.*, 2013). In this study, hydrazine-curcumin encapsulated nanoparticles were employed to “re-educate” TAMs by suppressing STAT-3 signaling, which triggers crosstalk between tumor cells and TAMs and is crucial for the regulation of malignant progression in breast cancer.

There is an emerging evidence that curcumin could exhibit anticancer effect through modulation of some essential components of the tumor microenvironment that regulates tumor progression (Zlotogorski *et al.*, 2013). Curcumin could affect the behavior of fibroblasts, such as proliferation, migration, and apoptosis (Zhang *et al.*, 2011). The immune modulation of curcumin in tumor microenvironment was also recently reported by Chang *et al.* (2012). They showed that enhanced cytotoxicity of CD8 (+) T cells toward tumors via blocking different immunosuppressors, including TGF- β , indoleamine 2, 3-dioxygenase, and regulatory T cells, when combined with adoptive therapy.

30.4 Clinical Trials of Curcumin as Metabolic Modulators in Cancer

Table 30.1 shows the current clinical trials of curcumin in the treatment and prevention of cancers. (The data were accessed in September 2014 from www.clinicaltrials.gov) Mostly, the ongoing and completed trials are phase I or II clinical trials, which evaluated the safety and efficacy. Maximum tolerated dose (MTD) for now might be, although different according to the clinical settings, 8 g/day when used alone. There are two clinical trials with a preventive setting in normal or high-risk populations. One is a phase I study, which evaluated MTD for preventing colon cancer in normal healthy subjects. The other is a randomized pilot clinical trial that studies a nanoemulsion formulation of curcumin in reducing inflammatory changes in breast tissue in obese women at high risk of breast cancer, such as atypical ductal breast hyperplasia, BRCA1/2 gene mutation, ductal breast carcinoma *in situ*, and lobular breast carcinoma *in situ*. The hypothesis is that curcumin may reduce inflammation in breast tissue and fat. This may affect the risk of developing breast cancer. Most of the clinical trials of a treatment setting are efficacy studies of curcumin alone or combination with other chemotherapeutic agents with progression-free survival being the primary endpoint in the patients with advanced stage pancreatic cancer or colorectal cancer. A variety of delivery systems have been developed and evaluated in clinical studies to improve the bioavailability of curcumin and to refine its therapeutic efficacy, including liposomal formulations, or encapsulation in polymeric nanoparticles. Some of the studies have been completed and waiting for the results, and others are still ongoing. Promising results are anticipated and further studies are warranted.

30.5 Conclusions and Future Perspectives

It is now believed that 90–95% of all cancers are attributed to lifestyle, with the remaining 5–10% attributed to faulty genes (Aggarwal *et al.*, 2009). One third of all cancer deaths in the USA are attributed to poor nutrition, physical inactivity, being overweight, and obesity (McGinnis and Foege, 1993). Food is one of the most important modifiable lifestyle aspects from patients’ perspectives. There is abundant evidence supporting that consumption of foods rich in fruits and vegetables decreased the occurrence of cancers. For nearly 30 years, epidemiologic studies have shown that dietary enhancement could prevent up to 90% of certain cancers including colorectal (Chan and Giovannucci, 2010), skin (Khan *et al.*, 2008), prostate (Syed *et al.*, 2008), breast (Bougnoux *et al.*, 2010), and lung cancers (Crangau and Camporeale, 2009).

Various scientific investigations have confirmed that curcumin possesses diverse and multiple molecular pathways of action involved in carcinogenesis and tumor formation (Zlotogorski *et al.*, 2013). All of the three central mechanisms of cancer progression in terms of the interplay between tumor microenvironment elements, disorganized inflammation, neovascularization, and immune modulation, are linked to the activation of NF- κ B. Curcumin’s inhibitory effect of NF- κ B related pathways has been extensively studied. Nevertheless, the research regarding the molecular mechanisms underlying the anticancer effects of curcumin through suppressing the crosstalk between the elements of tumor microenvironment is still in its infancy and, thus, there is so much to be elucidated. Further research is urgently required to confirm the anti-cancer effect of curcumin in modulating the tumor microenvironment.

Table 30.1 Clinical studies of curcumin for the treatment and prevention of cancers.

Intervention	Trial No.	Cancer types	Primary outcome	Phase	Status
Curcumin, 8g/day	NCT00094445	Pancreatic cancer	6-month survival	II	Completed
Nanoemulsion curcumin	NCT01975363	Breast cancer	Adherence, tolerability, safety	Pilot	Recruiting
Curcumin (1,2,3,or 4 g/day) + irinotecan	NCT01859858	Colorectal cancer	MTD	I	Recruiting
Surface-controlled water soluble curcumin	NCT01201694	Advanced cancers	MTD	I	Completed
RT with or without curcumin	NCT01917890	Prostate cancer	PFS	I/II	Recruiting
Curcumin + gemcitabine	NCT00192842	Pancreatic cancer	PFS	II	Completed
Preventive curcumin	NCT00027495	Colorectal cancer	MTD for prevention	I	Completed
Curcumin+CTX vs. CTX only	NCT01490996	Colorectal cancer	Completion of dose escalation > 2 cycle	I/II	Recruiting
Curcumin+taxotere vs. placebo+taxotere	NCT00765765	Prostate cancer, MCR	PFS	II	Recruiting
Curcumin, 2g/day	NCT02017353	Endometrial carcinoma	Inflammatory marker change	II	Recruiting
Curcumin vs. placebo	NCT02064673	Prostate cancer	RFS	II	Recruiting
Curcumin vs. curcumin+bioperine	NCT00113841	Multiple myeloma	NF-κB % change	Pilot	Completed

Abbreviations: CTX, chemotherapy; MCR, metastatic castration resistant; MTD, Maximum tolerated dose; PFS, progression-free survival; RFS, recurrence-free survival; RT, radiation therapy.

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Metabolomics of Green Tea

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31.1 Introduction

Natural products derived from medicinal plants are an abundant source of biologically active compounds, many of which have formed the basis for the development of nutraceuticals and pharmaceuticals (Raskin *et al.* 2002). Tea (*Camellia sinensis* L.) is a popular beverage worldwide, and, because of its possible health effects, it has received considerable attention as a medicinal herb (Khan and Mukhtar 2013). There are three main types of tea that differ according to the fermentation process; green (unfermented), oolong (semi-fermented), and black (fermented). Green tea constituents show various biological and pharmacological activities including prevention of cancer, reduction of body weight, alleviation of metabolic syndrome, prevention of cardiovascular diseases, and protection against neurodegenerative diseases (Yang and Hong 2013). The chemical components of tea vary according to species/cultivar, environment, growth, storage conditions, and leaf quality (Le Gall *et al.* 2004). In most cases, the quality and the health-promoting effects of tea are defined by their specific composition.

Metabolites, representative low-molecular-weight molecules (<1000 Da), are the result of the interactions of a system's genome with its environment, and are the end products of gene expression. The metabolome is defined as the total quantitative collection of low-molecular-weight metabolites present in a cell, tissue, or organism, that participate in the metabolic reactions required for growth, maintenance, and normal function (Fiehn 2002; Nicholson *et al.* 1999; Villas-Boas *et al.* 2005). Unlike the transcriptome and proteome that represent the processing of information during the expression of genomic information, the metabolome more closely represents the phenotype of an organism under a given set of conditions. Metabolomics, the measurement of global metabolite profile from a biological sample under different conditions, can help us to assess the quality of plant products, discover discriminant biomarkers, provide an enhanced understanding of disease mechanisms, elucidate mechanisms responsible for drug or herbal/dietary compound action, and predict individual variations in drug or herbal/dietary compound response phenotypes (Hur *et al.* 2013; Inoue *et al.* 2013; Lu, *et al.* 2013; Soga *et al.* 2006; Sreekumar *et al.* 2009; van Dorsten *et al.* 2006). In agricultural, nutritional, and medicinal research, a metabolomics approach is primarily applied to three areas of study: (1) chemical composition of foods and drinks, (2) metabolic responses to the intake of food or its components, and (3) biotransformation of dietary factors (Figure 31.1).

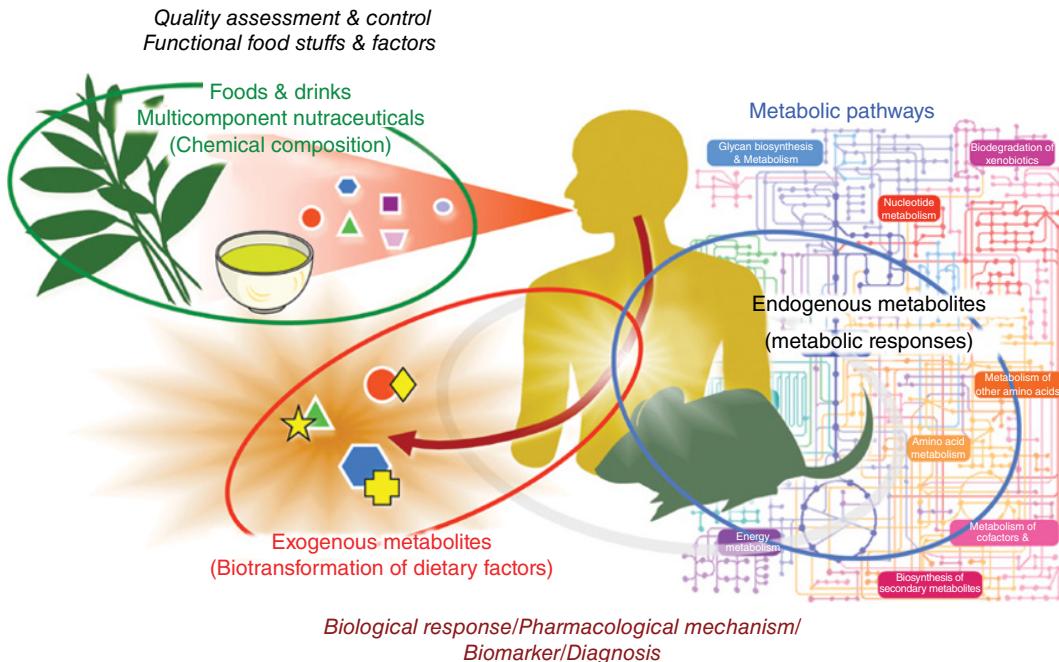


Figure 31.1 Targets of metabolomic approaches in tea research.

The functional biochemistry of plants is very diverse. The concentrations of many metabolites vary widely, and metabolomic analyses are required to determine all metabolites in plant extracts. MS and ^1H -NMR spectroscopy are representative analytical techniques useful for plant metabolomic research (Dunn *et al.* 2005; Krishnan *et al.* 2005). NMR allows the high-throughput analysis of crude extracts, and the quantitative detection of many different groups of metabolites, also providing structural information (Kim *et al.* 2010, 2011). However, NMR is less sensitive than MS-based approaches (Kim *et al.* 2011). MS coupled with pre-separation techniques, such as liquid chromatography: (LC)-MS or gas chromatography: (GC)-MS, is extensively used to investigate a wide range of molecules, including primary and secondary metabolites. The workflow of metabolic profiling includes sample preparation, analysis using various instruments, data processing, and data analysis (Figure 31.2). Chemometric methods including an unsupervised approach, principal component analysis (PCA), and another supervised approach, such as partial least-squares (PLS) or orthogonal PLS (OPLS)-discriminant analysis (PLS/OPLS-DA), and PLS/OPLS regression analysis, have been used to explore the relationships between the metabolome of diverse plant species and their genotype, origin, vintage, quality, or other specific attributes (Cuadros-Inostroza *et al.* 2010; Heuberger *et al.* 2010; Luthria *et al.* 2008; Okazaki *et al.* 2008). These approaches may also be useful for the unbiased evaluation of pharmaceutical properties of crude plant extracts and to identify specific bioactive compounds in extracts. In nutraceutical research, such techniques have been applied to identify subtle metabolic differences among individuals or different environmental conditions, including diet (Raskin *et al.* 2002; Scalbert *et al.* 2014). Herein, we describe recent applications of metabolomic approaches to study tea and its involvement in health.

31.2 Metabolic Profiling

Chemical composition affects tea quality and various biological activities. Metabolites of tea leaf strongly depend on genetic and environmental conditions. Metabolic profiling methods have been used to explore the relationships between the metabolome of diverse crude samples, such as tea chemical composition, and their specific attributes by determining the relative

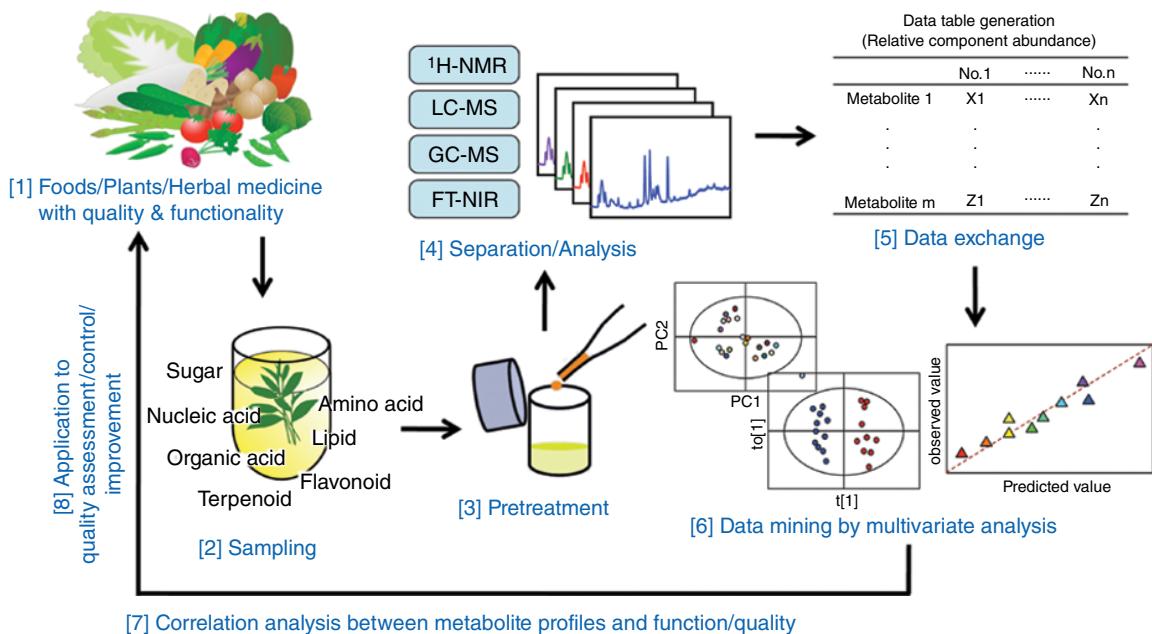


Figure 31.2 Scheme of a typical metabolic profiling experiment.

abundance of each metabolite to the total abundance of all metabolites. In these studies, MS, $^1\text{H-NMR}$ spectroscopy, and Fourier transform-infrared (FT-IR) spectroscopy have been used as analytical tools (Table 31.1). The rationale behind such techniques is that each sample's chemical composition is characterized using the multivariate spectra, and useful information concerning tea quality can be extracted by multivariate calibration and/or pattern recognition methods. NMR is a commonly used analytical tool in metabolomics research, and detects the specific resonance absorption profiles of metabolites in a magnetic field. One major advantage of NMR is non-invasiveness and non-reliance on analyte separation. NMR can be also used for the analysis of samples that are composed of diverse compound mixtures because the signal intensity is less affected by the other components in the sample. However, the major drawback of NMR is its lower sensitivity and detection coverage compared with MS. The availability of mass spectrometers is much greater than high-field NMR instruments, in part because of their lower cost. Thus, hyphenated chromatography-MS techniques have become increasingly used for metabolic profiling as well as better metabolite identification and quantification. However, unlike NMR and FT-IR spectroscopy, it is a destructive technique. FT-IR is not as sensitive as MS methods, but is widely used to obtain spectral fingerprints of biological samples. This technique has the benefit of enabling the rapid, reagentless, non-destructive analysis of complex biological samples, thus facilitating high-throughput screening and providing unbiased measurements of the whole system.

At present, because there is no universal instrument capable of measuring every type of metabolite, researchers should select the most appropriate method for their research, giving consideration to several factors including sensitivity, throughput, and cost (Putri *et al.* 2013).

Data analysis capable of detecting significant changes such that the data from biological samples can be validated is a crucial process in metabolic profiling. Generally, multivariate statistical analysis is used to clarify similarities and differences among samples on the basis of the multivariate data matrix (e.g., MS or NMR datasets). Such relationships are usually displayed as scatter plots (score plots) (Figure 31.3). Because hundreds of variables (peaks) are obtained, the relationships among samples must be theoretically interpreted on hundreds of dimensional axes (variables), but these relationships cannot be simply displayed. To visualize the sample characteristics, multivariate analysis can extract sample features by dimensional reduction. That is, hundreds of original variables are decreased to two or three synthetic variables, which are orthogonal with each other. This maximizes the statistical variance of samples, while leaving the original feature of samples largely unaffected (Trygg *et al.* 2007). The synthetic variables consist of

Table 31.1 Metabolic profiling of tea extracts.

Targets	Products (number)	Tools	References
Production origin/price and grade	Green tea (191)	$^1\text{H-NMR}$	Le Gall <i>et al.</i> 2004
Production origin/ manufacturing type	Green, oolong, black, yellow, white, and pur-erh teas (187)	$^1\text{H-NMR}$	Fujiwara <i>et al.</i> 2006
Climate	Green tea (4)	$^1\text{H-NMR}$	Lee <i>et al.</i> 2010
Altitude	Black tea (4)	$^1\text{H-NMR}$	Ohno <i>et al.</i> 2011
Manufacturing type/age	Pu-erh, black, and green teas (24)	LC-MS	Xie <i>et al.</i> 2009
	Tuocha (Black, green, or postfermented type; 20)	FT-IR	Xu <i>et al.</i> 2011
Shade culture/season	Green tea (4)	LC-MS/GC-MS	Ku <i>et al.</i> 2010a
Shade period/nutritional and sensory qualities	Green tea (4)	LC-MS	Lee <i>et al.</i> 2013
Fermentation process	Pu-erh tea (7)	LC-MS	Chen <i>et al.</i> 2013
	Pu-erh, black, green, white, yellow, and oolong teas (71)	LC-MS	Zhang <i>et al.</i> 2011
Postfermentation year	Pu-erh tea (30)	LC-MS	Ku <i>et al.</i> 2010b
Plucking position of leaf	Green tea (5)	$^1\text{H-NMR}$	Lee <i>et al.</i> 2011
Sensory quality	Green tea (53)	GC-MS	Pongsuwan <i>et al.</i> 2007
	Green tea (56)	LC-MS	Pongsuwan <i>et al.</i> 2008
	Green tea (53)	$^1\text{H-NMR}$	Tarachiwin <i>et al.</i> 2007
	Green tea (64)	FT-NIR	Ikeda <i>et al.</i> 2007
Health-promoting effect	Green tea (43)	LC-MS	Fujimura <i>et al.</i> 2011

**Figure 31.3** Multivariate statistical analyses used in metabolic profiling.

hundreds of original variables. An understanding of the contribution of each original variable to the synthetic variables leads to the identification of key variables that contribute to the similarities or differences among samples (Figure 31.3, loading plot).

PCA is an unsupervised approach and the most frequently used method in metabolomics for data mining (Figure 31.3, left panel). This model is depicted as a score plot and consists of two synthetic variables: principal component (PC) 1 (the greatest variance of data) and PC2 (the second greatest variance of data, orthogonal with PC1). This displays intrinsic groups of samples based on spectral variations. The corresponding loading plots show the contribution of each spectral variable to score formation.

The supervised multivariate technique is also used for identifying interesting metabolites. The PLS-based approach, which includes PLS, PLS-DA, OPLS, and OPLS-DA, can extract Y-correlated information from the X matrix (Figure 31.3, middle and right panels). The X matrix is an organized data matrix obtained with the non-targeted approach, while the Y matrix is the supervised data. If the Y matrix has specific variables, such as the parameter variables of a producing instrument or food quality evaluated by a sensory test, PLS and OPLS approaches are convenient methods for extracting meaningful metabolites correlated with Y variables. Particularly in the univariate Y, an OPLS approach allows us to easily interpret results compared with PLS. The orthogonal approach can remove the un-correlated data from the X to Y matrix where the number of latent variables correlated to Y is generally one. Because of features of the orthogonal method, users only focus on the first component to interpret the results. When searching for significant metabolites, researchers can use not only the loading matrix, but also the variable importance in the projection (VIP) (Figure 31.3, right panel). If the Y matrix does not have specific variables, but has some biases, a discriminant approach, such as PLS-DA and OPLS-DA, are frequently used (Figure 31.3, middle panel).

31.3 Tea Chemical Composition

On the basis of the previously mentioned analytical strategies, metabolic profiling of extracts from tea leaves has been performed to study various attributes (Table 31.1). Generally, the quality of teas is evaluated by professional tea tasters who evaluate product quality by leaves' appearance and aroma, color, and taste of the brew. Because the process of training a skilled tea taster may take years and is very expensive, it would be attractive to evaluate tea quality by some non-human technologies. As a promising alternative approach to the traditional methods of chemical and sensory analysis, metabolic profiling has been attempted for tea analysis. Fukusaki and coworkers succeeded in predicting the Japanese green tea ranking by metabolic profiling using four different analytical platforms (Ikeda *et al.* 2007; Pongsuwan *et al.* 2007, 2008; Tarachiwin *et al.* 2007). Furthermore, some metabolites, playing an important role in green tea's grade classification, were identified. These chemometric or data-driven approaches might have an advantage over the ordinary sensory test for classification and determination of tea quality. Recently, metabolites from a 50% aqueous methanol extract of green teas grown with different shade periods (0, 15, 18, and 20 days) were analyzed to investigate the effect of low light on their nutritional and sensory qualities (Lee *et al.* 2013). The shaded groups could be clearly distinguished from the control (0 day), and the 20-day group could be separated from the 15- and 18-day groups. The shade treatment increased quercetin-galactosylrutinoside, kaempferol-glucosylrutinoside, epicatechin-3-O-gallate (ECG), epigallocatechin-3-O-gallate (EGCG), tryptophan, phenylalanine, theanine, glutamine, glutamate, and caffeine levels but decreased quercetin-glucosylrutinoside, kaempferol-glucoside, gallic acid, and epigallocatechin (EGC) levels. This result, along with the sensory evaluation and color measurement data, suggests that shade treatment improves the nutritional and sensory quality of green tea. They also proposed a metabolomic pathway that could explain the relationship between low light and tea quality.

Green tea has various health-promoting effects. Although there are numerous tea cultivars, little is known about the differences in their nutraceutical properties. We performed metabolomic analyses to explore the relationship between the metabolome and health-promoting attributes of diverse Japanese green tea cultivars (Fujimura *et al.* 2011). We investigated the ability of leaf extracts from 43 Japanese green tea cultivars to inhibit thrombin-induced phosphorylation of myosin regulatory light chain (MRLC) in human umbilical vein endothelial cells. This thrombin-induced phosphorylation is a potential hallmark of vascular endothelial dysfunction. Among the tested cultivars, Cha Chuukanbonou Nou-6 and Sunrouge (SR) strongly inhibited MRLC phosphorylation. To evaluate the bioactivity of green tea cultivars using a metabolomics approach, metabolite profiles of all tea extracts were determined by LC-MS. Multivariate analyses revealed differences among green tea cultivars with respect to their ability to inhibit MRLC phosphorylation. In the SR cultivar, polyphenols were associated with its unique metabolic profile and its bioactivity.

In addition, using PLS regression analysis, we succeeded in constructing a reliable bioactivity-prediction model to predict the inhibitory effect of tea cultivars based on their metabolome. This model was based on certain identified metabolites that were associated with bioactivity. When added to an extract from the non-bioactive cultivar Yabukita, several metabolites enriched in SR were able to transform it into a bioactive extract. Recently, Ku and coworkers used metabolomic approaches to determine the effect of manufacturing type or cultivation method on the chemical composition of a single tea cultivar (green tea or pu-erh tea) (Ku *et al.* 2010a, b). They suggested that several polyphenolic compounds were associated with manufacturing type, cultivation method, or antioxidant activity. We investigated relationships between metabolomic data and health-promoting effects in 43 green tea cultivars. In the cultivar SR, certain polyphenolic constituents (delphinidin-glucoside/galactoside, quercetin-glucoside/galactoside, and theogallin) were associated with bioactivity. Although polyphenols have many health-promoting effects, the relationship between these compounds and the inhibition of MRLC phosphorylation in human endothelial cells remains unclear. These polyphenols differed from those reported by Ku *et al.* (Ku *et al.* 2010a, b). In addition, such polyphenols, especially anthocyanins, were barely present in the most consumed and distributed Japanese green tea cultivar. These facts indicate that a metabolomic approach is a useful tool for identifying unique bioactive factors. Such information may be useful for the development of markers to produce new cultivars with greater bioactivity, and to screen for bioactive tea cultivars.

31.4 Metabolic Responses to Tea Consumption

Metabolomics approaches have been reported as important and effective tools to examine the changes in endogenous metabolites of the whole system and potentially provide a better mechanistic understanding of biochemical and cellular events (Nicholson *et al.* 1999). Recently, effects of black and green tea consumption on human metabolism were investigated by ¹H-NMR-based metabolic profiling (van Dorsten *et al.* 2006). Green and black tea consumption (equivalent to 12 cups of tea per day) caused a different impact on endogenous metabolites in urine and plasma. Green tea intake increased the urinary excretion of several citric acid cycle intermediates more than black tea, which suggests an effect of green tea flavanols on human oxidative energy metabolism and/or biosynthetic pathways.

Hodgson and coworkers showed that GC-MS and LC-MS-based metabolic profiling of human plasma can enhance our understanding of the mode of action of exercise and green tea extract (GTE) beyond the physiological outcomes (Hodgson *et al.* 2013). Moderate-intensity exercise stimulated multiple metabolic pathways including lipolysis, glycolysis, as well as the activation of the citric acid cycle and the adrenergic system. Metabolite changes induced by GTE were more subtle and affected fewer pathways when compared with those induced by exercise alone. Seven-day GTE supplementation (1200 mg total catechins and 240 mg caffeine/day) mainly enhanced metabolites indicative of lipolysis and fat oxidation under resting conditions when compared with placebo. This effect was not enhanced during exercise. Furthermore, GTE did not stimulate the adrenergic system during rest and exercise because no increase in noradrenaline and related catecholamines was observed. This challenges catechol-*O*-methyltransferase inhibition as the putative mechanism of action of GTE *in vivo*. Yet GTE stimulated lipolysis under resting conditions, suggesting nonadrenergic mechanisms.

Green tea has been suggested to have beneficial health effects including protective effects against oxidative stress. Acetaminophen (APAP) is a widely used analgesic drug that can cause acute liver injury in overdose situations. Lu *et al.* explored the effects of GTE (500 or 1,000 mg/kg) on APAP-induced hepatotoxicity in liver tissue extracts by LC-MS and ¹H-NMR-based metabolic profiling of mice livers with GTE pretreatment 3 h or 3 days prior to APAP exposure or GTE exposure 6 h after APAP (Lu *et al.* 2013). GTE given prior to APAP ameliorated the APAP-induced hepatotoxicity in a dose-dependent manner whereas GTE given after APAP potentiated the toxicity. APAP exposure alone significantly altered multiple metabolite levels compared with the control. GTE pretreatment resulted in the metabolite levels returning to control levels or being less altered. In contrast, GTE given after APAP caused more changes in the metabolites over what was induced by APAP alone, indicating more severe hepatotoxicity. The changes in liver metabolites indicated perturbations of fatty acid metabolism, energy metabolism, bile acid metabolism, and phospholipid metabolism induced by APAP with differing effects on these metabolites depending upon the time of GTE exposure. The results indicate that the time at which GTE was given highly influenced the severity of APAP-induced toxicity. These findings highlight the need to understand the interactions between GTE with drugs, and support the importance of a metabolomic approach.

31.5 Biotransformation of Dietary Tea Components

Using LC-MS/MS metabolomic approach, Sang and coworkers identified more than 20 metabolites of tea polyphenols from human urine samples collected at different time periods after green tea consumption (Sang *et al.* 2011). It is known that phase II biotransformation (methylation, glucuronidation, and sulfation) and ring-fission metabolism represent the major metabolic pathways for tea catechins (Lambert *et al.* 2007). Consistent with previous observations, phase II metabolites of EGCG and ECG were undetectable in this study. EGC-glucuronide, methylated EGC-glucuronide, methylated EGC-sulfate, epicatechin (EC)-glucuronide, EC-sulfate, methylated EC-sulfate, as well as the glucuronide and sulfate metabolites of the ring fission metabolites of catechins were the major human urinary metabolites of tea polyphenols. Metabolite profile analyses of tea catechins provided by this study will help us to identify the metabolites that are representative of a 24-h time period after tea consumption. For example, Sang *et al.* found that most of the urinary EC and EGC metabolites were excreted within 9 h; however, the ring-fission metabolites and their glucuronide and sulfate conjugates are the major metabolites in the urine even at 12–24 h after tea ingestion (Sang *et al.* 2011). Because EC is also found in cocoa and many fruits, metabolites of EC and its related ring-fission product are not specific markers to reflect tea consumption, but they can be used as general markers to reflect fruit, chocolate, and tea consumption. Therefore, conjugated metabolites of EGC, methylated EGC, and its ring-fission products can be used as the exposure markers to reflect tea consumption. Using catechins and their metabolites to reflect tea consumption will be more accurate than the traditional method to follow tea consumption used in epidemiological studies, which is based on how many cups of tea are consumed by the subjects per day. These studies are actively performed as part of food metabolome analysis for identification of dietary markers to better reflect exposure to foods, including pur-erh and black teas (Xie *et al.* 2012; van Duynhoven *et al.* 2014), which helps to clarify their effects on health and diseases (Scalbert *et al.* 2014).

To elucidate the precise mechanism underlying the bioactivity of green tea polyphenol, high-resolution spatiotemporal information is needed. Although some studies have visualized its tissue distribution by fluorescence imaging, cerium chloride staining, and radioactive labeling assays (Fujimura and Miura 2014), spatiotemporal information has been lacking because of the absence of an analytical technique that can easily detect the localization of the naïve polyphenol. Conventional molecular imaging generally requires labeling steps that are time-consuming, expensive, and labor-intensive. The ability of these techniques to allow the discrimination of molecules is insufficient for simultaneous visualization of a target compound and its metabolites. A label-free molecular imaging technique could overcome these issues, but the development of such a technique has been a challenge.

Mass spectrometry imaging (MSI) is a new technology capable of determining the naïve distribution of ionizable biological molecules in tissue sections without any labeling on the basis of their specific mass-to-charge ratios. This technique can theoretically detect target molecules and their metabolites simultaneously in a single analysis, and is now widely used for *in situ* imaging of endogenous and exogenous molecules such as proteins, lipids, drugs, and their metabolites (Fujimura and Miura 2014). It is a tool potentially useful for the pathological analysis and understanding of diseases or pharmaceutical mechanisms. Matrix-assisted laser desorption/ionization (MALDI) is a commonly available ionization method used for MSI. MALDI-MSI can visualize macromolecules such as lipids and proteins/peptides. However, small molecules are not easily detected by MALDI-MSI because many matrix and/or matrix-analyte cluster ion peaks are observed in the low-mass range ($m/z < 700$). In contrast, Miura *et al.* recently reported that MALDI-MS with 9-aminoacridine (9-AA) achieved great improvement for the sensitivity of detection of endogenous low-molecular-weight metabolites in the negative ionization mode (Miura *et al.* 2010a, b). However, it still remains unclear whether such highly sensitive MALDI-MS techniques can visualize the most abundant and bioactive green tea polyphenol, EGCG.

A MALDI-MSI study showed that the EGCG peaks were not observed using 9-AA and major matrices, but 1,5-diaminonaphthalene (1,5-DAN) allowed for the detection of EGCG (m/z 457 [$M-H^-$]) in negative ionization mode (Kim *et al.* 2013). Furthermore, 1,5-DAN was useful to visualize the distribution of a single oral dose of EGCG in mouse tissue sections (Figure 31.4). Understanding the metabolic fates of bioactive dietary polyphenols is indispensable for determining their *in vivo* molecular mechanisms (Yang *et al.* 2013). However, both the functions of polyphenol metabolites and their localizations in different tissue micro-regions are unclear (Lambert *et al.* 2007). In contrast, 1,5-DAN-MALDI-MSI was able to visualize a spatially resolved biotransformation based on simultaneous mapping of orally dosed EGCG and its phase II metabolites such as its monosulfate (m/z 537) and monoglucuronide (m/z 633) forms (Figure 31.4). Interestingly, unlike in the liver, the localization patterns in the kidney compartments (pelvis, medulla, and cortex) were clearly different among EGCG and its phase II metabolites. Although the bioavailability of EGCG is very low (Lambert *et al.* 2007), this imaging technology could be used to visualize the *in situ* distribution of EGCG phase II metabolites in liver and kidney sections after oral dosing. This study will

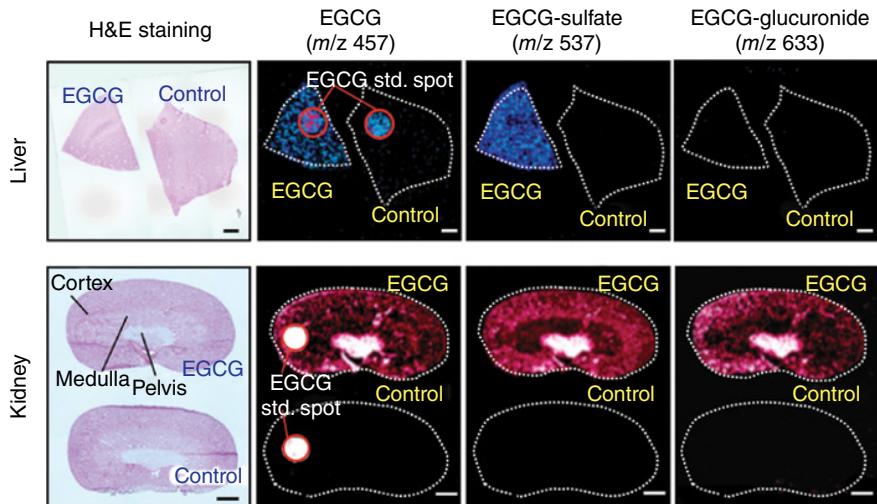


Figure 31.4 Visualization of dosed EGCG and its phase II metabolites within mammalian tissue micro-regions. Simultaneous visualization of EGCG and its phase II metabolites in liver (upper panel) and kidney (lower panel) sections. Two different images are shown in H & E staining and MALDI-TOF-MS: EGCG (m/z 457), EGCG-sulfate (m/z 537), and EGCG-glucuronide (m/z 633). An additional EGCG spot (gray circle) was visualized as the positive and internal control. Adapted from Kim *et al.* (2013) with permission from Nature Publishing Group.

contribute to unraveling both the biological consequences of the biotransformation of polyphenol and its mechanism of action, as well as help to accelerate the highly effective and efficient design of polyphenol-derived pharmaceuticals, multicomponent botanical drugs, dietary supplements, and functional foods.

31.6 Conclusion

GTE is a representative multicomponent botanical. Pharmacology of botanical-based nutraceuticals requires a “network” approach, in which multiple compounds interact with multiple targets *in vivo* with interdependent activities to achieve an optimal effect. The traditional approach to understanding the pharmacology of a multicomponent nutraceutical is to study the effects of a single component on a single biological activity, and gradually assemble those effects into an integrated picture. However, assembling the results obtained from such a reductionist approach to achieve a systems understanding of a concerted pharmacological intervention has proven impractical (Xue and Roy 2003). In addition, pharmacokinetic properties of a given compound in a multicomponent assay may be significantly different from that in a single compound assay because of drug–drug interactions. With such a complex “network” approach involving a large number of multi-parametric variables, it is technically challenging to identify the origin of each significantly changed metabolite in global metabolite pools and to assess the human endogenous responses to the exposure of GTE exogenous compounds (Xie *et al.* 2012). In this chapter, we described the applicability of recent metabolomics approaches to resolve such issues, especially in regard to defining the chemical composition of GTE, its metabolic responses, and biotransformation which are useful for quality control, health promotion, and chemotherapy. $^1\text{H-NMR}$, FT-IR, LC-MS, GC-MS, and MALDI-MS have advantages and disadvantages as metabolomics methods, and the choice should ultimately be determined by research goals and available laboratory equipment.

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Part V

Epigenetics

32

The Potential Epigenetic Modulation of Diabetes Influenced by Nutritional Exposures *In Utero*

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32.1 Introduction

Type 2 diabetes afflicts more than 300 million people worldwide (WHO 2015). Type 2 diabetes brings other health issues including increased risk of heart attacks, kidney failure, diabetic retinopathy, and diabetic foot (Kolata 1979; Porte and Schwartz, 1996).

32.2 Insulin Resistance

Insulin resistance is the common feature of many metabolic disorders, including Type 2 diabetes, obesity, cardiovascular disease, and hypertension (Reaven 2005). Insulin is a peptide hormone with 51 amino acids in total and is produced within the β -cells of the islets of Langerhans in the pancreas (Orci and Unger 1975; Steiner and Oyer 1967). Insulin orchestrates glucose homeostasis on one hand, by stimulating glucose uptake into peripheral tissues and on the other hand, by inhibiting glucose production by the liver. In this way, the normal blood glucose level is tightly maintained between 4 and 7 mM. Insulin resistance is a status when a normal amount of insulin cannot achieve the expected normal biologic response. At the early stage of insulin resistance, β -cell wills compensate by increasing insulin secretion in order to maintain blood glucose levels within a normal range. When insulin resistance develops, β -cell fails to produce the required insulin and the blood glucose levels rise (Clark *et al.* 1988, Donath and Halban 2004, Kloppel *et al.* 1985, Wajchenberg 2007).

The fat overload theory has been proposed for the development of insulin resistance over the past several decades (Griffin *et al.* 1999; Petersen *et al.* 1998; Roden *et al.* 1996). The role of free fatty acids was initially revealed based on several observations that insulin resistance is associated with elevated levels of free fatty acids in blood (Reaven *et al.* 1988). Fat is supposed to be stored in adipocytes in the form of triglycerides and the lipid leakage is responsible for insulin resistance in different tissues including skeletal muscle, pancreas, and heart, as well as liver (Boden *et al.* 2001; Krssak *et al.* 1999; Perseghin *et al.* 1997, 1999). Inflammation is the other theory illustrating insulin resistance. Plenty of

inflammatory factors secreted from adipocytes may interfere with the insulin signaling pathway and impair insulin action and glucose transport (Hotamisligil *et al.* 1996; Uysal *et al.* 1997; Vallerie *et al.* 2008; Yuan *et al.* 2001).

32.3 Skeletal Muscle

Skeletal muscle comprises 40–50% of the body mass and is the major site of substrate metabolism. Skeletal muscle is recognized as the primary site for postprandial glucose clearance (DeFronzo *et al.* 1985) and skeletal muscle is the major organ involved in the development of insulin resistance in Type 2 diabetic patients (DeFronzo 1988). Thus, defects in insulin-stimulated glucose transport in skeletal muscle account for the whole body insulin resistance noted in people with severe obesity or Type 2 diabetes (Dohm *et al.* 1988; Goodyear *et al.* 1995; Zierath *et al.* 1994).

32.4 Type 2 Diabetes

Type 2 diabetes is a multifactorial disease involving interactions between genetic and environmental, and nutritional factors. There are plenty of examples indicating there is a strong inheritance risk for Type 2 diabetes. For example, the people with first-degree relatives suffering from Type 2 diabetes will have a higher chance of developing Type 2 diabetes (Deo *et al.* 2006; Mills *et al.* 2010). Genome-wide association study (GWAS) provides further evidence that a number of specific genes are related to metabolic diseases (Brito *et al.* 2009; Franks *et al.* 2007a; Ruchat *et al.* 2010). *FTO* (rs9939609) has been associated with Type 2 diabetes by increasing obesity risk (Frayling *et al.* 2007). However, genetic variants identified in GWAS are unlikely to be responsible for the different susceptibilities to a disease in a population and genetic background could only explain a fraction of cases of metabolic diseases. Type 2 diabetes is more likely to be determined by the genotype and environmental interactions (Eichler *et al.* 2010; Franks *et al.* 2007b; Manolio *et al.* 2009).

32.5 Influence of High-Fat Diet

A high-fat diet is believed to contribute to insulin resistance based on the findings that accumulation of intracellular lipid metabolites from incomplete lipid oxidation could inhibit insulin signal transduction to glucose transport (Kim *et al.* 2000; Petersen *et al.* 2004; Ritov *et al.* 2005). Exercise is beneficial for improving insulin sensitivity by eliciting gene expression alterations that trigger structural and metabolic adaptations in skeletal muscle. Muscle contraction through physical activity drives adaptive responses to improve metabolic efficiency and oxidative capacity by changing gene expression profiles (Coffey and Hawley 2007).

32.6 Obesity

Obesity characterized with excess accumulation of white adipose tissue increases the prevalence of Type 2 diabetes. Conventional strategies for the management of obesity are often insufficient such as diet modifications and exercise behavior changes. Additionally, pharmacological options are limited (Matthews *et al.* 1998; Turner *et al.* 1999). When conventional strategies and drugs no longer work, many severely obese individuals opt to choose gastric bypass surgery as a means to reduce daily calorie absorption and lose weight. Several lines of evidence indicate that gastric bypass surgery can reduce comorbidities and improve clinical outcomes associated with obesity (Hammoud *et al.* 2009; Marsk *et al.* 2010; Sjostrom *et al.* 2004). Furthermore, the surgery dramatically improves insulin sensitivity and leads to the clinical remission of Type 2 diabetes (Greenway *et al.* 2002; Rubino *et al.* 2010; Sjostrom *et al.* 2007). Gastrointestinal peptide release has been proposed to participate in the improvement of glucose metabolism, but the underlying mechanism is not fully understood (Butner *et al.* 2010; Falken *et al.* 2011).

Plenty of evidence has showed environmental factors after birth can influence development of Type 2 diabetes. Nevertheless, more and more studies revealed the importance of nutritional conditions *in utero*. As early as two decades ago, David Barker showed environmental events and nutritional exposures *in utero* could have a lasting impact on adult disease (Barker *et al.* 1989). Barker *et al.* initially described the correlation of birth weight and the incidence of adult disease and proposed the “Barker hypothesis”: that adult chronic disease may originate from the adverse fetal environment. This was one of the first clues into why the *in utero* environment is important. Nowadays, the concept of “DOHaD”

(Developmental Origins of Health and Disease) has been widely accepted. It emphasizes the origins of human diseases and highlights the potential mechanism of pathogenesis and progression of metabolic diseases.

Epidemiology studies also showed that poor fetal growth was correlated with increased risk of hypertension, coronary heart disease and Type 2 diabetes. The most important one was the study of children born from the 1944–1945 Dutch famine that provided a link between maternal nutritional status and diseases in the offspring (Painter *et al.* 2005; Ravelli *et al.* 1976). During that time, people, including pregnant women, received as little as 400–800 calories per day. Those who exposed to the famine during early gestation had a higher risk to develop obesity and cardiovascular disease, whereas those who exposed only during late gestation produced lower obesity rates.

32.7 Intrauterine Growth Restriction (IUGR)

The intrauterine growth restriction (IUGR) rodent model with approximately 50% reduction in uteroplacental blood flow by ligating both uterine arteries (Simmons *et al.* 2001) is a classic animal model to mimic the adverse intrauterine environment and has been widely used to investigate the underlying biological mechanism. IUGR has been linked to the development of Type 2 diabetes in adulthood (Barker 1999). The underlying mechanism is still obscure. The Pdx-1, a pancreatic and duodenal homeobox 1 transcription factor that is critical for β -cell function and development, was reduced in β -cells of IUGR model (Stoffers *et al.* 2003).

Compared to the IUGR model, gestational diabetes mellitus (GDM) serves as another example to demonstrate the influences of intrauterine nutritional exposures on long-term effects of offspring. GDM has become a common medical complication in pregnancy and the prevalence of GDM varies between populations with a range of 1.7–11.6% (Schneider *et al.* 2012). Both epidemiologic investigations and animal studies have revealed long-term sequelae including cardiovascular abnormalities and metabolic syndrome in adult offspring exposed to intrauterine hyperglycemia (Dabelea *et al.* 1998; Metzger *et al.* 2008; Simeoni and Barker, 2009). Initially, epidemiologic studies illustrated that the Pima Indian population in Arizona had the highest prevalence of Type 2 diabetes among children and adults indicating that maternal hyperglycemia might contribute to the disease susceptibility in offspring (Pettitt *et al.* 1983). Further data supported that the offspring of diabetic mothers revealed high birth weight and had more chances to develop obesity, hypertension as well as dyslipidemia (Franks *et al.* 2006; Manderson *et al.* 2002).

The GDM rodent model can be established by administering streptozotocin that has the direct toxic effects on pancreatic β -cells. A high dose of streptozotocin destroys maternal β -cells and causes severe intrauterine hyperglycemia that can induce fetal growth restriction and small fetus (Van Assche *et al.* 2001). The offspring of diabetic mothers also showed higher levels of triglyceride and cholesterol at 26 weeks (Ma *et al.* 2012). We also found mean arterial pressure was increased at 26 weeks by exposure to intrauterine severe hyperglycemia in offspring (Ma *et al.* 2012).

However, the high glucose levels of diabetic patients or severe GDM women have been mostly well controlled during the pregnancy. In addition, an international multicenter Hyperglycemia and Adverse Pregnancy Outcomes (HAPO) study demonstrated that risk of adverse maternal, fetal, and neonatal outcomes continuously increased with maternal glucose levels, even mild hyperglycemia thought to be normal for pregnancy (Metzger *et al.* 2008). Thus, it is more realistic to study the effects of intrauterine mild hyperglycemia. Intrauterine mild hyperglycemia can also be induced by streptozotocin but at a lower dosage for 5 consecutive days (Van Assche *et al.* 2001). The offspring obtained from mildly diabetic mothers usually display normal glycemia and insulinemia and impaired glucose tolerance is only revealed under high glucose challenge (Van Assche *et al.* 2001). Our group has successfully established a rat model of intrauterine mild hyperglycemia induced by a single low dose of streptozotocin (Li *et al.* 2012). The birth weight of offspring obtained from mildly diabetic mothers was significantly higher than the controls (Li *et al.* 2012). The offspring of mildly diabetic mothers display normal blood glucose level at birth. Impaired glucose tolerance in adulthood was displayed under glucose tolerance test using glucose (2 g/kg body weight) given by gavage (Li *et al.* 2012; Yan *et al.* 2014). The offspring of diabetic mothers also showed higher level of triglyceride at 28 weeks (Zhang *et al.* 2013). We also found mean arterial pressure was increased from 12 weeks by exposure to intrauterine mild hyperglycemia in male offspring (Yan *et al.* 2014).

32.8 Environmental Factors and Epigenetic Modifications

All these data contribute the idea that early life environmental conditions can induce insulin resistance in offspring. It indicates that a certain developmental pattern has been programmed *in utero*. The next step is to investigate the potential mechanisms by which environmental factors can influence the levels of gene expression involved in insulin resistance.

A close relationship has been proposed to exist between environmental factors and epigenetic modifications. Several studies have been shown to support this hypothesis. For example, maternal care including licking and nursing behavior in rats could influence epigenetic changes of the glucocorticoid receptor promoter in offspring that might further affect offspring behaviors (Weaver *et al.* 2004). Monozygotic twins is another good example to illustrate the influences of environmental factors on different disease susceptibilities, indicating epigenetic differences that arise during aging may play an important role in disease development. A study by Fraga *et al.* provided evidence that young twin pairs had a similar distribution of epigenetic markers through the genome, whereas the amount and pattern of epigenetic markers of older twin pairs showed marked differences (Fraga *et al.* 2005).

What is “epigenetics”? The term *epigenetics* was first coined by the developmental biologist and evolutionist Conrad H. Waddington (1905–1975) and aimed to emphasize the potential mechanism driving the genotype to the phenotype during development. The definition has been changed and refined from time to time. The commonly accepted definition was described by Arthur Riggs and colleagues, “the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence”. DNA methylation and histone tail modifications are thought to be two major epigenetic modifications that can regulate gene expression at different layers. DNA methylation occurs on the 5 position of the pyrimidine ring of the cytosine. Particularly, 5-methyl cytosine followed by guanosine (CpG dinucleotide) is the dominant type of methylation pattern in mammals and CpG dinucleotides are usually clustered as CpG islands in the promoter regions of many genes. CpG islands modulate levels of gene expression by blocking transcription factor access to DNA. DNA methylation has been widely related to imprinting and mammalian development as well as genomic stability maintenance.

Non-CpG methylation (referring to methylated cytosine within CpA, CpT, or CpC) was anticipated to exist only in plants and embryonic stem cells. More non-CpG methylation than originally thought has been found in human embryonic stem cells based on a genome-wide, single-base-resolution method (Lister *et al.* 2009). More attention has been paid to revealing the potential physiological role of methylation in non-CpG contexts. Yan *et al.* reported that non-CpG methylation contributed to the total amount of DNA methylation in somatic tissues in mammals (Yan *et al.* 2011).

DNA methyltransferases (DNMT1, DNMT3A, and DNMT3B) have been identified to be responsible for catalyzing the addition of methyl groups to cytosine (Xie *et al.* 1999; Yen *et al.* 1992). The fundamental mechanism of DNMTs is not fully illustrated. Biochemical studies proposed that DNA methylation relied on the transient covalent complex formation (Santi *et al.* 1983). DNMTs have different functions in DNA methylation process. DNMT1 is believed to be responsible for maintenance of methylation during DNA synthesis, and DNMT3A and DNMT3B are required for *de novo* methylation (Bestor and Ingram 1983; Okano *et al.* 1999).

DNA methylation is established early in embryonic stage and cell type-specific DNA methylation patterns are created during the development. Environmental events and nutritional conditions may induce permanent DNA methylation mark changes *in utero* during the sensitive period and these adaptive changes will be “memorized” that may have a lasting impact on adult disease later (Barker *et al.* 1989). The potential plasticity of DNA methylation also enables reprogramming, depending on exposures to nutritional, chemical, and environmental factors.

DNA methylation can be modulated by intrauterine exposure of diet supplementation with methyl donors or mono carbon metabolites, such as folic acid and homocysteine (Cooney *et al.* 2002; Weaver *et al.* 2005). Studies also demonstrated that histone modifications could regulate expression of genes involved in glucose homeostasis in IUGR model. For example, significant decrease in H3 and H4 acetylation at the proximal promoter of *Pdx-1* has been reported in islets isolated from IUGR fetuses by which gene expression regulation would be interfered with the binding of a critical activator of *Pdx1* transcription (Park *et al.* 2008).

Individuals exposed the Dutch famine prenatally had relative lower DNA methylation of the gene of *IGF2* (*insulin-like growth factor-2*) (Heijmans *et al.* 2008), which was proved to be involved in growth and development (Baker *et al.* 1993) as well as altered methylation changes of other genes participating in metabolic disease (Tobi *et al.* 2009). These studies emphasized the importance of early intrauterine exposures on epigenetic alterations and these influences may be “memorized” and maintain throughout the whole lifespan. Additionally, epigenetic changes could also be transmitted to generation F2 and behave as transgenerational effects (Burdge *et al.* 2007; Painter *et al.* 2008).

Epigenetics is involved in normal development and the aberrant epigenetic marks participate in the disease progression. DNA methylation has been well studied and well discussed in tumors development (Bedford and van Helden, 1987; Cheng *et al.* 1997; Kim *et al.* 1994; Lin *et al.* 2001; Wahlfors *et al.* 1992). Epigenetic modifications may also provide a rational model to explain the mechanism by which environmental events (diet/nutrition, exercise, smoking, stress, etc.) affect metabolic diseases. Several groups started to focus on epigenetic influences on metabolic diseases and shed light on the understanding of the etiology of Type 2 diabetes and obesity.

32.9 Mitochondria and Energy Homeostasis

Mitochondria regulate energy homeostasis by metabolizing nutrients and producing ATP and are described as the “power plant” in cells. There is considerable evidence showing impaired mitochondrial function is associated with skeletal muscle insulin resistance in Type 2 diabetes and age-related insulin resistance (Petersen *et al.* 2003; Stump *et al.* 2003). Reduced mitochondria number and altered mitochondria morphology has been reported in skeletal muscle of Type 2 diabetic patients. Mitochondrial DNA (mtDNA) content is found to be decreased in obese subjects compared to lean subjects in skeletal muscle (Ritov *et al.* 2005).

32.10 Diabetes Progression

Barres *et al.* performed a MeDIP array (Methylated DNA immunoprecipitation followed by microarray technology) on *vastus lateralis* skeletal muscle obtained from Type 2 diabetic patients or normal glucose tolerant subjects. The 838 gene promoter regions were differentially methylated in Type 2 diabetes, of which 44 positive promoter regions were identified to be related to mitochondrial structure and function (Barres *et al.* 2009). The findings suggest that the differentially methylated changes may participate in Type 2 diabetes progression.

PGC-1 α (peroxisome proliferator-activated receptor γ coactivator 1 α) encoded by gene *PPARGC1* was first identified as a transcriptional coactivator regulating adipocyte differentiation through interacting with nuclear receptor PPAR γ (Puigserver *et al.* 1998). PGC1 α was the master regulator of mitochondrial biogenesis and respiration in skeletal muscle cells (Wu *et al.* 1999). Two studies based on the array method have shown PGC-1 α is downregulated in skeletal muscle obtained from Type 2 diabetic patients (Mootha *et al.* 2003; Patti *et al.* 2003).

Ling *et al.* showed PGC1 α might be important in human islet insulin secretion and the expression of *PGC1 α* in human islets could be regulated by epigenetic factors (Ling *et al.* 2008). Barres *et al.* reported more than a two fold increase in cytosine methylation of *PGC1 α* was revealed in skeletal muscle obtained from Type 2 diabetic patients compared to NGT subjects (Barres *et al.* 2009). In parallel with the increase in *PGC1 α* promoter methylation level in Type 2 diabetic patients, mRNA expression of *PGC1 α* was downregulated and negatively correlated with promoter methylation. A gene reporter assay provided direct evidence that gene expression could be markedly suppressed by introducing a single methyl group to cytosine of the *PGC1 α* promoter (Barres *et al.* 2009).

In order to elucidate the potential environmental and nutritional factors to alter DNA methylation status of *PGC1 α* , Barres *et al.* exposed primary human skeletal muscle cells to elevated concentrations of four different factors known to induce insulin resistance for 48 h: glucose, insulin, free fatty acids, or the inflammatory factor TNF- α . Free fatty acids and TNF- α , but not insulin or glucose, dramatically triggered hypermethylation of the *PGC1 α* promoter. As described earlier, fat overload and inflammation are two competing theories describing insulin resistance in skeletal muscle. The underlying mechanism is not fully understood. The findings suggest elevated free fatty acids and inflammatory factors may induce insulin resistance through epigenetic mechanisms. Further, silencing different DNMT isoforms in palmitate-treated cultured human skeletal muscle cells using siRNA technique showed silencing of DNMT3B prevented palmitate-induced hypermethylation of *PGC1 α* and defects of mtDNA content. Silencing of DNMT1 or DNMT3A failed to achieve the same effect as silencing of DNMT3B.

Regular physical activity reduces the risk for cardiovascular diseases, Type 2 diabetes, multiple cancers, depression, obesity, and musculoskeletal diseases (Thune *et al.* 1997). Acute gene activation induced by a bout of exercise was associated with a rapid change in DNA methylation in skeletal muscle. Exercise induced a marked hypomethylation on each respective promoter of *PGC1 α* , *pyruvate dehydrogenase kinase, isoenzyme 4 (PDK4)*, and *peroxisome proliferator-activated receptor δ (PPAR- δ)* (Barres *et al.* 2012). Caffeine mimics exercise-induced expression of genes related to mitochondrial function in L6 myotubes (Ojuka *et al.* 2003). A time-course study in L6 myotubes exposed by caffeine showed gene expression changes are associated with promoter demethylation and DNA hypomethylation is an early event (Barres *et al.* 2012). However, the potential demethylation mechanism is not yet elucidated.

Obesity is also a disease that results from interactions between genetic factors and environmental insults. Gastric bypass surgery has been proved to be beneficial to dramatically lose body weight and largely improve insulin sensitivity. The mechanisms for the normalization of insulin sensitivity and reversal of Type 2 diabetes after gastric bypass surgery are not fully understood. Changes in DNA methylation may provide a potential mechanism by which environmental influences are linked to changes in gene expression and the control of insulin sensitivity with obesity. Among the 14 genes analyzed, promoter methylation of 11 genes in obese subjects was restored to levels observed in the normal

weight subjects after the surgery in skeletal muscle suggesting obesity and surgery-induced weight loss have a dynamic effect on the epigenetic modifications (Barres *et al.* 2013).

DNA methylation is thought to be mitotically stable, and environmental events and nutritional conditions after birth were unlikely to alter DNA methylation changes in adult tissues (Reik *et al.* 2001). Nevertheless, studies mentioned previously show that DNA methylation could be dynamically remodeled, concomitant with alterations in insulin sensitivity, which make treatment strategies for metabolic diseases by modifying epigenetic marks possible.

32.11 Conclusion

Taken together, these studies provided additional evidence that DNA methylation may play an important role in the regulation of insulin sensitivity in Type 2 diabetes and obesity by sensing the environmental alterations. Environmental and nutritional factors have a dynamic effect on the level of DNA methylation in the promoter regions of key genes involved in lipid and glucose oxidation. Changes in DNA methylation may be an early event in reprogramming the metabolic profile in human somatic tissues.

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33

The Time has Come (and the Tools are Available) for Nutriepigenomics Studies

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33.1 Introduction: Great Strides in Deciphering Methylomes

We have seen exponential growth in epigenetic-related research since 2000. According to GENReport (Razvi, 2013), epigenetics-focused publications in PubMed increased from ~1000 per year in 2000 to >11,000 per year in 2012. About half of the publications are focused on DNA methylation and this trend will continue as interests in studying DNA methylation spans across multiple disease types and health and nutrition issues. Currently, 70% of the epigenetics research is made up of cancer/oncology studies while 12% are studies associated with metabolic diseases such as diabetes. The three dominant epigenetics assays are bisulfite-conversion based assays, deep sequencing/next-generation sequencing with or without bisulfite conversion, and methylation-specific restriction enzyme-based analyses.

DNA methylation, a form of epigenetic mechanism, has long been associated with genomic regulation, both in development (Smith and Meissner, 2013) and in response to environmental changes (Foley *et al.*, 2009). Because epigenetic changes are heritable and they can occur over an individual's lifetime, these changes can offer clues into human health and development (Dayeh *et al.*, 2014; Docherty *et al.*, 2014; Dominguez-Salas *et al.*, Foley *et al.*, 2009; Ghahramani *et al.*, 2014; Rönn *et al.*, 2013; Saffery *et al.*, 2012), and diseases. The recent drive to develop personalized biomarkers and therapeutic regimens, not only for genetic alterations but also for epigenetic alterations, fosters collaborations and team science to promote rapid growth in this research arena. As we understand the genesis of diseases, we can treat and prevent them better. More importantly, the same effort and development needs to take place in the arena of preventive medicine. In this chapter, I will outline key technologies and tools, and findings from epigenetic research leaders to support an approach that is resource friendly to profile epigenomics and nutriepigenomics in large cohort studies.

33.2 Recent Findings in Methylome Research and their Implications for Future Nutriepigenomic Research

33.2.1 Cohort Size and Data Reproducibility

The April 3, 2014 *Nature News-in-Focus* (Callaway, 2014) was entitled “Epigenomics starts to make its mark”. The article reports that, after close to a decade of little progress, epigenome-wide association studies (EWASs) are starting to make an impact. Properly powered studies with large case/control cohorts and with emphasis on reproducing findings made in one group of patients in separate cohorts of the same study start to yield mechanisms of disease. One of the highlighted studies performed genome-wide methylation analysis in blood and fat cells of a discovery cohort and multiple replication cohorts totaling more than 2500 subjects (Dick *et al.*, 2014). This study revealed the association between increases in methylation levels in three *HIF3A* loci with an increase in body-mass index (BMI) in adults of European origin. By their nature, EWASs are more challenging than genome-wide association studies (GWAS) as tissues and cell types have their unique epigenomes. Depending on the scope of the research question, these two design issues will also impact future nutriepigenomic studies.

33.2.2 Proxy/Surrogate Tissues

Non-invasive methods of collecting study samples, such as saliva and buccal cells via mouth swabs as well as urine samples, are preferred to invasive means such as drawn blood and tissue biopsy by study subjects. Saliva- or buccal cell-derived genomic DNA is an attractive alternative in genetic studies (Sun and Reichenberger, 2014). The opinion on whether one can use a more accessible tissue as a proxy/surrogate for a less accessible tissue in epigenomic epidemiologic studies is currently split. The BMI study (Dick *et al.*, 2014) found similar methylation changes in the hypoxia inducible transcription factor pathway CpG loci in blood cells and in adipose tissue. However, the LEGACY Girls study (Wu *et al.*, 2014) found high concordance between the methylation status of the repetitive element LINE-1 in white blood cells and saliva DNA and low concordance in Alu between these two tissue types as a result of environmental exposures. The human brain methylome study (Davies *et al.*, 2012) identified and correlated tissue-specific intra- and inter-individual methylation differences in multiple brain regions and in whole blood. The authors concluded that some inter-individual differences across brain regions were captured in the blood and proposed that peripheral tissues may be useful in revealing tissue-specific differences for epidemiological studies. These findings are a valuable resource for the research community as they charted a path to identify informative methylation markers in non- or less-invasive peripheral tissues that report on tissue-specific methylation status at sites difficult to sample.

33.2.3 Confounders of Methylome Profiles

Methylation marks change over the lifespan of an individual (Martino *et al.*, 2013; Steegenga *et al.*, 2014), and the level of these marks diverges even among identical monozygotic twins (Martino *et al.*, 2013; Saffery *et al.*, 2012). Horvath (Horvath, 2013) computationally derived a novel epigenetic clock to reveal important information associated with developmental biology, cancer, and aging research. He used a large body of publicly available methylome data from diverse organ sites from individuals of different age groups. A set of 353 clock CpGs correlates significantly with age (193 positively and 160 negatively) and these genes are enriched for key pathways for disease and development. Interestingly, Jaffe and Irizarry (Jaffe and Irizarry, 2014) identified that with aging, blood cell component changes. Cell type specific methylome profiles likely confound previously reported age-associated alterations in blood. In summary, a key step in methylation marker identification (Bock, 2009) is to computationally identify and optimize a small number of highly promising candidate loci. This often involves selecting for loci with widely varying methylation levels. If the intent is not to include age-associated methylation loci, one should screen the selected list of promising loci against gene/loci related to the effect of aging.

33.3 Strategies for Identifying and Optimizing a Small Number of Promising Methylation Markers

33.3.1 Methylome Profiling Protocols

There are many ways to profile the methylome; however, the goal is the same: to maximize genomic coverage at the discovery stage. The choice is affected by quality and quantity of genomic DNA and by financial and computation resources. A small selection of review articles are provided (Bock *et al.*, 2010; Boyle *et al.*, 2012; Lisanti *et al.*, 2013; Robinson *et al.*, 2010) as

a starting point. In general, it is difficult to know the functional consequence of observed methylation events as hypermethylation at a single CpG site can trigger gene silencing (Claus *et al.*, 2012) whereas promoter methylation in *CDKN1A* and *PDE7B* suppressed their transcriptional activity leading to perturbed insulin and glucagon secretion in Type II diabetic pancreatic islet b- and a-cells (Dayeh *et al.*, 2014). More often than not, methylation events do not result in gene expression changes (Horvath, 2013; Steegenga *et al.*, 2014). In the absence of a clear outcome of methylation events, additional layer(s) of information will be needed to identify functional methylation markers.

33.3.2 Integrating Transcriptional Information

Depending on the protocol used for methylome profiling, gene level information associated with individual CpG or with regions of interest is used to integrate methylation level with transcriptional activity for that gene. This approach has been used successfully by our group (Marcucci *et al.*, 2013) in identifying a group of seven genes whose summary score indicative of high DNA methylation and low expression is associated with better outcome in four sets of older as well as younger AML patients. The key innovation here is that DNA methylation marks were used to identify informative RNA transcripts resulting in a seven-gene score. This approach takes advantage of the availability of gene expression data to validate the methylation-derived signature in cohort studies lacking methylation data. Rönn *et al.* (2013) compared the adipocyte methylome and the transcriptome from subjects after 6 months of exercise training. About one-third of the gene regions showing differential methylation as a result of training showed gene expression changes. These included genes previously shown to be associated with obesity and type II diabetes. Thus genes response to exercise and relate to adipocyte metabolism are those exhibited both methylation- and expression changes. Using the combined analysis of differential methylation and gene expression in a retrospective study, Martino *et al.* (2014) evaluated the contribution of genetic and environmental risk of developing food allergy in children who developed IgE-mediated food allergy at 12 months to those who did not. They found 30% of the differentially methylated CpG loci were previously identified to be associated with food allergy. A set of 96 not previously implicated loci was mapped to the MAP kinase signaling pathway. These dysregulated methylation loci may have led to suboptimal CD4+ T-cell response to food early in life adding to the effect of the known allergy-associated methylation loci. Additional examples include studying the relationship between methylome and transcriptome in patients with nonalcoholic fatty liver disease (Murphy *et al.*, 2013) whereby functionally relevant differences in methylation can stratify patients with advanced disease or mild disease. Steegenga *et al.* (2014) also found discordant changes between methylation and gene expression in age-related genes in blood to represent genes involved in carcinogenesis and developmental processes. Findings from a few studies are being highlighted here. There are many other studies using this combined approach for methylation marker discoveries.

33.3.3 Genetic-Associated Epigenetic Changes

Bell *et al.* (2010) employed a microarray-based methylation-pulldown analysis and computation approach to extract absolute methylation values to examine genotype-epigenotype interactions in Type II Diabetes across linkage disequilibrium blocks in female subjects stratified according to disease susceptibility haplotype using known association loci. Increased methylation in the FTO obesity susceptibility haplotype was further localized to a 7.7 kb region of haplotype-specific methylation centered around a highly-conserved non-coding element shown to be a long-range enhancer. The finding showcased the power of integrating Genome-Wide Association SNP and methylation markers on revealing new disease-associated loci. Using a methylation beads array to retrospectively monitor methylation changes associated with food allergy development, Martino *et al.* (2014) classified differentially methylated loci with food allergy-associated SNPs and food-allergy-associated non-SNPs. Pathway analysis on the non-SNPs methylated loci uncovered MAP kinase signaling molecules not previously known to be linked to IgE-mediated food allergy in children. Scherf *et al.* (2013) studied the interaction between epigenetic deregulation and genetic susceptibility in three major lung cancer susceptibility regions. Their findings confirmed previous observation that hypermethylation of *TERT* and *CHRN4* (genes within the susceptibility regions) promoters resulted in significant expression changes. This combination approach revealed epigenetic alterations that are strongly associated with genetic susceptibility and therefore shed light on lung cancer tumorigenesis.

33.3.4 Other Approaches to Identify Functional Markers

Domingue-Salas *et al.* (2014) studied the effect of maternal periconceptional dietary intakes and plasma concentration of key methyl-donor pathway substrates on the methylation status of the infants' metastable epialleles. Metastable epialleles are epigenetic polymorphisms set up in early embryo, stably maintained in different tissues and these epigenetic marks are

different between individuals. These marks are potential targets to examine in nutriepigenomic studies. Clock genes (Horvath, 2013; Hughes *et al.*, 2012) or age-related genes (Steegenga *et al.*, 2014) are another source of functional marks. Horvath (2013) observed that the epigenetic clock in the breast tissue of an adult female was substantially accelerated in comparison to the clock for the rest of her tissue. The 353 clock CpGs are highly enriched for cell death/survival, cellular growth/proliferation, organismal/tissue development, and cancer by pathway analysis. These are gene ontology terms commonly associated with differentially methylated marks in cancer and disease. While studying the circadian and diurnal transcriptome of *Drosophila* brains, Hughes *et al.* (2012) noted that the genes downstream of clock genes, such as *TARDBP*, may play a part in initiation and progression of diseases such as neurodegenerative diseases. Also, age-associated genes as those described by Steegenga *et al.* (2014) are candidate methylation marks to be included in the analysis.

33.4 Validation of Methylation Markers Performance in Large Cohorts using Highly Targeted Assays

33.4.1 Validation Using Methylation-Based Assays

Global techniques for identification of promising methylation marks would be too costly and computation intensive to assess a large number of samples. Recent advances in technologies provide targeted approaches to perform large methylome validation studies. Two such approaches will be described.

- EpiTect™ Methyl II PCR Array System (Jiang *et al.*, 2012) is a real-time PCR based screening tool for regional DNA methylation of up to 94 gene targets starting with 2 µg genomic DNA without the need for bisulfite conversion step. This technology consists of two components: the DNA Restriction Kit and the Methyl II PCR Arrays (cataloged or custom). The DNA Restriction Kit uses the MethylScreen™ technology whereby sample DNA is: (1) Restricted with methylation-sensitive enzymes to yield 100% methylated DNA fragments; (2) Restricted with methylation-dependent enzymes to yield unmethylated DNA fragments; (3) Mock-restricted with no enzyme to yield total amount of input DNA for data normalization; and (4) Restricted with both methylation-sensitive and methylation-dependent enzymes to yield input DNA fragments not susceptible to digestion by the restriction enzymes. Comparison between the mock-digest and the double-digest represent the analytical window of the assay. The digests are mixed with qPCR Master Mix and dispensed into gene-specific primers of either cataloged or custom Methyl II PCR Array. The relative amounts of differentially methylated DNA species from the amplicons of interest are determined with comparative ΔC_T calculation. The efficiencies of the PCR primers are at least 80% of greater and methylated DNA can be detected as low as 6% of the total sample. This approach, however, is for screening as partial methylation information is derived only from subtracting methylated- and unmethylated fragments from total input DNA minus the unrestricted fraction.
- Paul *et al.* (2014) assessed the RainDance RainDrop™ BS-seq as a targeted validation platform for large cohort studies. The approach utilizes microdroplet-based PCR amplification to facilitate sensitive and specific amplification of up to 4000 targeted BS-seq loci followed by next-generation sequencing. This system can accommodate bisulfite-converted DNA input amount between 10–1500 ng, with and without whole genome amplification. The authors noted that an input amount of 100 ng provided analyzed data similar to the EWAS platform (Illumina Infinium™ HumanMethylation450 BeadChip™). The team summarized that this system can be used to study candidate methylation loci in an accurate and high-throughput manner. Nonetheless, the workflow involves key steps such as whole genome amplification, bisulfite conversion, microdroplet amplification, sequencing library generation and data analysis involving the bisulfite converted genome. All these steps required careful monitoring and quality control measures. It likely will require substantial optimizations before a research team can implement the assay on clinical samples with diverse quality.

33.4.2 Validation Using Gene Expression-Based Sequencing Panels as Readouts for Functional Methylation Markers

Sequencing instrument companies and sequencing service providers have focused heavily on pre-clinical, clinical, neonatal, pathology, and forensic diagnostic markets. Instruments, assays and library generation robots are constantly being launched to fill the needs of this space. One important product is the targeted content panels. The first to be launched was the DNA amplicon panels (both custom and fixed contents), followed by targeted exome panel and now the targeted RNA expression panels (both custom and fixed contents). As discussed in the previous paragraph, sample preparations for most methylation analysis assays

are challenging and require sizable amount of good quality genomic DNA as inputs. These challenges are amplified for studies with large sample size such as validating carefully selected methylation marks in population based nutriepigenomic studies. The rationale for using gene expression-based targeted panels for validating methylation markers are as follows: (1) Gene expression transcripts associated with functional methylation marks (i.e., methylation changes resulting in expression changes) capable of stratifying study subjects are candidates for this approach (see the detailed discussion on p. 4, Section “integrating transcriptional information”); (2) Although RNA is more labile than DNA, library preparation workflow for the sequencing of targeted gene expression panel is more straightforward than bisulfite-based or methylation enrichment-based protocols; (3) Targeted gene expression panels can tackle challenging samples such as FFPE as readouts can be short single-end 50bp runs; (4) Clinical labs have been using OncotypeTM DX to quantify the likelihood of disease recurrence in women with early-stage hormone estrogen receptor positive breast cancer and assesses the likely benefit from certain types of chemotherapy, and therefore have the workflow ready for total RNA as input samples; (5) Simultaneously provides quantitative gene expression information and gene sequence at base pair resolution; (6) Sample multiplexing leading to faster turnaround time and lower RNA inputs; and (7) Data analysis for targeted gene expression panel is automated whereas methylation analysis is still more challenging even though there are existing R packages for custom analysis. At the present time, there are several platforms to select from. Undoubtedly there will be more choices in the near future.

- Agilent SureSelectTM Custom RNA (Agilent Technologies, n.d.): This kit was launched in 2011 for targeted transcriptome analysis. It can capture 1 kb to 6 Mb content and is available for post-capture pooling and is available in sizes from 16 to 10,000 reactions.
- Illumina TruSeqTM Targeted RNA Expression (Illumina Technologies, 2015): Based upon a hybridization-extension approach to generate a customized panel of 12–1000 assays to target genes, isoforms, splice junctions and cSNPs from 400,000 pre-designed assays. Michael Hughes utilized this system (Life Technologies, 2015) to validate finding in his team’s previous global findings (Hughes *et al.*, 2012).
- Ion AmpliSeqTM RNA (Illumina, 2014): Based on highly multiplexed PCR similar to TaqMan assays but without the probes. Poole *et al.* (2014) summarized their experience using this approach.
- A non-kitted approach (Mercer *et al.*, 2014): RNA CaptureSeq.

33.5 Summaries

This is exciting times for all things epigenetics. The coming together of technical advances in next-generation sequencing and the endeavor to understand the interplay between the genome and the environment pushes the stable and readily accessible methylation marks to the forefront of epigenetic research. The realization of personalized medicine and preventive medicine will catalyze the use of these newly available tools and techniques to explore the interactions between nutrition and the genome and the epigenome. The targeted panel approaches will permit validations of global findings in the much needed population studies.

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34

Natural Phytochemicals as Epigenetic Modulators

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34.1 Introduction

More than six decades ago, the term *Epigenetics* was coined by British developmental biologist Conrad Waddington (Waddington 1968, 2012). Derived from the combination of Aristotelian word “epigenesis” and genetics, Waddington defined *epigenetics* as “the branch of biology which studies the casual interactions between genes and their products, which bring phenotype into being” (Waddington, 1942). The notion of *epigenesis* is underscored by the fact that the development of an organism from a fertilized zygote into an individual is determined not only by the information provided by their genes but also by other (epigenetic) factors metaphorically referred as *epigenetic landscape* (Goldberg *et al.* 2007). In the term epigenetics, *Epi* means above or over, and genetics reflects the involvement of genes and heredity. Therefore, in earlier studies epigenetic factors were summoned only when a phenomenon could not be explained by genetics. However, over the course of time, the understanding of epigenetics has transformed as its molecular underpinnings were gradually revealed. Today, epigenetics may be defined as the study of chemical changes to the genome that are heritable and modulate gene expression or cellular phenotype through mechanisms involving how DNA is packaged and expressed without any alterations in the gene nucleotide sequence itself (Henikoff and Matzke 1997; Hughes 2014).

Epigenetic mechanisms have been shown to be essential in regulating normal cellular functions and play important role during development. In mammals, DNA methylation is known to be associated with several key processes such as genomic imprinting, X-chromosome inactivation, and tissue specific gene expression (Illingworth *et al.* 2008; Jones and Takai 2001; Li *et al.* 1993; Payer and Lee 2008). Histone methylation and acetylation patterns were demonstrated to be closely associated with cognitive functions such as long term memory formation and storage (Jarome and Lubin 2013; Lubin *et al.* 2011; Peixoto and Abel 2013). Aberrant epigenetic alterations have been implicated in several pathologies such as cancer, metabolic syndrome, Alzheimer’s disease, and other neurological diseases (Bruce and Cagampang 2011; Gos 2013; Jones and Baylin 2002; Stilling and Fischer 2011). Unlike genetic changes in the genome such as mutations, epigenetic modifications are potentially reversible and can be modified by environmental factors. Epigenetic mechanisms

have been implicated in physiological responses to intrinsic and extrinsic environmental stimuli such as nutrition, radiation, and exposure to chemicals, toxins, and hormones (Jaenisch and Bird 2003; Suter and Aagaard-Tillery 2009). In this chapter, we focus on the role of natural phytochemicals as epigenetic modulators. We discuss in detail different mechanisms of epigenetic modifications in mammals, and present a comprehensive overview of the current state of knowledge on natural bioactive compounds and their influence on various epigenetic mechanisms.

34.2 Epigenetic Mechanisms in Mammals

Although a number of epigenetic mechanisms have now been identified, in mammals there are three major epigenetic mechanisms that are known to regulate gene expression. These include DNA methylation, modulation of chromatin structure by post translational modification of histone or non-histone proteins, and small non-coding microRNAs (miRNAs) that modulate gene expression by either inhibiting translation or causing targeted degradation of specific mRNAs (Choudhuri, 2011; Jaenisch and Bird, 2003). The latest advances in understanding these epigenetic mechanisms will be highlighted in the following sections.

34.2.1 DNA Methylation

Methylation of cytosines residues within the dinucleotide sequence-CpG and is one of the most widely studied epigenetic modifications in mammals (Issa and Kantarjian 2009). Forming an essential component of the cellular epigenetic machinery, DNA methylation in collaboration with histone modifications regulates gene expression by modulating DNA packaging and chromatin architecture (Dehan *et al.* 2009; Link *et al.* 2010). DNA methylation is a chemical modification that involves the transfer of a methyl (CH_3) moiety from the donor S-adenosyl methionine (SAM) to the 5' position of cytosine residue that precedes guanine in the CpG dinucleotide sequence, forming 5-methyl cytosine and S-adenosyl-L-homocysteine (SAH) (Bestor 2000; Gerhauser 2013; Issa and Kantarjian 2009; Santi *et al.* 1983). The mammalian genome has been reported to harbor 3×10^7 methylated cytosine, which residues mostly within CpG dinucleotide sequences (Bestor 2000). Although CpG sequences are unevenly distributed throughout the human genome, they are frequently enriched in gene promoters (often referred as *CpG islands*) and large repetitive sequences such as LINE and ALU retrotransposon elements (Bird 2002). DNA methylation is catalyzed by a group of enzymes known as DNA methyltransferases (DNMT) (Bestor 2000; Issa and Kantarjian 2009). There are three major DNA methyltransferases (Dnmt1, Dnmt3a, and Dnmt3b) in mammals. Evidences from phenotypic analyses of mice with mutant DNMT genes have provided useful mechanistic insights into the role and establishment of DNA methylation patterns during development (Bestor, 2000; Jaenisch and Bird 2003). Dnmt1 enzyme has been demonstrated to have a 5–30-fold more preference for hemi-methylated substrates and therefore is popularly designated as maintenance methyltransferase. It preserves the existing methylation patterns in the daughter DNA strands by adding methyl groups to hemi-methylated CpG sequences following replication. However, Dnmt1 has also been demonstrated to be involved in *de novo* methylation activity in embryo lysates and its sequence specificity was shown to be confined to the 5'-CpG-3' dinucleotide sequence with little dependence on sequence context or density (Yoder *et al.* 1997). Dnmt3a and Dnmt3b enzymes are essential for global *de novo* methylation as they preferentially target unmethylated CpG sequences (Okano *et al.* 1999). They have been shown to be highly expressed in developing mouse embryos and establish methylation patterns post implantation (Okano *et al.* 1999). Although Dnmt3L, the fourth family member, lacks intrinsic DNMT activity by itself, it colocalizes with Dnmt3a and Dnmt3b to establish genomic imprints in maternal germ line (Bourc'his *et al.* 2001; Hata *et al.* 2002) and facilitate methylation of retroposons (Denis *et al.* 2011). Dnmt2, another member of DNMT family, was found to lack any biochemically detectable DNMT activity and its deletion in mice had no obvious phenotypic effects on genomic methylation patterns or methylation of retroviral DNA (Okano *et al.* 1998).

Hypermethylation of CpG islands is usually associated with gene silencing. There are multiple routes through which DNA methylation can suppress transcription. A general mechanism is to exclude binding of proteins that modulate transcription through their DNA binding domains (Watt and Molloy 1988). For example, binding of chromatin boundary element binding protein CTCF to DNA is blocked by CpG methylation, which allows the enhancer to activate transcription (Bell *et al.* 1999; Ohlsson *et al.* 2001). This mechanism has been demonstrated to be essential for imprinting of the *Igf2* gene (Hark *et al.* 2000). Besides this, CpG methylation has been shown to block the binding of several other transcription factors, however, their biological consequences remain unknown (Tate and Bird 1993). Another mechanism for DNA methylation mediated gene repression involves binding of specialized DNA binding proteins to the methylated CpG stretches, which form repressor complexes with histone deacetylases (HDACs) and cause

chromatin compaction (Feng and Zhang 2001; Jones *et al.* 1998; Nan *et al.* 1998). In mammals, six methyl-CpG-binding proteins have been characterized to date, which include MeCp2, MBD1–4, and Kaiso. Studies demonstrate that all of them (except mammalian MBD3) possess a domain that specifically targets them to methylated CpG regions *in vitro* and *in vivo* (Hendrich and Bird 1998; Nan *et al.* 1993).

34.2.2 Histone Modifications

In addition to DNA methylation, post translational modification of N-terminal histone tails plays a significant role in epigenetic regulation of gene expression (Kornberg and Lorch 1999; Luger and Richmond 1998). A typical nucleosome unit consists of ~146 bp of DNA wrapped around an octamer of histones (H2A, H2B, H3, and H4) and represents the fundamental building unit of eukaryotic chromatin. A diverse array of covalent chemical modification of less structured, protruding N-terminal tails of core histones by methylation, acetylation, ubiquitination, phosphorylation, sumoylation, and ADP-ribosylation dictate the dynamics of chromatin state (Strahl and Allis 2000). Euchromatin is lightly packed form of chromatin where DNA is accessible for transcription whereas heterochromatin represents tightly packed chromatin state inaccessible to cellular transcriptional machinery. Most of the chemical modifications occur at Lysine (K), Arginine (R), and Serine (S) residues within the histone tails. These distinct histone modifications on one or more histone tails (often referred to as “Histone code”), which may act sequentially or in combination, are recognized by other proteins that signal further downstream events. A number of enzymes have been implicated in catalyzing (addition or removal) various histone modifications. Examples include histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs), histone demethylases (HDMs), histone kinases, and others. In brief, HATs catalyze the addition of acetyl group on the ϵ -amino group of lysine residues in the N-terminal tail of histones, which neutralize the positive charge, relax the chromatin, and facilitate the binding of transcriptional machinery to the DNA (Struhal 1998). To date, 25 HATs have been characterized which are divided into four families. Examples of these include GNAT (*hGCN5*, *PCAF*), MYST (*MYST*, *Tip60*), p300/CBP (*p300/CBP*, *SRC(SRC-1)*), and TAFII250 families (*TAFII250*) (Bannister and Kouzarides 2011; Gerhauser 2013). In contrast, HDACs catalyze the removal of acetyl groups from lysine residues resulting in the compaction of chromatin configuration, which represses transcription (Kuo and Allis 1998). HDACs are classified into four groups. HDAC-1, -2, -3, and -8 are members of Class I HDAC family, while HDAC-4, -5, -6, -7, -9, and -10 belong to the class II HDAC family. HDAC-11 belongs to the Class IV HDAC group. Sirtuins, which require NAD⁺ as a cofactor for their activity and are structurally unrelated to other HDAC classes, constitute the Class III HDAC family (Mottet and Castronovo, 2008; Sauve *et al.* 2006). HMTs catalyze the addition of methyl groups to lysine or arginine residues while HDMs act to remove them (Rice and Allis, 2001; Shi, 2007; Upadhyay and Cheng, 2011). Examples of histone lysine methyltransferase include EZH2 (Enhancer of zeste homolog 2), and those of histone lysine demethylase include LSD1 (Lysine specific demethylase 1) (Deb *et al.* 2014a; Kouzarides, 2007). Depending on the site of lysine methylation (K4, K9, K27, etc. in Histone H3) and methylation status (mono, di or tri methylation), histone methylation may have activating or repressive effect on gene expression (Bannister and Kouzarides 2011; Kouzarides 2007). H3K4, H3K36, and H3K79 methylation have activating effects on gene transcription whereas methylation of H3K9, H3K27, and H4K20 is generally associated with gene silencing or transcriptional repression (Bannister and Kouzarides, 2011; Suzuki and Miyata, 2006; Upadhyay and Cheng, 2011). A plethora of literature is available on each group of histone modifying enzymes, their mechanism of action, and various histone modifications, which is beyond the scope of this chapter.

34.2.3 Non-Coding RNAs

Recent evidences indicate that non-coding RNA (ncRNA) transcripts play a fundamental role in epigenetic regulation of gene expression and have been implicated in various epigenetic mechanisms such as transposon silencing, X-chromosome inactivation, DNA imprinting, and paramutation (Costa 2008; Peschansky and Wahlestedt 2014; Zhou *et al.* 2010). In humans, ncRNAs, which include microRNA (miRNA), small interfering RNA (siRNA), piwi-interacting RNA (piRNA), and so on, account for the majority of transcripts representing approximately 98% of all human transcriptional output (Mattick 2001; Szymanski *et al.* 2005). Based on the size, ncRNA can be classified into small ncRNA, which are generally less than 200 nucleotides in length and long ncRNA (lncRNA), transcripts that are more than 200 nucleotides in length. They can be divided into further subtypes based on their genomic origin and biogenic processes (Peschansky and Wahlestedt, 2014). Both types of ncRNAs have been shown to be essential *epigenetic modifiers*, constituting a hidden layer of complex internal signals controlling multiple levels of gene expression associated with development and physiology of an organism (De Lucia and Dean, 2011; Magistri *et al.* 2012; Ulitsky *et al.* 2011; Zhou *et al.* 2010). lncRNAs have been demonstrated to be involved in gene silencing via mechanisms

involving both histone modifications and DNA methylation. For example, the antisense lncRNA located in the *p14/p15/INK4* locus, *ANRIL*, was reported to cause gene silencing via recruitment of polycomb proteins (PcG) proteins (Kotake *et al.* 2011; Pasmant *et al.* 2007; Yu *et al.* 2008). Another well studied example includes the involvement of a 17kb lncRNA, *XIST*, in X-chromosome inactivation, which ensures X-linked gene dosage compensation in mammalian females (Mak *et al.* 2002; Plath *et al.* 2003; Silva *et al.* 2003; Zhao *et al.* 2008). This process involves the recruitment of mammalian PRC2 complex containing the histone methyltransferase EZH2 to the locus by a short repeat RNA (RepA) within *XIST* and deposition and spreading of repressive H3K27me3 marks throughout the X-chromosome. In addition to histone modifications, lncRNAs were also reported to mediate gene silencing through DNA methylation. One such example includes *Kcnq1ot1*, which in addition to interacting with PRC2 complex and G9a, has been implicated in the recruitment of Dnmt1 through a critical 890bp region to the CpG island of the imprinted genes (Mohammad *et al.* 2010).

Small ncRNAs particularly miRNAs regulate key epigenetic mechanisms. Short RNAs (50–200 nucleotides) were reported to be transcribed from H3K27me3 enriched PRC2 target genes and cause cell-type specific gene silencing in *cis* by stabilizing the PRC2 complex near the transcription site through interactions via formation of stem-loop structures (Kanhere *et al.* 2010). miRNAs are known to regulate various components of cellular epigenetic machinery particularly polycomb complexes and thus affect multiple downstream effects (Deb *et al.* 2013, 2014a; Sander *et al.* 2008; Varambally *et al.* 2008). One such example includes miR-214, which downregulates Ezh2 expression by targeting its 3'-UTR region and accelerates skeletal muscle differentiation and transcription of developmental regulators in embryonic stem cells (Juan *et al.* 2009). There are other miRNAs that have been implicated in the repression of Bmi1, a component of PRC1 complex (Godlewski *et al.* 2008; Liu *et al.* 2012; Wellner *et al.* 2009). DNA methylation has been also shown to be modulated by miRNAs. Dnmt1 and 3 have been reported to be targeted by the miR-29 family in lung cancer and leukemia cells (Fabbri *et al.* 2007; Garzon *et al.* 2009). In addition to the role of small ncRNAs as regulators of various epigenetic mechanisms, in many instances they are themselves targets of the same epigenetic processes, which may lead to further downstream changes. For example, in human breast tumorigenesis and metastasis decreased expression of a set of miRNAs was attributed to gene hypermethylation (Hsu *et al.* 2009; Lehmann *et al.* 2007; Lujambio *et al.* 2008). In summary, recent evidences suggest that ncRNAs have emerged as key regulators of epigenetic mechanisms and also that the modulation of these RNA transcripts by the same epigenetic processes may lead to major consequences.

34.3 Natural Phytochemicals and Epigenetic Mechanisms

Unlike genetic modifications, epigenetic states of the genes are reversible and can be altered by certain intrinsic and extrinsic factors. This characteristic of epigenetic mechanisms may lead to the development of abnormal phenotypes as well as regulate physiological responses to some environmental stimuli, diet, or therapeutic intervention (Jaenisch and Bird 2003). In past two decades, accumulated evidence shows that natural phytochemicals present in abundance in vegetables, fruits, and beverages, which were earlier known only for their antioxidant or chemopreventive effects, are also potent epigenetic regulators, which could target or revert abnormal epigenetic modifications in various human pathologies such as cancer (Davis and Ross 2008; Gerhauser 2013; Hauser and Jung 2008; Huang *et al.* 2011; Li *et al.* 2010a; Thakur *et al.* 2014; Verma *et al.* 2004). In this chapter, we focus on the role of some common natural phytochemicals as potent epigenetic modulators and discuss their targets in human pathologies, including cancer.

34.3.1 Apigenin

Apigenin is a plant derived flavonoid, chemically known as 4',5,7-trihydroxyflavone, known to be present in common fruits and vegetables such as parsley, onions, oranges, tea, chamomile, and wheat sprouts (Birt *et al.* 2001; Manach *et al.* 2004; Patel *et al.* 2007; Surh, 2003). Previous studies have confirmed that apigenin possess antioxidant, anti-carcinogenic, anti-inflammatory, and anti-mutagenic characteristics (Birt *et al.* 1986; Choi *et al.* 2014; Wei *et al.* 1990). However, the effect of apigenin on epigenetic related enzymes and their mechanisms was not recognized until recently. Apigenin treatment (20 and 50 μ Mol/l) was shown to cause a marked decrease in DNMT activity *in vitro* (Fang *et al.* 2007). Studies from our laboratory demonstrated that apigenin mediated growth arrest and apoptosis in prostate cancer cells was due to the inhibition of class I HDACs (Pandey *et al.* 2012). *In vivo* studies using PC-3 xenografts in athymic nude mice further confirmed that oral intake of apigenin (20 and 50 μ g/mouse/d over an 8-week period) reduces tumor burden, HDAC activity and HDAC -1 /-3 protein levels. HDAC-1 and HDAC-3 mRNA and protein levels were found to be significantly decreased in apigenin treated (20–40 μ M) PC-3 and 22Rv1 prostate cancer cell lines, which resulted in a global decline in histone H3 and H4 acetylation levels. A corresponding elevation in

p21/waf1 and bax levels was observed in both *in vitro* and *in vivo* studies, which resulted in the induction of downstream events, that is, apoptosis and cell cycle arrest. In a recent study by Paredes-Gonzalez *et al.* (2014), apigenin was shown to be reactive to the Nrf2 gene, which encodes a key transcription factor known for regulating the antioxidative defense system and skin homeostasis, in mouse skin epidermal JB6 P+ cells via epigenetic mechanisms. Hypermethylation of 15 CpG sites in a Nrf2 promoter was demonstrated to be reversed by apigenin treatment in a dose dependent manner. Furthermore, apigenin treatment resulted in decreased expression of Dnmt1, Dnmt3A, Dnmt3B, and HDAC (1–8) levels. However, the nuclear localization of Nrf2 was shown to be enhanced and there was increased expression of Nrf2 as well as its target gene NQO1 after apigenin treatment.

34.3.2 Curcumin

Curcumin [1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is the major active component of popular South Asian spice turmeric (*Curcuma longa*), belonging to *Zingiberaceae* (ginger) family. The majority of health benefits associated with turmeric intake has been attributed to curcumin for its beneficial characteristics, which include anti-microbial, antioxidant, anti-inflammatory, anti-allergic, and anti-carcinogenic effects (Bar-Sela *et al.* 2010; Prasad *et al.* 2014; Sharma *et al.* 2005; Teiten *et al.* 2010). At the molecular level, curcumin has been shown to modulate multiple intracellular pathways associated with proliferation, survival, invasion, apoptosis, and inflammation (Teiten *et al.* 2010). In the context of epigenetic pathways, several studies have reported curcumin to be a potent modulator of DNMTs, histone modifying enzymes such as HDACs and HATs as well as miRNAs (Fu and Kurzrock, 2010). *In silico* molecular docking studies of curcumin with Dnmt1 revealed that it can block or inhibit the catalytic thiol group of C1226 binding site in the enzyme resulting in decreased DNMT activity (Fu and Kurzrock 2010; Medina-Franco *et al.* 2011). This study was further validated by *in vitro* experimental studies, which showed curcumin to be a potent DNA hypo-methylating agent (Liu *et al.* 2009). Curcumin was reported to be an effective HDAC inhibitor in HeLa nuclear extracts with an IC_{50} value of 115 μ M. Docking studies performed for curcumin binding to HDAC-8 revealed curcumin to be a more potent HDAC inhibitor than known pharmacological inhibitors such as sodium butyrate and valproic acid (Bora-Tatar *et al.* 2009). Another study reported that in B-NHL cell line Raji cells curcumin treatment could reduce HDAC-1, -3, and -8 protein levels in a dose dependent manner and increase H4 acetylation levels (Liu *et al.* 2005). Consistent with earlier findings, studies by Chen *et al.* (2007) reported significant reduction in p300/CREB binding protein (CBP), HDAC-1 and HDAC-3 levels after exposure of Raji cells to curcumin. This decrease in HDACs and CBP levels were implicated in curcumin mediated repression of NF- κ B and Notch1 in Raji cells which finally result in decreased proliferation. Studies revealed curcumin to be a specific inhibitor of p300/CBP HAT, which has emerged as a novel target for cancer treatment (Balasubramanyam *et al.* 2004; Dekker and Haisma, 2009; Marcu *et al.* 2006). Curcumin treatment caused proteasomal degradation of p300 and other closely related CBP proteins with no such effect on HATs such as GCN5 and PCAF (Marcu *et al.* 2006). In addition, curcumin mediated inhibition of p300/CBP was found to block HDAC inhibitor MS-275 induced histone hyper-acetylation in PC3-M prostate cancer cells and peripheral blood lymphocytes.

The chemotherapeutic and chemopreventive effects of curcumin have been also closely linked to its ability to modulate miRNAs in cancer cells. A microarray based study of the effect of curcumin (10 μ M) on the miRNA profile in pancreatic cancer cells PxBc-3 showed significant changes in the expression of 29 miRNAs (11 upregulated and 18 downregulated) after 72 h treatment (Sun *et al.* 2008). Further studies confirmed that MiRNA-22, which has tumor suppressive function, was upregulated after exposure to curcumin and its downstream target genes SP1 and ESR1 were suppressed in these pancreatic cells. Ali *et al.* (2010) demonstrated that treatment of pancreatic cancer cells with curcumin and its analogue CDF could induce gemcitabine sensitivity via induction of miR-200 and inactivation of miR-21 expression. These studies underlined the importance of curcumin mediated modulation of miRNAs as an important mechanism for its biological effects.

34.3.3 (-)-Epigallocatechin-3-Gallate (EGCG)

EGCG, the major catechin found in green tea, is widely known for its health related benefits. It has been shown to have a multifaceted effect on various cellular targets at the molecular level including epigenetic pathways or enzymes (Butt and Sultan, 2009; Shankar *et al.* 2007; Thakur *et al.* 2014). Several studies have demonstrated that EGCG is a potent demethylating agent, which inhibits enzymes involved in DNA methylation as well as an effective histone modifying agent (Deb *et al.* 2014b; Li *et al.* 2010d; Nandakumar *et al.* 2011; Pandey *et al.* 2010). It is well known that CpG island hypermethylation at the promoter region leads to epigenetic repression of several critical tumor suppressor genes during tumorigenesis. Fang *et al.* (2003) reported that EGCG acts as a competitive inhibitor of DNMT ($K_i = 6.89 \mu$ M), which binds to the catalytic pocket and inhibit DNMT activity in a dose dependent manner. Furthermore, EGCG treatment (5–50 μ M for 12–144 h) was found to effectively reactivate methylation-silenced genes – p16(INK4a), retinoic acid receptor beta (RARbeta),

O(6)-methylguanine methyltransferase (MGMT), and human mutL homolog 1 (hMLH1), in human esophageal cancer KYSE 510 cells. EGCG was also reported to inhibit HDACs and increase permissive or active histone modifications such as histone acetylation at the target gene promoters. Studies from our laboratory showed that exposure of prostate cancer cells to green tea polyphenols (GTP) caused re-expression of the epigenetically silenced *GSTP1* gene, which correlated with the promoter demethylation due to DNMT1 inhibition and histone modifications at the promoter region (Pandey *et al.* 2010). However, GTP treatment did not show any global hypomethylation effect, which could result in genomic instability as the methylation status of LINE-1 promoter remained unaffected as demonstrated by methylation-specific PCR. GTP treatment decreased mRNA and protein levels of MBD1, MBD4, MeCP2, and HDAC 1–3 whereas acetylated histone H3 (LysH9/18) and H4 were found to be elevated. In another study, we demonstrated that GTP treatment caused cell cycle arrest and apoptosis by inducing proteasomal degradation of class I HDACs in human prostate cancer cells (Thakur *et al.* 2012). Studies by Li *et al.* (2010d) demonstrated that EGCG in combination with trichostatin A (TSA) could synergistically reactivate ER α expression in ER α negative MDA-MB-231 breast cancer cells by modulating histone methylation and acetylation pattern at the gene promoter. In addition, they also reported that treatment with EGCG and/or TSA contributes to transcriptional activation of ER α by causing a decreased binding of transcription repressor complex, Rb/p130-E2F4/5-HDAC1-SUV39H1-DNMT1 to the regulatory region of the gene.

EGCG has been reported to modulate polycomb proteins such as Bmi-1 and EZH2 (Balasubramanian *et al.* 2010; Choudhury *et al.* 2011; Deb *et al.* 2014b). EGCG alone or in combination with DZNep was shown to decrease P_cG proteins including EZH2, EED, SUZ12, MEL18, and BMI-1 via a mechanism involving proteasome associated degradation. The reduction in P_cG protein levels correlated with a decrease in repressive chromatin marks – H3K27me3, H2AK119ub, and HDAC-1 levels – whereas accumulation of acetylated H3 levels was found to be elevated. In a recent study, we reported that in breast cancer cells, EGCG or GTP treatment induced expression of epigenetically repressed *TIMP-3* gene is mediated by modulating epigenetic mechanisms involving EZH2 and class I HDACs independent of the promoter DNA methylation (Deb *et al.* 2014b). After EGCG or GTP treatment the protein levels of class I HDACs and EZH2 were significantly reduced. Interestingly, transcriptional activation of *TIMP-3* was associated with decreased EZH2 localization and H3K27me3 at the promoter with a concomitant elevation in H3K9/18 acetylation levels.

34.3.4 Genistein and Soy Isoflavones

Genistein is a phyto-estrogen belonging to the isoflavones category. Isoflavones, such as genistein, biochanin A, and daidzein, occur naturally in plant sources such as soybeans, fava beans, lupine, kudzu, psoralea, and so on (Coward *et al.* 1993; Kaufman *et al.* 1997). To date, numerous epidemiological and experimental studies have demonstrated the chemopreventive effects of genistein and other isoflavones on various cancer types such as breast and prostate cancer (Banerjee *et al.* 2008). In recent years, the role of genistein and other soy isoflavones as epigenetic modulators regulating gene expression has been widely reported by several studies. Genistein has been shown to be a more potent DNMT inhibitor as compared to biochanin A or daidzein. Fang *et al.* (2005) reported that genistein (2–20 μ M/l) could reactivate methylation-silenced genes such as retinoic acid receptor beta (RAR β), p16INK4a, and O6-methylguanine methyltransferase (MGMT) in esophageal squamous carcinoma cells KYSE 510 and prostate cancer cells LNCaP and PC3. The reactivation of such genes by direct inhibition of DNMT may be one of the factors contributing to the chemopreventive effects of soy isoflavones. In another study, Li *et al.* (2009a) demonstrated that genistein treatment in breast MCF10AT benign cells and MCF-7 cancer cells depletes telomerase (hTERT) activity through epigenetic modulation which involves genistein mediated decrease in Dnmt1, Dnmt3a, and Dnmt3b levels. Site specific hypomethylation in the hTERT promoter was found to increase the binding of E2F-1 transcription factor. Furthermore, genistein was shown to repress the hTERT promoter by chromatin remodeling, which involved an increase in trimethyl-H3K9 enrichment with a concomitant decrease in dimethyl-H3K4 chromatin marks. Studies by King-Batoon *et al.* (2008) showed that low, non-toxic doses of genistein (3.125 μ M, re-supplemented every 48 h for 1 week) could partially demethylate *GSTP1*, a tumor suppressor gene, in MCF-7, MDA-MB-468, and MCF10A breast cells. Similar *in vitro* studies other cancer types provide evidence that genistein is a potent demethylating as well as histone modifying agent, which could reverse the silenced state of critical tumor suppressor genes (Day *et al.* 2002; Majid *et al.* 2009a, b). However, *in vivo* clinical studies were inconclusive and did not fall in line with the studies performed in cell line models. In a randomized trial study involving thirty four healthy pre-menopausal women to investigate the estrogenic and methylation effect of soy isoflavones (40 or 140 mg daily) through one menstrual cycle, Qin *et al.* (2009) reported that considering all subjects or by dose there were no significant changes for any of the cancer related genes (p16, RASSF1A, RAR β 2, ER, and CCND2). In fact, RAR β 2 methylation was shown to increase post-treatment at circulating genistein levels greater than 600 ng/ml, unlike the effect observed for genistein levels below 600 ng/ml. Similarly, CCND2 methylation

was found to increase in subjects with post-treatment genistein levels above 200 ng/ml as opposed to the effects observed below this genistein level. Changes in the methylation of other genes p16, RASSF1A, and ER did not correlate with post-treatment genistein levels.

Genistein, daidzein, daidzein metabolite equol, and AglyMax have been reported to induce ER α -mediated histone acetylation via modulation of HAT activity (Hong *et al.* 2004, Majid *et al.* 2008) demonstrated that genistein-mediated epigenetic induction of p21WAF1/CIP1 and p16INK4a tumor suppressor genes in human prostate cancer cells involve chromatin modification via upregulation of HATs expression. Genistein was also shown to modulate HDAC activity. A study by Basak *et al.* (2008) demonstrated that AR downregulation in prostate cancer cell line LNCaP by genistein was attributed to the inhibition of HDAC6-Hsp90 co-chaperone function, which is required for AR protein stabilization. Genistein and other soy isoflavones are known to module miRNAs as well (Li *et al.* 2009b; Majid *et al.* 2010a; Parker *et al.* 2009; Sun *et al.* 2009). Parker *et al.* (2009) performed miRNA profiling of genistein treated and untreated UL-3A and UL-3B cell lines and found 53 miRNAs that were differentially expressed. Upregulation of miR-200 and let-7 by isoflavones was shown to downregulate ZEB1, slug, and vimentin, and therefore cause reversal of epithelial to mesenchymal transition (EMT) in gemcitabine resistant pancreatic cancer cells (Li *et al.* 2009b). In human uveal melanoma cells, genistein treatment was demonstrated to significant growth inhibition by targeting miR-27a and its target ZBTB10 (Sun *et al.* 2009).

34.3.5 Indole-3-Carbinol and Diindolylmethane

Indole-3-carbinol (I3C) is a hydrolysis product derived from a glucosinolate catalyzed by the plant enzyme myrosinase, which is further metabolized under the acidic conditions in the stomach to form dimer 3,3'-diindolylmethane (DIM) (Acharya *et al.* 2010; Aggarwal and Ichikawa, 2005; Minich and Bland, 2007; Rogan 2006). These phytochemicals are present in cruciferous vegetables, particularly in the *Brassica* genus, which includes broccoli, cabbage, cauliflower, Brussels sprouts, mustard, radish, and so on. Both these phytochemicals have been shown to exert anti-tumor effects by downregulating multiple cellular targets and inducing cell cycle arrest and apoptosis in various cancer types (Aggarwal and Ichikawa, 2005; Banerjee *et al.* 2011). A recent study by Li *et al.* (2010b) demonstrated that DIM selectively induced proteasomal degradation of class I HDACs (HDAC-1, -2, -4, and -8) with no effect on class II HDACs, both in human colon cancer cells *in vitro* and *in vivo* in tumor xenografts. Furthermore, depletion of class I HDACs was shown to relieve transcription repression of the cyclin-dependent kinase inhibitors p21WAF1 and p27KIP2, which ultimately resulted in G2 arrest and DNA damage triggered apoptosis. In another study, Beaver *et al.* (2012) examined the effects of I3C and DIM on prostate cancer cell lines LNCaP and PC3, which differ in their androgen receptor (AR) status. DIM was shown to inhibit HDAC activity in both cell lines; however, I3C demonstrated modest inhibition of HDAC activity in androgen sensitive LNCaP cells with no significant effect on androgen insensitive PC3 cells. A significant decrease in HDAC-2 protein levels was reported in both cell lines after DIM treatment but the levels of HDAC-1, HDAC-3, HDAC-4, HDAC-6, and HDAC-8 remained unaltered. Interestingly, a recent study by Busbee *et al.* (2014) reported that I3C and DIM may decrease activation, proliferation, and cytokine production by Staphylococcal enterotoxin B (SEB) induced T-cells *in vitro* and *in vivo* by acting as class I HDAC inhibitors.

In a study to examine the effect of DIM on miRNA expression profile between gemcitabine-sensitive and gemcitabine-resistant pancreatic cancer cells, Li *et al.* (2009b) reported that DIM treatment caused significant upregulation of members of the miR-200 and let-7 families. Furthermore, re-expression of miR-200 in gemcitabine-resistant cells was found to be associated with a corresponding downregulation of mesenchymal markers ZEB1, slug, and vimentin, and upregulation of epithelial marker E-cadherin, which led to the reversal of EMT phenotype to epithelial morphology. In another study, DIM treatment was demonstrated to increase the expression of miR-146a which correlated with reduced expression of EGFR, MTA-2, IRAK-1, and NF- κ B, ultimately resulting in the inhibition of pancreatic cancer cell invasion (Li *et al.* 2010c). In human breast cancer cells MCF-7 (estrogen receptor positive) and MDA-MB-468 (p53 mutant), DIM treatment was demonstrated to G2/M cell cycle arrest by downregulating the expression of Cdk-2, Cdk-4, and Cdc25A (Jin *et al.* 2010). In addition, DIM treatment was shown to cause upregulation of microRNA-21, which negatively regulates Cdc25A, ultimately resulting in decreased breast cancer cell proliferation.

34.3.6 Lycopene

Lycopene is the bright red carotene pigment belonging to tetra terpenoids and a phytochemical that occurs naturally in tomatoes, carrot, watermelon, papaya, cherries, and so on. It is a potent antioxidant and has been shown to modulate multiple genes involved in DNA repair, cell cycle control, and apoptosis in breast cancer cells (Chalabi *et al.*

2006, 2007). Studies by King-Batoon *et al.* (2008) reported the effects of lycopene on *GSTP1* gene in breast cancer cells. Lycopene (2 μ M, 1 week) was shown to induce *GSTP1* expression and demethylate *GSTP1* promoter in the MDA-MB-468 cell line but not in MCF-7 breast cancer cells. The expression of other genes such as *RAR β 2* and *HIN1* remained unaltered by lycopene treatment in MCF-7 and MDA-MB-468 breast cancer cells. Although this study has shown that lycopene may be DNA methylating agent but further studies are needed to decipher the lycopene mediated effects on epigenetic mechanisms.

34.3.7 Organosulfur Compounds

Vegetables such as onion, garlic, shallots, and so on, belonging to the *Allium* family possess various water and fat soluble organosulfur compounds (OSCs), which have been widely implicated for numerous health related benefits including cancer chemoprevention, improved immunity, cardiovascular health, and anti-microbial activity (Iciek *et al.* 2009; Nian *et al.* 2009; Thakur *et al.* 2014). Conversion of alliin to allicin and other alkyl alkane-thiosulfinate by the enzyme alliinase generate OSCs, which further decompose to more stable sulfur compounds such as diallyl sulfide (DAS), diallyl disulfide (DADS), and diallyl trisulfide (DATS). S-allylmercaptocysteine (SAMC) is the active metabolite derived from DADS, both of which are finally converted to allyl mercaptan (AM) and other metabolites (Lea *et al.* 1999). Accumulated evidences suggest that in human cancer cells these OSCs increase histone (H3/H4) acetylation, highlighting HDACs as their potential targets (Lea *et al.* 1999, 2001, 2002). In colon cancer cells Caco-2 and HT-29, DADS was reported to inhibit cell proliferation and cause G2 cell cycle arrest by inhibiting HDAC activity, inducing histone hyperacetylation as well as p21 (waf1/cip1) expression (Druesne *et al.* 2004). Similarly, a study by Nian *et al.* (2008) where several garlic derived OSCs were tested or screened *in vitro* for their ability to inhibit HDACs. They reported that AM was the most potent HDAC inhibitor. Further experiments, which include molecular modeling, structure-activity, and enzyme kinetic studies, confirmed competitive inhibition of HDAC-8 by AM ($K_i = 24 \mu$ M). Furthermore, treatment of human colon cancer cells with AM caused accumulation of acetylated histones in cellular chromatin and facilitated binding of transcription factor SP3, followed by recruitment of p53 at the p21(waf1) promoter.

34.3.8 Phenethyl Isothiocyanate (PEITC)

Isothiocyanates are metabolites of glucosinolates, which occur naturally in cruciferous vegetables (Link *et al.* 2010). PEITC is a metabolite of gluconasturtin obtained from water cress, which has shown significant beneficial health related effects particularly in cancer chemoprevention (Cheung and Kong 2010). Like other natural phytochemicals, PEITCs have been demonstrated to regulate epigenetic mechanisms by modulating DNA methylation and histone modifications. Studies by Wang *et al.* (2007) reported the dual action of PEITC treatment on DNA methylation as well as histone modifications in epigenetic re-activation of *GSTP1* gene in prostate cancer cells. They showed that exposure of LNCaP cells to PEITC inhibited HDAC activity and concurrently demethylate the promoter to restore the *GSTP1* levels to that found in normal prostate cells. In another study, exposure of mouse erythroleukemic cells to allyl isothiocyanates was found to cause increased acetylation of histones, with no significant effect on histone deacetylation (Lea *et al.* 2001).

34.3.9 Quercetin

Quercetin is a flavonoid or dietary polyphenol, which is abundantly present in citrus fruits, onion, and buckwheat. Studies showed that this bio-flavonoid is a potent free radical scavenger or antioxidant as well as a chemopreventive agent with pro-apoptotic and growth inhibitory effects on cancer cells (Gibellini *et al.* 2011). Tan *et al.* (2009) investigated the *in vitro* effects of quercetin on human colon cancer RKO cell line and showed that the hyper-methylation status of p16INK4a gene could be completely reversed after quercetin treatment in a concentration dependent manner. In another study, quercetin was shown to downregulate TNF-induced interferon-gamma-inducible protein 10 (IP-10) and macrophage inflammatory protein 2 (MIP-2) gene expression in murine intestinal epithelial cells. At the molecular level, quercetin treatment was found to cease recruitment of cAMP response element binding protein (CBP)/p300 and histone H3 phosphorylation/acetylation at the gene promoters, as well as inhibition of histone acetyltransferase activity (Ruiz *et al.* 2007). Studies by Lee *et al.* (2011) demonstrated that in human leukemic cells HL-60, quercetin treatment induced Fas-L related apoptosis, and activation of the ERK (extracellular signal-regulated kinase) and JNK (Jun N-terminus kinase) signaling pathways. Quercetin treatment was found to increase histone H3 acetylation, which in turn caused increased expression of Fas-L. Furthermore, quercetin treatment resulted in the activation of HAT and inhibition of HDAC; however, HAT activation was

found to be only associated with ERK and JNK pathways. Priyadarsini *et al.* (2011) reported a positive correlation between HDAC-1 and Dnmt1 inhibition by quercetin and its anti-tumor properties, such as pro-apoptosis, anti-angiogenesis, and anti-invasion. In a recent study, Ravichandran *et al.* (2014) proposed a refined pharmacophore model for the quercetin binding site of the SIRT6 protein. This study was an elaboration and in continuation from a previous study from the same research group, where they reported quercetin to be a potent SIRT6 inhibitor and identified a preliminary pharmacophore for quercetin binding (consisting of three hydrogen bond donor and one hydrogen bond acceptor) on the SIRT6 histone deacetylase (Singh *et al.* 2013).

34.3.10 Resveratrol

Resveratrol (trans-3,4',5-trihydroxystilbene), a plant derived polyphenolic compound present in grapes, wine, and root extracts of the weed *Polygonum cuspidatum*, has been identified as a potent anti-aging, anti-inflammatory, and chemopreventive agent affecting various molecular targets (Athar *et al.* 2009; Savouret and Quesne 2002). Focusing on epigenetic pathways related cellular targets, SIRT1, and acetyl transferase p300 were reported to be activated by resveratrol (Howitz *et al.* 2003; Rajendran *et al.* 2011; Wang *et al.* 2008a; Wood *et al.* 2004). Resveratrol was identified as a potent dietary activator of SIRT1, which lowers the K_m (Michaelis constant) for both acetylated substrate and NAD⁺. It was reported to stimulate SIRT1 dependent p53 deacetylation, which ultimately contributes to increased cell survival (Howitz *et al.* 2003). In another study by Wood *et al.* (2004), resveratrol was shown to activate sirtuins from metazoans *Caenorhabditis elegans* and *Drosophila melanogaster* and delay aging without any effect on fecundity. The anti-tumor effect of resveratrol was reported to be mediated partly by SIRT1 (Boily *et al.* 2009). Studies by Wang *et al.* (2008a) using SIRT1 mutant mice demonstrated that disruption of SIRT1 function p53 null background causes tumor formation and resveratrol mediated activation of SIRT1 reduced tumorigenesis. In addition, resveratrol was shown to have a negative effect on *Survivin* gene expression through histone deacetylation at the gene promoter and display a more profound inhibitory effect on Brca-1 mutant cells both *in vitro* and *in vivo* (Wang *et al.* 2008b). In prostate cancer cells, resveratrol was reported to cause downregulation of MTA1 (metastasis associated protein), destabilize the NuRD (Nucleosome remodelling deacetylase) complex, and thus allowed p53 acetylation. Furthermore, activation of p53 was shown to induce pro-apoptotic pathways causing apoptosis in prostate cancer cells (Kai *et al.* 2010).

34.3.11 Sulforaphane

Sulforaphane (SFN) is an isothiocyanate abundantly present in broccoli, watercress, broccoli sprouts, cabbage, and kale. SFN has been shown to modulate various physiological processes and affect various cellular pathways consistent with its chemopreventive effects, which include induction of cell cycle arrest, apoptosis, and xenobiotic metabolism (Clarke *et al.* 2008; Tomczyk and Olejnik 2010). Microarray based transcriptome analysis of human colon Caco-2 cells treated with SFN at physiological concentrations showed downregulation of the Dnmt1 gene as well as other genes known to be associated with carcinogenesis (Traka *et al.* 2005). Studies by Meeran *et al.* (2010) demonstrated that in MCF-7 and MDA-MB-231 breast cancer cells, SFN treatment cause dose and time dependent inhibition of hTERT (human telomerase reverse transcriptase) via an epigenetic mechanism involving DNA methylation and histone modifications. SFN treatment was shown to cause downregulation of Dnmt1 and Dnmt3a, which induced site specific demethylation at hTERT gene first exon facilitating the binding of CTCF associated with hTERT repression. Furthermore, ChIP analysis of hTERT promoter revealed that active histone chromatin marks such as acetyl-H3, acetyl-H3K9, and acetyl-H4 were increased whereas repressive chromatin marks, which include trimethyl-H3K9 and trimethyl-H3K27, were reduced after SFN treatment in a dose dependent manner. The SFN induced hyper-acetylation was reported to promote the binding of repressor proteins such as MAD1 and CTCF to the hTERT regulatory region. In another study, Myzak *et al.* (2004) reported that SFN metabolites – SFN-cysteine and SFN-N-acetylcysteine – were more potent HDAC inhibitors *in vitro* as compared to SFN or its glutathione conjugate. Furthermore, SFN treatment in HCT116 human colorectal cancer cells increased β -catenin-responsive reporter (TOPflash) activity in a dose dependent manner and inhibited HDAC activity. Consequently, there was an induction in acetylated histone levels bound to p21 (Cip1/Waf1) promoter. In human prostate epithelial cells BPH-1, LNCaP, and PC3, SFN treatment was shown to inhibit HDAC activity, which was accompanied by an increase in acetylated histone levels by 50–100% and a corresponding induction of p21 and Bax expression, which led to downstream events such as cell cycle arrest and apoptosis (Myzak *et al.* 2006b). In human breast cancer cells, SFN treatment was shown to inhibit HDAC activity but no change in H3 or H4 acetylation was observed (Pledgie-Tracy *et al.* 2007). Studies by Myzak *et al.* (2006a) provided first evidence for inhibition of *in vivo* HDAC activity and suppression of tumorigenesis in APC-minus mice.

Further studies demonstrated that daily SFN dose (7.5 μ M per animal for 21 days) in the diet significantly reduced the growth of PC-3 cells in male nude mice, which correlated with a decrease in HDAC activity and increased global acetylation levels. Furthermore, in human subjects, a single dose of 68 g broccoli sprouts caused a significant decrease in HDAC activity in peripheral blood mononuclear cells within 3–6 h after consumption (Myzak *et al.* 2007). In a recent study, Wong *et al.* (2014) studied the genome wide effects of SFN and DIM on promoter methylation in prostate epithelial as well as cancer cells using methyl-DNA immunoprecipitation followed by a genome-wide DNA methylation array. The study reported that SFN and DIM treatment induced widespread changes in promoter methylation patterns and reversed the status of several cancer-associated aberrantly methylated genes.

34.4 Conclusion and Future Perspectives

The role of natural phytochemicals as epigenetic modulators has been well supported by the studies described in this chapter. The fundamental role of epigenetic mechanisms in the regulation of gene expression and epigenetic aberrations implicated in various pathologies itself underlines the importance of compounds targeting them. Although recent advances in the field of *nutriepigenetics* have enhanced our understanding of the impact of various dietary components, particularly natural phytochemicals on the mammalian epigenome, especially in pathologies such as cancer, but much deeper studies are still needed to dissect their impact on global patterns of epigenetic modifications. Pre-clinical and clinical studies addressing the relevance and validity of *in vitro* experimental outcomes as well as analysis of safety profile, dose, bio-availability, and routes of administration for these compounds are required for designing better therapeutic strategies, along with appropriate design of clinical trials.

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Part VI

Peptidomics

35

Detection and Identification of Food-Derived Peptides in Human Blood: Food-Derived Short Chain Peptidomes in Human Blood

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35.1 Introduction

In nutrition, proteins and their partial hydrolysates, namely peptides, are conventionally considered merely an amino acid source (Grimble, 1994). However, Yoshikawa and his coworkers, who are some of the pioneers in the field of functional food research, have demonstrated in animal models that some food-derived protein hydrolysates exert anti-hypertensive activity upon oral ingestion (Yokoyama, *et al.*, 1992, 2000). Since then, evidence illustrating the beneficial effects of ingesting of proteins and peptides for purposes beyond utilizing their conventional nutritional properties has accumulated. Peptides in food-derived protein hydrolysates have been demonstrated to attenuate hypertension, hyperlipidemia, hepatitis, and colitis, as well as to improve skin, joint, and mental health (Hettiarachchy *et al.*, 2012; Mine and Shahidi, 2005). These benefits have been observed in animals as well as humans, suggesting that the oral ingestion of some peptides can have beneficial health-promoting effects. To elucidate the mechanisms underlying these effects, many *in vitro* studies based on enzyme reactions, cell culture systems, and suchlike, have been conducted, which have helped to elucidate the potential mechanisms of action. Furthermore, some conventional *in vitro* activity-guided fractionation assays have identified potentially active peptides. Peptides in food, however, can be further digested into smaller peptides and amino acids during digestion and absorption processes. In some cases, they may be metabolized into other compounds, which can lead to possible loss of activity. Even if the peptides escape peptidase digestion, some of them may not penetrate the intestinal epithelium. In contrast, metabolites of peptides in food – such as smaller peptides and modified peptides – might be changed to biologically active compounds. Thus, a peptide with *in vitro* activity in food may not necessarily initiate a biological response after oral administration. As such, it has been difficult to identify truly active peptides *in vivo* and to elucidate the underlying mechanisms of their action. To bridge this gap between *in vitro* and *in vivo* peptide activity in food, it is necessary to identify and determine which food derived-peptides are capable of reaching the target organs. We can obtain more information on the mechanism of peptide action by measuring the *in vitro* activity of food-derived peptides in a target organ or in blood than by measuring the *in vitro* activity of peptides in food. In this chapter, we introduce the recent advances in the identification of food-derived peptides in human blood. In addition, we discuss future prospects for mass spectrometry (MS)-based identification of a food-derived short chain peptidome.

35.2 Detection of Apparent Bioactive Peptides in Human Blood

As described previously, ingestion of some food-derived protein hydrolysates and fermented milk has been shown to attenuate mild hypertension in humans (Yoshikawa *et al.*, 2000). To identify the peptide active in this process, numerous studies have used *in vitro* activity-guided fractionation. In most of these studies, inhibitory activity against angiotensin converting enzyme (ACE) was used to screen for a potentially active peptide. ACE produces a hypertensive peptide hormone (angiotensin II) from the inactive precursor peptide (angiotensin I) and degrades a hypotensive peptide hormone (bradykinin) (Heeneman *et al.*, 2007). ACE-inhibitory peptides have been identified and suggested to be responsible for the hypotensive activity of some orally ingested food-derived substances. To confirm that these ACE-inhibitory peptides are absorbed, pre-column derivatization coupled with a column switching technique (Matsui *et al.*, 2002) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) in multi reaction monitoring (MRM) mode (Foltz *et al.*, 2007) have been used. In those studies, ACE inhibitory peptides were detected in animal and human blood (Foltz *et al.*, 2007; Matsui *et al.*, 2002) and vein tissue (Matsui *et al.*, 2004). In most cases, these ACE-inhibitory peptides were detected at concentrations in the low nM range after ingestion. However, these levels may be insufficient to induce a hypotensive effect by inhibiting ACE, because the IC_{50} against ACE in these peptides is in the μ M range. Therefore, it is possible that these peptides might act on other targets such as angiotensin II receptors (Yoshikawa *et al.*, 2013), or Ca^{2+} channel (Matsui *et al.*, 2005). Alternatively, other active peptides may be generated in the body from inactive precursor peptides in food. This possibility would not be detected by *in vitro* activity-guided fractionation. To address this issue, comprehensive detection and quantification of food-derived peptides in blood and target organs is necessary; such information could be obtained from the analysis of a food-derived peptidome in human blood.

35.3 Identification of Food-Derived Peptides in Human Blood

The concept of the peptidome is not new. Peptidome studies have effectively identified degradation products of endogenous proteins by matrix assisted laser desorption ionization (MALDI)-MS (Petricoin *et al.*, 2006). Food-derived peptides in human blood are predominantly di- and tripeptides. It is difficult to detect such low molecular compounds by the MALDI-MS. Recent developments in LC-MS, especially soft ionization methods such as electron spray ionization (ESI), have facilitated comprehensive, quantitative analysis of wide arrays of metabolites including amino acids, organic acids, and nucleic acids, in biological samples; this method is referred to MS-based metabolomics (Xiao *et al.*, 2012; Zhou *et al.*, 2012). MS-based metabolomics can provide information for understanding biological responses. However, peptides, even dipeptides, can differ in their structures. Tandem MS (MS/MS) analysis can provide additional structural information for peptides. The concentrations of food-derived peptides in blood and other biological samples are generally very low (in the range of a few μ M to nM); these samples also contain a variety of different compounds. In addition, it is also difficult to resolve hydrophilic peptides by reversed phase-high performance liquid chromatography (HPLC), the most powerful tool for resolving biological compounds, due to weak absorbance to the solid phase. It is still difficult to identify peptides by single LC-MS/MS analysis. However, if the structure of the food-derived peptide is available, LC-MS/MS in multi reaction monitoring (MRM) mode enables simultaneous identification of the food-derived peptides in biological samples with high sensitivity. The ability to identify food-derived peptides in a biological sample with precision and accuracy is crucial for food-derived peptidome analysis. Recent advances in identification of food-derived peptides in human blood are introduced in the following sections.

35.3.1 Identification of Food-Derived Peptides as Intact Forms

To accomplish food-derived peptidome analysis, peptides with diverse structures and low quantity must be isolated and identified from a complicated pool. Until recently, a food-derived peptidome has been considered an impossible feat. In 2005, this situation changed. After ingestion of 10 g of collagen peptide, the concentration of the peptide form of hydroxyproline (Hyp) increased by approximately 30 μ M from baseline (Iwai *et al.*, 2005; Shigemura *et al.*, 2011). This finding indicates that unexpectedly high amounts of food-derived peptides are present in human peripheral blood compared to previously suggested levels.

As shown in Figure 35.1, direct injection of a deproteinized fraction (75% ethanol-soluble fraction) of human plasma after ingestion of collagen peptide into a reversed phase-HPLC column yielded numerous peaks, especially in the non-absorbed range. Most of peaks were derived from non-peptide compounds. It was difficult to isolate food-derived peptides

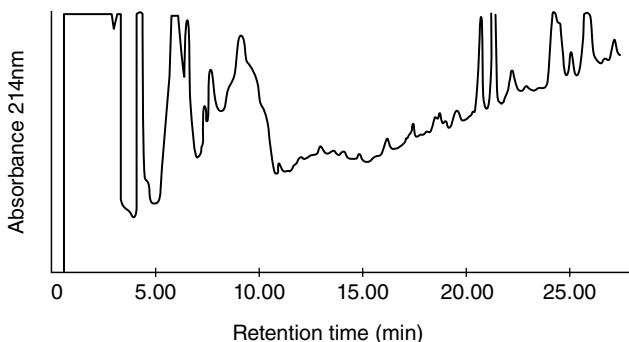


Figure 35.1 Reversed phase-HPLC patterns of 75% ethanol-soluble fraction of human serum obtained 60 min after ingestion of collagen peptide. Redrawn from Aito-Inoue *et al.* (2006).

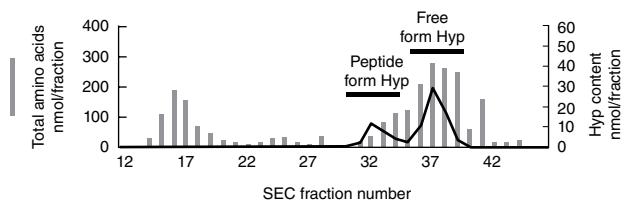


Figure 35.2 Fractionation of peptides in the ethanol-soluble fraction of serum collected 60 min after ingestion of collagen peptide by size exclusion chromatography (SEC). Bar indicates total amino acids after HCl hydrolysis. Line presents hydroxyproline (Hyp) content after hydrolysis. Redrawn from Iwai *et al.* (2005).

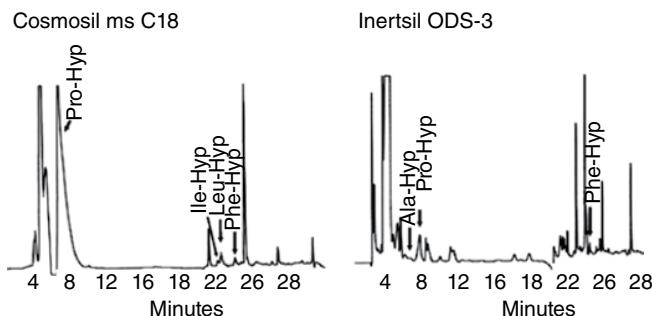


Figure 35.3 Isolation of food-derived collagen peptides by reversed phase-HPLC using two different columns. Collagen peptide fraction obtained by SEC as shown in Figure 35.2 was used. Absorbance at 214 nm was monitored. Each peak was subject to amino acid sequence analysis. Redrawn from Iwai *et al.* (2005).

by direct reversed phase-HPLC with the conventional ultraviolet (UV) absorbance detector. Next, an oligopeptide fraction was prepared by size exclusion chromatography (SEC) before the fractionation by reversed phase-HPLC. Elution of collagen peptide was monitored by detection of hydroxyproline (Hyp) after HCl-driven hydrolysis, as Hyp is exclusively contained in collagen. As shown in Figure 35.2, a fraction containing the peptide form of hydroxyproline (Hyp) was collected and subjected to fractionation by reversed phase-HPLC. Relatively fewer peaks appeared compared to the direct injection of the deproteinized fraction of plasma by reversed phase-HPLC (Figure 35.3). All peaks were collected and subjected to sequence analysis. Some Hyp-containing peptides were identified, as shown in Figure 35.3 (Iwai *et al.*, 2005). However, resolution of hydrophilic peptides by RP-HPLC is not high, and it is likely that some hydrophilic peptides may have been overlooked by this approach.

35.3.2 Isolation of Phenyl Thiocarbamyl Peptide for Sequence Analysis Based on Edman Degradation

To improve resolution and detection of hydrophilic peptides that could not be attained by reversed phase-HPLC, the precolumn derivatization technique has been developed. To accomplish this, phenyl isothiocyanate (PITC) has been used (Aito-Inoue *et al.*, 2006; Shigemura *et al.*, 2011). Derivatives of peptides, phenyl thiocarbamyl (PTC)-peptides (Figure 35.4), can be analyzed by peptide sequencer based on Edman degradation, for which the program is altered to begin from the cleavage reaction. As shown in Figure 35.5, specific derivatives were observed in the size-exclusion chromatography (SEC) fractions of plasma collected after ingestion of the collagen peptide. The major derivatives were identified as Pro-Hyp (peak a) and Hyp-Gly (peak b) by Edman degradation (Figure 35.6). These compounds without the derivatization are eluted in the non-absorbed fraction in reversed phase-HPLC, indicating the advantage of this approach. Using this method, some food-derived peptides in human blood after ingestion of collagen (Shigemura *et al.*, 2011), and elastin peptides (Shigemura *et al.*, 2012) were identified. Short chain peptide derivatives and amino acid derivatives in the final step of Edman degradation tend to be washed away from the sample disc of the automated peptide sequencer. For sequence analysis of short chain peptides (tri- and dipeptides) by Edman degradation, higher amounts (approximately 100 pmol or more) than the standard peptides/protein analysis are required. It is somewhat difficult to identify short chain peptides at concentrations below levels of 1 μ M in a sample.

PTC-peptides can be identified by MS/MS analysis. However, ion intensity of PTC-peptides by electron spray ionization (ESI) is not strong enough for sequence analysis, especially in the acidic sorbent. As shown in Figure 35.7, sorbent at alkaline pH can improve ionization intensity of PTC-peptides, while protonated phenyl thiohydantoin (PTH)-amino acid, degradation product of Edoman degradation, was also observed, indicating that PTC-peptide is not stable in ESI-MS. In addition, resolution of the PTC-peptides by reversed phase-HPLC in alkaline sorbent is, however, poor. Thus, for sequence analysis using MS/MS, other derivatization techniques are better suited.

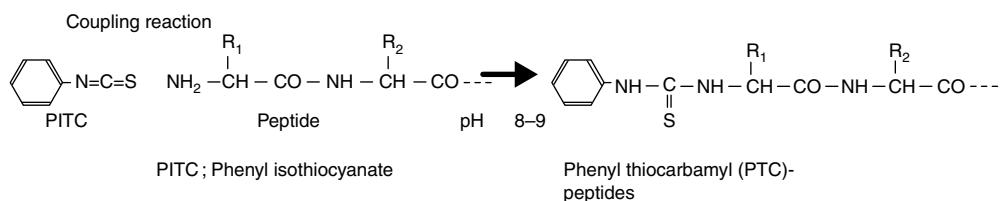


Figure 35.4 Reaction of phenyl isothiocyanate (PTC) with a peptide to form phenyl thiocarbamyl (PTC)-peptide.

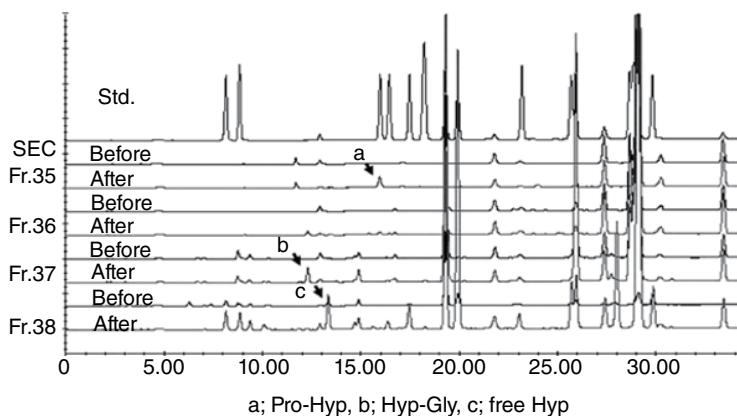


Figure 35.5 Detection of food-derived collagen peptides in human plasma before and 60 min after ingestion of collagen peptide by precolumn derivatization with PITC. Redrawn from Shigemura et al. (2011).

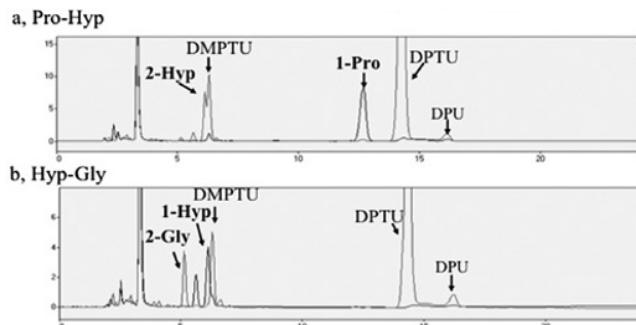


Figure 35.6 Sequence analysis of PTC-derivatives (a and b) in Figure 35.5. First and second cycles are shown. No signal was obtained in the third cycle. Redrawn from Shigemura et al. (2011).

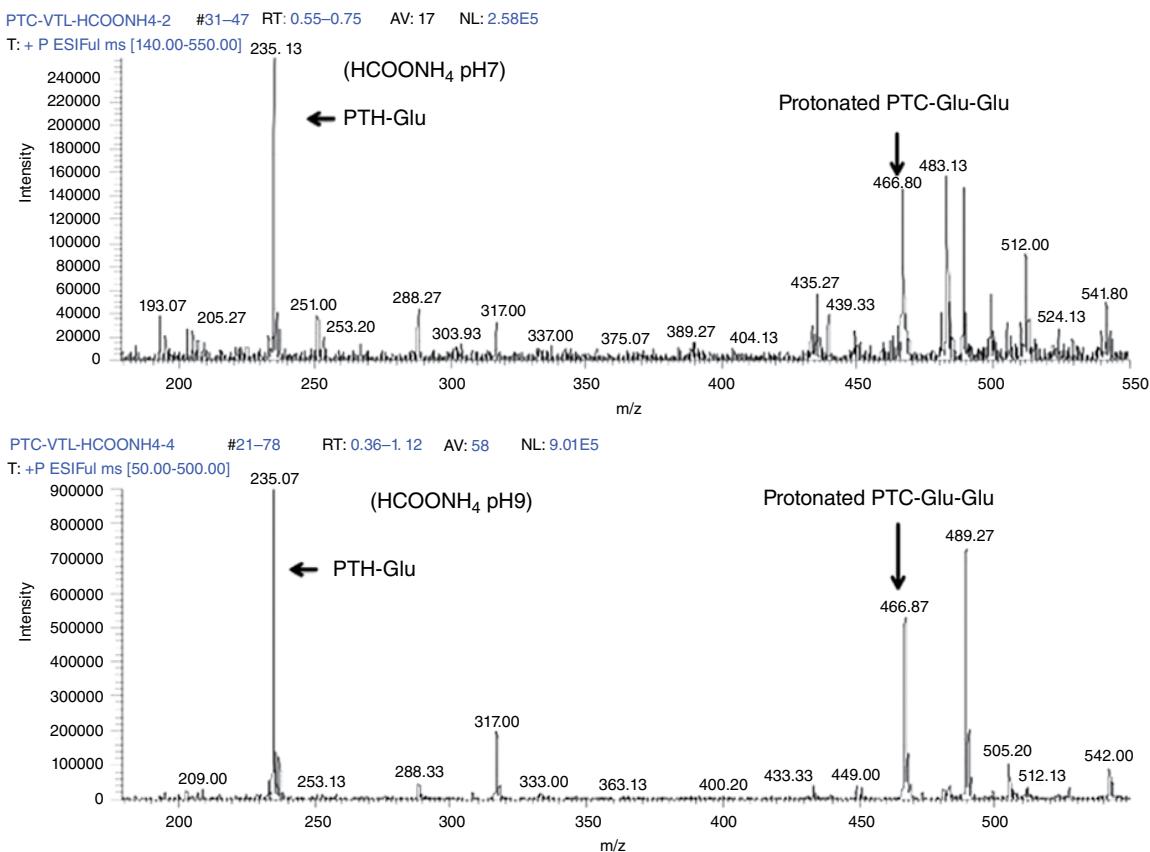


Figure 35.7 Electron spray ionization mass spectrometry (ESI-MS) patterns of phenyl thiocarbamyl (PTC)-Glu-Glu in neutral and alkaline sorbents. In addition to PTC-Glu-Glu ion, Edoman degradation product ion: phenyl thiohydantoin (PTH)-Glu appeared. (Unpublished data).

35.3.3 MS/MS Analyses of Derivatized Peptides

We have used 6-aminoquinolyl-N-hydroxysuccinimidylcarbamate (AccQ) for the ESI-MS/MS analysis of peptides (Shigemura *et al.*, 2012). As shown in Figure 35.8, the AccQ derivative of authentic peptides can be resolved by reversed phase-HPLC and give higher ion intensity with ESI-MS in the positive ion mode than the PTC-derivative (Figure 35.9, upper). The MS/MS analysis yielded fragments, which can be used for sequence analysis (Figure 35.9, lower left). All cases of MS/MS or MS/MS/MS analysis of the AccQ-peptides give fragment with $m/z = 171$ (Figure 35.9, lower right), which corresponds to the AccQ ion. Detection of this fragment helps to detect AccQ-peptides.

Using this approach, after ingestion of the hemoglobin peptide, a food-derived peptide was successfully identified in blood. Plasma was collected from a human volunteer before and after ingestion of the hemoglobin peptide. The deproteinated fraction was subjected to SEC to obtain the peptide fraction. An SEC fraction yielded a peak with $m/z = 171$ by ESI-MS/MS, specifically after ingestion (Figure 35.10, upper and middle). Based on its spectrum, this peptide could be identified as Val-Ala (Figure 35.10, lower). A few food-derived peptides in human blood have been identified using this approach. Another amino group-labeling reagent, 2,4,6-trinitrobenzene sulfonate (TNBS), has also been used successfully for this purpose (Nakashima *et al.*, 2013).

It is difficult to distinguish Leu and Ile residues by MS/MS analysis. In such cases, comparing retention time with synthetic peptides based on the suggested sequences can be used to identify the peptide. For example, Glu-Leu, Glu-Ile, (γ -Glu)-Leu, and (γ -Glu)-Ile can be resolved by reversed phase-HPLC. It is, therefore, important to check structure not only by MS/MS spectrum but also by retention time of authentic peptide.

35.4 Future Prospects

As shown in Figure 35.11, simultaneous determination of food-derived collagen peptides in human blood can be achieved by LC-MS/MS in MRM mode (Ichikawa *et al.*, 2010), if the structures of the peptides are available. However, except some major peptides present in large quantities in human blood, most of these peptides are difficult to identify by a single

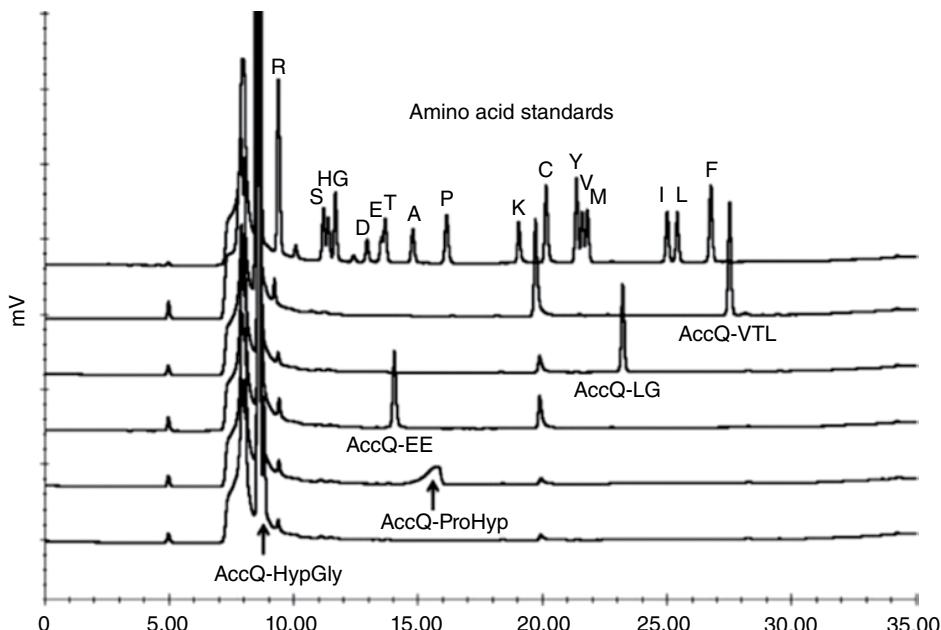


Figure 35.8 Resolution of AccQ-amino acids and some peptides. For amino acids, one letter abbreviations are used. (Unpublished data).

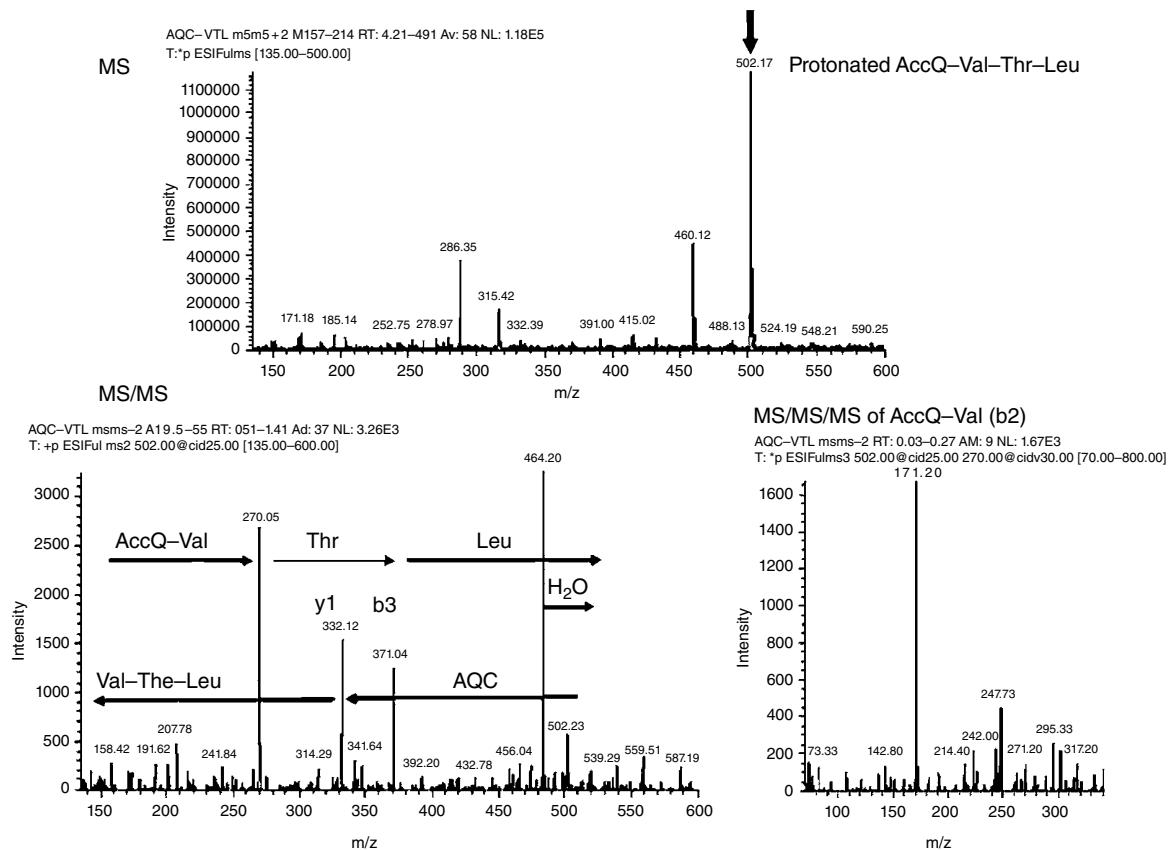


Figure 35.9 ESI-MS, MS/MS, and MS/MS/MS patterns of AccQ-Val-Thr-Leu. B2 ion by MS/MS was subjected to MS/MS/MS analysis. (Unpublished data).

LC-MS/MS analysis. Capturing of peptides using solid-phase extraction with cation-exchange resin (Aito-Inoue *et al.*, 2006) and sub-fractionation with size-exclusion chromatography (Iwai *et al.*, 2005), and so on is necessary. Even after these pretreatments, reversed phase-HPLC fraction frequently yields many peaks in the MS spectrum. As described previously, when peptides are derivatized with AccQ, MS/MS or MS/MS/MS of the derivatives yield fragment corresponding to AccQ with $m/z = 171$. If selected reaction monitoring for $m/z = 171$ is possible, selective detection of the AccQ derivatives can be obtained. MS/MS analysis of AccQ-peptide detected by selected reaction monitoring would provide rapid identification of peptides in a complex sample. To assist this approach, it is essential to develop software to automatically detect AccQ derivatives for sequence analysis.

Currently, the available sequence data for food-derived peptides in human blood and other biological samples is limited (Ichikawa *et al.*, 2010; Iwai *et al.*, 2005; Shigemura *et al.*, 2011, 2012). Most of these peptides are di- and tripeptides and resistant to peptidase digestion. Specific peptides can survive peptidase digestion and be absorbed into the blood and cells. Newly identified peptides could be registered to establish a food-derived peptidome database. The database would provide a ready-to-use MRM method pack for peptidome analysis of biological sample. It has been shown that even dipeptides can exert significant biological effects on cells (Matsui *et al.*, 2005; Shigemura *et al.*, 2009, 2011, 2012). A complete peptidome analysis would help to explore hidden potential functions of short chain peptides derived from orally ingested food peptides.

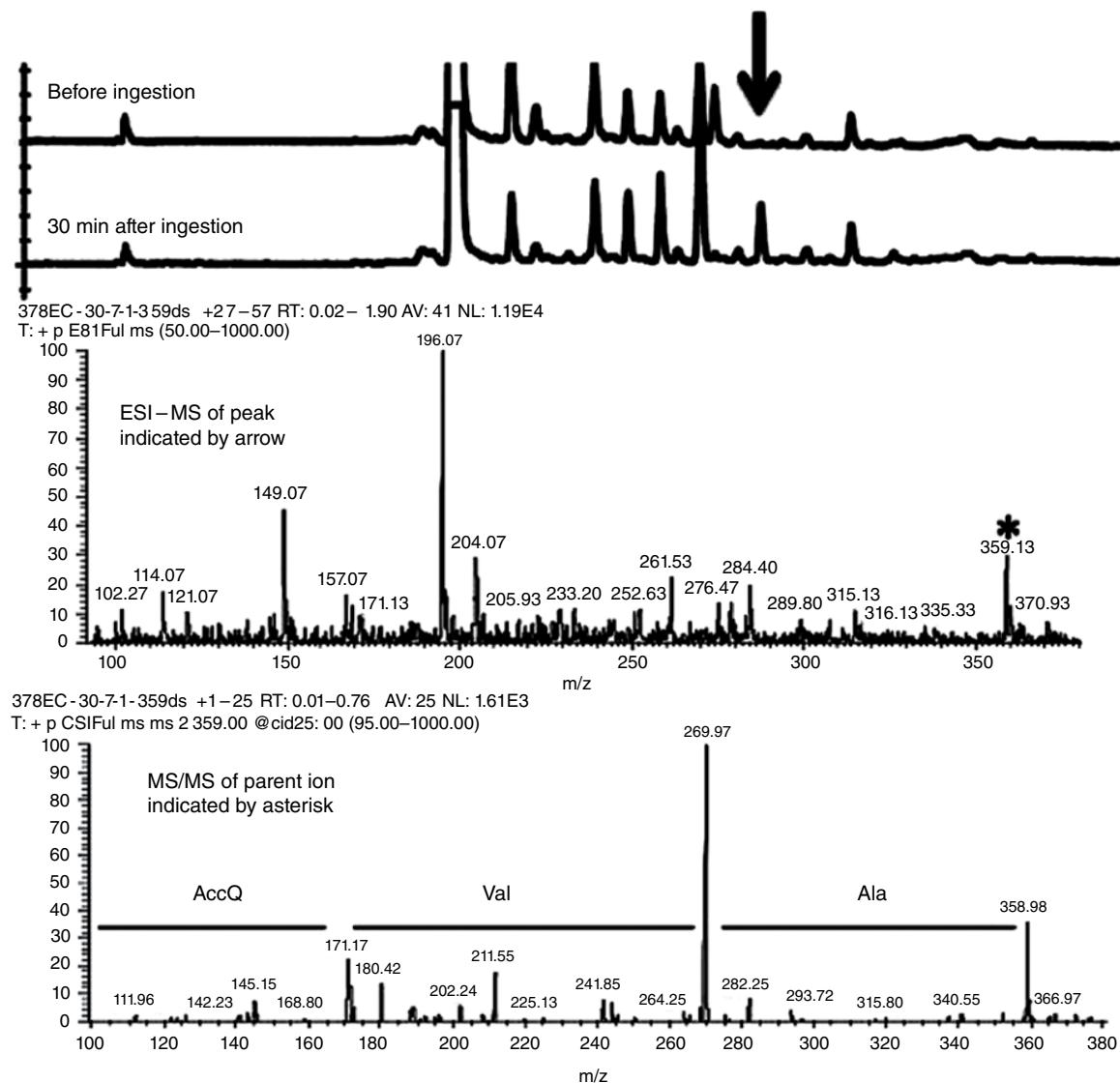


Figure 35.10 Identification of food-derived Val-Ala after ingestion of hemoglobin peptide. Peptide fraction obtained by SEC was subjected to precolumn derivatization with AccQ. AccQ derivative appeared after ingestion (arrow) was collected and subjected to ESI-MS and MS/MS. Peak marked with asterisk in MS spectrum was subjected to MS/MS analysis. (Unpublished data).

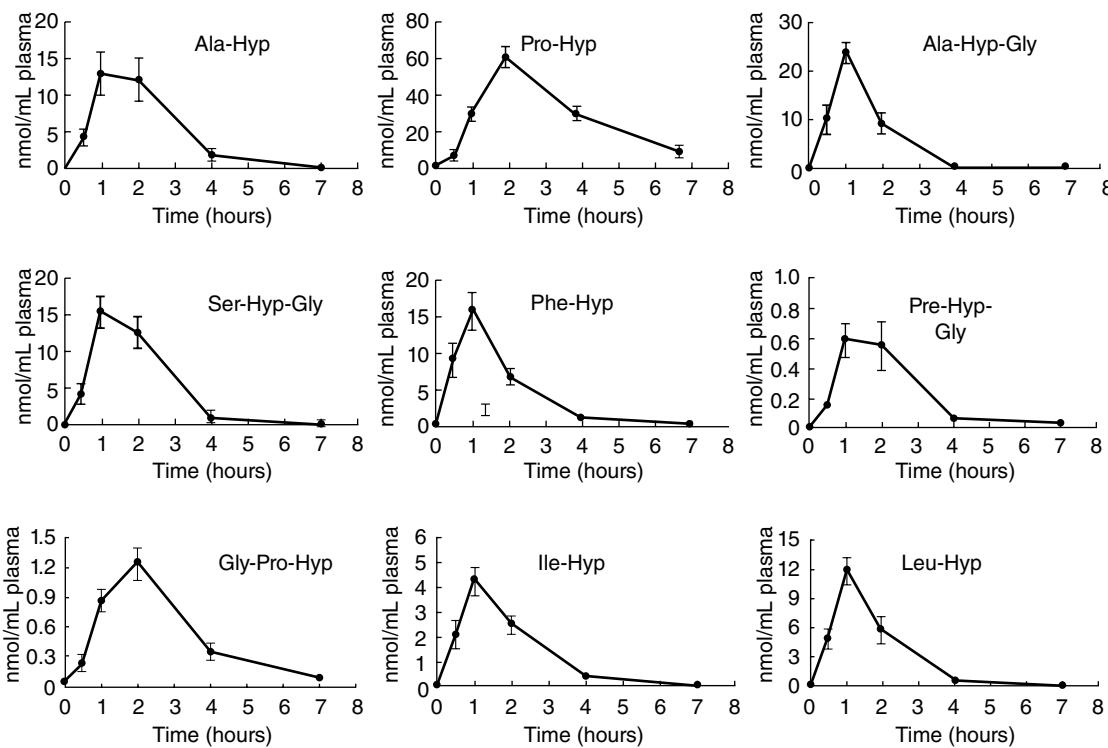


Figure 35.11 Contents of food-derived collagen peptides in human blood proteome. Each peptide was determined by LC-MS/MS in MRM. Redrawn from Ichikawa, et al. (2010).

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Part VII

Nutrigenomics and Human Health

36

Use of Omics Approaches for Developing Immune-Modulatory and Anti-Inflammatory Phytomedicines

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36.1 Introduction

The highly innovative development of the science and technology of the omics research era has created new research systems, including genomics, proteomics, lipidomics, and metabolomics, as well as the associated bio-informatics science, and databases. At the same time, progress in traditional western medical research appears to have reached a bottleneck, as single compound drugs are very costly to develop, synthesize, and/or engineer. If we are to see real and sustained progress in research and development for new drugs, we must find new and alternative approaches to utilize traditional remedies to develop advanced medicines.

Technology wise, instrumental systems for transcriptome, including the microarray of different messenger RNA, microRNA and other non-gene sequence-related RNA products, have already been developed; functional genomics studies using these systems have been remarkably successful. However, the candidate genes involved in specific functions often need further verifications for revealing their roles in the signal pathways. The difficulty may also arise from the high variety and seemingly unrelated responsive genes and complex signaling or regulatory systems involved.

Conventional proteomic analysis has its disadvantages, although two-dimensional (2D) gels can display viable candidate proteins for study. Up to 2000 proteins of biological systems can often be analyzed in sensitive 2D gel systems. There are other proteins, such as cytokines or chemokines of most leukocyte cells are expressed at relatively low levels, and often are not detectable by 2D gels. Recently, more sensitive methods, especially the advanced high throughput mass spectrometry-based omics technologies, have been (and are being) developed, leading to possible full proteome characterizations.

Metabolomics still faces a great challenge: a 2D or one-run display of the components of a metabolome has not been defined and cannot be systematically evaluated. Therefore, sequential analyses, for example, the LC/MS followed by NMR, were developed to address the “overall” or more comprehensive picture of metabolomes.

An increasing number of phytomedicinal studies, including some in traditional Chinese herbal medicine (TCM), have been considered to be metabolomic investigations. New strategies employing omics approaches may be especially useful for phytomedicinal research, as conventional phytomedicines often employ multiple components and they often are believed to interact with multiple molecular targets related to cellular and physiological (e.g., immunomodulatory) effects.

In order to successfully evaluate the effects of phytomedicines, various omics approaches are being systematically combined. New computational and cross-disciplinary analyses will be required for most experimental biology studies. Some examples of systematic and technical considerations, in terms of research into the immunomodulatory and anti-inflammatory effects using the omics approaches, are addressed in this brief review.

36.1.1 Needs and Importance of Systems Biology and Bioinformatics

Large numbers of scientists are investigating medicinal plants in search of regulatory genes and metabolites that can affect, modulate, or upgrade the biological and metabolic processes, which in turn can confer specific physiological or pharmacological functions. Recently, various high-output technologies, including genomics, transcriptomics, proteomics, lipidomics, and metabolomics, are employed in such a research effort (Fridman and Pichersky, 2005; Lockhart *et al.*, 1996; Pandey and Mann, 2000). Bioinformatics and systems biology approaches are considered by many as needed to organize, manage, process, and understand the vast amounts of data obtained in various omics studies (Lederman, 2009; Middleton *et al.*, 2007; Rapin *et al.*, 2006; Yao, 2002; Yip, 2008). In addition, systems biology is aimed at understanding complex biology by integrating omics data from various sources for network analysis, for evaluating the holistic system as a whole, as experimental results from omics studies are most often not obtained or isolated as a single set of data points or events (Ray *et al.*, 2002). By analyzing the omics data, bioinformatics tools can help upgrade new approaches for classifying and authenticating potential medicinal plants, identifying new bioactive phytochemicals or compounds, and even improving medicinal plant species or cultivars that can tolerate stressful environmental challenges.

The sophisticated human immune system, as we currently conceptualize it, is under the tight control of a complex network of regulatory genes, RNAs, modulatory proteins, and stimulatory metabolites. Past studies have often focused on understanding the roles of specific genes in immune responses. To associate expression changes with immunological conditions such as suppression, cancer, or autoimmunity, we can investigate the interrelationship of the up- and downregulation of genes or proteins patterns. By using microarray analysis and comparative genomics, Hutton *et al.* (2004) have identified genes and their regulatory elements responsible for maintenance, differentiation, and the general functioning of specific immune systems. In addition, most of the expression pattern of genes is related to the biological role and effects of the products of genes, and a similar statement may be made for protein expression (Staudt and Brown, 2000). Taken together, evaluation of gene and protein expression profiles may lead us to identify links between specific genes or proteins and the associated specific immuno-modulatory effects. Moreover, omics technologies may also be employed to address our views of the often-used concepts in immunology, such as: molecular dynamics in response to specific stimulations or alterations of the molecular state of targeted specific cells, in the hypothesis-driven research approach (Davies *et al.*, 2004). For instance, in the drug discovery process, pharmaceutical companies have used various microarray systems as screening tools to eliminate compounds that have molecular indications of toxicities before preclinical and clinical testing (Burns-Naas *et al.*, 2006). In basic research, omics technologies have dramatically and often unexpectedly improved our understanding on how drugs can regulate the immune system as well as of a variety of issues in mechanistic or hypothesis-driven research (Fisher *et al.*, 2004; Holladay *et al.*, 2002; Kinser *et al.*, 2004; Yin *et al.*, 2010). The data obtained from these studies not only may have significant impact on the future directions of those specific lines of research but also may improve our understanding of the specific immuno-modulatory regulation of given drugs.

The discipline of bioinformatics is the application of computational tools for biological sciences; its major aim is the management and interpretation of biological data (Swindells *et al.*, 2002). It has been an essential tool for fully integrating and multi-disciplinary understanding the processes in various biological areas (Kann, 2010). Among them, understanding omics data requires both common statistical and machine-learning methods, because the data are usually in high-dimensional form and complexity. Given the demand for studies on immunomodulatory effects of herbal medicines, this chapter introduces and summarizes the applications of some omics approaches and specific bioinformatics tools for investigating phytomedicines.

36.1.2 Omics Technologies

For systems biology research, technology platforms are often employed, including transcriptomics, proteomics, a number of metabolomics and others. They have enabled us to study living systems from a holistic or integrative perspective through revealing profiles of multiple biochemical components (Figure 36.1); it also opens up a unique opportunity to reinvestigate phytomedicines (Wang *et al.*, 2005). The revolution of genomics research and technology development has yielded

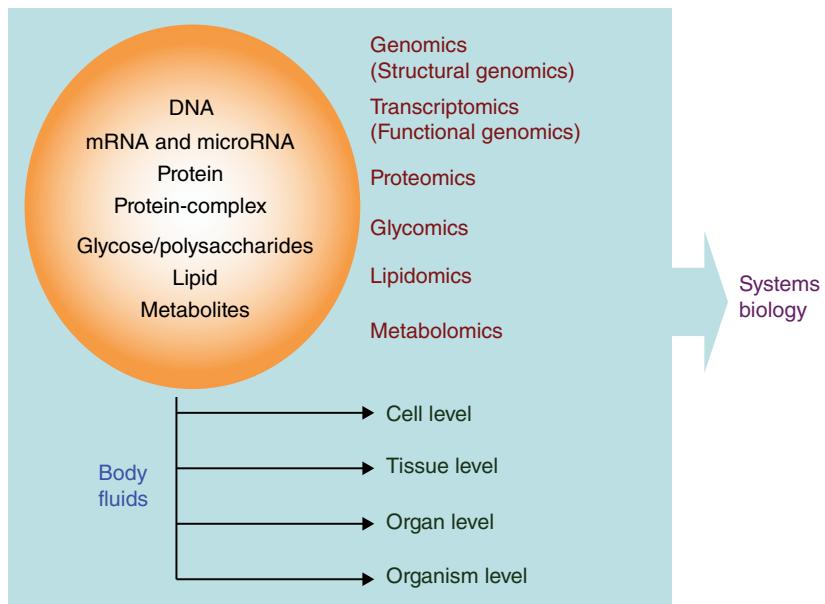


Figure 36.1 The different levels of measurement in a systems biology approach.

complete or draft DNA sequence maps for a spectrum of species including human, mouse and a series of model organisms. Having the genomic data available, many new “drug-able” targets based on transcriptomics study have been identified, opening up new insights into explanations of biological systems at a global scale. Additionally, through proteomics, we are witnessing the development of wonderful and multi-application tools for studying various signaling or mechanism systems at the level of proteins and protein–protein interactions (Cho, 2007; Wang *et al.*, 2005). In the meantime, studies on glycomics and bioactive polysaccharides are making great leaps in glycomics research; similarly, studies on regulation and metabolic control of a spectrum of lipids are creating new approaches for “lipidomics”. The recent wave of data from genomics and proteomics has precipitated the measurement of increasingly a group or spectrum of elements to provide a systems approach, especially at the level of metabolites and for the field of metabolomics.

Although omics are defined in several different ways today, in our opinion these systems provide “an integrated approach to study biological systems not only for the intracellular, but also at the cellular, organic, and the whole body or organismic levels or networks, through measuring and integrating the genomic, proteomic and metabolic data” in a global consideration manner (Figure 36.1) (Hart *et al.*, 2003; Lamers *et al.*, 2003; Wang *et al.*, 2005).

In the search for new phyto-medicines, the necessary purification of single active components has been in general successful, whereas synergistic effects of mixtures of components (e.g., crude plant extracts) remain difficult to evaluate. Utilizing omics technology, scientists hope to develop methods and models to detect and observe the effects of complex mixtures such as various plant tissue extracts traditionally used in herbal medicines. This applies especially to approaches employing metabolomics, which address comprehensive phytochemical profiling, bioactivity phenotyping, sophisticated bio-organic chemistry instrumentation, and new cross-talk experimental designs. This scenario needs not only advancement in natural product research, but also a revolutionary strategy in the development of molecular pharmacology-based herbal medicines (Wang *et al.*, 2005).

36.1.3 Phytomics

The term “phytomics” has been previously created based on the “omics-based approach” for studying chemical compositions in plant (Kung *et al.* 2003), specifically: using bioinformatics and/or statistics to address qualitative and quantitative aspects of chemical compositions or profiles of the plant metabolites of our interest; or to develop databases for addressing such aspects (Shyur and Yang, 2008).

36.2 Transcriptomics Study in Medicinal Plant Research

36.2.1 Application of DNA Microarrays in Toxicogenomics, Pharmacogenomics, and Functional Genomics Studies of Bioactivity from Medicinal Plants

Various research progresses on genomics-based identification of responsive gene clusters, gene families, or gene polymorphisms associated with immune system dysfunction has helped to address some basic issues in immunology, and have begun to expand our understanding of immune-related disease processes (Burns-Naas *et al.*, 2006). The application of omics technologies in toxicological research (toxicogenomics) provided new insights into mechanisms of action, as well as data likely to be useful for risk assessment (Barnatskii, *et al.*, 2005; Burns-Naas *et al.*, 2006). Gene chips or microarrays are already employed in immunotoxicology research to identify biochemical pathways that are altered by specific chemical exposures. For example, trichothecene mycotoxin deoxynivalenol has been shown in mice to modulate splenic early responsive genes, which are functionally related to immunity, inflammation, and chemotaxis (Ezendam *et al.*, 2004; Fisher *et al.*, 2004), indicating the importance of innate immune systems, including macrophages, granulocytes, neutrophils, and various soluble mediators released in the inflammatory response activated by the hexachlorobenzene treatment. For basic research, a number of mechanistic studies have been performed towards gaining a comprehensive understanding of the immunomodulatory properties of potential new drugs or drug leads. Thymic atrophy, for instance, appears to be mediated in part by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-induced apoptosis (Rhile *et al.*, 1996). Using an apoptosis-specific cDNA array combined with promoter analyses, specific and novel gene targets have been shown to enhance negative selection in the thymus and thus result in TCDD-induced thymic atrophy (Fisher *et al.*, 2004). In a separate study, cDNA microarray analyses were utilized to evaluate the TCDD regulation of Fas ligand (FasL) promoter activity through modulation via NF- κ B in thymic stromal cells and the subsequent initiation of the apoptotic pathway in thymic T-cells (Camacho *et al.*, 2005).

During the past decade we have witnessed a paradigm shift from utilizing single-target drugs to multi-target drugs (Chavan *et al.*, 2006; Wermuth, 2004). The concept of multi-targeted therapy was once believed to better represent the conventional herbal medicine treatments that often employ multi-component plant tissue extracts as natural products mixtures. However, very few phytomedicinal products have clear or systematic documentations comparable to that of chemically synthesized drugs as a single chemical compound. This situation has hampered our ability to predict precise or specific molecular targets, signaling or action mechanisms of activity, and possible side effects of "herbal drug" products (Chavan *et al.*, 2006). With these requirements for botanical and clinical uses, a validated genomics and metabolomics approach in combination can be applied to quantify specific chemical markers and, subsequently, to obtain chemically standardized extracts (Amagaya *et al.*, 2001). In addition, researchers have witnessed a wide range of molecular mechanisms governing various cellular and tissue behaviors. The genomics approach with integrations of large and diverse sources of gene, protein, and metabolite expression information will assist in making comprehensive and integrated predictions about the pharmacological effects of plant natural products (Ahmed *et al.*, 2003).

While numerous laboratories use genomics in their investigation of underlying mechanisms of immunotoxicity, few have employed genomic analyses as a screening tool. Many differentially expressed genes are known to play a role in apoptosis, host defense, cell growth, and differentiation, and trafficking of specific cells in body fluid systems. In the spleen, these may include the upregulation of IL-18, lymphotoxin B receptor, and colony-stimulating factor receptor, and downregulation of RANTES and histocompatibility antigens (Katz *et al.*, 2006; Kinser *et al.*, 2004; Luyendyk *et al.*, 2004; Pruett *et al.*, 2004). In the thymus, gene changes included the down-regulation of nuclear factor of activated T-cells, interferon gamma receptor, and T-cell transcription factor 7, and the upregulation of caspase 1 and ApoE. These findings are consistent with alterations previously observed in specific immune functions (Luyendyk *et al.*, 2004; Mondola *et al.*, 1987) and could further expand our knowledge at gene regulation level.

Applications of DNA microarray technologies in herbal drug research may be classified into three major areas. Firstly, it can be used in pharmacodynamics to aid the discovery of new diagnostic indicators and biomarkers for therapeutic response, elucidation of molecular mechanisms of herbal action, its formulations or its phytochemical components, and in identification and validation of new molecular targets for herbal drug development (Figure 36.2) (Chavan *et al.*, 2006; Clarke *et al.*, 2001). Secondly, it is applicable in toxicogenomics for predicting side effects of a medicinal herb or phytomedicine lead drug during preclinical activity and safety studies, conferring drug safety or resistance (Crowther, 2002). Thirdly, it is useful for botanical or plant identification and authentication of crude plant materials as part of an effort and regulatory system for standardization and quality control (Klapa and Quackenbush, 2003). Given these considerations, DNA microarrays may thus offer powerful predictive functions at different stages of a typical drug/phytomedicine discovery pipeline.

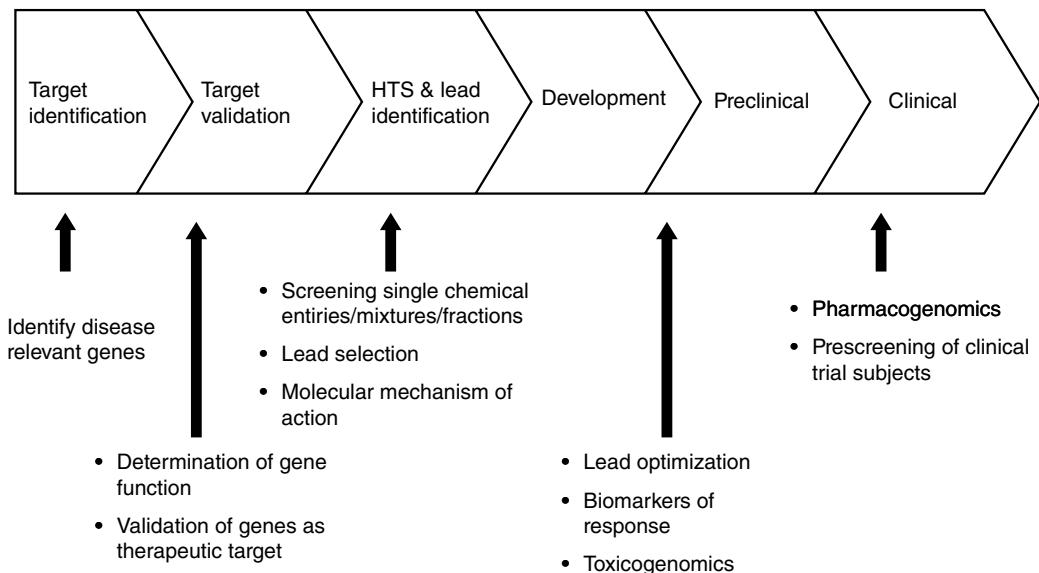


Figure 36.2 DNA microarray applications in natural product drug discovery and development Clarke et al. (2001).

36.2.2 Immuno-Modulatory Effects of Different Phyto-Compounds/Candidate Phytomedicines

With the increased demand for validated herbal products for medicinal use comes the need to better understand the molecular mechanisms of their biological activities. Although many reputed herbal drugs are investigated at the molecular level, it remains difficult to realize the exact targets of individual phytochemical components and how these molecules together or independently can contribute to specific immuno-modulatory effects. Here, we discuss findings from some of the recent studies on microarray-based gene expression aimed at elucidating immunoregulatory mechanisms of pure phytochemicals as well as specific herbal extracts.

36.2.2.1 Purified Compounds or Specific Phytochemical Groups

The Chinese medicinal herb root *Tripterygium hypoglaucum* has been subjected to cDNA microarrays containing 3000 human genes (derived from a leukocyte cDNA library) in order to study its role in apoptosis-inducing activity of plant alkaloids. Apoptosis induced by these *T. hypoglaucum* alkaloids was shown to be mediated through c-myc and NF-kappa B signaling pathways (Zhuang et al., 2004). In an animal model of aged rat, gene chip (Rat Genome U34A) analysis was applied to evaluate the gene regulatory pattern of *Epimedium* flavonoids in immune homeostasis. *Epimedium* flavonoids were found to reverse the “abnormal” or aging changes, allowing reconstruction of a beneficial equilibrium in gene expression and thus further remodeling of the immunohomeostasis in the aged rat (Chen et al., 2004). Taken together, results from these studies indicate that the expression pattern characterized by upregulation of specific apoptosis-promoting genes and downregulation of certain apoptosis-inhibiting genes can be considered as important genomic background of an immunohomeostasis imbalance (Chavan et al., 2006).

A traditional Chinese medicinal (TCM) herb prescription, Si-Jun- Zi decoction (SJZD), has been administered in a clinical setting to patients with disorders of the digestive system. Previous studies have indicated that the polysaccharides of SJZD are active components of the phyto-extract mixture in improving gastrointestinal function and immunity (Hu et al., 1983). SJZD polysaccharides also had a protective effect and enhanced re-epithelialization on wounded IEC-6 cells. To further elucidate this effect at the molecular level, an oligonucleotide microarray was employed to study differential gene expression of SJZD-treated IEC-6 cells. There was, indeed, increased expression of genes encoding for ion channels and transporters, known as critical to cell migration and restoration of wounded cells, suggesting a mechanism for re-epithelialization as well as improved immunity (Liu et al., 2005). These studies demonstrate the useful approach of functional genomics for research into modernization of TCM. Shikonin and its derivatives, from the TCM-claimed

medicinal herb *Lithospermum erythrorhizon*, have been shown to possess numerous beneficial pharmacological properties, including anti-inflammatory and anti-tumor properties (Chen *et al.*, 2002; Nakaya *et al.*, 2003). In our previous report, shikonin was shown to confer a potent bioactivity on suppression of TNF- α promoter activity (Staniforth *et al.*, 2004). Additionally, shikonin was found to mediate cytokine expression through inactivation of the RNA-activated protein kinase (PKR) pathway (Staniforth *et al.*, 2004). It was also suggested from the reports that regulation of TNF- α pre-mRNA splicing may constitute a promising target for future anti-inflammatory application (Chiu and Yang, 2007). Moreover, the functional genomic (DNA microarray) analysis on the cellular immunological effects of shikonin effectively distinguished the complex and specific bioactivities of this phyto-compound in human monocytes (Chiu *et al.*, 2010). Further, an ubiquitin pathway regulator, for example, Rad23A, was also identified as possible key regulators for this shikonin effect (Chiu *et al.*, 2010). A transcriptomics approach has therefore been instrumental in screening immune-modulatory effects of noteworthy phytocompounds. These studies have set useful examples for future systematization of key traditional herbal medicine-derived phytomedicines.

36.2.2.2 Medicinal Herbal Extracts

Screening of the human genome for TNF- α -inducible genes has been used to identify the anti-inflammatory effects of 5-Loxin, a standardized *Boswellia serrata* extract, in microvascular endothelial cells (Roy *et al.*, 2005; Singh and Atal, 1986). It was shown that 113 out of the 522 TNF- α -induced genes were responsive to 5-Loxin treatment. These genes are directly or apparently related to inflammation, cell adhesion, and proteolysis. These robust 5-Loxin-sensitive candidate genes were subjected to further evaluation for molecular signaling, and this processing led to the suggestion of the primary 5-Loxin-sensitive TNF- α -inducible pathways. Mechanistically, 5-Loxin can completely inhibit VCAM-1 expression, and TNF- α can cause inflammation by strongly upregulating the expression of this adhesion molecule VCAM-1 (Roy *et al.*, 2005).

Recently, genomics analysis has also evolved for evaluation of the efficacy of bioactive chemicals in natural health products as therapeutics, for instance, with regard to the alleviation of specific inflammatory activities in human airway epithelial cells (Katz *et al.*, 2006). In addition, one application of gene expression profiling in this research field may be the growing appreciation for the multiple and pivotal roles played by various dendritic cells (DCs) in initiating and regulating a spectrum of immune responses. These cells are responsible for recognizing and processing various antigens and their ultimate presentation to specific immune cell (e.g., T-cells) systems (Jin *et al.*, 2004; Nakamura, 2008; Yrlid *et al.*, 2000). It has been well established that DCs present in the epidermis (Langerhans cells: LCs) are required for the presentation of chemical allergens at the skin's surface, as well as for skin sensitization (Bergstresser *et al.*, 1980; Cumberbatch *et al.*, 1995). Investigations by Enk and Katz (1992) have revealed that topical exposure of mice to chemical allergens, but not to a non-sensitizing skin irritant, caused numerous changes in expression of cytokines and chemokines by LC and local epidermal cells. Among these changes recorded following allergen treatment was a rapid increase in LC expression of mRNA for interleukin-1 β (IL-1 β), a cutaneous cytokine necessary for the regulation of LC function and for skin sensitization (Enk and Katz, 1992; Halliday and Muller, 1987; Kimber and Cumberbatch, 1992; Kimber *et al.*, 2000). These results concluded that changes in the expressions of IL-1 β by LC in response to chemical allergens might hence provide a practical and efficient *in vitro* approach for identifying skin-sensitizing chemicals (Casati *et al.*, 2005).

Genome-wide analysis has been adopted as a less selective approach for measuring the holistic or global changes in gene expression (Pennie and Kimber, 2002; Ryan *et al.*, 2005; Wang *et al.*, 2006; Wang *et al.*, 2008; Yin *et al.*, 2010). It can be anticipated that the activation and functional maturation of DCs, as drastic cellular activities, are likely to be associated with changes in the levels of a spectrum of gene expressions that are responsible for: (1) intracellular metabolic processes; (2) control of cell motility (including those regulating intercellular communication and interactions with the tissue matrix); (3) cytokine and chemokine production; and (4) cell growth regulation and survival (Pennie and Kimber, 2002; Yin *et al.*, 2010).

Results of our previous studies on the immune-modulatory effects of a phytocompound mixture, extracted from the butanol fraction (BF) of a stem and leaf (S+L) extract of *Echinacea Purpurea* ([BF/S+L/Ep]), suggest that [BF/S+L/Ep] can effectively modulate DC mobility *in vivo* and related cellular physiology in the mouse immune system. In addition, [BF/S+L/Ep] modulated cell adhesion-, cell mobility-, cytokine-, and NF- κ B signaling-related activities in primary cultures of mouse DCs (Yin *et al.*, 2010). Similar study of Wang *et al.* (Casati *et al.*, 2005), have further shown that genes expressed in [BF/S+L/Ep]-treated human DCs revealed a key-signaling network involving a number of immune-modulatory molecules and lead to the activation of a downstream molecule, adenylate cyclase 8. These examples show that genomics approaches can be usefully employed for predicting candidate target molecules in future translational studies of phytochemicals, phytocompound mixtures, and medicinal herbal extracts.

36.2.3 Use of cDNA Microarray/Expression Sequence Tags (ESTs) for Evaluating Bioactivities of Medicinal Plants

A transcriptome is the set of all detectable RNA molecules, including mRNA, tRNA, rRNA, and non-coding RNAs (e.g., siRNA, microRNA, and long non-coding RNA [lncRNA]) produced in a group of test cells or tissues. By using the advanced transcriptomics, an organism's entire transcriptome can now be effectively analyzed for many experimental systems. Technically, transcriptomics is a technology to reveal genome-wide gene expression profiles, patterns, integrated or segregated features, or networks describing a global view or analysis of gene expression activities of the genome at the mRNA or regulatory RNA levels. These technologies comprise cDNA-AFLP, SAGE, cDNA microarray (or gene chip), oligonucleotide-microarray, and microRNA microarray (Lockhart *et al.*, 1996). Microarrays also have been used to detect gene expression changes of medicinal plants in a variety of developmental stages, geographic locations, natural growth environments, and/or cultivation conditions (Lockhart *et al.*, 1996). In phytomics studies, studies have aimed to identify the responsive genes that are regulated by active medicinal compounds, anti-pathogen infection, or adaptation to harsh environments (Carles *et al.*, 2005).

To design appropriate probe sequences for a DNA microarray efficiently, we need to consider the genome sequence information for a specific organism in its entirety or with a definable set or subset. However, since only very limited genomes of medicinal plants have currently been sequenced, one alternative is to gather the necessary transcriptome information, by generating or making use of existing expression sequence tags (ESTs) (Liu *et al.*, 1995; Wan *et al.*, 1996). Increasing numbers of EST libraries from medicinal plants such as *Panax quinquefolius* (Chen *et al.*, 2008), *Huperzia serrata* (Luo *et al.*, 2010), *P. Notoginseng* (He *et al.*, 2008), *Rehmannia glutinosa* (Sun *et al.*, 2010), and *Catharanthus roseus* (Shukla *et al.*, 2006) have been recently obtained. An automatic system for large scale EST sequence retrieval, assembly, and functional and pathway analyses has been established (Deng *et al.*, 2006). This system has been successfully applied to analyzing both plant (Thara *et al.*, 2004) and animal EST sequences (Boyko *et al.*, 2006; Deng *et al.*, 2006). These EST and annotation systems have provided a good foundation for design of suitable arrays for representative genomes or focused transcriptomics, hence providing valuable information for genomic research into phytomedicine.

36.2.4 Immuno-Modulatory Effects of Traditional Herbal Medicines Revealed by microRNA Analysis

The miRNAs are small, endogenous non-coding RNAs (20–22 nucleotides) that are now recognized as an important component of epigenetic gene regulation in mammals, which control an array of cellular processes such as cell differentiation, tissue development, body hematopoiesis, and immunity.

For instance, various cancer studies have shown that miRNAs interact with genes in diverse cellular pathways, resulting in differential gene expression profiles of normal and tumor tissues and distinguishable molecular patterns among different tumor types. Because epigenetic modifications are often reversible, developing drugs that can control epigenetic regulation thus represents a very promising and attractive avenue for treating or preventing cancers, including the development of medical foods, functional foods, or supplements as nutrition-based epigenetic modulators for cancer patients. Many plant secondary metabolites extracted as natural products from fruits, vegetables, teas, spices, and traditional medicinal herbs have been found to confer regulatory effects on the epigenetic machinery.

Curcumin, which is found abundantly in turmeric, functions as a strong anticancer agent in different cancer models through the modulation of miRNAs that target the *Nrf2*, *Neurog-1*, *RAR β 2*, *PTEN*, and *P53* pathways (Lee *et al.*, 2013; Shankar *et al.*, 2013). Epigallocatechin-3-gallate (EGCG) from green tea epigenetically controls a spectrum of molecular targets, some more preferentially towards cancer cells, including *RAR β* , *hTERT*, *GSTP1*, *p16*, *MGMT*, *hMLH1*, *MAGE-A1*, *Alu*, *LINE*, *BCL2*, *IL-6*, *IL-12*, *NF- κ B*, and *NOS-2* by mediation via miRNA regulation (Lee *et al.*, 2013). Genistein, rich in the soybean, has been reported to be miRNA activator for *ZEB1*, *ZBTB10*, and *EGFR* genes (Lee *et al.*, 2013).

In the case of human SW480 colon cancer cells, treatment with resveratrol was shown to decrease the levels of several oncogenic miRNAs targeting genes encoding Dicer1, a cytoplasmic RNase III- producing mature miRNAs from their immediate precursors, and tumor-suppressor factors *PDCD4* and *PTEN*. This study on miRNA indicated that resveratrol treatment significantly upregulated the expression of 22 miRNA and downregulated 26 miRNA species. A number of the downregulated miRNAs, including miR-17, miR-21, miR-25, and miR-92a-2, were shown to be constitutively upregulated in colon cancer. The level of miR-663 was significantly increased after resveratrol treatment, which is known to possess putative tumor-suppressor functions and target the *TGF1* transcript. Resveratrol can also upregulate expression of the components of the *TGF β* signaling pathway, including *TGF- β* receptors type I and type II, and downregulate the transcriptional

activity of canonical TGF- β key effectors proteins, SMADs. These findings suggest that miR-663 is an intention for the resveratrol action, which may contribute to a mode of its anticancer properties (Shankar *et al.*, 2013).

Diindolylmethane [DIM] treatment of gemcitabine-resistant human pancreatic cancer cells, MiaPaCa-2, Panc-1, and Aspc-1, resulted in an alteration in miRNA expression. DIM treatment caused upregulation of miR-let-7b, miR- let-7e, miR-200b, and miR-200c molecules. Furthermore, treatment of pancreatic cancer cells with DIM correlated with an upregulation of E-cadherin (an epithelial cell marker) and a downregulation of mesenchymal markers ZEB1 and vimentin. A recent study also showed that DIM treatment influenced the invasion capacity of pancreatic tumor cells via a miRNA-regulated mechanism. Treatment of these cancer cells with DIM caused upregulation of miR-146, which was correlated with a reduced expression of EGFR, MTA-2, and members of the NF- κ B signaling pathway (Shankar *et al.*, 2013). Ellagitannins can elicit their antitumor effects by modulating the transcription factors and signaling pathways that inhibit tumor cell proliferation and induce cell apoptosis. Specifically, exposure of liver cancer cells with BJA3121, an ellagitannin isolated from a plant *Balanophora japonica*, resulted in cell growth inhibition and alteration in expression of several miRNAs, including the upregulation of miR-let-7e, miR-370, miR-373, and miR-526b, and the downregulation of let-7a, let-7c, and let-7d. This pattern correlates with genes involved in cell differentiation and proliferation (Shankar *et al.*, 2013).

Microarray analysis of ginger- and zingerone-regulated gene expression profiles and pathways in mice with TNBS-induced colitis showed that three pathways, including oxidative phosphorylation, ribosome activity, and the taurine and hypotaurine metabolism, were downregulated by TNBS but upregulated by ginger and zingerone. These findings suggested that TNBS induced colitis may be mediated via upregulation of cytokine-related pathways, while ginger and zingerone ameliorated the TNBS-induced colitis in mice through downregulation of some cytokine-associated pathways (Hsiang *et al.*, 2013). A microarray-based mRNA expression profiling of CCRF-CEM (doxorubicin-sensitive acute T-lymphoblastic leukemia cell line) cells treated with casticin was performed in order to identify genes affected by this treatment. Networks related to NF- κ B, p38MAPK, histones H3 and H4, and follicle-stimulating hormones were identified to be likely involved (Righeschi *et al.*, 2012).

The cross examination of transcriptome and microRNA array analyses revealed that the topical treatment of shikonin *in vivo* affects the epithelial-mesenchymal transition (EMT) and the expression of microRNAs involved in the process, including 200a, 200b, 200c, 141, 205, and 429 microRNAs, in mouse skin wound healing model. Shikonin treatment downregulated expression of microRNA-205 and 200 family microRNAs, while expression of Sip1 (Zeb2) and Tcf8 (Zeb1), the targets of the 200 family microRNAs in EMT regulation were upregulated. Enhancement of these EMT activities resulted in faster healing of skin wounds in mice (Fathallah-Shaykh *et al.*, 2004).

36.3 Proteomics Studies on Research into Medicinal Plants

36.3.1 Use and Advancement of Analytical and Instrumentation Systems: Two-Dimensional Gel Electrophoresis (2-DE), Electrospray Ionization, Matrix-Assisted Laser Desorption/Ionization and Surface-Enhanced Laser Desorption

The Nobel Prize in Chemistry for 2002 was shared between scientists from two research expertise: mass spectrometry (MS) and nuclear magnetic resonance (NMR). These revolutionary breakthroughs have allowed chemical biology to become one of the most significant scientific disciplines in recent years. Scientists can now rapidly and reliably identify most proteins in a relatively small sample and readily produce three-dimensional display and/or images of expressed protein molecules with highly resolution. With these advancements, various experimental approaches and technologies were developed to obtain a better understanding of proteins and their regulatory effects on molecular and cellular functions of various biological systems (Cho, 2007; O'Farrell, 1975). Among them, technologies including two-dimensional gel electrophoresis (2-DE) analysis (Klose, 1975; O'Farrell, 1975), matrix-assisted laser desorption/ionization (MALDI), time-of-flight (TOF) (Smith *et al.*, 2007), and Surface-Enhanced Laser Desorption/Ionization (SELDI)-TOF MS (Cazares *et al.*, 2002) have been broadly used in proteomics studies on the research of medicinal plants.

36.3.2 Application of Proteomics for Research into Traditional Herbal Medicine

Proteomics technologies were applied to simultaneously study the function, organization, diversity, and the dynamic variety of total or a subset of proteins at the cellular or tissue levels (Cho, 2007). The current integrative approach used in proteomics is in line with the practice and holistic philosophy of traditional Chinese medicine (TCM). Recent advances in

multidimensional liquid chromatography, coupled with free-flow electrophoresis and capillary electrophoresis-based separation techniques, make it possible in separation of hundreds or even thousands of protein components in some medical plants (Gao *et al.*, 2007; Wen and Han, 2004). We may able now to explore an increased understanding of such complex mixtures and the reputed medicinal effects at the cellular and molecular levels through proteomics studies; it holds a key to the big demand for modernization and internationalization of a number of traditional phytomedicines (Wen and Han, 2004). In this article, some of the proteomics approaches in TCM research and development are addressed, highlighting the application in mechanistic investigation of specific phytomedicines. *Panax ginseng* and *Panax quinquefolius* are two of the valued herbs widely used in TCM. Conventional separation methods were unable to distinguish the different plant parts (main root, lateral roots, rhizome head, and epidermal tissues) between these two species. On the other hand, when 2-DE maps were employed, plant tissue samples containing distinct or common protein species (spots) can be easily discriminated or distinguished. Clearly, these potential protein biomarkers may also facilitate the identification processes for various medicinal plants that may be difficult to identify morphologically or anatomically (Lum *et al.*, 2002).

Numerous herbal medicines have been reported to confer immunomodulatory and anti-tumor effects in cancer cells (Cho and Leung, 2007a; Cho and Leung, 2007b; Koo *et al.*, 2007). Recent biological and pharmaceutical researches have shown that diosgenyl saponins may exert a large variety of biological functions, with a potential for use in cancer chemoprevention (Liu *et al.*, 2004). By using 2-DE, tryptic in-gel digestion, and MALDI-TOF MS analysis, Wang *et al.* (2006) suggested that dioscin, a saponin extracted from *Polygonatum zanlanscianense* Pamp., exhibited cytotoxicity towards human myeloblast leukemia HL-60 cells. This proteomics analysis also revealed that the expression of mitochondria-associated proteins was substantially altered in HL-60 cells upon dioscin treatment, suggesting that mitochondria were the major cellular and organelle target of dioscin cytotoxicity. Moreover, the results indicated that other pathways were likely also involved in detected dioscin cytotoxicity, including phosphorylation-based cellular signaling, RNA-related protein synthesis, and oxidative stress processes. The study demonstrated the benefits of using a proteomics approach in anticancer phytomedicine research (Cho, 2006).

36.4 Metabolomics Study on the Research of Medicinal Plants

Research into metabolomics, including both targeted and global metabolite profiling strategies, is rapidly becoming a popular and powerful approach of choice across a broad range of medical and biological sciences including systems biology, drug discovery, and molecular and cell biology (Shyur and Yang, 2008). Specifically for human metabolites, it is believed that at least 3000 metabolites that are essential for normal growth and development (primary metabolites) and >2000 secondary metabolites that are not essential for growth and development but may help fight off infection and other forms of stress on the body (Bren, 2005). In addition, metabolomics are now being generally considered a vital component of the systems biology approach, in which it can reflect and connect the genotypes with diverse yet specific phenotypes of specific types of cells, tissues, or organs (Bren, 2005). Within the past decade, the number of publications of metabolomics-related research articles has increased from roughly 40 in 2002 to 100, 170, 200, and >250 articles in the years 2004, 2005, 2006, and 2007, respectively. Now it is estimated that >300 articles, with a general aim or study on metabolomics were published annually in 2010. Owing to its remarkable versatility, metabolomics is rapidly becoming a universal tool and key component in medical research (Shyur and Yang, 2008). Combined with genomics and proteomics technologies, systems biology research using metabolomics investigates characteristic molecular signatures for disease diagnosis, prognosis, and therapeutics (Fiehn *et al.*, 2000). This section reviews the recent developments in technology platforms and experimental approaches for metabolomics studies in the research of immunomodulatory properties of potential medicinal plants.

36.4.1 Use of GC-MS, LC-MS, FT-IR, and NMR Technologies

At present, the term “metabolomics” often can be used interchangeably with “metabolite profiling” because the type of one-step, two-dimensional exhibition analysis used in genomics and proteomics experiments is not possible at the present time, as the complexity of chemicals in most biological systems, especially in plants, is highly diversified and can be enormous (Malyanker, 2007).

The two basic approaches in metabolomics can be classified as targeted and global metabolite analyses. Targeted metabolite analysis, (or metabolite profiling), as the name implies, targets mainly a subset of metabolites in test sample, instead of a complete, global metabolome analysis, often by using a particular set of analytic technique(s) such as gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS), and yields an

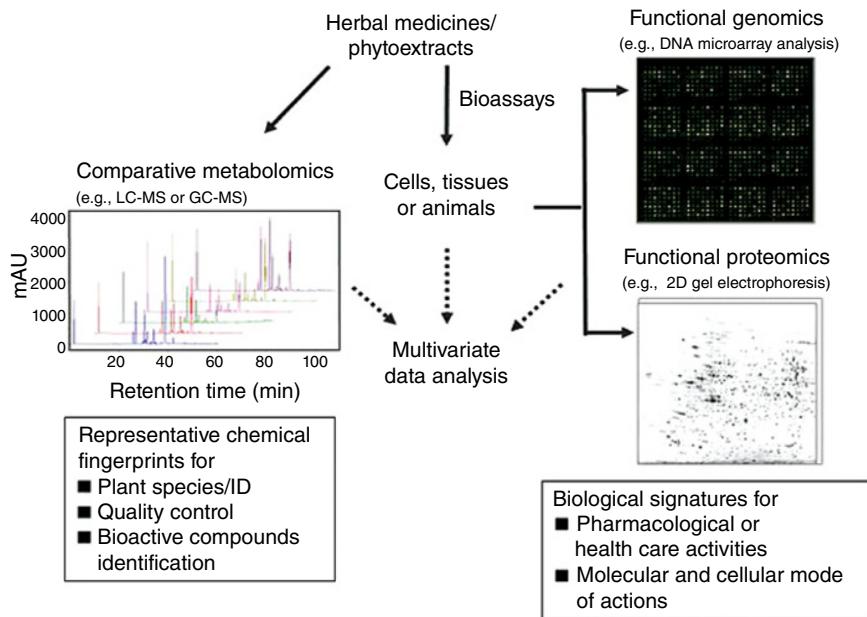


Figure 36.3 Key features of metabolomics technologies employed for research into phytomedicines. (Shyur and Yang, 2008, Reproduced with permission of Elsevier).

estimate of quantity (Sumner *et al.*, 2003). Metabolomics approaches using GC-MS, LC-MS, or 2D NMR are effective tools for quality control of medicinal plants or herbal medicine products (t'Kindt *et al.*, 2009; Zeng *et al.*, 2007). As shown in Figure 36.3 (Shyur and Yang, 2008), key aspects of the technology were assembled in many research institutions as “core labs/facilities” in the metabolomics approach for herbal medicine or other integrated research interest. Various other technical systems, methodologies or techniques, including thin layer chromatography (TLC), Fourier transform infrared spectroscopy (FT-IR), Raman spectroscopy and NMR (Biais *et al.*, 2009; Draisma *et al.*, 2010; Yang *et al.*, 2006) are also important research facilities in the metabolite analysis arsenal.

For secondary plant metabolites, mass spectrometry is currently the most broadly applied technology in metabolomic studies. Among the variety of MS techniques, GC-MS has long been popularly used in metabolite profiling of plant extracts (Horning and Horning, 1971; Serkova *et al.*, 2007). Rapid, high-resolution 2D GC \times GC-TOF MS has been employed in the phenotyping of natural rice variants (Pauling *et al.*, 1971) as well as for efficient quality control or analysis of herbal medicines (t'Kindt *et al.*, 2009). Recently, capillary electrophoresis-MS has also been developed as a metabolomics tool, capable of simultaneously analyzing over 1000 charged chemical species, a technique that is expected to create a number of obvious applications in processing and characterization of various biological samples (t'Kindt *et al.*, 2009). A shotgun approach using MALDI-TOF/TOF MS has recently been established for rapid analysis of negatively charged metabolites in mammalian tissues to: (1) facilitate the detection of low-abundant metabolites such as cAMP, cGMP, and IP₃; and (2) discriminate isomeric molecular species (Kusano *et al.*, 2007). In addition, novel instrumentation/equipment set ups developed recently, such as Fourier transform ion cyclotron resonance mass spectrometry (FT-MS), represents a quantum jump in the new capabilities of mass spectrometers for metabolite analysis. Due to the exceptionally high resolution of these instruments, metabolites with mass differences of less than 2 ppm can now be separated on a chromatographic time scale (van der Greef *et al.* 2004). The accurate results obtained can help reveal elemental compositions, which often enable unequivocal metabolite identification.

Remarkable recent developments in analytical biochemistry regarding the detection and characterization of compounds with small molecular mass, such as MS and high-field NMR coupled with user-friendly multivariate statistics, have led to highly efficient systems for comprehensive analysis of the metabolite data matrices generated by metabolomics experiments (Sun *et al.*, 2007). One-dimensional (1D) NMR spectrometry has shown its capability for high-output analysis and classification of chemically similar groups of test samples. At the same time, the large numbers of overlapping peaks

generated by such method may also hinder in some case accurate identification of specific metabolites. Recently, a replacement for the 1D ^1H NMR spectroscopic technology also has been developed: a 2D ^1H – ^{13}C NMR strategy (fast metabolite quantification, FMQ, by NMR), was developed for analyzing metabolites as multivariate statistical objects (Lindon *et al.*, 2007).

The new “hyphenated” techniques that combine in assay sequence various forms of liquid chromatography with NMR, such as HPLC-SPENMR, have effectively improved the sensitivity of NMR analyses and can be employed to characterize both high- and low-abundant metabolites in complex crude plant extracts (Lambert *et al.*, 2007; Lewis *et al.*, 2007).

36.4.2 Metabolomics Research in Medicinal Chemistry Studies

The great diversity of secondary plant metabolites are believed to have evolved through continuous interactions with challenging and predominantly hostile environments, including both abiotic and biotic stresses. When these features are coupled with characteristic species and agronomic differences, various phytochemicals as secondary metabolites generally can confer various specific bioactivities related to their biochemical structures (Clarkson *et al.*, 2006). These bioactivities apparently can help the host plants to defend specific plant pathogens and to reduce a spectrum of abiotic stresses, for example; drought, heat, and saline conditions. Interestingly, these secondary plant metabolites often were also found to confer potent and valuable bioactivities for defending human sickness, including viral, cancerous, and inflammatory diseases. Some well-known cancer chemotherapeutic drugs have been initially derived from plant secondary metabolites, such as paclitaxel (taxol), camptothecin (irinotecan, topotecan), and podophyllotoxins (etoposide, teniposide) (Schauer and Fernie, 2006; Shyur and Yang 2008). Recent re-recognition of the vast potential of plant secondary metabolites or natural products to serve as lead compounds for drug discovery and development, or as various general health care products, has renewed a lot of interest in pharmaceutical and nutraceutical research. *De novo* combinational chemistry has so far produced only a very limited number of novel drugs, the natural products and their derivatives are still considered by many scientists to be the primary source of leads for drug development (Singh and Bhat, 2003). In this area, the use of whole plants or their extracts as medicines gave way to the isolation of active phytocompounds, beginning in the early nineteenth century with the isolation of morphine from opium. In such a reductionist approach, however, single active phytocompounds may often be not identifiable because of their low abundance in test plant extracts, or alternatively, a spectrum of pharmacological efficacy traditionally observed arises only as a synergistic action of the multiple but specific ingredients present in a single plant or even from a multiple medicinal plant formulation, as in TCM (Newman and Cragg, 2007; Williamson, 2001).

In order to efficiently link the flood of experimental data and specific metabolites or general metabolite profiles information to biology and metabolism study systems, traditional bioinformatics is being combined with cheminformatics to generate a basic computational infrastructure for analysis of metabolomics (Lam *et al.*, 2010; Wishart, 2007a). A number of metabolomics databases, some based on both chemical and biological/biochemical data have been made publicly available (Wishart, 2007a). The Human Metabolome Database (HMDB) is currently the largest and most complete database in breadth and depth, offering spectral, physicochemical, clinical, biochemical, genomic, and metabolism information for a library of >2500 known human metabolites (Shulaev, 2006; Wishart, 2007b). Other databases include the BioMagResBank (BMRB) with an emphasis on NMR data (>270 pure compounds); the Madison Metabolomics Consortium Database (MMCD), which presents MS and/or NMR data on more than 10,000 metabolites (Wishart *et al.*, 2007); and the Golm Metabolome Database (GMD), which has been specifically designed for plant research and utilizes GC–MS data (Markley *et al.*, 2007). Additionally, Wishart has reviewed the development of algorithms and innovations in informatics concerning data reduction, normalization, and alignment that offer sufficient biological insight into metabolic profiles (Wishart *et al.*, 2007).

36.4.3 Metabolomics Approach Applied to Research into Immunomodulatory Effects of Phytomedicine

Most scientists generally agreed that chronic inflammation is a key factor in the development of many types of cancers. Natural products, especially from plants, were once popular choices in cancer therapeutics based on their immunosuppressive or anti-inflammatory effects (Kopka *et al.*, 2005; Kempen *et al.*, 2009; Martinez *et al.*, 2002; Singh and Bhat, 2003). Recently, metabolomics has been effectively used to characterize and monitor carcinogenesis activities in mouse models (Stewart *et al.*, 2002). In addressing oncology metabolomics, NMR was used to target biomarkers for prostate cancer by analyzing metabolites with anti-inflammatory effects in the development and progression of this cancer for better future

management (Griffin, 2006; Jordan and Cheng, 2007). This metabolomics approach has also been successfully implemented to monitor the metabolism in human brain, liver tumors, lymphomas, and colon cancers (Cheng *et al.*, 2005).

36.5 Lipidomics Study on the Research of Medicinal Plants

Plants have evolved bioactive lipids in parallel to animals, which have the potential to interfere with the physiological processes of the human lipidome. Recently, plant lipids have been recognized to play an important role in different intracellular signaling processes and in local hormonal regulation. Immune system modulation in mammals by dietary lipids is speculated to be through changes in the composition of membrane phospholipids, lipid peroxidation, modulation of eicosanoid production via linoleoic acid, and eicosapentanoic acid (which can lead to eventually production of anti-inflammatory resolvins and protectins), or alteration of specific gene expressions. Alteration of gene expression patterns in animals can be mediated by an array of lipid-protein interactions, such as molecular recognition at lipid receptors like cannabinoid (CB) receptors, G-Protein coupled receptor 55 (GPR55), transient receptor potential vanilloid receptor 1 (TRPV1) channel, peroxisome proliferating receptors (PPRs), sphingosine receptors, or lipoxin receptors. Any of these receptors could be a potential target for structurally distinct plant lipids, which are known to mediate a number of pharmacological effects. Lipidomics, which is a field of research analyzing lipids and lipid signals as well as their interacting moieties at a systems level, is expected to play a very important role in the elucidation of the potential pharmacological effects of plant lipids. There is increasing evidence to demonstrate that plant fatty acid amides (FAAs) and N-acylethanolamines (NAEs) can pharmacologically interfere with animal endo-cannabinoid system (Gertsch, 2008). A combined approach involving liquid chromatography-mass spectrometry-based lipidomics was used to study the effect of 3–6-year-old ginseng roots on glycemic and plasma lipid control in a rat model of Type 2 diabetes, which proved that ginseng roots show growth age-dependent therapeutic effects on hyperlipidemia and hyperglycemia in diabetic rats (Hu *et al.*, 2011). A myocardial lipidomics based on ultraperformance lipid chromatography coupled with quadrupole-time-of-flight mass spectrometry (UHPLC/Q-TOF MS) was developed to investigate changes of lipids in mouse heart tissues treated with three different dosage of Fuzi, a lateral root of Aconitum popularly used in traditional Chinese medicine (TCM) (Cai *et al.*, 2013).

36.6 Comparative and Bioinformatics Tools for Omics Studies

36.6.1 Ingenuity

A couple of functional genomics experimental approaches were employed in our previous studies on the modulatory effect of *Echinacea* plant extracts (e.g., the butanol-fractionated Leaf and Stem tissue extract designated as BF/S+L/Ep) on both mouse and human DCs (Wang *et al.*, 2006; Wang *et al.*, 2008; Yin *et al.*, 2010). Using the same defined phytochemical extracts in the study, we analyzed the genome-wide transcriptional response in the context of known functional activities and interrelationships among specific protein molecules and/or different cell phenotypes. Ingenuity Systems (www.ingenuity.com/), a structured network knowledge-based approach, provide us with good tools and insight into the regulation of bone marrow-derived dendritic cell activities relevant to the body's immune system. Figure 36.4 shows candidate molecular networks revealed by clustering analysis of the representative genes involved in the BMDC response to [BF/S+L/Ep] treatment (Yin *et al.*, 2010). The prototypical cell was constructed from 37 representative genes that responded to treatment with [BF/S+L/Ep] *in vitro* from 4 to 12 h. Genes whose expression was upregulated (more than doubled) or downregulated (to less than half) are shown. Selected regions of the network highlight three groups of genes, namely: Group 1: Immune response-related genes. Group 2: Adhesion molecules and cytoskeleton; cell movement related genes. Group 3: Cell cycle, cell proliferation and apoptosis related genes. Gene networks were analyzed using the Ingenuity Pathways program.

36.6.2 MetacoreTM

MetaCoreTM (www.genego.com/metacore.php) is another integrated knowledge database and software suite for pathway analysis of experimental data and gene lists. In the research of phytomedicines, it has also been used to evaluate the possible hierarchical control of microRNA expression from mouse tissues in order to identify trends of miRNA and mRNA expressions in response to targeted phytomedicinal treatment. Utilizing Metacore software, a prototypical network was constructed from six representative microRNAs that responded to treatment *in vivo* with specific phytochemicals

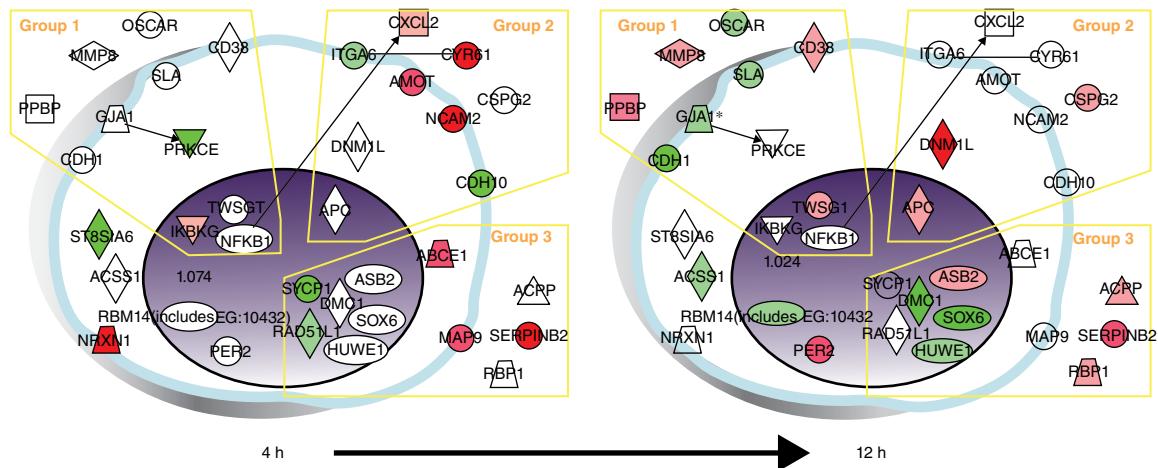


Figure 36.4 Pathway analysis of representative genes that responded to [BF/S+L/Ep] treatment. (Yin et al., 2010, Reproduced with permission of the authors).

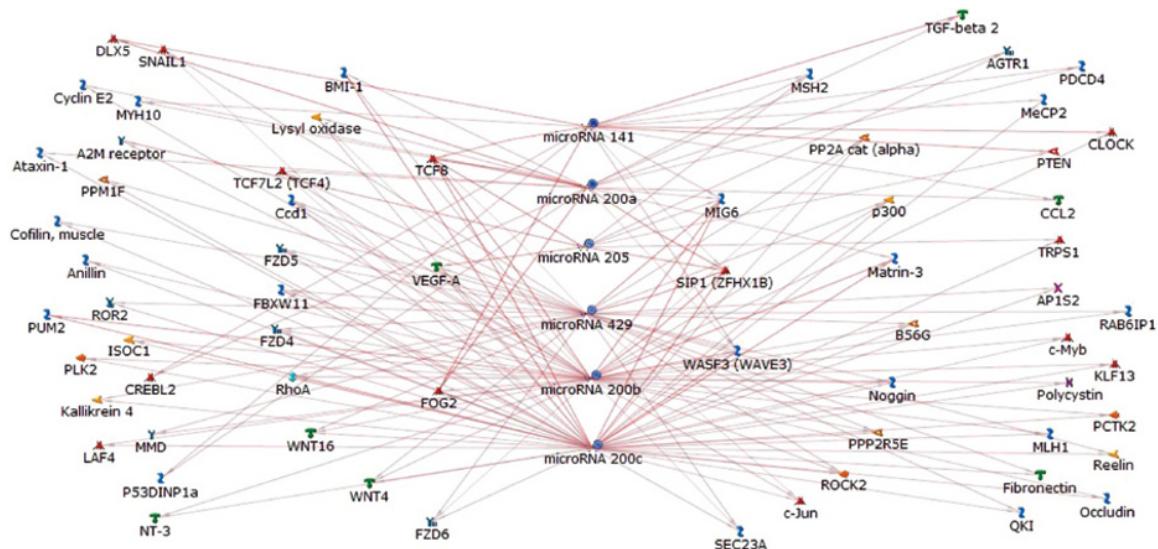


Figure 36.5 Pathway/network analysis of representative microRNAs, which are responsive in vivo to a specific single phytocompound treatment in inflamed mouse tissues.

(Figure 36.5). All selected microRNAs were found to be downregulated to less than half of the untreated levels, and are shown with nearby thermometer like symbols.

Specifically, connections (hits) within 6 microRNAs were employed as the parameter for this specific search. Arrows indicate the cross talks among five key molecules/pathways, (TCF8, VEGF-A, FOG2, MIG6, SIP1, and WASF3), and these are postulated to be regulated by treatment with a specific phytochemical from TCM formulation.

As seen in Figure 36.6, Putative signaling networks (microRNA-dependent inhibition of EMT) involved in modulatory effects of shikonin on skin tissue were predicted from the MetaCore software, six representative microRNAs and three differentially expressed genes that respond coordinately to an *in vivo* treatment with shikonin for 24 h are shown. Experimental data from transcriptomic array and microRNA array analyses are linked to and visualized on the maps as thermometer-like symbols, which indicate the change in expression levels of microRNAs or mRNAs (Yin et al., 2013).

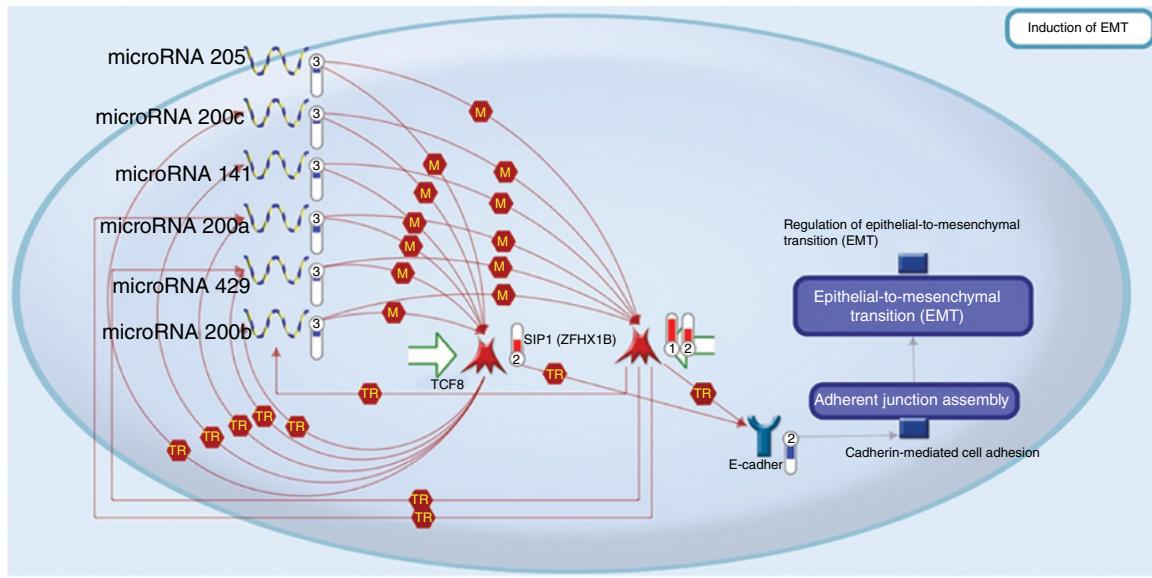


Figure 36.6 Combinational analysis of Transcriptome/DNA microarray and microRNA array datasets to predict possible effects of shikonin on mouse skin tissue. M: microRNA binding. TR: transcription regulation. TCF8 (Zeb1): transcription factor 8. SIP1 (Zeb2; Zfxh1B): Smad-interacting protein 1 (Yin et al., 2013). Wang et al. (2008). Reproduced with permission of the authors.

36.6.3 TRANSPATH

TRANSPATH (www.gene-regulation.com/index.html), a database system about gene regulatory networks, combines encyclopedic information on signal transduction with tools for visualization and analysis. By integrating with TRANSFAC, a database about transcription factors and their DNA binding sites, TRANSPATH can predict putative signaling pathways from ligand to target genes and their products, which may themselves be involved in a regulatory action.

For studying specific immunomodulatory effect of herbal medicine, the possible signaling pathways, networks or potential interactions among the responsive genes/target molecules in DCs treated with *Echinacea* extracts [BF/S+L/Ep] was assessed by using such TRANSPATH software. This bioinformatics analysis has predicted a key-signaling network involving a number of immune-modulatory molecules leading to the activation of a very important downstream regulatory molecule, the adenylate cyclase 8, effectively in regulating cAMP levels in mammalian cells. This analysis indicated two postulated key molecules/pathways, Adenylate cyclase (AC8) and calmodulin (CaM), responsive to the *Echinacea* extracts (Figure 36.7).

36.6.4 KEGG

KEGG (*Kyoto Encyclopedia of Genes and Genomes*: www.kegg.jp/kegg/) is a multifunctional bioinformatics resource for linking genomes to metabolic activities. It consists of 16 main databases and has been widely used as a reference knowledge base for biological interpretation of large-scale datasets generated by sequencing and other high-throughput experimental technologies. Among these databases, the KEGG DRUG database contains crude drugs (consisting of multiple chemical compounds) and formulas (consisting of multiple crude drugs) in the Traditional Chinese Medicine (TCM). In addition, KEGG PATHWAY and KEGG ENVIRON are also being organized to interpret and correlate relationships between genomic and chemical information of various natural products/metabolites from plants. For example, the biosynthetic pathway of stilbenoids, a group of phenolic compounds, was provided for revealing specific molecular interaction and different reaction networks (Figure 36.8). Although the knowledge on biosynthetic pathways of plant natural products is, in general, largely incomplete, the genomic information is expected to provide clues to missing enzymes and reactions for biosynthesis of specific plant secondary metabolites, the source for future modernized phytomedicines, either as pure compounds, fractionated phytochemical mixtures, or as crude plant extracts. Moreover, the genomic information may also uncover the architecture of biosynthetic pathways for generating chemical diversity of natural products.

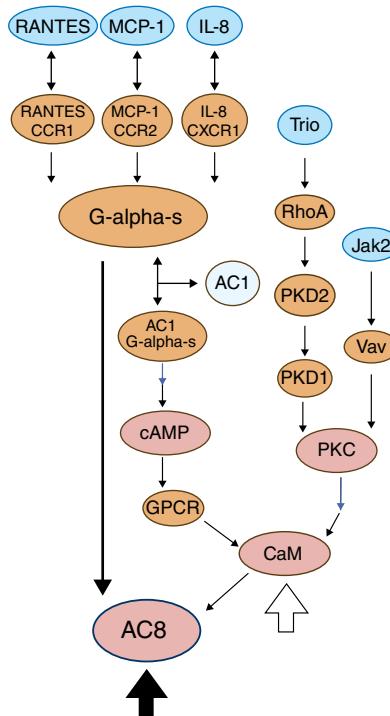


Figure 36.7 Bioinformatics analysis of [BF/S+L/Ep] bioactivity and the underlying candidate molecular signaling networks in human DCs. The 20 genes that were up- or downregulated at least five-fold over controls were analyzed. Specifically, connections (hits) within seven genes were employed as the parameter for the current search (Wang et al., 2008). Yin et al. (2013). Reproduced with permission of the authors.

36.7 Challenges and Perspectives

Traditionally, the pharmaceutical research and industries have focused on evaluating or monitoring individual gene, proteins as the target or basis for identifying new drugs. The quest for single molecules to modify single key factors in a disease process is now recognized as may not be able to provide a solution for a spectrum of diseases in which multiple cell types, target molecules and/or multiple pathways are known or believed to contribute to the diseases. Herbal extracts/mixtures as conventional phytomedicines may represent the combinational chemistry of the nature of traditional medicines, and encompass a vast repertoire of chemical entities that may have anecdotally and empirically found through long human culture history to confer a complex and yet integrated effect on numerous cellular components and functions, effecting medicinal activity. Various traditional herbal drugs may thus have good potential for re-invention and newly found use in the multi-target approach in treating various diseases. However, such potential of herbal drugs is undermined by difficulties in standardization, and the pharmacodynamics and pharmacokinetics studies of these multicomponent plants extract mixtures. Microarray analysis of gene expression profiles may be useful for elucidating such complex molecular mechanisms and networks underlying the multi-target pharmacological functions of herbal extracts and phytomedicine mixtures. Research into the patterns of gene expression at a range of stages during the treatment process may reveal key targets and mechanisms and help to identify biomarkers of either adverse or favorable response. A positive correlation between the transcriptional response induced by a putative or candidate herbal drug and the database profile of an existing pharmaceutical or therapeutic agent as a single chemical may provide us insight into the target specificity and mechanism of action, as well as in facilitating analysis of signaling pathways downstream of the specific target. This information could in turn be used to interpret possible bioactivity, function or effectiveness of test phytomedicines. In addition, various DNA, RNA, or protein microarrays may also be used for bioactivity-guided fractionation of herbal extracts, thereby narrowing in the active principles delivering the desired or observed effect. Microarrays may also improve the power for selection of

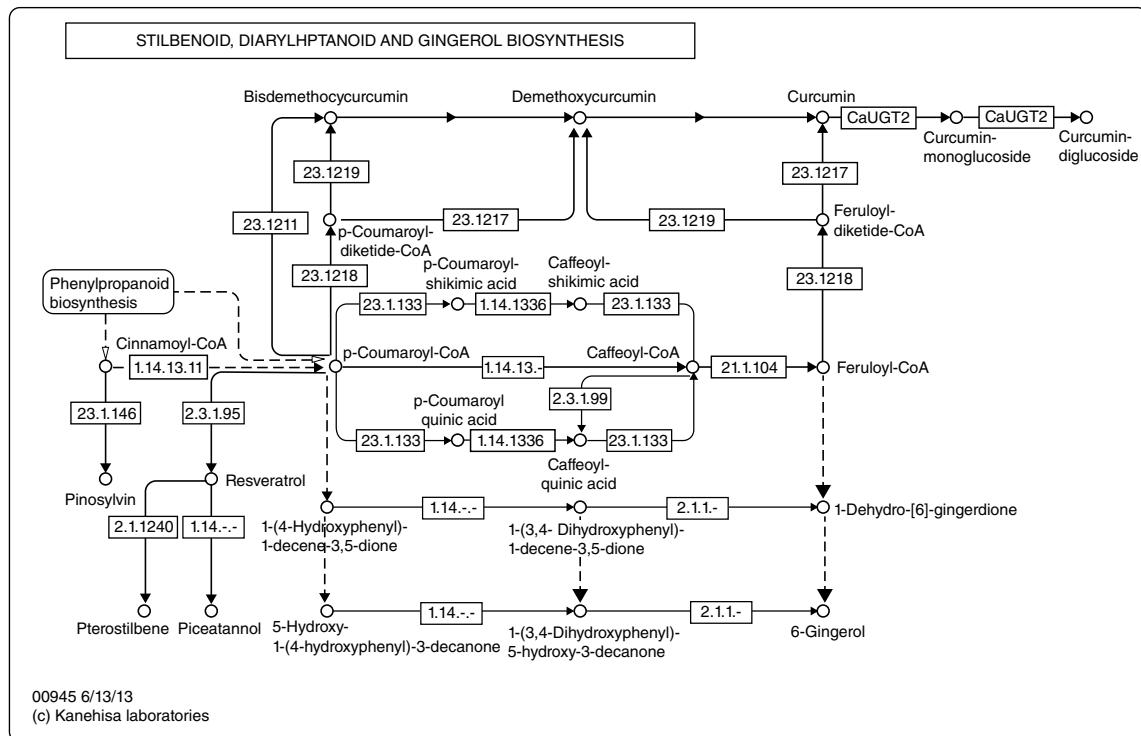


Figure 36.8 Stilbenoid, diarylheptanoid, and gingerol biosynthetic networks cited in KEGG. Adapted from www.kegg.jp/kegg/pathway/map/map00945.html.

biological targets and lead compounds up or down the drug discovery pipeline. Once useful transcriptome or/and proteome data from herbal drug candidates can be correlated with *in vivo* bioactivity or preclinical or “existing clinical” (as in some TCM) response outcomes (biomarkers) in defined biological systems, the best candidates can then be selected for further drug development (Chavan *et al.*, 2006).

Although some DNA microarrays have already offered impressive potential for pharmacodynamics and toxigenomics applications, they are still being considered to be in an exploratory stage and the data obtained from them will need validation by other biological experiments. Bioinformatics and statistical tools have a major role to play in analysis of the microarray results, whereby data from multiple experiments can and may need to be integrated to address complex biological activities, functions or effects. Another factor currently limiting microarray application is the cost of this technology (Chavan *et al.*, 2006). The challenge we face today is to develop or construct standardized, sensitive, reproducible microarray platforms, databases, and visualization methods for expression profiles that are affordable to most research scientists. With the use or development of improved, uniform, and sophisticated experimental designs, data management systems (Brazma *et al.*, 2001; Griffin and Kauppinen, 2007), statistical tools and upgraded algorithms for data analysis (Churchill, 2002; Fathallah-Shaykh, 2005), DNA microarrays hopefully can be more optimally used in herbal drug research. In spite of the vast potential offered by microarray and the related functional genomics and proteomics technology, the importance of integrating various *in vitro* biological assays, cell culture-based, and *in vivo* animal experimental systems cannot be ignored. Comprehensive strategy integrating information from diverse scientific experiments and technologies are expected to benefit and lead to molecule and cell evidence-based phytomedicines.

The integration of information from genomics, proteomics, and metabolomics is hoped to provide solid evidence-based rationales for systematic development of various modern phytomedicines, on top of the foundations of various traditional medicine cultures. The search for a specific, active single phytocompound may also be expedited when various metabolomics approaches are combined with a comprehensive array of bioactivity assay systems using standardized and normalizable mammalian cell, tissue, and animal models.

Where a “complete metabolome-exhibition” system is currently not available, HPLC-, GC-, and LC/MS-based metabolite-profiling systems, alone or in combination, may already offer a good description or authentication tool for comparative and qualitative analyses and definition of the unique, distinctive, or combinational profile features of the conventional herbal medicine formulations, as elegantly demonstrated recently by Lam *et al.* (2010). These and the improved or newly developed metabolomics technologies in linkage may also be usefully applied to discovery and development of new phytomedicines, as single phytochemicals or their mixtures, or as fractions or the whole preparation of the crude extracts of various medicinal plant tissues. Our challenges together, as scientists and healthcare-takers are to coordinate and integrate our intellectual thrusts, talents, and efforts to address and target specific medical and medicinal research areas – for example, for anti-inflammation and related chronic or cancerous diseases, for future research and development of advanced phytomedicines – may be pursued more effectively as an international program.

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The Application of Algae for Cosmeceuticals in the Omics Age

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37.1 Introduction

Many concerns have contributed to the increasing attention and demand for various skin care products, such as a growing population of elder individuals over younger ones, skin damage by environmental and pollution threats, and eagerness to improve the current life standard. It is believed that individuals with an attractive appearance have distinct social advantages in comparison to those who are perceived as less attractive. Driven by an aging population looking for innovative anti-aging solutions, the global cosmeceutical industry in 2011 is recorded to reach \$13.1 Bn and this is projected to double in the near future. The use of microalgae by-products in cosmeceuticals can be traced back to 1989 when microalgae was successfully cultivated to produce oxygen that would support microbial metabolism to break down organic materials along with the release of nutrients. Since then, microalgae have been widely explored, mainly for their oil properties for further use as a biofuel feedstock (Brennan and Owende 2010; Mercer and Armenta 2011). However, the use of microalgae for biofuel production is still in its infancy and is yet to grow due to the high maintenance and production cost. The oil products derived from microalgae are of low-value with high cost of production (US\$1.13 per kg as reported by Borowitzka, 2013). In contrast, secondary metabolites obtained from microalgae have shown higher economical value; high value chemicals obtained from the first commercialized algae ranged from US\$ 300–1500 per kg (Cardozo *et al.* 2007; Fleurence 1999). Fresh water algae like *Haematococcus* are reported to produce carotenoids and astaxanthins that are widely used in aquaculture and also used in innovative anti-inflammatory and antioxidant application. *Spirulina* and *Nannochloropsis* have been used as nutrient supplements and also in skin care application, respectively. The market price for dried *Spirulina* ranges from US\$15–50 kg⁻¹ while extracted and purified form of lipid from *Nannochloropsis* cost US\$ 260 l⁻¹ (Gellenbeck 2012). Microalgae possesses various metabolites or bioactive molecules that are potentially useful for dermatological interest such as preventing aged skins (Gorouhi and Maibach 2009), healing of skin burn (Badiu *et al.* 2008), and eliciting the production of collagens to maintain water balance (Wijesinghe and Jeon 2011). There are anti-cancer properties found in *Scenedesmus* sp. (Qin *et al.* 2008), antioxidant properties found in *Chlorella vulgaris* (Mannavanan *et al.* 2012), and also other bioactive compounds found in microalgae (Mayer *et al.* 2011). It is expected that these components that protect the survival of microalgae, when applied on skin, can also provide a barrier against outside threats and thus improve skin condition. Therefore, the research of new sources of bioactive compounds is needed and expected with huge economical return.

The major concern to new sources is the efficacy. Unlike the employment of plant materials in most skin care products in the past, the formulation of skin care products nowadays requires more concrete evidence of functional activity of an ingredient. This could be obtained from various types of investigation or refinement supported by the latest omics technologies, including *in-vitro* bioassays, gene expression, testing of antioxidant activities, human clinical test, and immune functions. To increase the efficiency of any skin care products, nanoscience emerges as a new technology due to the ability to deliver smaller size materials for more effective dermal penetration (Basavaraj 2012). For manufacturing skin care products, the source of raw material has always been an important aspect to consider. The ever-changing environmental conditions contribute to the different qualities and quantities of raw materials. Therefore, it is very difficult to maintain the quality of end product in one batch of cultivation compared to another. In the case of drug production, the USA and Europe demand that drugs that are to be produced for the market use the same system used for last clinical trial, in order to avoid discrepancy in drug production. This involves a big investment with no guarantee of return (Dove 2002). Cosmeceutical production with microalgae is less stringent as its quality maintenance can be more easily monitored with the advancement in close microalgae cultivation (Brown 1991; Grobbelaar 2000; Richmond 2004; Tate *et al.* 2012). In fact, the use of microalgae in skin care products (e.g., *Arthospira* and *Chlorella*) is already established in the skin care market (Stoltz *et al.* 2005). Some examples of successful cosmeceutical products from microalgae are *Dermochlorella DG*® from CODIF Research and Nature (Britany, France), XCELL-30® from Greensea (Meze, France), Alguronic Acid® from Agenist (San Francisco, CA, USA), and Alguard® from Frutarom Ltd (Martins *et al.* 2014).

The formulation of cosmeceuticals involves the integration of scientific findings of various perspectives and selection of appropriate combinations of ingredients in the formula. For example, Protulines, a protein-rich extract from *Arthospira*, is included in the Exymol anti-aging skin care product in order to exert a tightening effect and to prevent stria formation.

37.2 Metabolomics

Metabolites are the end products of cellular regulatory process and “metabolome” refers to a set of low molecular weight metabolites. In order to survive in a competitive environment, freshwater and marine algae have developed defense strategies that result in significant level of bioactive compound diversity elicited from different metabolite pathways (Jamers *et al.* 2009; Obata and Fernie 2012). Hence, microalgae are a primary source of bioactive compounds that could be used as functional ingredients. To enhance the production of bioactive compound, various bioprocessing factors have been used, for example, light, temperature, pH, carbon source, salinity, and so on (Griffiths *et al.* 2012; Tate *et al.* 2012; Wen and Chen 2003), and various extraction methods have been tested for efficient extraction of the oil (Halim *et al.* 2012; Lee *et al.* 2010; Mercer and Armenta 2011) and metabolites (Serrive *et al.* 2012) from microalgae. Metabolomic approaches by HPLC, NMR, and GC-MS enable the illustration of chemical structural and chain conformation of some complex bioactive compounds, and this approach is particularly important for high throughput screening (HTS) in natural product discovery (Fan and Lane 2011; Moco *et al.* 2007; Yang and Zhang 2009). Metabolomic profiling has also been applied to different growth stages of microalgae in order to explore excretion of different types of metabolites to maximize its production (Su *et al.* 2012). However, it is estimated that more than 30,000 species of microalgae are present but only a few hundred of the species have been explored and cultivated for industrial purposes (Gouveia *et al.* 2008). Metabolomic approaches offer an advantage in effective de-replication strategies to eliminate the same natural compounds that are already isolated and studied by using a powerful database search tool such as NAPRALERT, Beilstein, AntiBase, MarinLit (containing ~24,000 marine compounds isolated from approximately 6000 species), Chapman and Hall’s *Dictionary of Natural Products* (~170,000 compounds from both marine and terrestrial organisms), and NAPROC-13 (information about over 6000 natural compounds) (Martins *et al.* 2014).

Most of the secondary metabolites from microalgae are targeted for pharmacology and therapeutic usage and relatively fewer metabolites are identified for use in cosmeceutical purposes (Table 37.1).

37.3 Genomics

The microalgae being extensively studied were the *Chlamydomonas reinhardtii* from which a complete sequence draft was available from 2003 and the whole genome sequence was then published in 2007 (Merchant *et al.* 2007). Genome properties of diatom *Phaeodactylum tricornutum* (Scala *et al.* 2002), red alga *Cyanidioschyzon merolae* (Nozaki *et al.* 2007), and *Galdieria sulphuraria* (Barbier *et al.* 2005) have also been studied in the discovery of different cellular metabolisms.

Table 37.1 A list of algae and its application in various fields.

Boactive compounds	Sources	General usage	Reference
<i>Fatty acids:</i>			
Eicosapentaenoic acid (EPA)	<i>Monochrysis lutheri</i>		
Docosahexaenoic acid (DHA)	<i>Pseudopedinella</i> sp.		
Myristic acid	<i>Coccolithus huxleyi</i>		
Palmitic acid	<i>Nannochloropsis salina</i>		
9-hexadecenoic acid	<i>Nannochloropsis</i> sp.		
Stearidonic acid	<i>Monodus subterraneus</i>		
Timnodonic acid	<i>Chlorella minutissima</i>		
Clupanodonic acid	<i>Prasinophyceae</i>		
	<i>Heteromatrix rotunda</i>		
	<i>Cryptophyceae</i>		
	<i>Cryptomonas maculata</i>		
	<i>Cromonas</i> sp.		
	<i>Asterionella japonica</i>		
	<i>Navicula incerta</i>		
	<i>Navicula sprophila</i>		
	<i>Chaetoceros</i> sp.		
	<i>Phaeodactylum tricornutum</i>		
	<i>Pavlova</i> spp.		
	<i>Pavlova salina</i>		
	<i>Pavlova lutheri</i>		
<i>Steroids</i>			
Cholest-5-en-3b-ol	<i>Pyramimonas cf. cordata</i>		
Cholesta-5,22E-dien-3b-ol	<i>Attheya ussurensis</i>		
24-Methylcholesta-5,22E-dien-3b-ol	<i>Sargassum oligocystu</i> (brown algae)		
24-Methylcholesta-5,24-(28)-dien-3b-ol	<i>Sargassum carpophyllum</i> (brown algae)		
24-Methylcholest-5-en-3b-ol			
24-Ethylcholesta-5,22E-dien-3b-ol			
24-Ethylcholest-5-en-3b-ol			
22-dehydrocholesterol			
29-hydroperoxystigmasta-5,24(28)-dien-3b-ol			
24 (S) / 24 (R)-hydroxy-24-vinylcholesterol			
Ostreasterol			
3 β ,28 ξ -dihydroxy-24-ethycholesta-5,23Z-dien			
2 α -oxa-2-oxo-5 α -hydroxy-3,4-dinor-24-ethylcholesta-24-(28)-ene			
24-ethylcholesta-4,24(28)-dien-3,6-dione			
24 ξ -hydroperoxy-24-vinylcholesterol			
Fucosterol			
		<ul style="list-style-type: none"> Precursor of a group of eicosanoids, hormone like substances such as prostaglandins, thromboxanes and leucotrienes. Activity against a range of Gram-positive and Gram-negative bacteria. Used as fish feed 	(Desbois <i>et al.</i> 2009; Wen and Chen, 2003) (Volkman <i>et al.</i> 1991)
		<ul style="list-style-type: none"> Biochemical properties are species-specific, thus serving as both a chemotaxonomic biomarker for distinguishing members of algal taxa. Potentially used for cell growth. Potentially cause a cytotoxic effect A potential anti-fungal agent 	(Ponomarenko <i>et al.</i> 2004) (Permeh <i>et al.</i> 2011) (Tang <i>et al.</i> 2002)

Table 37.1 (Continued)

Boactive compounds	Sources	General usage	Reference
Carotenoids	<i>Laminaria</i> sp.	• Used as structural stabilizers for protein assembly in photosystems.	(Christaki <i>et al.</i> 2011;
Phyloene	<i>Haematococcus Pluvialis</i>	• Used as inhibitor for ROS activities.	Guerin <i>et al.</i> (2003);
Lycopene	<i>Dunaliella salina</i>	• Could directly provide photoprotection against UV light photo-oxidation in the skin.	Pinto <i>et al.</i> 2000)
δ -carotene	<i>Spirulina maxima</i>	• β -carotene was also shown to modulate UVA-induced gene expression in human keratinocytes	(Woo <i>et al.</i> 2010)
γ -carotene	<i>Gonyaulax polyedra</i>	• Provitamin A activities, converting to retinol by enzyme	(Heo and Jeon, 2009)
α -carotene	<i>Chlorella vulgaris</i>	• β -carotene 15,15-monoxygenase.	(Sinha <i>et al.</i> 1998)
Lutein	<i>Isochrysis galbana</i>	• Astaxanthin plays a key role in melioration/prevention of several human pathological processes, such as skin UV-mediated photo-oxidation, inflammation, prostate and mammary carcinogenesis, ulcers due to Helicobacter pylori infection and age-related diseases.	(Garcia-Pichel <i>et al.</i> 1992)
Zeaxanthin	<i>Diacronema vinkianum</i>	• Anti-inflammatory and antioxidant	
Cantaxanthin	<i>U. pinnatifida</i> ,	• Photoprotective effect against UV-B induced cell damage.	
Astaxanthin	<i>S. fulvellum</i> ,	• Antioxidants and protect against oxidative stress induced by UV-B radiation.	
Violaxanthin	<i>L. japonica</i> ,	• UV-protective compounds	
Fucoxanthin	<i>H. fusiformis</i>	• Gelling, viscosifying and emulsifying properties.	
Scytonemin	<i>S. siliquastrum</i>	• As bulking agents, laxatives, suppositories, capsules, tablets, and anticoagulants.	
	<i>Scytonema</i> sp.		
	<i>Chlorogloeopsis</i> sp.		
Phycocolloids	<i>G. gracilis</i>		
Agar (α (1–4)-3,6-anhydro-L-galactose) and (β (1–3)-D-galactose residues)	<i>G. bursa-pastoris</i>		(Marinho-Soriano, 2001)
Carrageenan	<i>Phaeophyceae</i>		
Alginate			
Mycosporine-like amino acids (MAAs)	<i>Porphyra umbilicalis</i>	• Sunscreen in aquatic organisms	(Whitehead and Hedges, 2005)
Palythine	<i>Porphyra rosengurtii</i>	• Antioxidants	(de la Coba <i>et al.</i> 2009).
Asterina	<i>Gracilaria birdiae</i>	• Reduces sunburn formation	(Cardozo <i>et al.</i> 2011)
Palythinal	<i>Gracilaria domingensis</i>	• Photoprotective compounds	
Shinorine	<i>Gracilaria tenuistipitata</i>		
Porphyra			
Palythene			
ShinorinePalythinePorphyra-334			

(Continued)

Table 37.1 (Continued)

Boactive compounds	Sources	General usage	Reference
<i>Polyhalogenated monoterpenes</i>	<i>Portieria hornemannii</i>	Pharmacological activities:	(Fuller <i>et al.</i> 1992)
Plcocalide A	(red algae)	antimicrobial, anti-	(Burja <i>et al.</i> 2001)
Borophycin	<i>Plocamium, Chondrococcus</i>	tubercular, and anticancer	
Cryptophycin	and <i>Ochtoide</i>	activities	
	<i>Nostoc linckia</i>	Antimicrobial activities	
	<i>Nostoc spongiaeforme</i>		
	var. <i>tenue</i>		
<i>Phlorotannins (polyphenol)</i>	<i>Cystophora</i>	Antibacterial activities	(Ahn <i>et al.</i> 2007;
2-bromotriphlorellol-A hepta-	<i>Phaeophyceac</i>	Antioxidant, anti-aging,	Chandini <i>et al.</i> 2008;
acetate	<i>E. radiata</i>	whitening, anti-allergic,	Kang <i>et al.</i> 2004;
Triphlorellol A	<i>E. bicyclic</i>	anti-inflammatory, etc.	Wijesinghe and Jeon,
Eckstololon	<i>E. kurome</i>	UV-B protection, tyrosinase	2011)
Fucodiphlorellol G	<i>A. nodosum</i>	inhibitory activity/	(Kim <i>et al.</i> 2006; Yoon
7-phloro eckol	<i>S. marginatum,</i>	whitening effect, inhibitory	et al. 2009)
6,6-bieckol	<i>P. tetrastomatica</i>	of matrix metalloproteinases	
Phlorofucofuroeckol A	<i>T. conoides</i>	(MMPs) to reduce wrinkle	
Phloroglucinol	<i>Ecklonia Cava</i>	formation	
Eckol	<i>Ecklonia stolonifera</i>		
Dieckol	OKAMURA		
Polyketide	<i>Symbiodinium</i> sp.	Pharmaceutical	(Kobayashi and Ishibashi,
Amphidinolide B	<i>Cyanobacteria</i>	Antitumor	1993)
Cyanobactin		Cytotoxic	(Sivonen <i>et al.</i> 2010)
Indole alkaloid norharmane	<i>Cyanobacteria</i>	Cancer cell growth inhibitory	
9H-pyrido(3,4-b)indole	<i>Anabaena cylindrica</i>	Enzymatic inhibition	(Volk, 2008)
	<i>Anabaena inaequalis</i>	Increase insulin secretion	
	<i>Anabaenopsis siamensis</i>	Induction of apoptotic cell	
	<i>Chroococcus minutus</i>	death	
	<i>Nodularia harveyana</i>	Involve in co-mutagenic	
	<i>Nostoc carneum</i>	activity.	
	<i>Nostoc commune</i>		
	<i>Phormidium foveolarum</i>		
Calothrix A & B	<i>Cyanobacteria</i>	Anti-malaria activity	(Rickards <i>et al.</i> 1999)
	<i>Calothrix</i> sp.		
Dolastin 10 and 15	<i>Cyanobacteria</i>	Anti-tumor/anticancer	(Tan, 2007)
	<i>Lyngbya</i> sp.		
	<i>Oscillatoria</i> sp.		
	<i>Symploca</i> sp.		
<i>Neuroactive substances</i>	<i>S. macrocarpum</i>	• Promoted neurite	(Hur <i>et al.</i> 2008)
Sargaquinoic acid and		outgrowth and supported	
sargachromenol		the survival of neuronal	
		PC12D cell.	
		• Apoptotic effect <i>in vitro</i>	
		and <i>in vivo</i> to treat psoriasis	

A more advanced study, transcriptomics, involves the exposure of RNA transcripts information to the gene expression information. The study of transcriptional response can be divided into two categories: (1) the technique that involves no prior knowledge such as differential display, suppression subtractive hybridization (SSH), and serial analysis of gene expression (SAGE); and (2) the technique that involves the prerequisite of knowledge about a gene sequence such as micro-arrays. SAGE has been used less frequently compared to microarray, and the examples are transcriptome profiling of marine coccolithophore *Emiliania huxleyi* (Dyrhman *et al.* 2006) and gene expression stimuli by cadmium stress in *Chlamydomonas reinhardtii* (Rubinelli *et al.* 2002). SSHs have been used mostly for monitoring or detection of toxic algae

species. A microarray has been developed for *Chlamydomonas reinhardtii* that contains 10,000 sequences of oligonucleotide, which covers almost its full genome (www.chlamy.org). Since then, gene expression in *Chlamydomonas reinhardtii* under different stress stimuli was tested to unravel the underlying metabolic pathway in transcription level. Examples studied include light-regulated genes and the involvement of phototropin (Im *et al.* 2006), chloroplast RNA stability mutant (Erickson *et al.* 2005), oxidative stress (Ledford *et al.* 2007), sulfur starvation (Zhang *et al.* 2004), phosphorus deprivation (Moseley *et al.* 2006), and toxicity to TNT (Patel *et al.* 2004). The most common genomic application in microalgae is related to increasing lipid production for biofuel, and the microalgae studied were *Nannochloropsis gaditana* (Randor Radakovits, 2013) and *Dunaliella tertiolecta* (Rismani-Yazdi *et al.* 2011). To date, only 20 strains of microalgae have been genetically manipulated with few successful stable transgenes, but most of the transgenes are of the transient expression; the techniques used for microalgae transformation are summarized by Qin *et al.* (2012).

Despite limited studies of genomics reported on microalgae, some breakthroughs in natural product exploration using metagenomics approaches have been reported. By the use of total environmental DNA extraction, metagenomics enables direct access to the genomes observed in different environmental conditions (Brady 2007) and also the genomes of difficult cultivated microorganism for effectively natural product discovery (Li and Qin 2005). Genome mining is also used for the discovery of novel natural products by analysis of genome sequences for genes encoding proteins, gene clusters and novel pathways for several known natural compounds (Galm and Shen 2007). Genome mining also allows the development of combinatorial biosynthesis in order to obtain new molecules that would otherwise be difficult to synthesize using other methods (Piel 2006).

In cosmeceutical practice, microarrays have been reported to be able to screen gene expression in green alga *Haematococcus pluvialis* under antaxanthin-inductive culture conditions (Eom *et al.* 2006). A recent growing field of nutrigenomics uses genetic testing to detect mutations related to skin health and this is followed by rectification by personalized medicine, such as nutraceuticals or skin creams enriched by cosmeceuticals (Subbiah 2010). Global gene expression profiling, such as microarrays, has also been used for identification of skin aging problems including matrix production, barrier, lipid synthesis, antioxidant capacity, and hyperpigmentation (Osborne *et al.* 2012). The application of genomic technology with metabolomic clinical testing on human skin cell culture has led to the identification of cosmetic compounds such as niacinamide, Pal-KTTKS, hexamidine, retinyl propionate, and sodium dehydroacetate. The use of these compounds in cosmetic skin care product can aid improvement in aging skin, improved appearance of fine lines, wrinkles, and age spots.

37.4 Proteomics

Application of proteomics in microalgae could also be beneficial to the cosmeceutical industry. Information about genome structure and regulation is complemented by the proteomics approach, thus making it an important study following genomics and transcriptomics. Proteomics supports the understanding of many cellular processes and network functions by providing information on post-translational modification including subcellular localization of gene products. Not many proteomic studies have been reported on microalgae, although few studies have reported on optimizing viable proteomic workflows for algae. The analysis of proteomic of most microalgae species is difficult since these organisms could contain substances such as high oil body content and high dynamic range of protein abundance that could sometimes hinder the analysis of low abundance proteins (Hurkman and Tanaka 2007). Therefore, important consideration should be given to the optimization of its extraction method (Nguyen *et al.* 2011). Research has been reported on protein preparation methods in several species of microalgae to increase the detection of the highest number and quality of protein spots with clear background (Wang *et al.* 2009; Wong *et al.* 2006). The key reagents and experimental setting for a 2D system were quantitatively evaluated using *Haematococcus pluvialis* as a model (Wang *et al.* 2003). Protein identification is based on its peptide sequence. After protein extraction and digestion following the 2D system, the proteins can then go through sequencing by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis or LC-MS/MS or MALDI-TOF/TOF. In theory, the 2D system enables identification of more proteins compared to 1D. However, in the proteomics study of red alga (*Gracilaria changii*), only 15 proteins were properly identified from 500 spots obtained due to lack of information on database about the unidentified spots (Wong *et al.* 2006). A similar problem was reported by Contreras *et al.* (2008) who only managed to identify 10 out of 46 spots and 14 out of 140 spots from *Scytoniphon gracilis Kogame* and *Ectocarpus siliculosus* (Dillwyn) Lyngb. Although 2D proteomics is a good tool for identifying protein, it still has limitations in detecting and resolving some proteins that are too large (>100 kDa), too small (<10 kDa), basic (pI > 10), and hydrophobic (Rampitsch and Srinivasan 2006). Nevertheless, a report on plastid and periplastid-targeted proteins of *Bigelowiella natans* revealed up to 302 proteins (Hopkins *et al.* 2012).

Proteomics analysis that allows further analysis of genetic diversity of different algae strains has been reported, such as in red alga (Wong *et al.* 2006), brown algae (Contreras *et al.* 2008), unicellular alga (Le Bihan *et al.* 2011), and chlorarachniophyte alga (Hopkins *et al.* 2012). Recent proteomics studies on microalgae have focused on environmental stress signaling; examples are heavy metals (Cid *et al.* 2010), phosphate and nitrogen limiting conditions (Sun *et al.* 2012), cadmium (Kim *et al.* 2005), oxidative stress (induced by sodium acetate and ferrous sulfate) (Wang *et al.* 2004), low temperature stress in *Chlamydomonas* sp. (Kan *et al.* 2006), and copper (Ritter *et al.* 2010). There are also studies that focus on proteins involved in lipid metabolism as these are a major component for biofuels (Nguyen *et al.* 2011) and sterol biomarkers (Volkman *et al.* 1994).

The understanding of proteomics dynamics for simple protein discovery or targeted analyses could be achieved using quantitative proteomics. The methodologies are more complex than normal protein identification using 1D or 2D SDS-PAGE. In this approach, proteins or peptides must be labeled isotopically and further differentiated by mass spectrometry, normally LC-MS (liquid chromatography-mass spectrometry). There is a variety of quantitation methods for such studies; however, the most commonly used are iTRAQ, iCAT, and SRM or MRM.

In the iTRAQ (Isobaric tags for relative and absolute quantitation) approach, isobaric tags are cleaved from peptide during MS/MS (Thompson *et al.* 2003). Up to eight tags are commercially available for use in one application. Although all isobaric tags have identical masses and chemical properties, each tag consists of a mass reporter tag with unique number of ¹³C substitutions for balancing purposes. iTRAQ modifies primary amino groups using isobaric tags (Ross *et al.* 2004; Wiese *et al.* 2007) that appear as single peaks in MS scans, providing relative quantitative protein information (Wiese *et al.* 2007). iTRAQ is the only quantitative method that requires MS/MS, hence it is not possible to quantify unlabeled peptides. As certain protein samples are complex in nature, iCAT (isotope-coded affinity tag) would be a better choice for these samples. Not only can iCAT be used to reduce sample complexity, it can also identify low-abundance proteins and peptides in the complex sample (Schmidt *et al.* 2005). However, only cysteine residues would be tagged as iCAT tags containing the sulfhydryl-reactive chemical group, which it will only label as free thiols on cysteine residues. Labeling is much more specific in this approach as cysteine residues may not be present in all peptides and thus reducing sample complexity. Due to its specific tagging of the cysteine residue, labeled peptide purification has to be performed via affinity column chromatography.

Specific peptides in a complex mixture can be measured by designing an experiment such as SRM (single reaction monitoring) or MRM (multiple reaction monitoring) to obtain maximum sensitivity for detection of target compounds. A targeted or modified peptide with a known mass can be studied by triggering dependent product ion scan to confirm a peptide sequence (Anderson and Hunter 2006; Cox *et al.* 2005). SRM and MRM utilize selective ion monitoring to detect or screen molecules that have undergone a thorough characterization during the discovery phase (Kitteringham *et al.* 2009). The focus is only on analytes of specific masses. Precursor m/z and fragment m/z (SRM or MRM transition) for many common compound molecules can be predicted by selecting a specific tryptic peptide as stoichiometric representative of the cleaved protein and quantitated against a spiked internal standard (Anderson and Hunter 2006; Cox *et al.* 2005). Peptide selection is crucial to ensure the success of SRM/MRM experiment as tens to hundreds of peptides were produced by tryptic digestion from each targeted protein (Picotti *et al.* 2007). To determine its quantity only a few representative peptides are targeted per protein to ascertain the presence of a protein in a sample (Lange *et al.* 2008). SRM and MRM are by far the simplest and cost-effective quantitation methods.

With the advance in proteomics, some crucial enzymes, amino acids and proteins contributing to pharmaceutical agents have been identified and used as developing pharmaceutical agents (Harnedy and FitzGerald 2011). Monoclonal antibodies (mAbs) offer great potential for various diseases, such as chronic disease, infectious agents, and cancers. As antibody identification and engineering technology have advanced, identifying an antibody with desired binding characteristics is no longer rate-limiting, but producing it in sufficient quantities has become the major challenge. There are more than double the number of mAB-based therapeutics approved for use by US Food and Drug Administration (FDA) today (Franklin and Mayfield 2005). Amongst the list, Avastin™ (bevacizumab; Genentech, Inc), and Xolair® (Omalizumab; Genentech/Novartis); these would require >20 times the total world production of therapeutics mAbs in 2002, which is approximately 1000 kg. Cost of goods (COG) prior to purification for mAbs often exceeds USD 150 per g (Dove 2002). Limited production and high cost production of these types of molecule are examples of drawback to existing production system. Eukaryotic algae, *Chlamydomonas reinhardtii*, has been used to produce the therapeutic proteins, which greatly reduced its cost (USD 0.09/l) (Franklin and Mayfield 2005). Some useful proteins for cosmeceuticals are listed in Table 37.2.

Other useful peptides that have not been reported in algae, such as signal peptides Palmitoyl pentapeptide-4 (Pal-KTTKS), were found to increase collagen I, III, and IV, elevated glycoaminoglycan synthesis, and hence increase elastin, proteoglycan, glycosaminoglycans, and fibronectin proliferation (Gorouhi and Maibach 2009; Osborne *et al.* 2008).

Table 37.2 Bioactive proteins compound of microalgae that are suitable for cosmeceutical application.

Proteins	Algae	Role	Reference
Pepsin	<i>Porphyra yezoensis</i>	ACE-inhibitory, anti-mutagenic, blood sugar reducing, calcium precipitation inhibition, lowering cholesterol, antioxidant and improved hepatic function	(Harnedy and FitzGerald 2011)
Essential amino acids	<i>Ulva pertusa</i>	Anti-hypertensive, anti chlosterolemic, and antioxidant properties	(Houston 2005)
Lectins	<i>Eucheuma serra</i>	Mitogenic, cytotoxic, and anti-bacterial activities, prevent tooth decay	(Hori <i>et al.</i> 2007; Sugahara <i>et al.</i> 2001)
Hexapeptide	<i>Ulva</i> sp.	Mitogenic activities	(Ennamany <i>et al.</i> 1998)
Chlorella derived peptides	<i>Chlorella vulgaris</i>	Inhibit solar ultraviolet B induced matrix metalloproteinase -1 (MMP-1) level in skin fibroblast cells	(Chen <i>et al.</i> 2011)
Glycoprotein (ARS-2)	<i>Chlorella vulgaris</i>	Anti-tumor activities	(Hasegawa <i>et al.</i> 2002)
C-Phycocyanin	<i>S. platensis</i>	Block the proliferation of chronic myeloid leukemia cell line	(Subhashini <i>et al.</i> 2004)

37.5 Conclusion

While bioactive compounds from microalgae have great potential in cosmeceutical application, several technical challenges limit the development of microalgae for cosmeceutical application. The serious barrier in the discovery of novel bioactive compound is the low culturability of microalgae. The bioactive compounds are then subjected to screening for their potential application in the cosmetic industry, which involves high throughput screening at large sample sizes, hence the importance of microalgae cultivation. Moreover, the large cost incurred in clinical screening for cosmeceutical benefits has prevented the exploration of many microalgae species that could potentially have cosmeceutical benefits.

Despite the challenges and the many unknowns about the omics application of algae in cosmeceutical products, the lure of the anti-aging battle and the demand for a more healthy skin appearance are both expected to continually drive research further into production of more effective and innovative algae cosmeceutical products in the future. This could be largely attributed to the potentially high economic return, the content of anti-inflammatory and antioxidant substances, anticancer properties, and particularly, the presence of metabolites or bioactive molecules in algae that are reported to be able to prevent skin aging, improving skin burn healing, and eliciting the production of collagens.

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38

Gut Microbiome and Functional Foods: Health Benefits and Safety Challenges

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38.1 Introduction

The human intestinal tract is colonized by different microbial species. Each microorganism within the gut encodes unique metabolic functions, which, taken together, comprise the gut microbiome. Indeed, the metabolic potential of the gut metagenome, or the sum of microbial encoded genes is about 100-fold that of the human genome with different functional activity between microbial species, and it represents a metabolic potential impact on host health. The close association between host physiology and this diverse intestinal microbiota is clearly demonstrated by several studies (Guarner and Malagelada 2003). However, signals between the gut microbiota and human immune system too can get lost in translation leading to the activation of pathological processes (Mackie 2002). Certain microbial proteins share a considerable structural homology with human proteins, for example leptin in the mammalian system: The gut satiety hormones involved in controlling food intake and to which auto antibodies are raised in metabolic disease (Proal *et al.* 2009; Zhou *et al.* 2008). Such observations raise the possibility that aberrant gut microbiota profiles may play a pathological role in these chronic diet-associated diseases. Ever since, the human genome has evolved closely with its microbial counterpart over the course of evolution, resulting in many shared or co-metabolic pathways and through these shared metabolic pathways the mammalian host derives energy and nutrients from its diet. Foods that are conventionally consumed as part of a usual diet, and are demonstrated to have physiological benefits and/or reduce the risk of chronic disease beyond basic nutritional functions are defined as *Functional Foods*.

“Let food be the medicine and medicine be the food”: a phrase given by Hippocrates over 2500 years ago is receiving a lot of interest today as scientists, clinicians, and consumers realize the many health benefits of foods. Several diseases related to obesity, chronic gastrointestinal conditions, diarrhea, lactose intolerance, and inflammatory bowel diseases are increasing all over the world (Fuller 1989). Functional foods provide an opportunity to improve the human health, reduce health care costs, and support economic development. Several probiotics based dairy products have been developed to meet these challenges. Examples are low cholesterol fresh milk, omega-3-milk, low lactose and lactose-free products, and milk products that can control or manage hypertension and immune functions (Bellisle *et al.* 1998).

The market for probiotic based food and beverages is increasing exponentially. Although the concept of probiotic functional food came into existence recently, when food products were targeted to play a significant role in therapeutic aids and health supplements, but the origin of cultured dairy products is presumed to be as old as the beginning of civilization (Hardy 2000; Roberfroid 1999). Numerous traditional soured milks or cultured dairy products such as kefir, koumiss, leben, yogurt, and curd were developed in ancient times and used for staying healthy. Dairy products like curd and yogurt are consumed regularly to maintain the balance of gut flora generally upset due to metabolic disorders like constipation, diarrhea, dyspepsia, cystitis, chronic ulcerative colitis, and mucous colitis. Intestinal gut flora exist in a dynamic balance, but this may be disturbed under certain conditions such as prolonged diseases, food and water contamination, and excessive use of antibiotics (Kwak 2001; Vyas and Ranganathan 2012). Besides improving the gut micro-biome probiotics provide immunologic enhancement, anticarcinogenic properties, and play an important role for diabetics and the obese. Other potential benefits include the regulatory role of probiotics in allergic disease demonstrated by a suppressive effect on lymphocytes' proliferation and interleukin-4 generation *in vitro* (Shiby and Mishra 2013). Also, the immune inflammatory responses to dietary antigens in allergic individuals are alleviated by probiotics, this being partly attributable to enhanced production of anti-inflammatory cytokines and transferring growth factor- β (Turnbaugh *et al.* 2006). The health issues can be overcome by consuming probiotic based dairy products and probiotics therapy (Toma and Pokrotnieks 2006). This chapter highlights the importance of gut microbiome in health and its symbiosis with diet, the importance of functional food in maintaining microorganism balance in the gut, and the challenges underlying in the usage of functional food to combat disease onset due to bad gut microbes.

38.2 Microbiome Symbiosis

Microorganisms within the human intestine ferment carbohydrate sources, which reach the colon in small chain fatty acids (SCFAs), acetate, propionate, and butyrate (Conterno *et al.* 2011). These small organic acids have been shown in animal studies to regulate incretin or gut hormone production, thereby controlling satiety and food intake (Cani *et al.* 2009). They also stimulate the gut hormone glucagon-like peptide-2, which is involved in maintaining gut barrier function, a defense mechanism that can impede the uptake of inflammatory compounds such as lipopolysaccharide (LPS) from the gut lumen that trigger the low-grade chronic inflammation and subsequent insulin resistance associated with obesity and other metabolic diseases (Tappenden *et al.* 2003). Microbial conjugation of bile acid (BA) and the enterohepatic circulation is a core activity of human gut microbiota and is thought to directly regulate cholesterol levels in the blood. Studies state that these deconjugates (bile acids) emerge as important cell-signaling molecules, interacting with nuclear receptors such as Farnesoid X receptor (FXR), Pregnan X receptor (PXR), and Peroxisome proliferator-activated receptor alpha (PPAR α), as well as G-protein coupled receptors such as TGR-5, which regulate inflammation, xenobiotic detoxification, lipid metabolism, cholesterol uptake and biosynthesis, gut metabolism, glucose homoeostasis, and thermogenesis (Sayin *et al.* 2013).

Human dietary interventions, including analysis of the gut microbiota and their metabolic activities, are needed to confirm which gut bacteria are involved in metabolism and how their modulation through dietary means alters BA profiles entering the enterohepatic circulation showing subsequent impact on health (see Table 38.3 later). Other metabolites derived from microbial metabolism in the gut have also been shown to reduce the risk of metabolic disease in animal studies. The neurotransmitter γ -aminobutyric acid is also produced by gut bacteria including certain lactic acid bacteria (Barrett *et al.* 2012). Gamma-amino butyric acid given orally is also a strong regulator of inflammation and immune function, and has been shown to reduce the risk of metabolic disease by improving insulin sensitivity through reduced inflammation in laboratory animals on a high-fat, obesogenic diet (Tian *et al.* 2011). Certain gut bacteria, most notably the *Bifidobacteria*, have been shown to produce folate; a key metabolite that lowers circulating levels of homocysteine. Animal studies have shown that feeding folate-producing *Bifidobacteria* can increase plasma folate concentrations, and that for co-administration of the *Bifidobacterium* strain with a substrate for its growth within the intestine, the prebiotic inulin can increase plasma folate concentrations further in a synergistic manner (Pompei *et al.* 2007). Gut bacteria may also interact with dietary fats; studies have shown that both the quantity and type of dietary fat can reshape the microbial community structure within the gut (Fava *et al.* 2013). Conversely, it has been shown in a study that *Bifidobacteria* capable of biohydrogenation of fatty acids, when administered to pigs or mice along with α -linoleic acid, can increase concentrations of beneficial fats, eicosapentaic acid (EPA), docosahexaenoic acid (DHA) DHA, and conjugated linoleic acid concentrations in extra-intestinal organs

including adipose tissue, liver, and brain (Wall *et al.* 2010). The decrease in gut microbiota or its alteration by external materials may lead to various acute and chronic infections, as discussed in detail next.

38.2.1 Diarrhea (Infectious and Antibiotic Associated)

Rotavirus-induced diarrhea is still a major problem and frequent cause of death, especially in children and people in developing countries. Protection by probiotic bacteria and yeasts with immune stimulatory properties and shortening of acute infections is perhaps the best way to combat the recurrent infections. Beneficial effects such as decreased frequency of infections, shortening of the duration of episodes, less shedding of rotaviruses, or an increase in the production of rotavirus-specific antibodies have been demonstrated for *Lactobacillus rhamnosus* GG (LGG), *L. casei* Shirota, *L. reuteri*, *Bifidobacterium animalis* ssp. *lactis* Bb-12, and a number of other probiotic strains (Mastretta *et al.* 2002). Other reported studies were not able to show the effectiveness of *L. rhamnosus* GG in infants and in severe dehydrating diarrhea (Costa-Ribeiro *et al.* 2003). In another study undertaken in France, 287 children (aged 18.9 ± 6.0 months) in day-care nurseries were administered daily either unfermented jellied milk, conventional yogurt, or a probiotic yogurt product containing 108 cfu/ml *L. casei* sp. alternatively for 1 month. The mean duration of diarrhea shortened from 8.0 to 5 to 4.3 days by consumption of conventional yogurt, and probiotic product while the incidence of diarrhea remained constant by unfermented milk (Pedone *et al.* 1999). Furthermore, this study was expanded to a randomized, controlled multi-center clinical trial with a total of 928 children (aged 6–24 months) where administration of fermented milk containing *L. casei* for 2 months lowered the frequency of diarrhea (Pedone *et al.* 2000). Another Finnish study on children from day-care centers, who consumed milk containing a probiotic *Lactobacillus rhamnosus* strain had 16% less days of absence from day care due to diarrhea and other gastrointestinal, respiratory tract infections (Hatakka *et al.* 2001). Another report on addition of Bb-12 *L. reuteri* ssp. to infant formulas did prevent infectious diseases in 204 undernourished Peruvian children and in Israeli child-care centers (6–24 months) (Oberhelman *et al.* 1999; Weizman *et al.* 2005). On the other hand, analogous studies were performed more seldom in adults and overall the beneficial effects were less pronounced (Pereg *et al.* 2005). Investigations on the effect of probiotic bacteria on traveler's diarrhea showed inconsistent results, possibly due to differences between probiotic strains, the traveled countries, the local micro flora, specific (eating) habits of the travelers, or the method of administration of the probiotic (before or during travel, as a capsule or a fermented milk product). Whereas some studies revealed less or shortened episodes of diarrhea in subjects consuming the probiotic, others found no such effect (Hilton *et al.* 1997). Although *in vitro* and animal studies provided good evidence that probiotic strains inhibit growth and metabolic activity as well as the adhesion to intestinal cells of enteropathogenic bacteria like *Salmonella*, *Shigella*, or *Vibrio cholerae*, other studies have also reported positive effects in humans (Ritchie and Romanuk 2012).

Nowadays, large numbers of antibiotic-resistant pathogenic strains are developing causing antibiotic associated diarrhea, so alternative therapies are required to pacify this problem. Therapeutic use of certain probiotic strains on antibiotic treatment in most studies reduces the frequency and duration of episodes of antibiotic-associated diarrhea and the severity of symptoms (Duman *et al.* 2005). The oral administration of a fermented milk product (200 g/d) containing 105–107 cfu/g *Bifidobacterium animalis* ssp. *lactis* and *Lactobacillus acidophilus* 4 weeks before and during a *Helicobacter pylori* eradication therapy led to significantly less episodes of diarrhea (7% versus 22% of the subjects) compared with the placebo group (Armuzzi *et al.* 2001). An antibiotic treatment may result in life-threatening pseudo membranous colitis, which is associated with abundance of anaerobic toxigenic bacteria (e.g., strains of *Clostridium difficile*). Application of probiotics significantly decreases the number of relapses in successfully treated *Clostridium difficile* infections (McFarland *et al.* 2006). Other curing methods, like chemo and radiotherapy used in treatment of cancer and other diseases, causes severe disturbances of indigenous intestinal microflora accompanied by diarrhea or increased cell counts of fungi (*Candida albicans*) in the gastrointestinal tract and other organs. Both side effects were ameliorated by the use of probiotic bacteria.

38.2.2 Lactose Intolerance

The prominent health effect of fermented milk products is the enhancement of lactose digestion and the avoidance of intolerance symptoms in lactose intolerance population. The fermented milk products ingested with live bacteria contain microbial β -galactosidase perform lactose hydrolysis in the small intestine. Moreover, it has been recently demonstrated in mice that during this transit living *Streptococcus thermophilus* or *Lactobacillus casei* defensis are also able to perform the lactose hydrolysis and last but not least primary or adult-type hypolactasia (the reason for

lactose intolerance) is not a disease, but rather the normal physiological situation where gastrointestinal complaints of lactose maldigestion like flatulence or diarrhea can be reduced by probiotics (Zhong *et al.* 2004).

38.2.3 Inflammatory Intestinal Diseases

The term inflammatory bowel disease (IBD) is implied for a variety of incurable immune mediated diseases with unknown etiology that results in chronic relapsing inflammation of the gut. However, probiotics may be useful in controlling IBD by modulating the useful intestinal microbiota. A study reports reduction in *Enterococci* and *Clostridia* causing colon cancer upon consumption of probiotic *L. salivarius* UCC 118, which establishes beneficial modifications to the intestinal microbiota, resulting in reduced inflammation. It is stated that use of probiotic strain *L. salivarius* UCC118 is safe (Bai and Ouyang 2006). Studies in experimental animals give a clue about the potential application of *lactobacilli*, *bifidobacteria*, *lactococcus*, or other probiotic nonpathogenic strains of *Escherichia coli* (e.g., strain Nissle 1917) to treat colitis. Likewise, patients with inflammatory bowel diseases (Crohn's disease and ulcerative colitis, necrotizing enterocolitis, diverticulitis, or inflammation of an ileal pouch responded positively too (Mimura *et al.* 2004). It has been proven that probiotic use was associated with a decreased expression of inflammatory markers with increased IgA secretion, lower drug consumption, and overall a higher quality of life of the patients during treatment of colitis (Bibiloni *et al.* 2005). In recent times more positive study outcomes have been reported concerning the potential mechanisms like regulation of intestinal flora (Chapman *et al.* 2007).

38.2.4 Immune Modulation

Microorganisms and their cell-wall components (peptidoglycans, lipopolysaccharides), DNA and metabolites have shown immunomodulatory properties. Modulation of the systemic and secretary immune response is well established in mice and other experimental animals. Functions like inhibition of bacterial translocation increased proliferation in organs of the immune system (Peyer's patches, spleen); stimulation of phagocytes/macrophages and natural killer cells increased release of cytokines (IFN α , IFN γ , INF α) and defensines11, shifts in the Th1/Th212-balance towards less allergy/atrophy, increased production of specific antibodies and increased resistance and prolonged survival during co-administration of viruses, toxins, and bacteria (rotavirus, *Klebsiella pneumoniae*, *Salmonella typhimurium*, *Shigella*, *Vibrio cholerae*, *Listeria monocytogenes*). Similar effects on parameters of the cellular and humoral immunity has also been proven in human studies (Chermesh and Eliakim 2006). Stimulation of the immune system by itself does not necessarily imply a positive health effect. In healthy gut, the immune system develops a balance between mucosal immunity and tolerance to antigens, but in cases like food allergies this balance is impaired. Probiotic supplementation may help to normalize the symptoms of food allergy by modulating the immune response through modification of the intestinal flora towards improvement. Controlled clinical studies showing therapeutic effects of probiotics, protection against infections or reduction of allergic reactions but still the investigation of the mechanisms are needed to be studied in detail (Chapman *et al.* 2007).

38.3 Functional Food Intervention of Gut Microbiota

Probiotics play an important role in the gut system. A study done by Dumas *et al.* (2006) reported the impact of choline metabolism disruption on the development of metabolic disease and non-alcoholic fatty liver disease in mice prone to disease (129S6) and in mice resistant to disease (BALBc) using NMR-based metabolomics. These investigators found that low plasma levels of phosphatidylcholine and increased urinary excretion of methylamines (dimethylamine, trimethylamine – TMA, and trimethylamine-N-oxide – TMAO) was associated with insulin resistance and a fatty liver phenotype. Importantly, these metabolites result from the host microbiota's co-metabolic processing of choline and phosphatidylcholine. The microbial conversion of choline into methylamines is induced by high-fat feeding and mimics the disease effects of choline-deficient diets. Other studies have shown that high-fat, low fermentable fiber diets, typically used to induce metabolic disease in laboratory animals, decimate the gut microbiota. Cani *et al.* (2007) showed that high-fat, low fermentable fiber feeding leads to significant die off in dominant and putatively beneficial gut bacteria including *Bifidobacteria*, butyrate-producing Firmicutes and polysaccharide degrading Bacteroidetes are present. Interestingly, high-fat feeding did not change numbers of Gram-negative sulfate-reducing bacteria (SRB) in these experiments. The beneficial gut bacteria was accompanied by translocation of bacterial cell wall lipopolysaccharide (LPS) across the gut wall, either through increased permeability due to reduced tight junction control or carried with fat absorbed from the gut. LPS is an inflammatory cell wall constituent of Gram-negative

bacteria and triggers an inflammatory cascade through Toll-like receptor-4 or CD14, and NF- κ B signaling molecules. Interestingly, Zhang *et al.* (2010) showed more recently that in another murine model of metabolic disease (ApoA-I knockout mice on high-fat diet), the SRB, *Desulfovibrionaceae*, showed higher relative abundance in animals with insulin resistance, especially insulin resistant animals fed high-fat diets. In this study too, numbers of *Bifidobacteria* were decimated by high-fat feeding SRB, which convert mainly dietary sulfate to H₂S. These are considered to play an important role in human H₂S metabolism and have been implicated in inflammatory bowel disease. Moreover, it has become apparent that probiotics or probiotic effects are strain specific with particular probiotic health effects, for example immune modulation, production of antimicrobial compounds, or the ability to lower cholesterol being present in one strain and absent in another strain belonging to the same species even. A number of mechanisms have been proposed including binding of cholesterol within the gut lumen or incorporation of cholesterol into bacterial cell walls. Although a few of these putative mechanisms have been shown in human subjects, they have been used *in vitro* as screening tools in an attempt to improve probiotic efficacy and investigated the ability of a *Lactobacillus reuteri* strain NCIMB 30242 microencapsulated in a yoghurt formulation to reduce the cholesterol levels in hypercholesterolaemic individuals (Jones *et al.* 2012). Importantly, this strain had been selected as a putative cholesterol-lowering probiotic because of its bile salt hydrolase activity. These authors found that consuming the yoghurt containing the microencapsulated probiotic *L. reuteri* NCIMB 30242 twice daily for 6 weeks with a combined daily dose of about 1011 viable bacteria, decreased serum total cholesterol and LDL-cholesterol by 4.81 and 8.92%, respectively, compared with the placebo. Moreover, the subjects receiving the probiotic had lower serum levels of plant sterols, a surrogate marker for cholesterol absorption, and higher plasma levels of deconjugated BA, leading the authors to suggest a novel cholesterol-lowering mechanism for this strain through reduced cholesterol absorption via the action of deconjugated BA on hepatic FXR α , a nuclear receptor responsible for controlling lipid absorption from the gut by regulating the expression of lipid transporters on the gut wall. Thus, it appears that by right choice of probiotic strain it may be possible to lower disease risk through improved profiles of biomarkers especially plasma cholesterol levels. New observations on how diet shapes both the composition and metabolic output of the gut microbiota place developments in functional food, establishing a scientific view for efficacious functional food design.

38.4 Types of Functional Foods and Their Effects

The growing interest in food having physiological benefits, reducing chronic disease, and optimizing health is lead to the term “functional food” (Table 38.1). The concept was first developed in Japan in the 1980s when, faced with escalating health care costs, the Ministry of Health and Welfare initiated a regulatory system to approve certain foods with documented health benefits in hopes of improving the health of the nation’s aging population (Arai 1996). These foods, which are eligible to bear a special seal, are now recognized as Foods for Specified Health Use (FOSHU) (International Life Science Institute 1999). As of July 2002, nearly 300 food products had been granted FOSHU status in Japan. In the USA, functional foods have no such regulatory identity. However, several organizations have proposed definitions for this new food category. In 1994, the National Academy of Sciences’ Food and Nutrition Board defined functional foods as “any modified food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains” (Thomas and Earl 1994). The International Life Sciences Institute defines them as “foods that, by virtue of the presence of physiologically- active components provide a health benefit beyond basic nutrition” (International Life Science Institute 1999). In a 1999 position paper, the American Dietetic Association defined functional foods as foods that are “whole, fortified, enriched, or enhanced,” but more importantly, states that such foods must be consumed as “... part of a varied diet on a regular basis, at effective levels” for consumers to reap their potential health benefits (American Dietetic Association 1999). On the basis of available information, functional foods are categorized as mentioned in the following.

38.4.1 Probiotics and Prebiotics

The term *probiotic* is derived from the Greek word meaning “for life”. The majority of probiotic microorganisms belong to the genera *Lactobacillus* and *Bifidobacterium*. However, other bacteria and some yeast also have probiotic properties. *Lactobacilli* and *Bifidobacteria* are Gram-positive lactic acid-producing bacteria that constitute a major part of the normal intestinal micro flora in humans. *Lactobacilli* are non-spore forming rod-shaped bacteria. They have complex nutritional requirements and are strictly fermentative, anaerobic, and acidophilic. They are found in several habitats that are rich in carbohydrate-containing substrates such as human and animal mucosal membranes, on plants or material of plant origin, sewage and fermented milk products, and fermenting or spoiling food. *Bifidobacteria* constitute a major part of the normal

Table 38.1 Types of functional foods used for health benefits.

Functional components	Source	Potential benefits
Carotenoids		
Alpha-carotene/beta-carotene	Carrots, fruits, vegetables	Neutralizes free radicals, which may cause damage to cells
Lutein	Green vegetables	Reduces the risk of macular degeneration
Lycopene	Tomato products (ketchup, sauces)	Reduces the risk of prostate cancer
Dietary Fiber		
Insoluble fiber	Wheat bran	Reduces risk of breast or colon cancer
Beta-glucan	Oats, barley	Reduces risk of cardiovascular disease Protects against heart disease and some cancers; lowers LDL and total cholesterol
Soluble fiber	Psyllium	Reduces risk of cardiovascular disease Protects against heart disease and some cancers; lower LDL and total cholesterol
Fatty Acids		
Long chain omega-3 Fatty acids: DHA/EPA	Salmon and other fish oils	Reduces risk of cardiovascular disease Improves mental, visual functions
Conjugated linoleic acid (CLA)	Cheese, meat products	Improves body composition Decreases risk of certain cancers
Phenolics		
Anthocyanidins	Fruits	Neutralizes free radicals; reduces risk of cancer
Catechins	Tea	Neutralizes free radicals; reduces risk of cancer
Flavonones	Citrus	Neutralizes free radicals; reduces risk of cancer
Flavones	Fruits/vegetables	Neutralizes free radicals; reduces risk of cancer
Lignans	Flax, rye, vegetables	Prevention of cancer, renal failure
Tannins (proanthocyanidines)	Cranberries, cranberry products, cocoa, chocolate	Improves urinary tract health Reduces risk of cardiovascular disease
Plant Sterols		
Stanol ester	Corn, soy, wheat, wood oils	Lowers blood cholesterol levels by inhibiting cholesterol absorption
Prebiotics/Probiotics		
Fructo-oligosaccharides (FOS)	Jerusalem artichokes, shallots, onion powder	Improves quality of intestinal microflora; gastrointestinal health
Lactobacillus	Yogurt, other dairy	Improves quality of intestinal microflora; gastrointestinal health
Soy Phytoestrogens		
Isoflavones: daidzein genistein	Soybeans and soy-based foods	Alleviates menopause symptoms, such as hot flushes, protects against heart disease and some cancers; lowers LDL and total cholesterol

*Source: International Food Information Council.

intestinal microflora in humans throughout life. They appear in the stools a few days after birth and increase in number thereafter. The number of *Bifidobacteria* in the colon of adults is 10¹⁰–10¹¹ cfu/g, but this number decreases with age. *Bifidobacteria* are non-motile, non-sporulating rods with varying appearance and most strains are strictly anaerobic.

According to the Food and Drug Association, USA Probiotics are defined as “live microorganisms when administered in adequate amount confer health benefits to the host” (Ohashi and Ushida 2009). Probiotics can be taken as food supplements that beneficially affect intestinal microbial balance. Administration of the probiotics for value added products should have following characteristic features:

- Probiotic organisms should preferably be of human origin for the survival of the strain at the site; its proliferation and colonization, which is found to be a natural inhabitant of the intestine.
- Probiotics should be tolerated by the immune system of the host and not be pathogenic, allergic, or mutagenic/carcinogenic.

- For successful application in foods, the probiotic used should also be technologically compatible with the food manufacturing process.
- In addition to that, the foods containing the probiotic bacteria must maintain the characteristic sensory attributes of the fortified traditional food.

The probiotic bacteria protect the host against pathogens by two mechanisms; the barrier effect or colonization resistance, and changes occurring in host's own defense mechanism. Infection caused by *Salmonella* spp., *Listeria monocytogenes*, *Escherichia coli*, *Shigella flexneri*, *Botrytis*, *Yersinia* spp., and certain viruses such as reovirus and poliovirus, can be cured by the use of the most beneficial strains of probiotic organisms, thus it has to be present in an gut environment as stable microflora. The barrier effect of probiotics in small intestines needs some protective strains at cellular target of the pathogen. Few adhesive strains of lactic acid bacteria produce a better mucosal barrier effect against disease causing pathogens in small bowel than non-adhesive ones. The intestinal luminal environment, epithelial and mucosal barrier function, the mucosal immune system, and numerous cell types involving epithelial cells, dendritic cells, monocytes/macrophages, B-cells, and T-cells, are influenced by probiotics. *Lactobacilli* strains are also able to secrete bacteriocins (antibacterial substances) that are active against *Salmonella typhimurium*. Probiotics can modulate the host immune system, both at a local mucosal immune level and systemically. LAB is a natural inhabitant of gastrointestinal tract. The strains of *Lactobacilli* and *Bifidobacteria* species commonly used in the dairy industry for manufacturing probiotic milk products are presented in Table 38.2. These products are functionally active and affect the disturbed physiology of gut micro flora. Probiotics are used as health supplements with promising results for therapeutic use functions by changing the cell growth and differentiation, antagonism against pathogens, and innate immunity against infection. This has raised the research potential to identify safe and effective treatments for immune stimulation, reduction of blood lipids, and serum cholesterol, overcoming lactose intolerance, correcting gastrointestinal disorders, inhibiting *Helicobacter pylori* responsible for peptic, gastric, and duodenal ulcers, as well has anticancerous properties, diminishing the recurrence of superficial bladder tumors, reduction of harmful intestinal microbial enzyme activity, decreasing fecal mutagenicity, reduction of the duration of rotavirus diarrhea, and production of vitamins with the possibility that it stimulates the antibody and cell-mediated immune responses (Table 38.3). In patients suffering from hyperacidity or gastric ulcers, probiotics may contribute a lot to the control of *H. pylori* infections. The recent epidemiological reports reveal that the consumption of whey-based medium fermented by *Lactobacillus johnsonii* La1 strain is noticed associated with lower incidence of ulcers and bladder cancer to some extent in specific populations.

38.4.2 Proteins and Peptides

Proteins are polymers of amino acids while peptides are small chunks of amino acids. The health promoter activity of these proteins and peptides are well established (Arai 1996). These proteins in the gut decrease cholesterol re-absorption and increase the excretion of indigestible substances in our digestive tract through feces, toxin, and bile. Therefore, proteins are considered as health promoters. Furthermore, digestion of protein leads to the production of bioactive active peptides, which have demonstrated a wide range of useful activities including antimicrobial, antifungal, blood pressure lowering effects, cholesterol lowering effects, enhancement of mineral absorption, immunomodulatory effects, and localized effects on the gut (Arai 1996). The research on food derived bioactive compounds and the effect on the gut after digestion is in its infancy, but it is imperative to increase our understanding of food derived bioactive peptides during the normal digestive

Table 38.2 Different probiotic products and their significance.

Probiotic dairy products	Micro organism	Therapeutic Significance
Acidophilus milk, paste, and butter milk, lactose free milk	<i>L. acidophilus</i> , <i>S. thermophilus</i> , <i>L. delbrueckii</i> subsp. <i>bulgaricus</i> .	Lactose maldigestion Maintenance of normal intestinal flora
Probiotic dahi, mishiti doi, shrikhand	<i>L. acidophilus</i> , <i>L. lactis</i> , <i>L. lactis</i> subsp. <i>diacetylactis</i> , <i>B. bifidum</i> , <i>S. thermophilus</i>	Removal of toxic amines, strengthening of immune systems
Special yoghurt, Biogurt, Bifighurt	<i>B. bifidum</i> , <i>L. acidophilus</i> , <i>S. thermophilus</i> , <i>L. delbrueckii</i> subsp. <i>bulgaricus</i>	Enhanced digestibility, prevents constipation
Cultured drink, Progurt,	<i>S. lactis</i> subsp. <i>diacetylactis</i> , <i>S. lactis</i> subsp. <i>cremoris</i> , <i>L. acidophilus</i> , and/or <i>B. bifidum</i>	Reduction of serum cholesterol Removes dietary procarcinogens

Table 38.3 Diseases and benefits resulting from probiotics.

Locus	Disease	Health effect
Mouth and teeth	Caries and gingivitis	Reduction of gingivitis by <i>L. reuteri</i> , Colonization of the teeth' surface by <i>lactobacilli</i> from a "bio-yogurt" less caries after ingestion or oral vaccination with heat-killed <i>lactobacilli</i>
Stomach	<i>Helicobacter pylori</i> infection	Inhibition of growth and adhesion to (duodenum), infection in mucosal cells decrease in gastric <i>H. pylori</i> concentration less side effects during antibiotic therapy
Small bowel	Bacterial overgrowth	Normalization of the small bowel microflora, lesser frequency of diarrhea, decreased release of toxic N-metabolites
Intestinal microflora plus host metabolism (liver, kidney)	Decreased detoxification/ excretion of toxic metabolites due to liver/ renal failure; hepatic encephalopathy	Increased bifidobacteria cell counts application of lactose and shift from a preferably protein- to a carbohydrate - metabolizing microflora, less toxic and/or putrefactive metabolites, improvement of hepatic encephalopathy after administration of bifidobacteria and lactulose
Urinary tract infections	Irritation or inflammation of the vagina, urethra, bladder, ureter, kidney, or cervix due to infections by endogenous microflora (from mostly candidiasis) and imbalances in local microflora	Restoration of an imbalanced microflora tract by selected <i>lactobacilli</i> Decreased incidence and increased curing rates in bacterial vaginosis and vaginitis due to the local or oral application of lacto and bacilli; decreased incidence or recurrence of urinary tract infections or exogenous bacteria, no effects

process (Inaba *et al.* 2002, Okamoto *et al.* 2003). Health inducing peptides are already on the market in Japan, in Europe a sour milk drink product containing bioactive peptide is available. In the USA, a milk powder that contains antihypertensive peptides is also available as functional food for blood pressure reduction. Similarly, alpha-lactoalbumin, whey protein, and fungal immunomodulatory protein, are commercially available on the market with diverse health benefits (Hsieh *et al.* 2003; Sahelian 2005). The bioactive peptide derived from different milk proteins during gastrointestinal digestion or milk fermentation with proteolytic enzymes exerts a number of physiological effects *in vivo* on gastrointestinal, cardiovascular, endocrine, immune, nervous, and other body systems (Korhonen and Pihlanto 2007). Currently, milk derived peptides acting as a source of antioxidants in health promotion and disease prevention has been recognized (Elias *et al.* 2008; Power *et al.* 2014). Whey protein component, lactoglobulin, lactalbumin, and serum albumin were studied and proved to have anticancer potential. Lactoferrin acts as inducer of apoptosis, inhibits angiogenesis, and modulates carcinogenic metabolizing enzymes; lactoferrins also act as iron scavengers (Zammer 2002).

38.4.3 Carbohydrates and Fibers

Carbohydrates and dietary fibers are good for health. The gut function, brain, and muscle activities are well coordinated by proper usage of carbohydrates inside the body. Recent reports from the World Health Organization and the Food and Agriculture Organization of the United Nations on carbohydrates in humans suggest that: An optimum diet should contain at least 55% of energy from carbohydrates and 20–35 g dietary fiber per day for all those over 2 years of age, a wide range of carbohydrate-containing food should be consumed so that the diet is sufficient in essential nutrients and dietary fibers (Newell-McGloughlin 2008). Dietary fibers are found in plant food (fruit, vegetables, and whole grains) and play a key role in maintaining a healthy gut. There are two types of fibers, soluble and insoluble (Dobbing 1989). Soluble fiber are soluble in water and found in beans, oats, fruits and help to lower blood fat and blood sugar. Insoluble fibers are insoluble in water and passes through digestive tract. High fibers content in food allow slow and steady digestion in gut and also help in weight maintenance. Researchers have reported that a diet of grain-free whole food with carbohydrate from cellular tuber, leaves and fruits may produce a gastrointestinal microbiota consistent with our evolutionary condition, potentially explaining the exceptional macronutrient-independent metabolic health of non-westernized population and the apparent efficacy of modern "Paleolithic" diet on satiety and metabolism. Galacto-oligosaccharides (GOS) as functional food constituents play a special role as prebiotics in gastrointestinal (Sangwan *et al.* 2012). The microbiota of small and large intestine are maintained by digested carbohydrate in human gut (Zoetendal *et al.* 2012).

38.4.4 Lipids and Fatty Acids

The presence of omega-3 and omega-6 in fish oil has led to it being recognized as a functional food because of its ability to reduce blood pressure and risk of other cardiovascular disease. The main omega-3 fatty acid is fish oil is docohexaenoic (DHA) and eicosapentaenoic acid (EPA) (Augustsson *et al.* 2003). Linoleic and linolenic acid also provide increased cardiovascular benefits and are abundant in fish oil, vegetable oil, (canola, soyabean, and sunflower), and nuts such as peanuts and almonds. The role of n-3 fatty acid in decreasing breast prostrate and colon cancer is well established (Augustsson *et al.* 2003; De Deckere 1999). Conjugate linoleic acid (CLA) reduces development of adipose tissue and weight loss, and reduces severity of chronic kidney disease. Further research studies showed soya bean oil has greater antioxidant capacity due to a high tocopherol content compared to peanut oil (Nicoletta *et al.* 2003). Wheatgerm oil has gained popularity in diets as well in cosmetics due to its high content of vitamin E, A, D, and B1, B2, B3, and B6 essential fatty acids and proteins. The wheatgerm oil has long been used for healing and reducing scars, stretch marks, damaged skin; retarding the effect of aging, relieving and healing eczema, psoriasis, and sunburns; and treating muscle fatigue from overexertion (Charrois *et al.* 2001).

38.4.5 Flavanoids and Lycopene

The role of plant flavanoids and catechin as antioxidants used in treatment of various inflammatory metabolic diseases is well established (Arts *et al.* 2001; Murphy *et al.* 2003; Zeisel 1999). Catechins are strong antioxidants that inhibit damage to DNA and blood vessels, thereby reducing the risk of cancer development and cardiovascular disease. Cranberry juice contains high level of epicatechin polymers that prevent adhesion of bacteria and viruses to the urinary tract (Murphy *et al.* 2003). Tomatoes are considered as very rich source of lycopene; similarly bright colored food such as canned pizza sauce, spaghetti sauce, barbecue sauce, and ketchup are also rich in lycopene. Lycopene reduces free radical generation and helps turn nutrients into energy (Ohnishi and Yakoyama 2004). Increasing consumption of lycopene-containing food products can reduce blood pressure in hypertensive patients by reducing plaque formation (Ohnishi and Yakoyama 2004). The chemopreventive role of lycopene present in tomato and tomato puree is well studied (Polívková *et al.* 2010). Carotenoid, which is responsible for the red color of tomato, has attracted attention because of its role in the prevention of chronic diseases in which oxidative stress is the major etiological factor; such as cancer, cardiovascular disease, neurodegenerative disease, and hypertension (Waliszewski and Blasco 2010).

38.4.6 Vitamins

The folic acid and relative B group vitamins, particularly folate (B9), may give considerable protection against serious diseases such as cancer, heart disease, and birth defects (Newell-McGloughlin 2008). Micronutrient malnutrition, the so-called hidden hunger, effects more than half of the world's population; mainly women and pre-school children in developing countries (US FDA 2003). The clinical and epidemiological evidence show that minerals and vitamins play a significant role in the maintenance of optimal health and are limiting in diet (Meisel 2005). Vitamin B6 in conjunction with B12 plays an important role in protecting against cardiovascular disease. Research on Vitamin E shows that 100 IU per day for 2 or more years reduces adverse outcome related to heart disease by 37% in men and 41% in women (Ryan-Harshman and Aldoori 2005). Vitamin C supplementation for 10 years or more reduced the chance of cataract (Gale *et al.* 1996). Higher Vitamin C intake among elderly provides protection against cognitive impairment and cerebrovascular disease (Gale *et al.* 1996).

38.5 Regulations and Safety of Functional Food

Manufacturing of a functional product, mainly probiotics, has played a key role in growing awareness towards the health benefits of probiotic therapy. It should be assured that structure/function or health claims for the products are made under the supervision of experts in the field, and claim against the products is truthful and not misleading according to FDA approval. Proper vigilance in identifying, typing, and cataloguing all bacteria is necessary. Inappropriate methods are still frequently applied for the identification of species and strains of *lactobacilli* and *bifidobacteria*, and harmonization is needed. Certain reports on the risk of infection with probiotic are similar to that of infection with commensal strains, mostly in immune compromised hosts (Prantera *et al.* 2002).

The probiotic strains can significantly affect the gut micro flora and enhance the improvement of disease infections. However, risk factors have been identified for *lactobacilli* that are involved in the production of some proteinases and glycosidases and platelet aggregation. Increase in gut permeability by probiotic microorganism above the threshold level could cause an allergy or inflammation. The pharmaceutical industry is widely using probiotic microorganisms without

any health issues for consumers. This has raised the importance of regulations and safety measures for the commercialization of probiotic products. Full characteristics and appropriate molecular profiles of currently used probiotics generated by standard operating procedures should be undertaken. Molecular techniques such as 16S ribosome sequence analysis for speciation and genomic DNA fragment analysis for strain differentiation (e.g., by fluorescent amplified fragment-length polymorphism analysis or pulse field gel electrophoresis PFGE profile is important done for probiotic strains. The applied research information of probiotic strain characteristics should be made freely available to researchers and should include comparable data on non-probiotic strains of the species. Such information and methodological guidance would help to understand better the etiology of the *lactobacilli* or *bifidobacteria* risk infections.

Information to consumers about the relationship between diet and health in food regulations has been provided in 1990, 1994, and 1997. The first of these is the Nutrition Labeling and Education Act of 1990 (NLEA). The NLEA allows statements on food labels that characterize the relationship of any food or food component with a disease or health-related condition. Such “health claims” must be pre-approved by the FDA before their use. Under the NLEA, the FDA was mandated by Congress to review 10 diet-disease relationships, eight of which were eventually approved as health claims (Table 38.4). The NLEA also enables the authorization of new health claims after submission of a petition to the FDA. Because of the complexity and expense involved in the petition process, however, as of July 2002, only five additional health claims have been approved under NLEA in response to food industry petitions (Table 38.5). To expedite the health

Table 38.4 Diet-disease relationship mandated for review by FDA under NLEA and currently approved as health claims.

Diet-disease relationship	Model claim
Calcium and osteoporosis	Regular exercise and a healthy diet with enough calcium help teens and young adults White and Asian women maintain good bone health and may reduce their risk of osteoporosis
Sodium and hypertension	Diets low in sodium may reduce the risk of high blood pressure, a factors associated with many diseases
Dietary fat and cancer	Development of cancer depends on many factors. A diet low in total fat may reduce the risk of some cancers
Dietary saturated fat and cholesterol and coronary heart disease	While many factors affect heart disease, diets low in saturated fat and cholesterol may reduce the risk of this disease.
Fruits, vegetables and grain products that contain fiber (particularly soluble fiber) and coronary heart disease	Diets low in saturated fat and cholesterol and rich in fruits, vegetables and grain products that contain some types of dietary fiber (particularly soluble fiber) may reduce the risk of heart disease, a disease associated with many factors
Fruits and vegetables and cancer	Low fat diets rich in fruits and vegetables may reduce the risk of some types of cancer, a disease associated with many factors

Table 38.5 Health claims approved by the Food and Drug Administration following petitions submitted by the food industry.

Food component	Approved health claim
Sugar alcohols and dental caries	Frequent eating of foods high in sugars and starches as between meal snacks can promote tooth decay. The sugar alcohol [name of product] used to sweeten this food may reduce the risk of dental caries.
Foods that contain fiber from whole oat products and coronary heart disease	Diets low in saturated fat and cholesterol that include soluble fiber from whole oats may reduce the risk of heart disease
Soy protein and coronary heart disease	Diets low in saturated fat and cholesterol that include 25 g of soy protein a day may reduce the risk of heart disease. One serving of [name of food] provides 6.25 g of soy protein.
Plant sterol/stanol esters and coronary heart disease	Plant sterols: Foods containing at least 0.65 g per serving of plant sterols, eaten twice a day with meals for a daily total intake of at least 1.3 g, as part of a diet low in saturated fat and cholesterol may reduce the risk of heart disease. A serving of [name of the food] supplies grams of vegetable oil sterol esters Plant stanol esters: Foods containing at least 1.7 g per serving of plant stanol esters, eaten twice a day with meals for a total daily intake of at least 3.4 g, as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease. A serving of [name of the food] supplies grams of plant stanol esters.

claims approval process and thus hasten the availability of health messages to consumers, Congress enacted the FDA Modernization Act (FDAMA) in 1997. This legislation streamlines the FDA pre-approval process by enabling the use of so-called “authoritative statements” on food labels as health claims. Such statements must be published by certain US Government bodies responsible for the protection of public health such as the NIH, the Centers for Disease Control and Prevention, or the National Academy of Sciences.

38.6 Safety Challenges of Functional Food

The general safety of lactic acid bacteria and *Bifidobacteria* is proved by their long past of consumption in traditional fermented milk products without any harmful effects on human health. The Food and Drug Administration of the USA considers *lactobacilli* and *Bifidobacteria* used for food production to be “generally recognized as safe” (GRAS) with the exception of one strain belonging to the *L. rhamnosus* species. The fermented milks and LAB beverages association of Japan has developed a standard, which requires a minimum of 107 viable *bifidobacteria* cells/ml to be present in fresh dairy products (Gasser 1994). The criteria developed by National Yoghurt Association (NYA) of the United States specifies 10⁸ cfu/g of LAB at the time of manufacture as a prerequisite to use the NYA “Live and Active Culture” Logo on the containers of the products. In Germany, all but two strains of *lactobacilli* and *bifidobacteria* are classified as “1” (absolutely safe) by the “Berufsgenossenschaft der chemischen Industrie”. Moreover, beneficial strains of probiotic bacteria have been proven to be free of risk factors and can be used for transferable antibiotic resistances, cancer-promoting and/or putrefactive enzymes and metabolites, hemolysis, activation of thrombocyte aggregation, or mucus degradation in the mucus layer of the gastrointestinal tract (Nagpal *et al.* 2012). Despite the absence of a pathogenic potential, lactic acid bacteria were found in <0.1% (enterococci 1%) of clinical samples from severe infections (endocarditis, meningitis, or bacteraemia). These bacteria originated from the indigenous microflora, whereby in many cases the translocation was facilitated by underlying disease, lesions, or inflammations in the oral cavity and in the gastrointestinal tract, or by an impaired immune system. Two incidences of pathogenic effect have been published concerning food probiotics: a *Lactobacillus* strain was isolated from a liver abscess, which was undistinguishable from the food probiotic *L. rhamnosus* GG [98]. In a second case the contents of a probiotic capsule (*L. rhamnosus*, *L. acidophilus*, and *Streptococcus faecalis*) was accidentally ingested after a tooth extraction, which lead to endocarditis. Probiotic bacteria were recovered from the clinical sample. Also, in several cases probiotic yeast, *Saccharomyces boulardii*, was found in fungaemia, due to an immunocompromised state or due to other infections. However, there is no evidence for a high risk due to the ingestion of probiotic products in comparison with conventional products. This conclusion is supported by a study from Finland, where the consumption of *L. rhamnosus* GG has increased considerably during the last two decades without an increase in the incidence of infections by *lactobacilli*. Moreover, studies in immunocompromised persons (HIV-positive subjects, patients with leukemia) did not show undesired effects, but rather positive effects such as, for example, lower incidence of *Candida* during chemotherapy. Health risks due to over dosage or long-term ingestion have also not been observed.

38.7 Functional Foods and Nutrigenomics

The importance of functional food and other food ingredients in prevention of chronic diseases and to improve the quality of life is undergoing extensive research. Academic, government, and private research institutes around the globe are devoting substantial efforts to identifying how functional foods and food ingredients might help in reducing healthcare costs and improving the quality of life for many consumers. An emerging discipline that will have a profound effect on future functional foods research and development efforts is *nutrigenomics*, which investigates the interaction between diet and development of diseases based on an individual's genetic profile (Fogg-Johnson and Meroli 2000). Interest in nutrigenomics was greatly augmented by the recent announcement that a rough draft of the complete sequence of the human genome had become available. In February 2001, the complete sequence of the human genome was announced by Venter and colleagues (Celera Genomics Sequencing 2001). This technological breakthrough could eventually make it feasible to tailor a diet for an individual's specific genetic profile. Nutrigenomics will have a profound effect on future disease prevention efforts, including the future of the functional foods industry. Another technology that will greatly influence the future of functional foods is biotechnology (Gura 1999). Recent examples of biotechnology-derived crops, which have tremendous potential to improve the health of millions worldwide, include golden rice and iron-enriched rice (Institute of Food Technologists 2010). These grains are genetically engineered to provide enhanced levels of iron and β-carotene that could, in turn, help prevent iron deficiency

anemia and vitamin A deficiency-related blindness worldwide (Anderson *et al.* 2005). In the future, other foods enhanced with other nutritive or nonnutritive substances may even help to prevent chronic diseases such as heart disease, osteoporosis, or cancer (Falk *et al.* 2002). The acceptance of biotechnology by consumers (currently a major issue in Europe) will be important if the potential of this powerful methodology is to be realized. Similarly, healthy people who are aware from the effect of balanced intestinal flora and the importance of healthy immune system are curious about the benefit of functional food. Functional food may prevent complaints due to occasional imbalances of an otherwise balanced system. Health benefits, like prevention of occasional gastrointestinal complaints, common infectious diseases (e.g., colds), or atopic diseases of healthy people, as well as normalization of a decreased intestinal motility or reduction of certain long-term risks (cancer, ischemic heart disease) is surely of interest for the common population. But consumption of functional food should be carefully undertaken with balanced nutrition for a healthy lifestyle.

38.8 Conclusions

Recent studies have proved that functional foods hold promise for public health. There is a need for time to increase the promotion of functional foods and structure/function claims on the basis of strong scientific evidence. Confusion also exists about claims applied to foods and those applied to dietary supplements. With the addition to foods of ingredients usually found only in dietary supplements, such confusion has increased. Although claims about the potential health benefits from functional foods or food ingredients must be communicated effectively to consumers, the differences between health claims and structure-function claims must also be more widely addressed to allow consumers to understand the differences in the scientific bases of such claims. Any health benefits attributed to functional foods should be based on sound and accurate scientific criteria, including rigorous studies of safety and efficacy. Interactions with other dietary components and potential adverse interactions with pharmaceutical agents must be clearly imparted. Further, the established and scientifically approved health promoting probiotics and their usage in disease treatment is very promising. Although awareness of probiotics intake has increased the markets of functional food products greatly, the manufacture of dry cultured products containing viable bacteria is still in early stages of development. The formulation of suitable blends containing healthful ingredients such as dietary fiber, natural vitamins, and folic acid also offers new possibilities for this widely expanding research area. There is a need to intensify research to distinguish the therapeutic benefits of viable and nonviable organisms in different functional foods apart from the general nutrient enhancement achieved. Although from the ongoing research more of promising health effects of probiotics are being observed, more verifiable molecular characterization and clinical studies are needed to demonstrate the safety, efficacy, and limitations of a putative probiotic, to find the actual response and long-term consumption effect on the gut microbiome, and to establish the importance of probiotics as functional foods.

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Conflict of Interest

Authors declare there is no conflict of interest.

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An Overview on Germinated Brown Rice and its Nutrigenomic Implications

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39.1 Diet and Health: The Role of Staple Foods and Nutrigenomic Implications

Over the years, the role of staple foods in providing adequate nutrition has been acknowledged. Historically, the choice of staple foods used to be based on availability, proximity to communities, and palatability. Health benefits were never considered in such choices. Even when scientific advances set in it took many decades for the focus on staple foods to shift towards purely health benefits (Kearney, 2010; Kuhnlein and Receveur, 1996; Weststrate *et al.*, 2002). Admittedly, in much of the earlier part of the twentieth century, nutrition research focused on functions of the major macromolecules (carbs, proteins, and fats), their deficiency states and how those could be managed. In the later part of the twentieth century, focus shifted towards provision of staple foods with enhanced health benefits (DellaPenna, 1999; Graham *et al.*, 1999; Nestel *et al.*, 2006; Vergères, 2013; Weststrate *et al.*, 2020). Iron, vitamin A, and other minerals were fortified in staple diets this way (Drewnowski, 2005; Stoltzfus and Dreyfuss, 1998; Tontisirin *et al.*, 2002; WHO *et al.*, 1997).

Advances in nutrition research, especially in the post-human genome era, have resulted in the evolution of newer fields of nutrition research. With this came the realization that diets were important variables in the causation of disease and health maintenance (Carrera-Bastos, *et al.*, 2011; WHO and FAO, 2002); diets have the capacity to interact with the human genome at different levels, and could result in changes in the transcriptome, proteome, and metabolome. The use of molecular biology tools to study interactions between diets and any of the aforementioned aspects of the genome is termed *nutrigenomics* (Afman and Müller, 2006; Müller and Kersten, 2003; Mutch *et al.*, 2005; Vergères, 2013). These interactions at the diet-genome interface have tremendously advanced our understanding of the roles played by diets in health and disease (Figure 39.1).

Dietary signals elicit changes in transcriptional factors following interactions with receptors resulting in transcriptional (transcriptomics) and translational (proteomics) changes that affect metabolic content of cells (metabolomics) in different ways. When the resulting changes produce favorable metabolic outcomes, disease prevention and health promotion are the end result (Müller and Kersten, 2003). Nutrigenomics has recently been the focus of much attention within the nutritional sciences in an attempt to understand the role of diets in regulation of the genome and what diets could be used to improve health. Studies in this area have given birth to the understanding that environmental factors, especially dietary, not only regulate the genome in those that directly experience them but also across generations (Feil, 2006; Mathers *et al.*, 2009). Such transgenerational studies are thought to be the underlying reason for some hitherto considered evolutionary changes

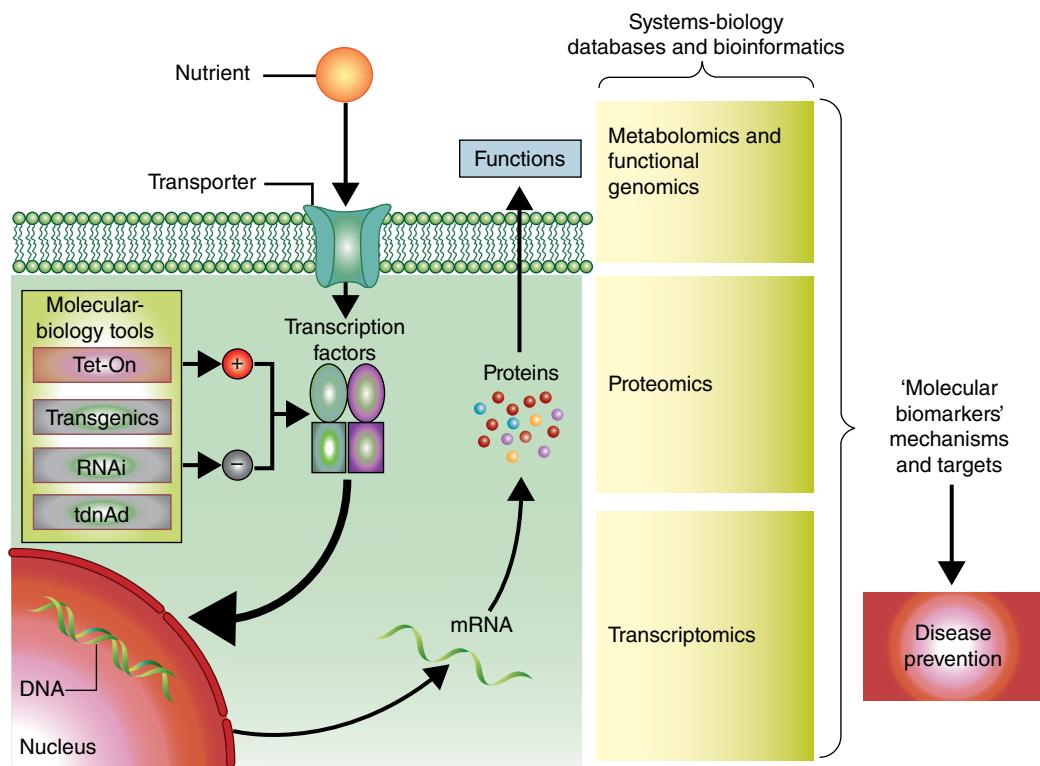


Figure 39.1 Schema showing nutrigenomic tools used to study nutrient-gene interactions and disease mechanisms. Nutrient sensing by cells can lead to changes in expression of genes, mostly mediated by transcriptional factors, with changes in levels of mRNA (transcriptomics), proteins (proteomics), and metabolic contents (metabolomics). Use of molecular biology tools to modulate the nutrient sensing of cells can add to understanding on bioactivity. Overall, nutrigenomic studies increase understanding on molecular markers influenced by nutrients and targets for disease prevention and health promotion. Müller and Kersten (2003). Reproduced with permission from Nature Publishing Group.

observed in humans across generations. Furthermore, the genetic basis of most diseases is now believed to have epigenetic links due to environmental factors. As such, observational studies and genome-wide association studies (GWAS) have indicated that only about 10% of chronic diseases can be explained by genetic susceptibility and that over 90% of cases are traceable to environment especially dietary factors (Whitehead and Whitehead, 1999).

Obesity, metabolic syndrome, cancers, Type 2 diabetes, and other chronic diseases have been on the increase despite advances in healthcare delivery and management of these diseases (Mathers and Loncar, 2006; Strong, *et al.*, 2005). It is likely that dietary and other environmental factors are contributing to such increases in disease incidents across generations (Mathers *et al.*, 2009; Skinner *et al.*, 2010). As more studies unravel these hypotheses, it remains to be speculated that dietary factors, especially staple diets, may have a huge role in such disease incidents. Considerations of the role of diet in the causation of chronic diseases, its potential for disease prevention, and finally the transgenerational implications of diets and dietary habits, underline diet as an important variable in the overall global burden of diseases. This is more so when diets are consumed in the form of staple foods; the continuous nature of consumption of staple foods on a daily basis means the components of such diets will impact on the health of individuals continuously over the long term. Therefore, this calls for action on choice of staple diets that not only improve health of individuals but also could improve health across generations through epigenetic modifications. The enormous consumption of rice across much of Asia and Africa suggests that it is a staple food that deserves considerable attention in order to safeguard the health of over half the world's population, who, oftentimes consume this staple because of availability and affordability (Khush, 2005). In this regard, the health implications of the commonly consumed rice (polished rice) have received considerable attention, although the outcome does not inspire as much confidence. This has prompted calls for better alternatives.

Already, it is documented that chronic diet-related diseases are the leading cause of death globally, with a huge burden on much of Asia and Africa, where there are limited resources to provide state-of-the-art healthcare facilities (Abegunde *et al.*, 2007; Yach *et al.*, 2004). With the threat of food security in many parts of the developing world, stakeholders are championing a cause for more food availability and sustainability in feeding the world's population. Not only should these agencies support the provision of any food, they should support in addition the provision of healthy foods to avert moving from one crisis (hunger) to another (metabolic diseases). This is very important for the developing countries where white rice is the main staple food, which recently has been linked with development of chronic diseases, like Type 2 diabetes (Hu *et al.*, 2012; Sun *et al.*, 2010; Nanri *et al.*, 2010).

39.2 Health Implications of White Rice and Brown Rice Consumption

As can be recalled, white rice is a major staple food for people in low to middle income countries and recently it has been linked to the development of Type 2 diabetes. The incidence and prevalence of Type 2 diabetes in those countries is projected to increase (International Diabetes Federation, 2013), and though a direct link has not been made, excessive white rice consumption may be contributing to the growing trend. In fact, despite the evidence linking white rice to diabetes, Kadoch believes it may not be a direct link and that adoption of a Western lifestyle is largely to blame for the burden of diabetes (Kadoch, 2012). In a review, Hu argued that multiple factors are responsible for the growing incidence and prevalence of diabetes in Asian countries (Hu, 2011). However, it is also possible that excessive white rice consumption is contributing to some extent in view of recent evidence linking it to diabetes and its daily and enormous consumption in Asian countries.

Sun *et al.* (2010) reported an increased risk of diabetes among white rice consumers, and that replacing one-third of daily serving with brown rice will reduce the risk of developing Type 2 diabetes. Nanri *et al.* (2010) also reported an increased risk of Type 2 diabetes in women who consumed white rice, although Zhang *et al.* reported not finding a similar association in middle aged Chinese men and women (Zhang *et al.*, 2011). A meta-analysis involving over 350,000 subjects followed up for 4–22 years, however, showed an increased risk of Type 2 diabetes among Asians who consumed white rice instead of brown rice (Hu *et al.*, 2012). It still remains controversial, however, especially since Kempner had used white rice to manage cardiometabolic diseases (Kempner *et al.*, 1958; Nuttall, 1983). Interestingly, fruits and vegetables were included as part of the diet, and they may have cancelled out any negative effects of white rice (Liu, 2003). High glycemic index of white rice is likely the reason behind the worsening of cardiometabolic risks due to its consumption (Barclay *et al.*, 2008; Jenkins *et al.*, 2002; Miller *et al.*, 1992). High glycemic index will promote postprandial hyperglycemia, glucose-induced oxidative stress, and eventually, cardiometabolic risk (Ludwig, 2002; Rebolledo and Dato, 2005). The risks associated with white rice consumption based on the findings so far suggest that less consumption of white rice may be helpful towards reducing risk of cardiometabolic diseases, especially Type 2 diabetes. Furthermore, epigenetic studies have demonstrated that dietary factors and habits can induce intrauterine molecular reprogramming events in growing fetuses with consequent increase in the risk of chronic diseases (Mathers *et al.*, 2009; Skinner *et al.*, 2010). Hence, it is likely also that epigenetic events due to excessive white rice consumption are promoting the transgenerational risk of cardiometabolic diseases. These risks indicate the need for healthier alternatives for white rice in order to lower chronic diseases burden among white rice-consuming countries.

39.3 Germinated Brown Rice: Bioactives, Functional Effects, and Mechanistic Insights

Rice grown on the paddy usually has chaff (husk), which when dehulled yields brown rice. Milling of brown rice to remove the outer bran layer and germ will then produce white rice (mainly the endosperm) that is commonly consumed. The outer bran layer removed during milling contains bioactive compounds that have disease-preventing potential (Wu *et al.*, 2013) (Figure 39.2).

Over 3 billion people are thought to be dependent on white rice as a staple food, especially in Asia and Africa where the burden of chronic disease is on the increase (Khush, 2005; Yach *et al.*, 2004). Brown rice is not consumed as much as white rice, partly due to its hard texture. This wholegrain food, however, has been reported to contain bioactives like γ -aminobutyric acid (GABA), γ -oryzanol, dietary fiber, acylated steryl glycoside (ASG), minerals, and vitamins, mostly in the bran layer (Table 39.1). To overcome the problem of hard texture and improve palatability, germination of brown rice is commonly carried out (Imam *et al.*, 2012a; Wu *et al.*, 2013). Germination entails soaking brown rice in water with/without the use of

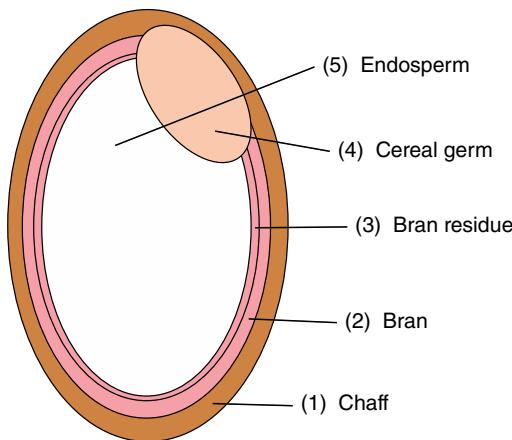


Figure 39.2 Schema of rice grain showing the various parts as it is derived from the paddy. The chaff is dehulled to access the bran layer, which is often removed together with the germ to get the white rice (mainly endosperm) that is commonly consumed. Source: Wikipedia, <http://en.wikipedia.org/wiki/Rice>

Table 39.1 Germinated brown rice bioactives and their potential bioactivities.

Nutrients	Biological activities in germinated brown rice	Reference
Acylated steryl glycoside	Antioxidant Hypocholesterolemic Anti-hyperglycemic Hypotensive effect	Imam <i>et al.</i> (2012a)
GABA	Accelerating metabolism in brain, and preventing headaches or depressions after effects of cerebral arteriosclerosis and cerebral apoplexy Preventing climacteric disorder Preventing presenile derangement such as insomnia and mental irritation	Patil and Khan (2011)
Dietary fiber	Activating renal function Relieving constipation. Preventing cancer of colon Regulating blood sugar levels	Patil and Khan (2011)
Inositol	Accelerating fat metabolism Preventing fatty liver Preventing arteriosclerosis	Patil and Khan (2011)
Ferulic acid and other phenolics	Antioxidant including scavenging super oxides Suppressing melanogenesis	Patil and Khan (2011)
Phytic acid	Antioxidative effect Preventing cardiovascular disease Preventing platelet aggregation	Patil and Khan (2011)
Tocotrienols	Scavenging super oxides Protecting skin from ultraviolet rays	Patil and Khan (2011)
Magnesium	Preventing heart diseases	Patil and Khan (2011)
Potassium	Lowering blood pressure	Patil and Khan (2011)
Zinc	Activating reproductive function Preventing arteriosclerosis	Patil and Khan (2011)
Gamma-oryzanol	Antioxidative effect Preventing skin aging Hypocholesterolemic	Patil and Khan (2011)
Prolylendopeptidase inhibitor	Preventing Alzheimer's disease	Patil and Khan (2011)



Figure 39.3 Photo of germinated brown rice. The sprouts indicating germination are indicated by dark rings. The sprouts appear when enough stimuli have been applied to induce germination of the seeds. The rice grains in the photo were germinated over 24 h, similar to what was reported in Ismail *et al.* (2014). Photo courtesy of Siti Farhana Fathy and Der-Jiun Ooi, Universiti Putra, Malaysia.

seed stressors like anoxia treatment, sodium hypochlorite, and hydrogen peroxide (Ismail *et al.*, 2014; Wu *et al.*, 2013). The outcome of the germination process is a grain that is less hardy and more palatable. Interestingly, during the process of germination, activation of hydrolytic enzymes, notably amylase, not only breaks down seed molecules like amylose that give the seed its lower texture, but also catalyzes the production of more bioactive compounds in the seed (Imam *et al.*, 2012a; Wu *et al.*, 2013). The increased bioactive composition of the germinated brown rice is a reflection of the increased demands for seed growth (Ismail *et al.*, 2014). However, arresting the germination of the grain at the appropriate time (evidenced by appearance of sprouts, Figure 39.3) by removing optimal germination conditions maintains the high bioactive composition, which can be consumed for health benefits.

Numerous studies have demonstrated the improved bioactive composition of brown rice following germination (Table 39.2). In fact, we have used a combination of three effective methods of germination (anoxia treatment, sodium hypochlorite, and hydrogen peroxide) that yielded significantly high levels of bioactives (Ismail *et al.*, 2014). In particular, a comparison of the GABA content of our germinated brown rice after 24 h of germination indicated that it was higher than what others reported for germination times of 48 and 72 h (Imam *et al.*, 2013a; Roohinejad *et al.*, 2010). This improved bioactive composition is believed to be the reason behind the increased bioactivity of germinated brown rice compared to brown rice. Also, the improved texture of germinated brown rice over brown rice means it is likely to be patronized more than brown rice, and white rice for health reasons. Already, there have been reports that germinated brown rice incorporated into bread and other food products had good texture and was received positively than if brown rice was used (Imam *et al.*, 2012a; Kim, 2013).

Consumption of staple foods like germinated brown rice, with potentially better health benefits due to higher amounts of bioactive compounds, can be a way to provide continuous availability of health-promoting bioactive compounds. It is also a convenient way to manage chronic diseases through daily diets. This is even more so for parts of the world where economic constraints make it difficult for individuals to have access to state-of-the-art healthcare facilities. Staple foods with functional properties will have more benefits to these people through health promotion and disease prevention, than any medication or treatment modality they cannot afford when they become ill.

The documented functional effects of germinated brown rice include antidiabetic, antioxidant, weight reducing, hypocholesterolemic, antidepressant, cognitive-enhancing, hepatoprotective and immunomodulatory properties (Imam *et al.*, 2012a; Patil and Khan, 2011; Wu *et al.*, 2013). Considering these properties of germinated brown rice, it could potentially produce better outcomes especially for cardiometabolic diseases than pharmacological agents, which often have single pharmacological action and many side effects. Also, metabolic changes due to disease processes cause underlying associated transcriptional or translational perturbations, which may not be the target of most pharmacological agents. There are now more studies targeting the interaction of food and its components with the genome (transcriptome, proteome, and

Table 39.2 Changes in concentrations of bioactives during germination of brown rice grains. Patil and Khan (2011). Reproduced with permission from Springer Science + Business Media.

Bioactive	Changes during germination	Reference(s)
<i>Gamma aminobutyric acid (GABA)</i>	↑ GABA	Banchuen <i>et al.</i> (2009, 2010); Charoenthaikij <i>et al.</i> (2010); Imam and Ismail (2013); Imam <i>et al.</i> (2013a); Jannoey <i>et al.</i> (2010); Karladee and Suriyong (2012); Komatsuzaki <i>et al.</i> (2007); Li <i>et al.</i> (2008, 2012); Maisont and Narkrugsa (2010); Oh <i>et al.</i> (2010); Roohinejad <i>et al.</i> (2011); Songtip <i>et al.</i> (2012); Watchararpaiboon <i>et al.</i> (2010)
<i>Dietary fiber (DF)</i>	↑ DF, ↑ soluble fiber, ↓ insoluble fiber	Banchuen <i>et al.</i> (2009); Lee <i>et al.</i> (2007); Jayadeep <i>et al.</i> (2011); Li <i>et al.</i> (2008, 2012); Maisont and Narkrugsa (2010); Oh <i>et al.</i> (2010)
<i>Acylated steryl glycoside (ASG)</i>	↑ ASG	Imam <i>et al.</i> (2013a,b); Usuki <i>et al.</i> (2008)
<i>Phenolics and antioxidants</i>	↑ phenolics and antioxidant activity	Imam <i>et al.</i> (2012b, 2013a); Li <i>et al.</i> (2008) Maisont and Narkrugsa (2010) Azmi <i>et al.</i> (2013); Imam and Ismail (2013); Jongjareonrak <i>et al.</i> (2009); Kim <i>et al.</i> (2011); Sani <i>et al.</i> (2012); Sawaddiwong <i>et al.</i> (2008); Tian <i>et al.</i> (2004)
<i>Proteins</i>	↑ proteins	Banchuen <i>et al.</i> (2009); Lee <i>et al.</i> (2007); Moongngarm <i>et al.</i> (2010); Watchararpaiboon <i>et al.</i> (2010)
<i>Fats</i>	↑ fat	Banchuen <i>et al.</i> (2009); Lee <i>et al.</i> (2007); Watchararpaiboon <i>et al.</i> (2010)
<i>Vitamins</i>	↑alpha-tocopherol, ↑alpha-tocotrienol, ↑gamma-oryzanol, ↓gamma-tocopherol, ↓gamma-tocotrienol, ↑ thiamine	Britz <i>et al.</i> (2007); Jayadeep <i>et al.</i> (2011); Watchararpaiboon <i>et al.</i> (2010)
<i>Gamma- oryzanol</i>	↑ oryzanol	Imam <i>et al.</i> (2013a); Imam and Ismail (2013); Oh <i>et al.</i> (2010)
<i>Amylose content</i>	↑free sugars, ↓amylose	Banchuen <i>et al.</i> (2009); Jayadeep <i>et al.</i> (2011); Lee <i>et al.</i> (2012); Maisont and Narkrugsa (2010); Musa <i>et al.</i> (2011); Songtip <i>et al.</i> (2012); Xu <i>et al.</i> (2012)
<i>Minerals</i>	↑ magnesium and chloride	Ismail <i>et al.</i> (2014)

↑ = increased, ↓ = decreased. Germination of brown rice to reduce its hard texture also potentiate the bioactive composition of the rice grains, and the higher amounts of bioactives is believed to confer germinated brown rice with its enhanced bioactivity.

metabolome), in an attempt at regulating the underlying perturbations responsible for diseases not just metabolic changes that are a consequence. Hence, nutrigenomic studies underlying the health benefits of germinated brown rice have provided some mechanistic insights into its functional properties. Relevant studies are reviewed in Table 39.3.

39.3.1 Nutrigenomic Effects of Germinated Brown Rice on Obesity and Cholesterol Metabolism

Cardiovascular diseases (CVDs) remain the most significant cause of morbidity and mortality globally (Yach *et al.*, 2004). There have been advances in the management of CVDs, but the growing burden of the disease has prompted search for better alternatives to currently available drugs. In fact, many of the drugs used in the management of CVD cause serious side effects (Bellosta *et al.*, 2004). Close links have been unraveled between diet and the risk factors for CVD. Hence, dietary factors can promote cardiometabolic risk through hypercholesterolemia and increased oxidative stress, while diets with opposite effects will lower such risks (Mann, 2002). Germinated brown rice has been studied extensively for its cardioprotective effects, including its ability to reduce risk factors like dyslipidemia, excess plasminogen activator inhibitor-1, oxidized low density lipoprotein (LDL), and hypertension, mediated through its bioactives (Imam *et al.*, 2012a; Wu *et al.*, 2013). We have demonstrated that germinated brown rice lowered cholesterol and reduced the risk of CVD through regulation of hepatic cholesterol-related genes including LDL receptor (LDLR), apolipoprotein A (APOA), adiponectin, lipoprotein lipase (LPL), peroxisome proliferator-activated receptor gamma (PPAR γ), v-akt murine thymoma viral oncogene (Akt), and ATP-binding cassette

Table 39.3 Summary of Nutrigenomic mechanisms involved in germinated brown rice functional effects.

Bioactivity	Underlying nutrigenomic mechanism	Reference
Antiobesity	Suppression of fatty acid synthesis and fat deposition in adipose tissues via transcriptional regulation of adipogenic (C/EBP α , PPAR γ , and SREBP-1c), FAS, aP2, LPL, and inflammatory (IL6 and TNF) genes	Ho <i>et al.</i> (2012, 2013); Imam <i>et al.</i> (2013b)
Hypocholesterolemia	Reduction in total cholesterol and LDL and increase in HDL via transcriptional regulation of hepatic LDLR, adiponectin, LPL, PPAR γ , AKT and ABCA 1 and apolipoprotein A genes	Imam <i>et al.</i> (2013a, 2014)
Antihyperglycemia	Reduction in blood glucose through suppression of gluconeogenic (fbp and pck) genes	Imam and Ismail (2013)
Antioxidative	Increase in antioxidant status and reduced oxidative stress via transcriptional regulation of antioxidant (SOD, catalase and IGF2), and anti-apoptotic (AKT, NF-K β , ERK1/2, JNK, p53 tumor suppressor gene, and p38 MAPK) genes	Azmi <i>et al.</i> (2013); Imam <i>et al.</i> (2012b,c,d)
Activity against menopause-related problems	Transcriptional regulation of bone metabolism genes, including BMP-2, SPARC, RUNX-2, Osx, <i>periostin</i> , <i>Postn</i> , <i>Col1&2</i> , and <i>CGRP</i> . Upregulation of uterine expression of estrogen related genes (ER- β , CaBP9k, C3, HSP70, and IL4 receptor)	Muhammad <i>et al.</i> (2013a, b)

ABCA: ATP binding cassette; AKT: v-akt murine thymoma viral oncogene; aP2: adipocyte fatty acid-binding protein; BMP-2: *bone morphogenic protein-2*; C3: complement protein; CaBP9k: calcium-binding protein; CGRP: *calcitonin receptor gene*; Coll 1&2: *collagen 1&2*; C/EBP α : CCAAT/enhancer binding protein; ER- β : estrogen receptor-beta; ERK1/2: extracellular signal-regulated kinase 1/2; FAS: fatty acid synthase; HDL: high density lipoprotein; HSP70: heat shock protein 70 kDa; IL4: interleukin 4; IL6: interleukin 6; JNK: c-Jun N-terminal kinase; LDL: low density lipoprotein; LDLR: LDL receptor; LPL: lipoprotein lipase; NF-K β : nuclear factor beta; Osx: *osteoblast-specific transcription factor osterix*; p38 MAPK: mitogen activated protein kinase; Postn: *osteoblast specific factor*; PPAR γ : peroxisome proliferator-activated receptor γ ; RUNX-2: *runt-related transcription factor 2*; SPARC: *secreted protein acidic and rich in cysteine*; SREBP-1c: sterol regulatory element-binding protein-1c; SOD: superoxide dismutase.

(ABCA)1 (Imam *et al.*, 2013a; Imam *et al.*, 2014). Increased expression of hepatic APOA may underlie the increased high density lipoprotein (HDL) concentrations observed following germinated brown rice supplementation (Imam *et al.*, 2013a), while upregulation of hepatic adiponectin indicate an increased synthesis of adiponectin as the basis for the increased circulating adiponectin levels observed in rats (Torimitsu *et al.*, 2010). Adiponectin is known to improve metabolic indices and reduce the risk of cardiometabolic disease and complications (Declerq *et al.*, 2010; Rabe *et al.*, 2008). Additionally, LDLR is responsible for cholesterol uptake and clearance by the liver, and hence its upregulation in the liver partly explains the lower LDL levels observed due to germinated brown rice (Imam *et al.*, 2013a). Also, upregulation of hepatic Akt, and downregulation of hepatic LPL and PPAR γ are suggestive of cholesterol metabolism-enhancing abilities of germinated brown rice in the liver (Imam *et al.*, 2014). These changes possibly explain the improved cholesterol metabolism, including lower levels of total cholesterol and LDL, and higher levels of HDL, seen when germinated brown rice is consumed. These multiple mechanisms of cholesterol regulation by germinated brown rice indicate how effective it could be towards reducing hypercholesterolemia and eventually CVD risk. Overall, these effects indicate a potent cardioprotective recipe.

Furthermore, germinated brown rice has been reported severally to reduce weight, partly through promoting breakdown of fat as we have demonstrated and also through regulation of adipogenic genes (Ho *et al.*, 2012; Imam and Ismail, 2013; Imam *et al.*, 2014). Gluconeogenesis promotes breakdown of fat and protein sources for the production of glucose, and in diabetes, this process becomes hyperactive (Magnusson *et al.*, 1992). Germinated brown rice was shown to suppress gluconeogenesis and reduce weight irrespective of its effect on gluconeogenesis likely by promoting breakdown of fat (Imam and Ismail, 2013). Conversely, Ho *et al.* have reported that adipogenic (CCAAT/enhancer binding protein [C/EBP α], PPAR γ , and sterol regulatory element-binding protein-1c [SREBP-1c], fatty acid synthase [FAS], adipocyte fatty acid-binding protein [aP2], and LPL), and inflammatory genes (IL6 and TNF) are suppressed by germinated brown rice (Ho *et al.*, 2012, 2013). Changes in the adipose tissue that promote build-up of fat deposits are closely linked with inflammation (Wellen and Hotamisligil, 2003), which germinated brown rice was shown to suppress and eventually reduce obesity. Overall, the findings so far suggest that germinated brown rice reduces weight through multiple transcriptional

mechanisms that eventually promote fat catabolism and prevent fat deposition. Reduced rates of obesity are important among the low and middle income countries to lower risks of chronic disease, especially with sedentary lifestyles that promote cardiometabolic disease risks among these people becoming more common. In particular, risk of cardiometabolic disease is reported to be higher among Asian populations, where white rice is consumed enormously, even at lower body mass indices (Hu, 2011). Communicable diseases are still a problem and the rising burden of non-communicable diseases in these countries may be too much to bear. Germinated brown rice may be a healthier choice of staple for these countries.

39.3.2 Nutrigenomic Effects of Germinated Brown Rice on Oxidative Stress

Oxidative stress is implicated as an underlying factor in many chronic diseases and their complications (Aruoma, 1998). White rice promotes oxidative stress through glucose-induced toxic changes, while brown rice and germinated brown rice have antioxidant properties that are likely mediated through multiple mechanisms. High amounts of antioxidants including GABA, phenolics, ASG, and vitamins may explain the potent antioxidant effects of germinated brown rice. Several studies have shown high antioxidant potentials for germinated brown rice extracts, which contribute towards its effects against lipid peroxidation and other oxidative changes (Imam *et al.*, 2012a). Usuki *et al.* demonstrated that germinated brown rice reduced oxidative stress in type 2 diabetes through induction of insulin-like growth factor 1 (Usuki *et al.*, 2011). In addition, we have shown that upregulation of hepatic antioxidant genes including catalase and superoxide dismutase (SOD) by germinated brown rice bioactives may underlie its antioxidant effects (Imam *et al.*, 2012b; Imam *et al.*, 2012c; Imam *et al.*, 2012d). In particular, germinated brown rice was shown to upregulate SOD2, which is strongly associated with antioxidant and antiapoptotic effects (Kannan and Jain, 2000). Similarly, germinated brown rice was able to reduce oxidative stress in neuronal cells via transcriptional regulation of antioxidant (SOD and catalase) and apoptosis (AKT, nuclear factor kappa beta [NF- κ B], extracellular signal-regulated kinase [ERK] 1/2, c-Jun N-terminal kinase [JNK], p53 tumor suppressor gene, and p38 mitogen activated protein kinase [MAPK]) genes. Apoptosis is often the end result of oxidative stress, and may underlie oxidative stress complications. Regulation of both processes indicates that germinated brown rice may be useful in diseases where oxidative stress-induced apoptosis is a problem, including neurodegenerative and cardiometabolic diseases (Chandra *et al.*, 2000; Kannan and Jain, 2000).

Furthermore, the reduced oxidation of LDL by germinated brown rice may greatly contribute towards reduced risk of CVD, as we have demonstrated (Imam *et al.*, 2014). Plasma F2-isoprostanes, important markers of oxidative stress with significant clinical implications, were equally suppressed by germinated brown rice. Elevated levels of F2-isoprostanes and oxidized LDL (ox-LDL) are closely related, and reflect the high levels of oxidative stress and increased risk of cardiometabolic diseases, while reduced levels can greatly lower such risks (Berliner and Heinecke, 1996; Moore *et al.*, 1995). Overall, oxidative stress promotes damage through reactive free radical species, which can be scavenged by antioxidants. When left unchecked, oxidative stress through excess free radicals may eventually lead to disease conditions and complications (Aruoma, 1998). The strong antioxidant effects of germinated brown rice demonstrated by its ability to transcriptionally regulate oxidative stress-related genes, therefore, suggest it could reduce the risk of oxidative stress-related chronic diseases including CVD, diabetes, and neurodegenerative diseases.

39.3.3 Nutrigenomic Effects of Germinated Brown Rice on Glycemic Control

White rice produces excess postprandial glycemia, and can increase risk of cardiometabolic diseases and complications (Ludwig, 2002; Miller *et al.*, 1992). Brown rice and germinated brown rice, however, through their many bioactive compounds could produce lower postprandial glycemia mediated locally in the gut (dietary fiber), and systemically via regulation of glucose metabolism pathways (GABA, oryzanol, ASG, and phenolics) (Imam *et al.*, 2012a; Imam and Ismail, 2013). Reports of the glucose-lowering effects of germinated brown rice have been available for some time now, but the nutrigenomic mechanisms underlying such effects were not known until recently.

We have demonstrated that germinated brown rice through the effects of its bioactive compounds on key gluconeogenic genes (fructose-1,6-bisphosphatase and phosphoenolpyruvate carboxykinase) can regulate plasma glucose in type 2 diabetes (Imam and Ismail, 2013). As can be recalled, gluconeogenesis is activated in diabetes to produce more glucose because the cells sense low glucose levels because of insulin resistance (Magnusson *et al.*, 1992). Gluconeogenesis is generally a challenging process to regulate in diabetes because of the nature of its regulation. In our study, the transcriptional level of gluconeogenic genes due to germinated brown rice was similar to what was observed for metformin, a standard drug used to manage glycemia in diabetes, but glycemic control due to germinated brown rice was better. This was indicative of the multiple mechanisms of action of germinated brown rice towards reduction of glycemia; in addition

to what was observed in the study, lower glycemia may have been contributed by dietary fiber and high amylose content of germinated brown rice, with resultant lower insulin response, while other transcriptional and non-transcriptional mechanisms not reported could have contributed to plasma glucose reduction (Imam *et al.*, 2012a). In the same study, brown rice produced similar glycemic control as metformin, suggesting that the higher amounts of bioactives in germinated brown rice contributed towards better activity.

39.3.4 Nutrigenomic Effects of Germinated Brown Rice on Menopause-Related Problems

There are an increasing number of women that reach menopause due to better living standards and improved healthcare delivery. With increased longevity, there is need to improve quality of life of these women, especially since hormonal imbalances cause not only physical but also emotional and psychological disturbances in addition to increased risk of diseases (Barrett-Connor, 1992). Additionally, other life stresses including bereavement, loneliness, depression and anxiety may complicate the lives of these women. Hormone replacement therapy (HRT) has been used for some of these menopausal problems. However, HRT has not been effective at managing all menopause-associated problems, and may even increase the risks of CVD, cancers and other diseases (Beral *et al.*, 2002). These issues necessitate search for newer alternatives, and germinated brown rice has shown promise in this regard. In the past, germinated brown rice was reported to improve memory and cognition, possibly because of its rich GABA content (Mamiya *et al.*, 2004; Mamiya *et al.*, 2007; Zhang *et al.*, 2010). GABA is an inhibitory neurotransmitter that may elicit anti-depressant and anti-anxiolytic effects (Kalueff and Nutt, 2007), and may produce similar properties due germinated brown rice, although it is likely that other bioactive compounds play a role in these effects.

Germinated brown rice was able to regulate uterine expression of estrogen-related genes (estrogen receptor-beta (ER- β), calcium-binding protein (CaBP9k), complement protein (C3), heat shock protein 70 kDa (HSP70), and interleukin (IL)-4 receptor), suggesting that it may have some estrogen-like activity on the uterus. Moreover, increase in glandular and luminal epithelial cells of the uterine and vaginal wall suggested that germinated brown rice may prevent against vaginal dryness, atrophy, and discomfort. Also, osteoporosis is a serious problem in menopause, and germinated brown rice was shown to up-regulate genes involved in bone formation in an osteoporosis animal model. It was able to transcriptionally regulate bone metabolism genes in ovariectomized animal model, including bone morphogenic protein-2 (BMP-2), secreted protein acidic and rich in cysteine (SPARC), runt-related transcription factor 2 (RUNX-2), osteoblast-specific transcription factor osterix (Osx), periostin, osteoblast specific factor (Postn), collagen 1&2 (Col1&2), and calcitonin gene-related peptide (CGRP) (Muhammad *et al.*, 2013a; Muhammad *et al.*, 2013b).

The absence of any documented side effects due to germinated brown rice indicates that it could be used to improve menopausal problems by women without risking complications like cancers and CVD, as reported for HRT. Also, regulation of cardiometabolic risk by germinated brown rice is beneficial for menopausal women since they are at risk of such diseases due to loss of estrogen (Carr, 2003; Rosano *et al.*, 2007). The implications of these observations for menopausal women are potentially enormous and may reverse some of the problems associated with menopause due to estrogen deficiency.

In aggregate, germinated brown rice bioactives mediate its effects by regulating transcription of genes and their products in various disease processes. The resultant improvement in metabolic indices through multiple mechanisms may be beneficial in many disease processes. Also, synergism of the bioactives in germinated brown rice likely mediates its functional effects, in agreement with the concept of food synergy; the effects of the germinated brown rice as a whole may be contributed by the effects of the individual compounds as well as their arrangement in the germinated brown rice matrix (Jacobs and Tapsell, 2007; Jacobs *et al.*, 2009). Hence, the overall effect of the food may not be explained by the effect of only one of its bioactives. This is in agreement with our findings on the effects of germinated brown rice bioactives on the expression of PPAR γ ; individual bioactive compounds upregulated the expression of the gene, while in combination they downregulated its expression (Imam *et al.*, 2013b). These multiple mechanisms underlying the effects of germinated brown rice may mean better metabolic outcomes when compared with single bioactive compounds or even pharmacological agents. Germinated brown rice could, therefore, serve as an efficient alternative to white rice, and as an adjuvant for the management of many chronic diseases especially when consumed on a long term to provide continuous supply of bioactives. Consumption of germinated brown rice instead of white rice in rice-consuming countries may reduce the overall burden of disease in view of the health benefits of germinated brown rice documented thus far and the risks of cardiometabolic diseases due to white rice consumption. There is need for more long term studies and policy changes in rice-consuming countries in order to provide the health benefits of germinated brown rice to as many people. Importantly, very little resistance will be expected from these populations since white rice is already their staple food.

39.4 Conclusions

Germinated brown rice is a health food developed as a consequence of trying to improve the hard texture of brown rice and became a healthier substitute for white rice. Its enhanced bioactive composition has been shown to produce better functional effects than brown rice and white rice, including antihyperglycemic, hypocholesterolemic, and antioxidant effects. Nutrigenomic studies have provided insights into how these properties are mediated by germinated brown rice bioactives likely through food synergy. Notably, transcriptional regulation of genes related to gluconeogenesis, cholesterol metabolism, oxidative stress and apoptosis are linked to its effects towards cardiometabolic diseases, while modulation of bone metabolism and uterine genes underlie its effects in menopause.

39.5 Future Considerations

Thus far, a lot has been documented regarding the nutrigenomic implications of germinated brown rice. However, these findings have been reported using *in vitro* or animal models. Already, clinical trials on germinated brown rice have been reported, which showed that germinated brown rice is able to improve glycemic control and hypercholesterolemia (Imam *et al.*, 2012a). These findings from human studies suggest that nutrigenomic insights from preclinical studies may be the underlying mechanisms for germinated brown rice bioactivity in humans. Furthermore, mechanistic basis into bioactivity of germinated brown rice have mostly dwelled on transcriptional regulation of genes (transcriptomics), and not much has been reported on translational (proteomics) and metabolomic changes it induces. There is a need for post-transcriptional studies to map out the entire mechanisms involved in germinated brown rice bioactivity. Nutrikinetic and nutridynamic studies together with those on metabolomic fingerprinting and profiling (Cevallos-Cevallos *et al.*, 2009; van Duynhoven *et al.*, 2012) could provide insights into the fate of germinated brown rice bioactives and whether the bioactives or their metabolites are responsible for germinated brown rice bioactivity. Such information may pave way for development of targeted health products, using germinated brown rice as functional food ingredient, with specific functional properties.

White rice is consumed by half the world's population of mostly low and middle income countries, and is linked to cardiometabolic risks. It is likely that white rice consumption is contributing to the growing burden of these problems and especially diabetes in countries where it is consumed as a staple food. There are reports of the implications of maternal and paternal lifestyle factors, including diet on the risk of disease in their offspring, and white rice may be contributing in this way to the rising trend in metabolic disease incidents (Ling and Groof, 2009). Future studies are needed to evaluate any links between white rice consumption and epigenetics events with transgenerational implications on disease risk.

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Conflict of Interest

None.

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Novel Chromium (III) Supplements and Nutrigenomics Exploration: A Review

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40.1 Introduction

Type 2 diabetes mellitus is growing at epidemic proportions in the society. The number of global cases of type 2 diabetes is projected to reach 350 million by the year 2030 (Ogden *et al.* 2006). Although this increase in prevalence of diabetes has been often attributed to the increase in obesity in the population and our sedentary lifestyle, diabetes is a multifactorial disease, influenced by diverse factors that include our diet/nutrition and our genetic susceptibility. Consequently, varying the diet/nutrition may affect genetic susceptibility and can alter disease progression. In this regard, recent findings in the area of genomics and epigenetics provide intriguing examples by which dietary components can influence gene expression in a variety of diseases.

Insulin resistance, defined as an impaired responsiveness of the body to insulin is a prediabetic condition that is observed long before the development of full-blown diabetes mellitus and offers an attractive avenue for intervention to delay the onset of diabetes (Muonio and Newgard 2008). Recent data from the National Institute of Diabetes and Digestive and Kidney Diseases, the National Institutes of Health has indicated that the prevalence of insulin resistance among adults 20 years or older was in the range of 35%, whereas about 50% of those aged 65 or older are afflicted with the syndrome. This amounts to an estimated 79 million of Americans aged 20 or above having the prediabetic condition. Early identification and treatment of prediabetes therefore offers an avenue to delay the progression of diabetes and related cardiovascular disease (Gillies *et al.* 2007). Although dietary modulations and exercise have proven to be of value in combating insulin resistance or delaying the development of full blown diabetes, these measures are difficult to achieve. Pharmacological agents that augment insulin sensitivity are limited and the use of those that are available such as the glitazones and biguanides are marred with several adverse complications. In this context, the use of nutritional supplements and micro-nutrients has been extensively investigated as potential approaches to improve insulin sensitivity and delay the progression to type 2 diabetes mellitus. Among nutritional supplements, trivalent chromium (III) has received attention based on its ability to improve cardiometabolic symptoms.

40.2 Trivalent Chromium, Insulin Regulation, and Signaling

Trivalent chromium (III) has been long known to improve carbohydrate and glucose metabolism and therefore was believed to be “essential” (Freund *et al.* 1979; Jeejeebhoy *et al.* 1977; Mertz and Schwarz 1959). Although the “essential” role of chromium (III) in human health has been questioned (Landman *et al.* 2014; Vincent 2013), both clinical and preclinical studies have demonstrated that the supplementation of chromium (III) has beneficial effects in subjects with diabetes and cardiovascular disease, and suggested in several recent reviews (Hummel *et al.* 2007; Lau *et al.* 2008; Suksomboon *et al.* 2014; Vincent 2010; Wang and Cefalu 2010). In contrast to human studies on diabetic subjects (which is affected by dose restriction, comorbidities, concurrent use of antidiabetic medications, and several other variables), animal studies using both genetic and nutritional models of diabetes have consistently demonstrated a beneficial effect of chromium (III) in alleviating insulin resistance, diabetes, and lipid anomalies. However, the molecular basis of the beneficial effects of chromium (III) is yet unclear.

As inorganic chromium (III) is poorly bioavailable, low molecular weight organic chromium (III) complexes have been synthesized, characterized, and studied for insulin-potentiating activity. The most popular chromium (III) compound available in the market is chromium (III) picolinate, which is available as an independent chromium (III) supplement and also as a component of multivitamins and energy drinks. Concerns about the potential toxicities of the picolinate ligand in chromium (III) picolinate (Bagchi *et al.* 2002; Hepburn *et al.* 2003), prompted us to synthesize and characterize a novel low-molecular weight chromium (III) complex, chromium (III)-D-phenylalanine (Yang *et al.* 2005). We argued that the phenylalanine ligand would be more bio-friendly and the D-isomer would delay metabolism as compared to the L-isomer. Our studies demonstrated that this novel complex was capable of upregulating insulin-stimulated insulin signal transduction via affecting effector molecules downstream of the insulin receptor, as evidenced by enhanced levels of tyrosine phosphorylation of insulin receptor substrate-1 and the phosphorylation of Akt (Tyr-308 and Ser-473), and elevation in the enzyme activity of phosphoinositide 3-kinase both in cultured cells (adipocytes and hepatic cells) and in the liver and muscle of genetic and nutrition rodent models of insulin resistance (Dong *et al.* 2008; Kandadi *et al.* 2011; Hua *et al.* 2012; Sreejayan *et al.* 2008; Yang *et al.* 2005, 2006). Interestingly, in addition to augmenting the effectors that propagate insulin signals, chromium (III) treatment augmented the effectors that negatively regulate insulin signaling. For instance, the phosphorylation of JNK, which was elevated under insulin resistant conditions, was attenuated by chromium (III) (Kandadi *et al.* 2011). Similarly, phosphorylation of serine-307 residue on the insulin receptor substrate which has been shown to attenuate insulin signaling (Aguirre *et al.* 2000) and accelerate the degradation of the insulin receptor substrate-1 protein (Sykiotis and Papavassiliou 2001), was significantly attenuated by chromium (III) both *in vivo* and *in vitro* (Sreejayan *et al.* 2008). The functional consequence of augmented insulin signaling is elevated muscle glucose uptake, which is mediated by the muscle glucose transporter GLUT-4. Interestingly, our studies also showed that chromium (III) improved GLUT-4 translocation to the muscle under insulin resistant condition and consequent upregulation in glucose uptake in skeletal muscle of obese mice (Sreejayan *et al.* 2008). Mechanistic studies into these effects revealed that chromium (III) treatment attenuated endoplasmic reticulum stress in the liver of obese mice. Emerging evidence suggests that endoplasmic stress plays a pivotal role in the development of insulin resistance and may represent a unifying mechanism in the pathophysiology of insulin resistance and Type 2 diabetes (Nakatani *et al.* 2005).

40.3 Regulatory Pathways

Despite the aforementioned effects of chromium (III) on insulin signaling, inhibitors of the insulin signaling pathway failed to fully block the potentiation of insulin-stimulated glucose uptake caused by insulin. Furthermore, because chromium (III) exhibited a transient effect on cellular glucose uptake we were interested in studying the effects of chromium (III) supplementation on adenosine-monophosphate-activated protein kinase (AMPK), which has gained attention for its regulatory role in cellular energy homeostasis (Hardie *et al.* 2003; Kemp *et al.* 2003). AMPK, which is activated by elevated cellular AMP levels, has been shown to regulate fatty acid oxidation in the heart and skeletal muscle and has emerged as an important regulator of glucose metabolism (Rutter *et al.* 2003). Our studies revealed that chromium (III) stimulates the phosphorylation of the α -catalytic subunit of AMPK at Thr-172, as well as the downstream targets of AMPK, including acetyl-CoA carboxylase (at Ser-212) and eNOS (at Ser-1177) (Zhao *et al.* 2009). Additionally, both the glucose uptake and AMPK phosphorylation mediated by chromium (III) were inhibited by the AMPK inhibitor compound C. It is noteworthy that, whereas both wortmannin (PI3K inhibitor) and compound C attenuated chromium (III)-induced glucose uptake in cardiomyocytes, neither of these compounds by themselves blocked the glucose uptake, suggesting the possibility of synergistic effects of both these pathways.

Based on our studies with D- chromium (III) phenylalaninate, and because brewer's yeast (from which the chromium (III)-based "glucose-tolerance factor" was first identified) contained a variety of amino acids associated with chromium (III) (Urumow and Wieland 1984), we designed, synthesized, and characterized a number of novel chromium (III) complexes (Figure 40.1). Among these novel molecules, chromium (III) dinicotocysteinate was found to be most efficacious in augmenting insulin signaling (unpublished studies). This compound was subsequently tested for its safety (Sreejayan *et al.* 2010) following which Jain and coworkers demonstrated that this novel molecule lowered blood glucose and inflammatory markers and elevated adiponectin levels in Zucker diabetic fatty rats (Jain *et al.* 2011). Recent randomized, double blind, placebo controlled human studies revealed that chromium (III) dinicotocysteinate supplementation lowers insulin resistance by reducing tumor necrosis factor- α , insulin, and oxidative stress levels in diabetic subjects (Jain *et al.* 2012), based on which this molecule was eventually commercialized.

Despite the aforementioned beneficial effects of chromium (III), and despite the strong association of a genetic component in the pathophysiology of diabetes mellitus, few studies have investigated in depth the effect of chromium (III) at the genetic level. Rink and coworkers used microarray studies to assess the gene expression associated with treating niacin-bound chromium (III) to type 2 *Lep^r db* obese diabetic mice (Rink *et al.* 2006). These studies further validated the DNA microarray data by carrying out real-time PCR analysis of the candidate genes. Of note is that of the over 45,000 genes studied in this genome-wide analysis, only the expression of very few genes was altered by chromium (III) supplementation, suggesting a rather specific role of chromium (III) in modulating gene expression. These studies revealed that treatment of diabetic mice with niacin-bound chromium (III) resulted in the upregulation of genes encoding proteins involved in glycolysis, muscle contraction, muscle metabolism, and muscle development were upregulated in the mice receiving the chromium (III) supplement. Specifically, supplementation with chromium (III) upregulated the myogenic genes including calsequestrin, a calcium mediator for muscle contraction; tropomyosin-1, a myosin regulator; and key enzymes necessary for glycolysis, such as enolase and glucose phosphate isomerase-1. Calsequestrin is an abundant calcium-binding protein found in the sarcoplasmic reticulum of skeletal and cardiac muscle. It sequesters calcium in the endoplasmic reticulum and provides a depot for calcium, which is made available to the cells during contraction. Our earlier studies had demonstrated that chromium (III) D-phenylalanine alleviates obesity-associated cardiac contractile defects in obese mice (Dong *et al.* 2007). In these studies, we showed that myocytes obtained from obese mice exhibited depressed peak shortening, reduced maximal velocity of shortening/relengthening, prolonged time-to-peak shortening and time-to-90% relengthening (TR90), reduced electrically stimulated rise in intracellular calcium and slower intracellular calcium decay. Interestingly, treatment of chromium (III) rectified reduced maximum velocity of shortening/relengthening and all mechanical and intracellular calcium anomalies. What was all the more interesting, especially in the context of the aforementioned studies by Rink and coworkers, is that the sarco(endo)plasmic reticulum Ca(2+) ATPase activity and Na(+)–Ca(2+) exchanger expression, which was depressed in obese mice, was reversed by chromium (III) treatment. These studies suggest that chromium (III) may not only be altering calsequestrin in the fat tissue, it may also be affecting calcium ATPase activity and calcium exchanger in the heart muscle by altering the expression of these genes. The increase in tropomyosin-1 gene in response to chromium (III) further suggests that chromium (III) treatment modulates intracellular calcium levels.

The enzymes glucose phosphate isomerase and enolase 3 are involved in glycolysis and have been reported to be downregulated in adipose tissues of obese subjects (Baranova *et al.* 2005). The upregulation of these key genes involved in the glycolytic pathway by chromium (III) further sheds light on the regulatory role of chromium (III) on glucose metabolism. Recent studies show chromium (III) supplementation normalized glycogen content in liver of diabetic mice, which was accompanied by an increase in activity of hepatic glycolytic enzymes including glucokinase, phosphofructokinase, and pyruvate kinase, and a suppression of the activity of gluconeogenic enzymes such as glucose-6-phosphatase and phosphoenolpyruvate carboxykinase (Sundaram *et al.* 2012). Collectively these studies provide credence to the notion that supplemental chromium (III) may be mediating some of its beneficial effects by altering the metabolism of glucose.

It is of note that Rink and coworkers demonstrated the upregulation of myogenic genes in fat tissue (Rink *et al.* 2006), suggesting that this would consequently result in reduced fat content of the tissue over time. Although this is an interesting hypothesis no studies have been performed to assess whether chromium (III) triggered a myogenic response in fat cells. In addition to upregulating muscle specific genes in adipocytes, the niacin-bound chromium (III) treatment resulted in the downregulation of cell death-inducing DNA fragmentation factor (CIDEA) and uncoupling protein-1 (UCP1) in the adipose tissues. These adipose tissue proteins represent key component involved in the thermogenic role of brown adipose tissue. CIDEA is widely expressed in brown adipose tissue (Lin and Li 2004) and genetic deficiency of this protein results in a lean phenotype in mice that is resistant to diet-induced obesity and diabetes (Zhou *et al.* 2003). Furthermore knockout of this protein increases the metabolic rate and lipolysis in brown adipose tissue suggesting a critical role for this protein

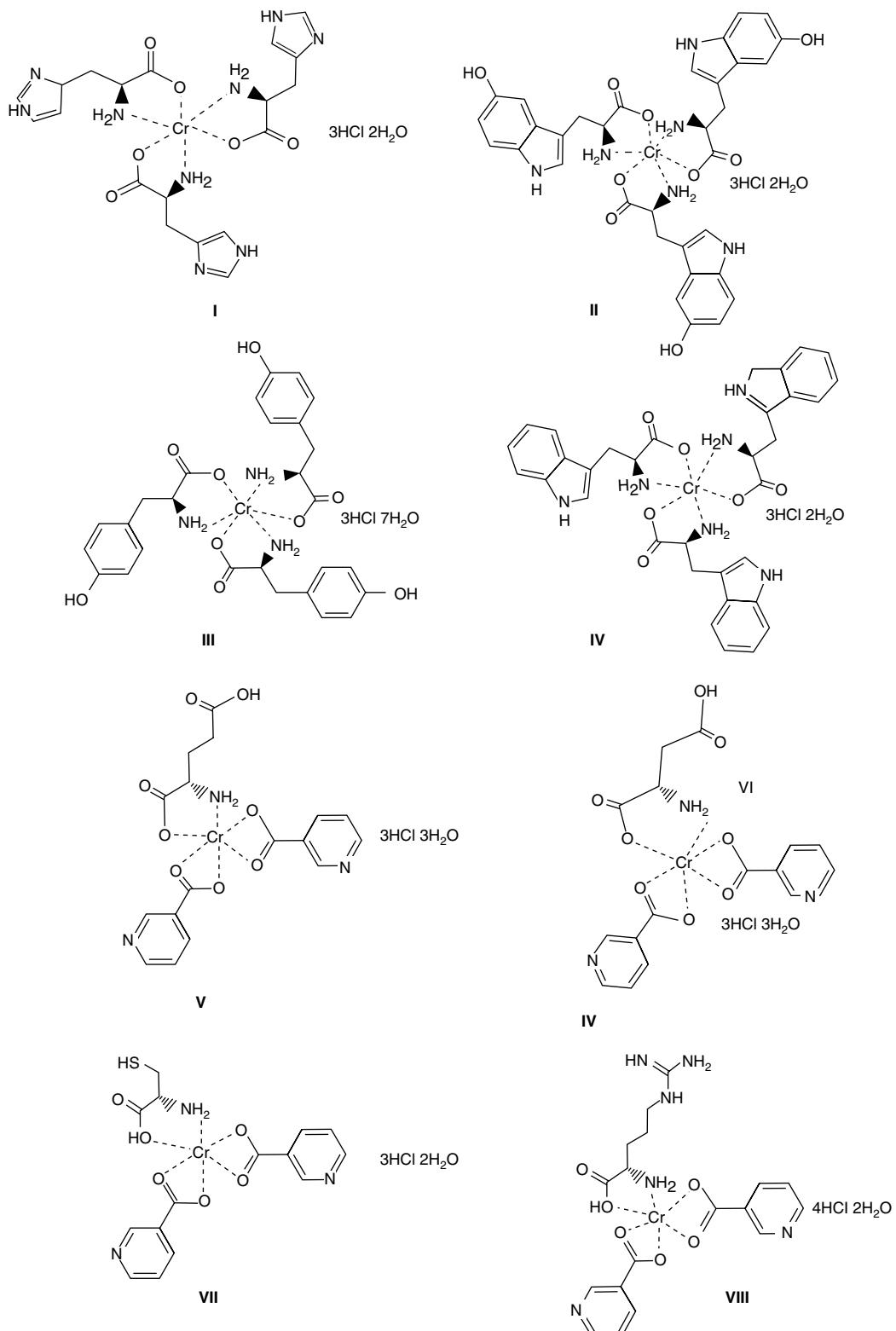


Figure 40.1 Novel low-molecular weight chromium (III) amino acid complexes. I. Chromium (III) histidinate II Chromium (III) hydroxytryptophanate III. Chromium (III) tyrosinate IV. Chromium (III) tryptophanate V. Chromium (III) dinitocinoglutamate VI. Chromium (III) dinitocinoaspartate VII. Chromium (III) dinitocotinoaspartate VIII. Chromium (III) dinitocotinocysteinate.

in energy modulation and adiposity (Zhou *et al.* 2003). Brown adipose tissue containing a large number of mitochondria and expressing UCP1 is metabolically active through dissipating energy as heat in cold temperatures (Cinti 2002; Keipert and Jastroch 2014). The upregulation of UCP1 by chromium (III) may further explain the beneficial profile of chromium (III) in regulating the energy balance in the cell. On a related note, Qiao and coworkers studied the effect of chromium (III) complexes on cultured skeletal muscles and found that while these complexes increased glucose uptake, they also increased the levels of uncoupling protein-3 in the skeletal muscles (Qiao *et al.* 2009).

40.4 MicroRNAs

In addition to genes, recent studies have suggested that chromium (III) can alter microRNAs (miRNAs) as well. miRNAs are small noncoding RNAs that bind to complementary 3'UTR regions of target mRNAs, causing the degradation of transcriptional repression of the target. The regulatory role of miRNAs in carbohydrate and lipid metabolism has been an area of recent interest (Maegdefessel 2014). Interestingly, recent studies show that miRNAs are potential therapeutic targets in the induction of brown adipocyte lineage differentiation from myoblast and white adipose tissue (Zhou and Li 2014). In a recent study, Zhang and coworkers showed that the TianMai Xiaoke tablet, a key ingredient of which is chromium (III) picolinate, can alter a variety of miRNAs in diabetic subjects (Zhang *et al.* 2014). Of these upregulated miRNAs, noteworthy are miR-375 and miR30d, which has been shown previously to play a pivotal role in stimulating insulin secretion by the islets (Poy *et al.* 2004; Tang *et al.* 2009).

Finally, there is a growing body of literature to suggesting a role of epigenetic factors linking genes and environmental factors in the pathogenesis of diabetes mellitus. Although no direct studies have reported a causal role of chromium (III) in modulating the epigenetics two studies suggest such possibility. Padmavathi and coworkers investigated the impact of maternal chromium (III) restriction on WNIN/GR-Ob rats, a mutant obese rat strain with impaired glucose tolerance (Padmavathi *et al.* 2011). These authors found that maternal chromium (III) restriction increased insulin resistance in the offspring as evidenced by increased fasting plasma glucose, fasting insulin, and homeostasis model assessment of insulin resistance. Previous studies by the same group (Padmavathi *et al.* 2010) reported that chronic maternal chromium (III) restriction increased adiposity and impairment in lipid metabolism in offspring. Taken together, these studies suggest that deficiency of chromium (III) may have role in affecting epigenetic alterations associated with the pathology of diabetes mellitus.

40.5 Summary and Conclusions

In summary, the investigation into the genetic alterations by trivalent chromium (III) in the context of the pathophysiology of diabetes mellitus is in its infancy. Further detailed genome-wide studies, microRNA analysis and epigenetic studies will help in identifying the mechanistic basis of chromium (III)'s reported benefits in cardiometabolic diseases. Such understanding will further aid in the design and development of novel strategies to counter or control insulin resistance and diabetes.

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Part VIII

Transcriptomics

41

Transcriptomics of Plants Interacting with Pathogens and Beneficial Microbes

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41.1 Introduction

Plants are continually engaged in complex interactions with beneficial and detrimental microorganisms. They live in dynamic environments and are subject to frequent pathogenic and environmental stresses. Several diverse, self-defense pathways have evolved in plants to allow them to cope with these stresses. Some of these pathways are well studied in model organisms such as *Arabidopsis thaliana*, but defense signaling in other plants and interactions (cross-talk) between these pathways and their prioritization are currently not well understood. These pathways have often been considered autonomous or linear with a few well-studied genes. However, most likely pathways rather present highways within complex networks, where transcriptional changes of nearly every gene are deployed by several pathways. Interestingly, plants interacting with beneficial microbes show very few transcriptional responses (Zamioudis and Pieterse 2012).

Since the emergence of agriculture approximately 10,000 years ago, farmers have grappled with many pests and pathogens causing poverty, starvation, and social upheaval (Bebber *et al.* 2013; Flood 2010; Oerke 2006; Strange and Scott 2005). It is estimated that 30–40% of crop yields are lost annually, while from this estimation, 10–16 % of crop production is destroyed by pest and pathogens (Chakraborty and Newton 2011; Flood 2010; Oerke 2006). In 2007, the United States Environmental Protection Agency (EPA) reported that more than \$39.4 billion were spent globally on pesticides (approx. 2.4 million tons), including mostly herbicides, fungicides, and insecticides (Grube *et al.* 2011). Agrochemicals may also kill beneficial microbes (e.g., beneficial endophytic fungi) and remain as residues in almost 40% of foods, according to FAO-WHO reports (Maksimov *et al.* 2011). Concerned by negative effects and environmental issues of synthetic fertilizers and pesticides, scientists have been trying to find alternative ways to facilitate plant growth in agriculture, horticulture, and silviculture (Glick *et al.* 2007). Among these alternatives, biological control agents could be more economically and environmentally friendly strategies for protecting plants and crops against diseases in the long run. However, these require intricate knowledge of plant-microbe interactions and will often fail if plants are already under high disease pressure. Instead these biocontrol agents should be used as a preventative measure or at a stage when pathogens are still relatively low in numbers. Thorough investigations of the currently poorly-characterized soil microbial communities associated

with plants, especially beneficial microbes in the rhizosphere, will generate knowledge that should contribute towards growing healthy plants (Barea *et al.* 2005b).

There is a real interest in using environmentally friendly techniques in agriculture to achieve high quality and quantity of agricultural products. For this purpose, the application of beneficial microorganisms is an important alternative method (Russo *et al.* 2012). The replacement of synthetic chemicals with beneficial microbes to reduce negative effects on the environment previously has been proved (Dobbelaeere *et al.* 2003; Russo *et al.* 2012). A combination with traditional chemical pesticides could be most effective, where beneficial microorganisms can be used for plant growth promotion, as a preventative measure against pathogens, or when disease is still relatively low, while chemical pesticides may still need to be used when pathogens overpower the plants. In this chapter, we will provide an overview of interactions of plants with pathogens and beneficial microbes and provide examples how rapid transcriptional responses in the plant can lead to successful plant defense or mutual benefits with microorganisms.

41.2 Plant Defense Responses against Pathogens

Plants are used as nutrient sources by a lot of different pathogens, including bacteria, fungi, oomycetes, viruses, and parasitic plants (Wirthmueller *et al.* 2013). Recent reviews have highlighted the most important fungal, bacterial, and viral diseases (Dean *et al.*, 2012; Mansfield *et al.*, 2012; Scholthof *et al.* 2011, respectively). In these surveys, 10 pathogens from each class were identified as important diseases on the base of scientific and economic importance (Table 41.1).

On the basis of pathogenic life style and source of energy, pathogens are classified as biotrophs, necrotrophs, and hemibiotrophs. Biotrophs derive nutrients from the host's living tissues while necrotrophic pathogens kill the host tissue and feed on contents of dead cells. Hemibiotrophs typically start as a biotroph but then switch to a necrotrophic life style at a later stage (Ottmann *et al.* 2009). Plant pathogens from various species secrete structurally unrelated effectors into the plants' apoplast and cytoplasm to develop infection and to suppress the plants' immune systems. Many necrotrophic pathogens employ cell wall-damaging enzymes and a wide range of host-selective or non-selective toxins, which cause cell death (Gijzen and Nürnberger 2006; Ottmann *et al.* 2009; van't Slot and Knogge 2002; Wirthmueller *et al.* 2013; Wolpert *et al.* 2002). Biotrophs, on the other hand, often remain undetected by the plant's immune system.

In response to biotic attacks, plants have their own mechanisms of defense; such as mechanical, biological, chemical, and indirect ways leading to enhanced plant resistance. In general, plant defense mechanisms are divided into two groups: constitutive and inducible defense (Wittstock and Gershenzon 2002). Constitutive (pre-formed) defenses are present in plants before being attacked by pathogens. These include physical barriers, like wax, cuticle, cell walls, stomata, and lenticels. Furthermore, plants produce toxic chemicals, such as secondary metabolites including alkaloids and terpenoids (Guest and Brown 1997; Wittstock and Gershenzon 2002). Cell walls provide a physical barrier in early stages of a pathogen attack (Vorwerk *et al.* 2004) and contain antimicrobial proteins and secondary metabolites (Darvill and Albersheim 1984; De Lorenzo and Ferrari 2002; Thomma *et al.* 2002).

The activation of plant defense mechanisms has been well-studied. In response to being attacked by a wide variety of microbial pathogens and pests, plants induce numerous defense mechanisms (Glazebrook 2005; Odjakova and Hadjiivanova 2001). For example, pathogens may secrete glycohydrolases to destroy the plant cell wall. Soon after that, the degraded

Table 41.1 The top 10 most important fungal, bacterial, and viral plant pathogens.

Fungal pathogens	Bacterial pathogens	Viral pathogens
1. <i>Magnaporthe oryzae</i>	1. <i>Pseudomonas syringae</i>	1. <i>Tobacco mosaic virus</i>
2. <i>Botrytis cinerea</i>	2. <i>Ralstonia solanacearum</i>	2. <i>Tomato spotted wilt virus</i>
3. <i>Puccinia</i> spp.	3. <i>Agrobacterium tumefaciens</i>	3. <i>Tomato yellow leaf curl virus</i>
4. <i>Fusarium graminearum</i>	4. <i>Xanthomonas oryzae</i>	4. <i>Cucumber mosaic virus</i>
5. <i>Fusarium oxysporum</i>	5. <i>Xanthomonas campestris</i>	5. <i>Potato virus Y</i>
6. <i>Blumeria graminis</i>	6. <i>Xanthomonas axonopodis</i>	6. <i>Cauliflower mosaic virus</i>
7. <i>Mycosphaerella graminicola</i>	7. <i>Erwinia amylovora</i>	7. <i>African cassava mosaic virus</i>
8. <i>Colletotrichum</i> spp.	8. <i>Xylella fastidiosa</i>	8. <i>Plum pox virus</i>
9. <i>Ustilago maydis</i>	9. <i>Dickeya (dadantii and solani)</i>	9. <i>Brome mosaic virus</i>
10. <i>Melampsora lini</i>	10. <i>Pectobacterium carotovorum</i>	10. <i>Potato virus X</i>

products can be detected by cell surface-resident danger-associated molecular pattern (DAMP) receptors (Wirthmueller *et al.* 2013). In addition, plants are able to recognize evolutionarily conserved pathogen molecules, such as bacterial flagellins and fungal chitins, collectively named pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs), by the innate immune system that is equipped with plant pattern recognition receptors (PRRs). Identification of PAMPs or DAMP by PRRs activates PAMP-triggered immunity (PTI), which is an extracellular recognition defense mechanism (Dodds and Rathjen 2010). PTI protects plants against non-host pathogens and inhibits disease caused by virulent pathogens (Ponce de León and Montesano 2013).

At the intracellular level, plant resistance (R) genes and related proteins such as nucleotide-binding (NB) proteins and nucleotide-binding and leucine rich repeat (NB-LRR) domain-containing proteins distinguish pathogenic effector proteins (now referred to as avirulence (Avr) proteins) directly or indirectly. This can lead to effector-triggered immunity (ETI), which is highly specific and strong to biotrophs and hemibiotrophic pathogens (Boyd *et al.* 2013; Dodds and Rathjen 2010; Glazebrook 2005; Jones and Dangl 2006; Ponce de León and Montesano 2013). PTI and ETI gene expression patterns and responses are relatively similar while their intensities are different (Dodds and Rathjen 2010). A number of cellular events associated with both PTI and ETI are supplemented by downstream responses like rapid influx of calcium ions, reactive oxygen species (ROS) production, activation of mitogen-activated protein kinases (MAPKs), reprogramming of gene expression, cell wall reinforcement and hypersensitive response (HR) (Dodds and Rathjen, 2010). They also have an effect on signaling pathways, mediated by salicylic acid (SA), jasmonates (JA), ethylene (ET), and the ubiquitin proteasome system, which have important roles in subsequent defense responses (Dodds and Rathjen 2010; Trujillo and Shirasu 2010).

In the case of biotrophic pathogens, the SA signaling pathway mediates resistance in plants, typically by HR and programmed cell death that limits the spread of the pathogen, but can also lead to systemic acquired resistance (SAR). However, in the case of necrotrophs, programmed cell death is prevented and JA- and ET-dependent pathways protect plants against pathogens. The same pathway can be activated by beneficial soil microbes, leading to priming or induced systemic resistance (ISR), a state that allows plants to mount defenses more rapidly and/or stronger against pathogen or insect attack (Choudhary *et al.* 2007; Conrath *et al.* 2006; Glazebrook, 2005; Heil and Bostock; Van Wees *et al.* 2008).

Research on the plant defense response to viruses was initially focused on the SA pathway and the subsequent HR and consequent programmed cell death that can provide effective resistance against these obligate biotrophic pathogens. Another pathway, the RNA interference pathway (RNAi), which is conserved among plants, fungi, insects, and animals. It can provide specific resistance to viruses by degrading double-stranded RNA and leaving small interfering RNA molecules that act specifically against viral sequences (Soosaar *et al.* 2005). RNAi is also involved in gene regulation, including the production of microRNA that upon pathogen attack may degrade transcripts (gene silencing) of a negative regulator of plant defense or that act directly against pathogen sequences. Interaction between hormone signaling and innate immunity pathways (PTI/ETI), as well as RNA silencing pathways was studied by Zvereva and Pooggin (2012). There is growing evidence that PTI/ETI and RNA silencing pathways interact.

41.3 Transcriptomics during Plant-Pathogen Interactions

Pathogen resistance can be achieved by making use of a multitude of transcriptional regulators that reprogram the transcriptome to favor defense responses. In this symphony, a blend of signaling hormones SA, JA, and ET, have sophisticated and important roles in transcriptional regulation within large gene networks (Moore *et al.* 2011; Pieterse *et al.* 2009). For example, by using type III secretion systems (TTSS), bacterial plant pathogens transfer 15–30 effectors per strain into host cells. These effectors are able to kill the plants often by mimicking or inhibiting their functions (Beth Mudgett 2005; Grant *et al.* 2006), while eukaryotic plant pathogen effectors are currently poorly understood. Effectors can act either in the extracellular matrix or inside the plant cell. Correspondingly, plant transmembrane or cytoplasmic receptors, such as receptor kinases, detect PAMPs and trigger kinase cascades to amplify and transmit the signal to activate transcription factors and Mediator subunits, which then establish transcription of pathogenesis-related (PR) defense genes that ultimately help plants to defend themselves (Atkinson and Urwin 2012; Zhang and Klessig 2001). Plant transcriptional regulators consist not only of DNA binding transcription factors that have activation and repression roles, but also of cofactors, like Ca^{2+} , which are indirectly able to co-activate or co-repress transcription through interaction with DNA binding transcription factors (Moore *et al.* 2011).

Several families of transcription factors like WRKY, ERF, MYC, and MYB are involved in plant defense (Alves *et al.* 2013; Moore *et al.* 2011). For example, pathogen infection in *Arabidopsis* and subsequent accumulation of SA trigger transient cellular reduction sensed by Cys residues of the nonexpressor of pathogenesis-related protein 1 (NPR1), which

can change molecular conformation that directly effects its localization and transcriptional activity. NPR1 regulates more than 2200 immune-related genes in *Arabidopsis* (Moore *et al.* 2011). The family of transcription factors containing a bZIP domain is among the largest families of transcription factors in plants. One of the well-known members of this family comprise the TGA transcription factors, which have both positive and negative regulatory properties in relation to basal resistance and PR-1 induction in *Arabidopsis* (Alves *et al.* 2013; Moore *et al.* 2011). It has been reported that SA induces the recruitment of TGA factors to the PR-1 promoters in *Arabidopsis* and tobacco (Butterbrodt *et al.* 2006; Johnson *et al.* 2003). WRKY transcription factors consist of 74 members in *Arabidopsis* that respond to pathogen attack and SA accumulation. They have been divided into three groups based on their domain and structures with both positive and negative regulatory functions. For example, WRKY33 has a positive regulatory function and induces SAR while WRKY7 has a negative regulatory role (Eulgem and Somssich 2007; Pandey and Somssich 2009). The MYB family of transcription factors typically contains hundreds of members, which are classified on the basis of MYB repeats (Feller *et al.* 2011; Raffaele and Rivas 2013). In this family, R2R3 MYB transcription factors regulate plant immunity against microbial pathogens, such as AtMYB30, which is involved in abscisic acid (ABA) signaling, programmed cell death, and HR; or AtMYB96, which is induced by ABA and is involved in SA biosynthesis (Dubos *et al.* 2010; Seo and Park 2010; Vailleau *et al.* 2002).

Recently, the role of Mediator subunits in transcriptional regulation has been highlighted. Mediator subunit protein complexes provide a bridge between RNA Polymerase II and DNA-binding transcription factors (An and Mou 2013). The role of specific subunits in plant defense has been studied in detail. For example, PFT1/MED25 not only is involved in early flowering and abiotic stress signaling, but also is required for successful JA signaling in plants (Kidd *et al.* 2009).

Transcriptome profiling in roots in response to pathogen attack has been less studied. Yi Chung Chen *et al.* (2014) recently reported that *Fusarium oxysporum* attack to *Arabidopsis* roots mostly lead to repression of many genes and had very little in common with transcriptional defense responses in infected leaves. One of these repressed genes encodes the transcription factor ERF72 whose mutants showed resistance against *F. oxysporum*. Gene expression profiling of *Verticillium longisporum*-infected *Arabidopsis* roots lead to induction of defense-related genes independent of changes in SA, JA, ABA, or auxin levels (Iven *et al.* 2012), suggesting that other signaling compounds maybe required to mount root defenses.

41.4 Plant Responses during Interactions with Beneficial Microbes

While plant-pathogen interactions and their transcriptional responses in plants have been studied in great detail, this is not the case for plant beneficial microorganisms. This is probably because interactions with beneficial microorganisms form part of a plant's normal growth. For 150 years it has been known that bacteria and fungi promote plant growth and suppress plant pathogens (Berg 2009). Generally, bacteria and fungi have a variety of saprophytic and symbiotic relationships with other organisms, which can be both detrimental (pathogenic) and beneficial (mutualistic) (Barea *et al.* 2005b). Plants in natural and agricultural environments are continuously exposed to these microorganisms.

Plants roots penetrate in the soil to acquire inorganic nutrients and water supply. Therefore, as the soil is a major source of microorganisms, most of the plant-microbes interactions happen in the rhizosphere. This region is directly influenced by root exudates and associated soil microorganisms, which play a major role in nutrient cycling by degrading and mineralizing organic materials, releasing and transforming organic and inorganic nutrients. They are able to effect plant growth and nutrient uptake by production of growth stimulators or inhibiting substances (Marschner *et al.* 2011). Alternatively, soil microorganisms' growth and activity are mainly dependent on the availability of carbon sources, which are provided by plants (De Nobili *et al.* 2001; Demoling *et al.* 2007).

Plant growth-promoting rhizobacteria (PGPR) are symbiotic or free-living bacteria that exist in the rhizosphere. They are able to promote plants' growth by producing bioactive factors, biocontrol agents, growth promoters, and enzymes; in other words, PGPR can influence plant health and growth, disease suppression, and nutrient accessibility (Babalola 2010). PGPRs are able to control plant diseases (biological control) through mechanisms like antagonism, competition, producing antibiotics and antimicrobial peptides, siderophores, inducing systemic disease resistance (ISR), and improving nutrient uptake (Anith *et al.* 2004; Babalola 2010; Maksimov *et al.* 2011). Beneficial microorganisms such as nitrogen-fixing bacteria, endo- and ectomycorrhizal fungi, PGPB, and plant growth-promoting fungi (PGPFs) have important roles in plant development and chemicals' replacement (Raaijmakers *et al.* 2009; Russo *et al.* 2012). Generally, five classes of beneficial microorganisms can be distinguished in soil, including: (1) detritus decomposers; (2) N₂-fixing bacteria; (3) arbuscular mycorrhizal fungi; (4) PGPR and PGPF; and (5) fungal and bacterial antagonists of root pathogens (Barea *et al.* 2005a, b).

Symbiotic N₂-fixation is a well-known process, exclusively driven by rhizobial bacteria such as *Rhizobium* spp., *Sinorhizobium* spp., *Bradyrhizobium* spp., *Mesorhizobium* spp., and *Azorhizobium* spp. They are able to convert N₂ to ammonium (NH₄⁺) in legumes' root nodules, but there are also an astonishing number of free-living N₂-fixing bacteria that are associated with plants (Barea *et al.* 2005b). Mycorrhizal symbiosis is a wide-spread microbial plant mutualistic interaction where fungi establish an external mycelium, which provides a bridge connecting the root to large areas of surrounding soil microhabitats. Arbuscular mycorrhiza (AM) association is the most important type of mycorrhizal interaction where arbuscules grow inside root cells that resemble haustoria formed by biotrophic fungal pathogens. AM symbiosis increases the supply of water and mineral nutrients such as phosphate, ammonium, zinc, and copper to the plant. They also enhance other beneficial microorganisms' activity such as N₂-fixing bacteria and phosphate solubilizing bacteria, and provide protection against biotic and abiotic stresses (Barea *et al.* 2005b).

On the basis of their mechanisms, PGPR have been divided into two main groups, direct and indirect plant growth promotion (Glick 1995; Figueiredo *et al.* 2011). The first group of PGPR directly enhances plant growth nutrient cycling. Therefore, they have biofertilization and phytostimulation functions, like symbiotic nitrogen fixation conducted by free-living diazotrophs (bacteria and archaea that fix atmospheric N₂) such as *Azotobacter* spp., *Bacillus* spp., and *Klebsiella* spp., *Azospirillum* spp. (Babalola 2010; Boddey and Dobereiner 1995; Riggs *et al.* 2001). Also, some of the PGPR solubilize mineral phosphate. For example, some strains of *Bacillus* spp., *Enterobacter* spp., and *Pseudomonas* spp. have the potential to solubilize inorganic P and mineralize organic P to increase P availability to plants (Babalola 2010; de Freitas *et al.* 1997; Rodríguez and Fraga 1999). There are several reports that show the contribution of PGPR in making other soil minerals available to plants, including K, Fe, Mg, Mn, Zn, and Cu (Puente *et al.* 2004; Rana *et al.* 2012). Some PGPR, such as *Azospirillum* spp., *Pseudomonas* spp., and *Bacillus* spp., are able to produce phytohormones like indolacetic acid (IAA), gibberellins and cytokinins, and change root patterns and stimulate plant growth (Babalola 2010; Broadbent *et al.* 1971; Cassán *et al.* 2013; Pan *et al.* 1999).

The second group plays indirect roles in plant growth by being involved in the biocontrol of plant pathogens (Barea *et al.*, 2005b). Biological disease control functions by competition against pathogens (e.g., by production of siderophores) and/or the production of metabolites and proteins that affect the pathogens directly, for instance antibiotics, cell wall-degrading enzymes such as chitinase, cellulases, amylases, and glucanases, and hydrogen cyanide (Weller 1988; Enebak *et al.* 1998; Maksimov *et al.* 2011). In addition to the previously described strategies, PGPR can protect plants against fungal, bacterial and viral diseases, insect attack, and even nematodes by causing ISR (Ramamoorthy *et al.* 2001). Generally ISR, which is activated by PGPR in plants, will be followed by the accumulation of PR proteins such as peroxidases, β -1,3 glucanases and endochitinases, synthesis of phytoalexins, and other secondary metabolites in plants (Ramamoorthy *et al.* 2001).

Although research about PGPF is currently less comprehensive compared to PGPR, it is clear that many fungi, such as *Trichoderma* spp., *Fusarium equiseti*, and *Penicillium simplicissimum*, often as endophytes, are also highly effective in promoting plant growth by phytohormones and ISR (Elsharkawy *et al.* 2012; Hossain *et al.* 2007; Meera *et al.* 1994; Waqas *et al.* 2012).

41.5 Transcriptomics during Beneficial Plant-Microbe Interactions

Immune signaling in plants is triggered by receptor-mediated perception of both pathogenic and beneficial microbes. Recently, it was described that MAMP-triggered immune signaling in the roots is very similar to what was observed in the leaves, and the initial interactions of roots with beneficial microbes also include a ROS burst (Millet *et al.* 2010; Zamioudis and Pieterse 2012). However, to establish a mutualistic interaction with the plant, beneficial microbes need to prevent, weaken, or overcome host immune responses. For example, flagellin is normally a common bacterial effector protein leading to rapid defense responses, but *Rhizobium* colonizing the legume *Lotus japonicus* has evolved flagellin epitopes that avoid recognition by the plant (Lopez-Gomez *et al.* 2011).

Several studies have shown that the transcriptome changes by beneficial microbes' colonization in roots are relatively mild, especially if compared to the massive transcriptional reprogramming that occurs during pathogen attack (Pieterse *et al.* 2014). JA and ET signaling pathways are two key hormones for ISR triggered by beneficial microbes (Van Wees *et al.* 2008). Several key regulatory proteins have been identified. For example, NPR1 plays an important role in both pathogenic and beneficial interactions, depending on the activation of different hormone-dependent defense pathways. Another central transcription factor required in early signaling steps of ISR in *Arabidopsis* is MYB72 (Van der Ent *et al.* 2008). It also interacts with other transcription factors such as EIN3 and EIL3, which are part of the ET response pathway involved in ISR (Van Wees *et al.*, 2008). MYB72 was found to be upregulated during normal interactions with soil microbes (compared to axenically-grown plants) (Carvalhais *et al.* 2013) and is also required for regulation of iron uptake (Sivitz *et al.*, 2012).

MYC2, a basic-helix-loop-helix transcription factor, has been identified as a master regulator of JA signaling in *Arabidopsis* (Anderson *et al.* 2004; Kazan and Manners 2013). MYC2 forms an essential part of ISR, triggered by soil beneficial microbes. Among other functions, it acts a positive regulator for ABA signaling, JA-mediated insect defense and ISR, but as a negative regulator for JA-mediated defense against necrotrophic pathogens. In the absence of biotic or abiotic stresses, JAZ repressors have a strong affinity to MYC2, MYC3, and MYC4 (Cheng *et al.* 2011; Fernández-Calvo *et al.* 2011), but during signaling JAZ repressors are degraded leading to activation of MYC2 as a transcription factor (Kazan and Manners 2013). MYC2 also requires interaction with the Mediator complex subunit MED25 to target promoters (Çevik *et al.* 2012; Chen *et al.* 2012; Kidd *et al.* 2009). In *Arabidopsis*, it has been shown that priming leading to ISR is dependent on MYC2 function and other AP2/ERF transcription factors (Pieterse *et al.* 2014; Van der Ent *et al.* 2009).

41.6 Knowledge on Modulation of Host Immunity by Pathogens and Beneficial Microbes May Lead to New Resistance Strategies

It is interesting to note that research in plant-microbe interactions has focused on detrimental interactions of plants with mostly virulent pathogens. This is because research funding was biased towards studies targeting agronomically important plant diseases. However, there is a mounting body of evidence that pathogens are the exception to plant-microbe interactions and that interactions with commensal or beneficial microorganisms is not only more common, but essential to the plant's normal growth and development. For example, plants in the absence of mixed microbial communities only displayed half the biomass when compared to plants grown in the presence of soil microbes (Carvalhais *et al.* 2013). *Fusarium oxysporum* has been studied in detail as a plant pathogen (e.g., by Yi Chung Chen *et al.* 2014) but other strains of this fungus may colonize the plant as an endophyte without causing any damage or even by providing benefits via cross-protection. Interestingly, *myc2*, *coi1*, and *pfl1/med25* *Arabidopsis* mutants, which are all compromised in JA signaling, show resistance to the pathogenic *F. oxysporum* (Anderson *et al.* 2004; Thatcher *et al.* 2009; Kidd *et al.* 2009, respectively). The reason appears to be that this hemibiotrophic fungus strongly upregulates the JA pathway in *Arabidopsis*, which suppresses the SA pathway, a pathway leading to ROS burst, HR, and programmed cell death, which would have been the appropriate response to deal with the initial biotrophic lifestyle of this fungus.

The concept of pathway "hijacking" has been shown to occur in other fungal and bacterial patho-systems (de Torres-Zabala *et al.* 2007; Spoel *et al.* 2007) and, most recently, also in virus-infected plants (Shuey 2014). In these cases, hemibiotrophic or biotrophic pathogens induce the JA pathway, which not only is ineffective against these pathogens but also antagonizes the SA pathway. Similarly, the bacterial pathogen *Pseudomonas syringae* produces the jasmonate mimick coronatine to weaken SA-mediated defenses (Brooks *et al.* 2005). A similar "trick" was recently found for *Cucumber mosaic virus* (CMV) infection of *Arabidopsis* (Shuey 2014). While an incompatible CMV isolate led to SA-dependent gene expression and resistance, a compatible isolate was able to overcome resistance by inducing JA signaling. The same strategy can be used by beneficial microorganisms. JA signaling prevents cell death by inducing enzymes and compounds to detoxify cells from the initial ROS burst. For example, *Piriformospora indica* suppresses SA-dependent plant defenses by activating JA-mediated signaling via MYC2 and JAR1 (Jacobs *et al.* 2011). More examples on the modulation of host immunity by beneficial microbes have been reviewed by Zamioudis and Pieterse (2012).

These examples show that by studying detrimental and beneficial plant-microbe interactions we can learn more about the way microbes successfully overcome or divert plant defense pathways. Conversely, studying incompatible interactions where the pathogen does not complete its lifecycle would be advantageous in order to learn more about successful defensive strategies by plants.

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42

Transcriptomic and Metabolomic Profiling of Chicken Adipose Tissue: An Overview

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42.1 Introduction

Chickens are both a popular food animal species and a valuable model organism that has been used for centuries. A number of features make chickens particularly attractive as a model for studies relevant to human nutrition and obesity. Like humans, chickens have low rates of lipogenesis in adipose tissue. Phenotypic selection can be used to rapidly produce lines of chickens that differ in fatness, and broiler chickens are naturally prone to obesity. Development *in ovo* creates novel means through which to manipulate the embryo and its diet, and to study mechanisms that regulate adipose development and promote fatness in juveniles. The chicken genome is highly conserved with humans; therefore, insight into control of adipose metabolism in chickens can be extrapolated to corresponding pathways in humans. Transcriptomic analysis of chicken adipose tissue provides a means to discover novel pathways that may yield new insight into mechanisms that promote or prevent obesity. Coupling transcriptomics with metabolomics offers the possibility to develop a systems level view of adipose tissue function, and how it can be controlled by diet.

42.2 Chicken as a Model Organism

Domestic chickens arose from wild red jungle fowl more than 8000 years ago, primarily driven by their use for eggs or meat. Although most commonly thought of as a food animal, chickens have a long history as a model organism. Chick embryos were examined by the ancient Egyptians, and Aristotle systematically described embryonic development using chicken eggs as early as 300 BC. The majority of what is known about vertebrate developmental biology arose from or was catalyzed by studies using chicks over the past ~2000 years. Chickens offer many of the same practical advantages as rodents as a vertebrate model for studies of human health. *In ovo* maturation occurs in approximately 21 days, comparable to the gestation time of rodents. Large numbers of fertilized eggs can be produced and incubated simultaneously, enabling large-scale experiments with generous sample sizes. Incubators capable of housing hundreds of eggs are relatively inexpensive. Fertilized eggs can be refrigerated and held in a dormant state for several days, providing flexibility to the timing of incubation and to experimental studies. Eggs for many breeds can be acquired through local poultry producers, often at no cost because of the large scale of commercial production.

Feed can be purchased through local agricultural vendors (e.g., Farmers' Co-Op) or made in house from grains and mineral supplements, making it simple to create experimental diets (e.g., varying the source of fat or mineral content in the diet). Development *in ovo*, rather than *in utero*, creates the ability to manipulate the early developmental milieu by direct injection of nutraceuticals, pharmacological agents, nutrients, or chemicals of interest into the egg. Because the developing embryo consumes the yolk sac as energy during the final stages of development, *in ovo* injection can be used to manipulate the diet prior to hatch.

42.3 Chicken Genome and Genetic Diversity

The chicken was the first agricultural animal to have its genome sequenced due to its importance as a food animal worldwide. Chickens have approximately the same number of genes as humans, the majority of which show a high level of sequence conservation. The comparable number of genes is packed into a genome that is approximately half the size of the human genome due to a dearth of repetitive elements in chickens. Sequencing revealed that the chicken genome is around seven times more diverse, based on the frequency of single nucleotide polymorphisms (SNP), than that of humans, despite being approximately half the size in terms of base pairs. Recombination rates are relatively high in chickens. On average, recombinations occur 2.5 times more frequently in chicken than in humans, and five times more frequently than in mice. The propensity for shuffling of genetic information facilitates a rapid adaptation to selection for traits of interest.

42.4 Chicken as a Model for Studies of Adipose Biology and Obesity

Rodent models continue to be the *de facto* choice for obesity research and they have been extremely informative, particularly for elucidating the effects of individual genes through transgenic and knockout models. At the same time, monogenic models of excess fat gain or loss are not representative of most of the human population in whom obesity results from complex interactions between genetics, diet, and lifestyle. A number of traits make chickens a viable model for studies of nutrition, adipose biology, and lipid metabolism. Like humans, but unlike rodents or pigs, chickens rely on liver rather than adipose tissue for the majority of de novo lipid synthesis (Chicken-of-Tomorrow Contest 1951; Leveille *et al.* 1975; Hockin 2009; O'Hea and Leveille 1968; Ramsey and Rosebrough 2003). Most metabolic genes are conserved with humans, and a number of the quantitative trait loci (QTLs) that have been linked to fatness in chickens contain genes implicated in human susceptibility to obesity or diabetes (Hu *et al.* 2010; Nadaf *et al.* 2009). The process of adipogenesis is highly conserved between chickens and humans, including PPAR γ orchestration of the transcriptional changes necessary to produce mature, lipid-storing adipocytes (Wang *et al.*, 2008). The similarity is manifested by the fact that *in vitro* differentiation of preadipocytes can be achieved using hormonal induction protocol comparable to that used for rodents and humans (Ramsey and Rosebrough 2003). Chickens are naturally hypoglycemic and their adipose tissue is refractory to insulin, consistent with the early stages of insulin resistance. Additionally, chickens present advantages over rodents as models in which to study adipose metabolism in juveniles. Adipose tissue develops during the embryonic stage, and fat pads in the thigh and neck regions can be visualized as early as embryonic day 12. Fat pads can be dissected from developing embryos and used *ex vivo* for studies of adipocyte metabolism during the very early stages of tissue development, which is not feasible with rodent models. Amniotic fluid is consumed prior to hatching and provides the first meal for the chick. The composition of this meal can be manipulated by direct injection of nutrients, phytochemicals and other compounds directly into the egg, creating the ability to perform studies of very early effects of diet on adipose metabolism. These types of experiments have particular potential for studies of abdominal adipose tissue development, which begins shortly after hatch. The egg can also be injected at earlier stages to investigate the effects of chemicals and other compounds in the embryonic milieu on adipose development, for example, to study the effect of embryonic exposure to environmental obesogens such as bisphenol A. Comparable studies in rodents require the maternal diet to be manipulated, which does not necessarily result in comparable changes in milk consumed by the offspring.

42.5 Natural and Selected Models of Differential Fatness

42.5.1 Broilers

In recent decades, chickens have been subjected to human-driven selection mainly for food purposes by laying eggs or providing meat. Modern laying chickens were produced through selection for the ability to divert energy into efficient egg

production, and as a result are relatively small in size and lean (Hockin 2007). By contrast, today's meat-type (broiler) chickens are a result of selection for rapid growth. Up until the 1950s, the majority of chicken production occurred in private farms and backyards rather than in large commercial production companies. In 1945, the American grocer A&P (Atlantic & Pacific Tea Company) organized the national "Chicken of Tomorrow" contests to encourage meat-type poultry breeders to develop a more efficient broiler, to meet the rising demand for an easy-to-prepare source of animal protein as more women entered the workforce (Chicken-of-Tomorrow 1951). Intensive selection for rapid growth continued, and today's broiler chickens reach market age in half the time and weigh two times more than their predecessors, despite the need for less feed (Emmerson 1997). Inadvertently, selection significantly increased carcass fat which, from the industry's perspective, wastes feed and increases costs to producers. However, excessive fatness relative to other chicken breeds makes broilers a novel model for studies of human obesity. In particular, they represent a valuable model for juvenile obesity because rapid fat deposition begins in the first few weeks of life, well before sexual maturity. As in humans prior to adolescence, fat accumulation occurs through a combination of adipocyte hyperplasia and hypertrophy. As adults, broilers are more than twice as fat, based on weight of abdominal adipose tissue relative to body weight, than both egg-type chickens and wild-derived meat-type chickens.

42.5.2 Selected Lines

Much of what is known regarding mechanisms of fattening and the regulation of adipose metabolism in chickens has resulted from the study of genetic lines that differ in fatness due to phenotypic selection. Divergent phenotypic selection is a tool that can be used to produce genetic lines of that differ according to a desired trait by selecting parents with extreme values for a phenotype of interest. Divergent selection is particularly effective in chickens due to the underlying genetic diversity and rapid rates of recombination within the genome. The most dramatic example is the set of high weight (HWS) and low weight (LWS) lines developed by Siegel based on selection for body weight at 8 weeks of age. After 73 generations of selection, body weight differed between these two lines by approximately eight-fold. High body weight chickens showed increased feed efficiency, feed consumption, increased carcass fat, increased muscle size and number, and egg size, but decreased plasma growth hormone, immunocompetence, and egg production (Siegel and Wolford 2003). Because of the importance of hepatic lipogenesis in avians, lines that differ in fatness can be produced by selecting on circulating VLDL level, which reflects the supply of fatty acids available for uptake and incorporation into adipocyte triglyceride stores. Direct selection on abdominal fatness is more difficult due to the inability to measure it directly in live birds, but has also been used to produce lines that differ significantly in adiposity (adipose mass relative to body weight) (Cartwright *et al.* 1986). The French Fat line (FL) and Lean Line (LL) chickens, produced in this manner, differ approximately two-fold in despite comparable levels of feed intake. Adipocyte hypertrophy and hyperplasia are apparent in FL versus LL as early as 2 weeks of age, well before increased hepatic lipogenesis that is not detectable until at least 5 weeks of age (Hermier *et al.* 1989). Collectively, lines of domestic chickens represent a novel set of models with which to investigate adipose development and to identify factors and pathways that promote excess fat deposition.

42.6 Transcriptomics and Metabolomics as Tools for the Studies of Adipose Biology in Chicken

Chicken adipose tissue presents the opportunity to understand pathways that underlie obesity in humans because of its relevance as a model organism and the ease with which diet and developmental milieu can be manipulated. Transcriptomics represents a discovery-based approach through which genes and pathways that control adipose function can be uncovered and characterized in the absence of a priori hypotheses. A number of custom cDNA- and long oligonucleotide-based micro-arrays have been fabricated for chicken after its genome was sequenced. The array collection designed to interrogate chicken gene expression includes broad format arrays designed to be useful for any tissue (a 13K cDNA transcriptome array (Yerramsetty *et al.* 2013), 20K long oligo chicken genome array (Lu *et al.* 2010), Agilent (X Li BMC Genomics 2008) as well as those focused on specific tissues (3011 lymphocyte array (Dettmer *et al.* 2007), a 3072 intestinal array (Wenk 2005), an 11K heart specific array (Patti *et al.* 2012), a 14,718 macrophage specific array (Lang *et al.* 2010), and a 5K immune related array (de Leenheer and Thienpont 1992; Yerramsetty *et al.* 2013)). The most comprehensive is the Affymetrix GeneChip Chicken Genome Array, which targets 32,773 transcripts corresponding to over 28,000 chicken genes. The array also contains probes targeting over 600 transcripts from viruses that are known to infect chicken and are important commercially. RNAsequencing (RNAseq) continues to supplant microarrays for studies of large scale gene

expression. RNAseq effectively counts transcripts and is much more sensitive than microarrays, which rely on hybridization efficiency and labeling strategies to provide a relative readout of transcript abundance. RNAseq also enables transcript discovery, while microarrays only interrogate transcripts corresponding to probes represented on the array. To date, RNAseq has not been used for studies of chicken adipose tissue.

As an emerging field in systems biology, metabolomics and lipidomics techniques are continually being applied to an increased number of biological systems, and the chicken's status as a model organism for biological study makes it an ideal candidate for such analyses. Despite this, there is a relative dearth of studies involving chicken metabolomics and lipidomics (El Rammouz *et al.* 2010; Hermo *et al.* 2014; Shen *et al.* 2014), with even fewer are studies dealing specifically with chicken adipose tissue (Ji *et al.* 2012). As with all biological systems, chicken adipose tissue contains a variety of small molecule metabolites (Ji *et al.* 2012). Recent advancements in technology, specifically with regards to mass spectrometry, have paved the way for new and enlightening studies into cellular metabolism that can provide information relating to metabolic pathways, global physiology, and other cellular processes involving small molecules. The gold standard of metabolomics and lipidomics would be the holistic study of the entire small molecule content of a cell, and these entities are generally considered to be all compounds less than 1–2 kDa in mass. However, this pool of molecules, that is, the metabolome and lipidome, comprise a wide array of chemical structures with widely varying functionality from amino acids and nucleotide triphosphates to coenzymes and lipids. The disparate chemical properties of the molecules that comprise the metabolome and lipidome presents a unique analytical challenge in that no one extraction technique or detection method is a catch-all for all molecules; and instead, techniques are currently chosen based on molecular subset of most interest (Dettmer *et al.* 2007). This has led to bifurcation of the small omics technologies into the two sub-disciplines of metabolomics and lipidomics. Metabolomic methods typically involve the analyses of water-soluble metabolites, while lipidomics studies focus on the analysis of the lipid content, although some overlap of the molecular species is observed between the analyses. To date, lipidomics technologies are much more developed than metabolomics since lipids are biosynthesized from the combination of a defined set of headgroups and lipids, which makes their properties more uniform compared to those of the small molecule metabolites on whole. This simplifies lipid analyses; however, lipidomics methods often are still further subdivided based on head group, water solubility, and/or polarity further requiring different techniques for proper identification (Wenk 2005).

Metabolomics studies fall into two broad classes, targeted and untargeted analyses (Patti *et al.* 2012). Targeted metabolomics is designed to look specifically for known compounds or biomarkers of interest. These are often carried out on instruments, such as triple quadrupole mass spectrometers, where selectivity can be achieved using tandem mass spectrometry wherein each metabolite is detected using very specific parent mass and fragmentation patterns. These experiments, while limited in the overall scope of molecules detected, are very useful for quantification via the use selected reaction monitoring protocols and internal standards (Lang *et al.* 2010; Yerramsetty *et al.* 2013), which have become the gold standard for medical diagnostics and Pharmacokinetics (de Leenheer and Thienpont 1992). Untargeted metabolomics involves analyzing samples in a "shotgun" approach where a broad mass to charge (m/z) window is scanned as the sample is analyzed to detect as many molecules as possible. The advancement in the sensitivity and mass accuracy of high-resolution mass spectrometers is making untargeted analyses more routine, and it is now becoming feasible to achieve good levels of quantitation during untargeted experiments (Lu *et al.* 2010). The high resolution of the instrument often allows compounds to be identified by mass alone, while LC retention time data yields an extra level of specificity.

Although analytical technologies for metabolomics and lipidomics have rapidly advanced, the software to process the data and the bioinformatics capabilities are still lacking. A considerable amount of processing software and scripts, such as MetaboAnalyst (Xia *et al.* 2009, 2012) (www.metaboanalyst.ca), XCMS (Benton *et al.* 2010; Smith *et al.* 2006; Tautenhahn *et al.* 2008) (<http://metlin.scripps.edu/xcms>), and MAVEN (Clasquin *et al.* 2002; Melamud *et al.* 2010) (<http://genomics-pubs.princeton.edu/mzroll>), have been developed to identify features from mass spectrometric data (see <http://metabolomicssociety.org/index.php/resources/metabolomics-software> for broad list). These packages typically function to align chromatographic data, integrate ion peaks, and perform some level of statistical analysis (Sugimoto *et al.* 2012). To address the bioinformatics issues, there have been several projects such as LIPID Metabolites and Pathway Strategy (www.lipidmaps.org), the Human Metabolomics Database (www.hmdb.ca), and the METLIN Metabolite Database (<http://metlin.scripps.edu>) that have come about in an attempt to catalog lipids and metabolites and to provide databases of standard from mass spectrometric analyses. Further, resources such as the *Kyoto Encyclopedia of Genes and Genomes* (Kanehisa and Goto 2000; Kanehisa *et al.* 2014) (www.genome.jp/kegg/), MetaboAnalyst, and the Interactive Pathway Explorer_ENREF_15 (Letunic *et al.* 2008; Yamada *et al.* 2011) (iPath, <http://pathways.embl.de/>), are useful for mapping compounds to their relevant biochemical pathways.

42.7 Insight into Control of Adipose Tissue Growth and Metabolism in Chickens from Transcriptomics and Metabolomics

Liver has been the focus of most studies into control of adipose expansion in avians because of its role as the primary lipogenic organ. Considerably less is known about regulation of adipose tissue growth and metabolism. Global transcriptome profiling of adipose tissue from lean versus fatty chicken models has begun to reveal insight into mechanisms that control adipose deposition in chickens. Resnyk *et al.* (2013) used the Del-Mar 14K Chicken Integrated Systems microarray (NCBI GEO Platform # GLP1731), a custom microarray synthesized using a library of ~ 14,000 ESTs sequenced from chicken cDNA libraries, to identify differentially expressed genes in adipose tissue of French FL and LL lines. Expression was profiled at 1, 3, 5, 7, 9, and 11 weeks of age to characterize patterns of expression across the window of post-hatch adipose deposition. More than 3000 genes showed statistically significant age-dependent patterns of gene expression in these lines, regardless of genetic background, reflecting developmental changes in the tissue and the switch from adipocyte hyperplasia to hypertrophy as the primary mechanism of fattening (at approximately 7 weeks of age). The majority of genes that differed significantly between the two lines did so in an age-dependent manner. Expression profiling links excessive fatness to differences in lipid metabolism in adipose tissue. Despite its lesser contribution to lipogenesis (compared to liver), genes that regulate fatty acid synthesis (e.g., fatty acid synthase) were expressed at higher levels in FL adipose tissue. These differences were not observed until >5 weeks of age, suggesting that perpetuation of excess fat deposition includes contributions from *de novo* lipogenesis within white adipose tissue.

Transcriptomics and metabolomics/lipidomics are complementary tools that, when combined, can be more informative than either single platform. The combination of data types is particularly valuable for studies of metabolism, which is central to adipose biology. Metabolomics data can be used to validate that changes in gene expression have functional consequences on how the tissue allocates energy. They can also prompt further investigation of specific changes in expression of genes that may otherwise be overlooked. Combined transcriptomic and metabolomic studies further suggest that lipogenesis and fatty acid metabolism contribute to differences in fatness between broilers and leaner lines of chickens. Ji *et al.* (2014) used Affymetrix arrays to compare adipose tissue of fatty broilers to tissue from two lean lines of chickens (meat-type Fayoumi and egg-type Leghorn) with distinct genetic backgrounds, to increase the robustness of comparisons to fattier broilers. Adiposity differed significantly between lines, with broilers approximately 2.2 times as fatty as Leghorns and 1.5 times fattier than Fayoumi. Consistent with differences between FL and LL, genes that mediate *de novo* lipogenesis were expressed at higher levels in broiler adipose tissue. Conversely, genes that regulate the catabolism and oxidation of fatty acids were upregulated in the two lean lines relative to broilers, including components of both the peroxisomal and mitochondrial pathways for fatty acid oxidation. Small molecule metabolomic analysis of adipose tissue revealed that that carnitine, and acetylcarnitine, which play roles in fatty acid oxidation, along with many amino acids were present at significantly greater levels in adipose of both lean lines versus broilers (Ji *et al.* 2014). Some of the same changes were reported in LL versus FL, including upregulation of pyruvate dehydrogenase kinase 4 (PDK4), a kinase that promotes utilization of fatty acids by phosphorylating and inactivating pyruvate dehydrogenase. By comparing broilers to two distinct lean strains, these authors identified both pathways that were commonly activated in lean lines versus broilers and those that were specific to either Leghorns or Fayoumis, suggesting that leanness can be maintained through multiple mechanisms. They also highlight the potential relevance of fatty acid utilization in adipose tissue as an important component of leanness.

Transcriptional and metabolomic profiling have been used to characterize the homeostatic control of metabolism in chicken adipose tissue (Ji *et al.* 2012). Young (21 day-old) broiler chicks were either fed *ad libitum*, fasted for 5 h, or fed but injected with an anti-insulin antibody to neutralize the effect of insulin on metabolism. Affymetrix-based transcriptomics analyses revealed that the loss of insulin action had only modest effects on chicken adipose tissue. In contrast, fasting significantly altered the expression of >1200 genes relative to the fed group, including those in pathways related to carbohydrate, amino acid and lipid metabolism, and synthesis. Integration of metabolomic and transcriptomic data highlighted the effects of fasting and insulin neutralization on tissue amino acid metabolism. Genes involved in proteolysis and amino acid degradation were increased in fasting and were paralleled by decreased tissue levels of numerous amino acids.

Broilers of this age are rapidly forming new adipocytes. Fasting has significantly downregulated genes in each major step of adipocyte differentiation, from mesenchymal stem cell commitment to an adipocyte fate through the final stage of lipid filling. These results suggest that formation of new adipocytes in the growing broiler is dynamically regulated by energy sensing, or by secondary signals (e.g., abundance of specific metabolites) that are produced in response to fasting. They also show that although *de novo* lipogenesis rates are relatively low in chicken adipose tissue, this pathway is also regulated by signals related to energy sensing. In addition to suppressing lipogenesis, fasting upregulated rate-limiting

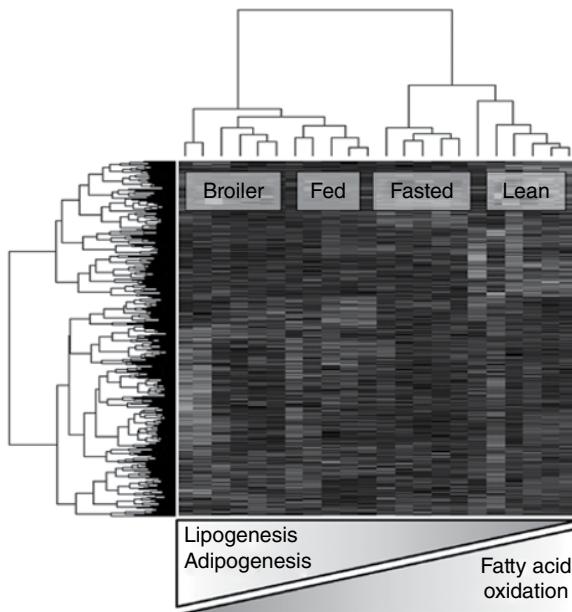


Figure 42.1 Gene expression signatures of leanness and fatness in chicken adipose tissue. Hierarchical clustering illustrates that expression profiles of fasted broilers are more similar to those of lean Leghorns than fed broilers, despite differences in genetic background. Adipose tissue of broilers and of the fed state is characterized by relatively higher expression of genes involved in lipid synthesis and adipocyte differentiation, while tissue of fasted broilers and of lean lines expressed higher levels of genes involved in lipid catabolism and fatty acid oxidation (Ji *et al.* 2012, 2014).

genes for fatty acid oxidation, including the rate-limiting enzymes in both mitochondrial and peroxisomal oxidation pathways (carnitine palmitoyltransferase 1A (CPT1A), and acyl-Coenzyme A oxidase 1 (ACOX1). Tissue concentration of β -hydroxybutyrate, a ketone product of beta oxidation, was also increased in adipose tissue of fasted versus fed chickens, providing biochemical evidence that fasting increased adipose fatty acid oxidation. Qualitatively, adipose tissue from fasted broiler chickens showed similar gene expression patterns to those of both LL versus FL and of Leghorn and Fayoumi compared to broilers (Figure 42.1). Based on these similarities, transcriptomic studies of chicken adipose tissue suggest that the balance between fatty acid synthesis and oxidation within white adipose tissue contribute to the control of fat mass. Further, they suggest that adipogenesis is regulated according to energy availability, at least in the young, growing bird. The ease with which the diet of the developing chick can be manipulated creates possibilities to evaluate the effect of nutraceuticals on pathways identified by these studies, and on their potential therapeutic role against obesity in humans.

The ability to discover unexpected pathways that are associated with a trait(s) of interest is one of the main benefits of transcriptomics. Transcriptome studies often identify hundreds of genes that are differentially expressed, raising the challenge of extracting biological meaning from the data. Pathway discovery is enabled by informatics-based geneset enrichment tools, such as the Database for Annotation, Visualization, and Integrated Discovery (DAVID). These tools take as input a set of genes, such as a list of differentially expressed genes, annotate them functionally using Gene Ontology (GO) terms and known pathway (e.g., KEGG) membership, and then identify functions and pathways that are statistically over-represented compared to what would be expected by chance based on comparisons to the species' corresponding genome or to all genes on the array used. For example, pathway analysis was used to discover that expression of many genes in the Wnt signaling pathway differs between broilers and leaner Fayoumi and Leghorn lines (Ji *et al.* 2014). Geneset enrichment analyses often reveal unanticipated pathways that are linked to the trait or experimental condition of interest. These types of tools have revealed a number of unexpected pathways that may contribute to fat deposition and regulate adipose metabolism in chickens. For example, pathways associated with blood coagulation, acute phase signaling and prothrombin activation, none of which have clear ties to adipose deposition, were significantly overrepresented in the set of genes differentially expressed between the French FL and LL chickens (Resnyk *et al.* 2013). As adipose transcriptomic data continue to be acquired from various chicken models, convergent information from multiple studies will establish the pathways that underlie fattening and control adipose metabolism in chicken.

42.8 Conclusions and Future Directions

In conclusion, expression profiling reveals that metabolism of adipose tissue of chickens is dynamically regulated, despite its modest contribution to lipid synthesis relative to liver. Identifying the molecular mechanisms that control adipose metabolism may present valuable new means through which fatness can be reduced to improve efficiency and reduce feed costs and the consequences of excess fatness to the broiler industry. Given the similarities between lipid metabolism in chickens and humans, these findings also highlight the value of the chicken as a model organism for studies of obesity in humans.

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43

Nutritional Transcriptomics: An Overview

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43.1 Introduction

Today, the role of food and nutrition in human health, and especially prevention of illness, is gaining recognition. Micronutrients are essential for optimal human health. Diseases of modern society, such as diabetes, heart disease, and cancer, have shown to be effected by dietary patterns.

Nutrition and genetics both play a significant function in human health as well as the development of chronic diseases. The risk of disease is often associated with genetic polymorphisms, but the effect is dependent on dietary intake and nutritional status. To understand the link between diet and health, nutritional research must cover a broad range of areas, from the molecular level to whole body studies. Therefore, it provides an excellent example of integrative biology requiring a “systems biology” approach. The interaction between the human body and nutrition is an extremely complex process involving multi-organ physiology with molecular mechanisms on all levels of regulation (genes, gene expression, proteins, and metabolites). Both the challenges and promises that are presented by the merger of “biomics” technologies and mechanistic nutrition research are enormous, but will ultimately develop in a new concept, namely, nutritional systems biology. The newly emerging approaches are nutrigenomics and nutrigenetics, aiming to determine an individual’s personal nutrition requirement and why this often is different from the measures set for a population. Although the term nutrigenomics is relatively new, the concept has been around for some time. Perhaps the most familiar example is lactose intolerance, which is a condition resulting from an inadequate production of lactase in the small intestine due to genetic variation in the lactase gene. Individuals with lactose intolerance are unable to efficiently break down the primary milk sugar (lactose) from dairy products. Consequently, the dietary recommendation is to limit lactose-containing foods or to use lactase supplements or lactose-free dairy products to prevent gastrointestinal discomfort (Figure 43.1).

In other words, nutrigenomics describes the scientific approach that integrates nutritional sciences and genomics and incorporates the application of other high-throughput “omics” technologies such as transcriptomics, proteomics and metabolomics to investigate the effects of nutrition on health. In fact, using the human genome data base as well as that of model organisms, nutrigenomics studies the genomewide effects of food components on gene expression (transcriptomics), the complete collection of proteins at a given nutritional state (proteomics) and the entire metabolite pattern occurring under a defined nutritional condition (metabolomics). In fact, omics in nutrition should be organized in an integrated

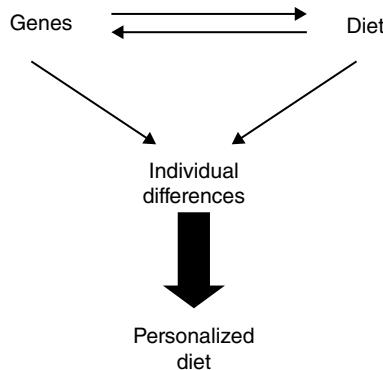


Figure 43.1 Personalized medicine and personalized nutrition.

fashion to elucidate biomarkers. Applied wisely, omics will promote an increased understanding of how nutrition influences metabolic pathways and homeostatic control; how this regulation is disturbed in the early phase of a diet-related disease and to what extent individual sensitizing genotypes contribute to such diseases (Figure 43.2).

Eventually, omics will allow effective dietary-intervention strategies to recover normal homeostasis and to prevent diet-related diseases. Moreover, nutrigenetics indicates genetic variation (i.e., SNPs, CNVs, and VNTRs) that can affect how nutrient and non-nutrient bioactive are assimilated, partitioned, and utilized to impact metabolism and physiology. It is a great potential to improve health by understanding the interaction between nutrients/foods and body physiology, and thus improve dietary prevention and treatment of diseases affecting people in rich as well as poor societies.

43.2 Molecular Nutrition

Nutrients may influence gene expression directly as ligands for nuclear receptors or by inducing epigenetic modifications. However, nutrients are also essential building blocks, may act as coenzymes in chemical reactions, can be converted into bioactive products, inhibit oxidation of other molecules, or serve as energy sources. Dietary factors could interact with multiple biological processes. Nutrients interact with genes and alter functional outcomes; nutrients may induce epigenetic changes; nutrients may influence gene expression as ligands for nuclear hormone receptors; nutrients may post-translationally modify proteins; and nutrients may change the metabolomic signature in the blood. It seems reasonable to take advantage of well-designed studies to execute molecular nutrition in health and diseases. Identification of a link between an exposure and disease often initiates with an epidemiological study. An example is the association between obesity and elevated concentrations of several plasma amino acids suggesting that specific amino acids may contribute to regulation of body weight (Murtaugh *et al.* 2007; Rosell *et al.* 2006; Spencer *et al.* 2003). No epidemiological study can alone provide an absolute answer about the effect of the exposure on the outcome. When an association between a risk factor and the outcome is supported by evidence from a large number of observational studies, basic sciences about biological mechanisms, and experimental epidemiology, causality is strengthened and dietary guidelines may be provided (Jeffery and Keck 2008).

The human body consists of more than 200 diverse cell types with the same DNA sequence, although unique gene expression patterns. The distinction in gene expression among the cells is mainly directed by epigenetic modifications, including changes in DNA methylation and histone modification. DNA methylation is one of the major epigenetic modulators (Handel *et al.* 2010); it can suppress gene expression by modulating the access of the transcript machinery to the chromatin or by recruiting methyl-binding proteins. However, recent studies support the concept that environmental factors affect metabolic functions through epigenetic modifications (Cedar and Bergman 2009).

The daily ingestion, absorption, digestion, transport, metabolism, and excretion of nutrients and food bioactives involve many proteins such as enzymes, receptors, transporters, ion channels, and hormones. Variations in genes encoding proteins that affect any of these processes can alter both the amount of the protein produced as well as how well that protein functions. If a genetic variation leads to altered production or function of these proteins then nutritional status might be affected.

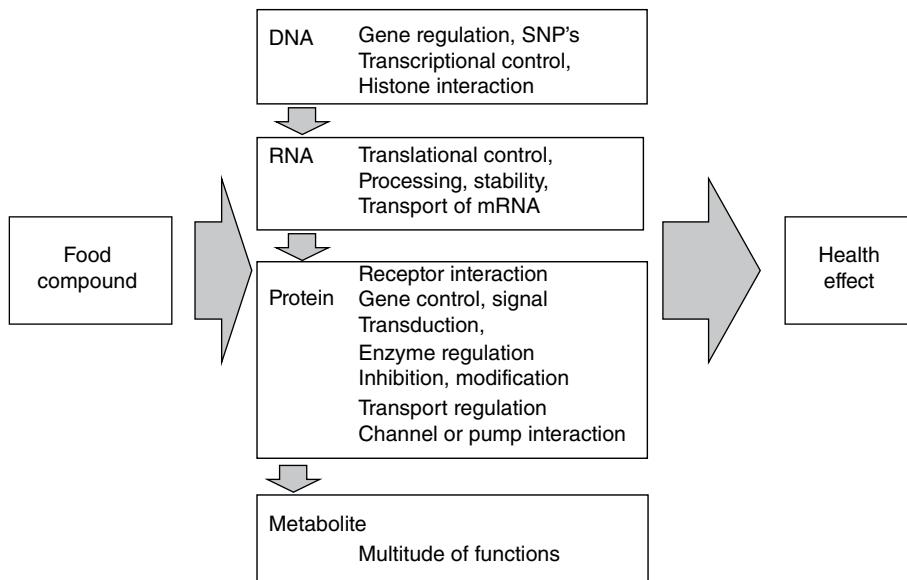


Figure 43.2 Nutritional genomics. Van Ommen (2004). Reproduced with permission of Elsevier.

Disorders such as obesity, diabetes, cancer, and cardiovascular disease that take several years – or even decades – to develop have multifactorial etiologies. The concept of diet–gene interactions involves a genetic variant modulating the effect of a dietary factor on a specific phenotype or health outcome measure such as serum lipid concentrations, high blood glucose or obesity. Conversely, diet–gene interactions can refer to the dietary modification of the effect of a genetic variant on the phenotype or health outcome measure.

43.3 From Nutrients to Genes Expression Profiling

Transcription factors are the main agents through which nutrients influence gene expression. *Transcriptomics* refers to the complete collection of gene transcripts in a cell or a tissue at a given time, and may be used to study gene transcription in response to dietary changes (Muller and Kersten 2003). The nuclear hormone receptor superfamily of transcription factors is probably the most important group of nutrient sensors, which influence gene expression. Numerous nuclear hormone receptors, bind nutrients, and undergo a conformational change that results in the coordinated dissociation of co-repressors and the recruitment of co-activator proteins to facilitate transcription activation (Panagiotou and Nielsen 2009). Moreover, epigenomics, as the study of the complete set of epigenetic modifications in a cell or a tissue at a given time, suggesting that environmental factors may influence the epigenome (Fraga *et al.* 2005). Changes in DNA-methylation levels among humans with metabolic diseases are associated with alterations in expression of genes (Ling *et al.* 2007). Until recently, epigenetic modifications have been considered erased during gametogenesis or early embryogenesis. However, novel findings have shown that epigenetic marks are not always cleared between generations (Morgan *et al.* 1999). Altogether, several lines of evidence indicate that some of the effects of diet and physical activity are induced via epigenetic modifications. Of note, the study of microRNAs is often classified to be part of epigenetics. MicroRNAs are small non-coding RNA molecules derived from hairpin precursors, usually between 20 and 30 nucleotides in length. They can mediate post-transcriptional silencing for about 30% of protein-encoding genes in mammals (Bartel 2004).

Transcriptomics is mainly used for three different purposes in nutrition research; first, it can provide information about the mechanism underlying the effects of a certain nutrient or diet; second, transcriptomics can help to identify genes, proteins, or metabolites that are altered in the pre-disease state and might act as molecular biomarkers; third, transcriptomics can help to identify and characterize pathways regulated by nutrients (Muller and Kersten 2003). However, an important challenge in human transcriptomics studies is the inaccessibility of human tissues. Blood, subcutaneous adipose tissue, and skeletal muscle are among the tissues, which can be relatively easily collected. Thus, animal studies can be good supplements to human studies to understand how nutrients affect gene regulation in a variety of tissues. Thus, overall, it seems reasonable to

emphasize that transcriptomic technology has finally achieved a level of technical maturity to generate high quality experimental data and robust biological information. The “signatures” imply characteristic patterns of differential gene expression, are effectively measures of cell phenotype, and can be used to look for novel biomarkers in cells that have been exposed to different levels of micronutrients. Approaches designed to identify characteristics gene expression signatures are being developed and tested widely in the context of medical research, in particular cancer research (Fowke *et al.* 2004; Murtaugh *et al.* 2007; Palaniappan *et al.* 2003). It is worth noting that the effects of nutrition on health and disease cannot be figured out without thoughtful consideration of how nutrients perform at the molecular level. The completed of large genome projects has clearly drawn attention to the importance of genes in human nutrition (International Human Genome Sequencing Consortium 2002; Venter *et al.* 2002; Waterston *et al.* 2002). In fact, micronutrients are potent dietary signals that influence the metabolic programming of cells and have an important role in the control of homeostasis. Moreover, genetic predisposition is an important contributor to the main causes of mortality linked to diet (Francis *et al.* 2002; Willett 2002).

43.4 Biological Actions of Nutrients

Micronutrients are essential regulators of important metabolic and physiological processes in humans. Micronutrient deficiencies cause specific illnesses. Suboptimal intakes may contribute to the development and severity of chronic diseases. Micro- and macronutrients have important effects on gene and protein expression as well as on metabolism. The molecular structure of a nutrient determines the specific signaling pathways that it activates. Small changes in structure can have a deep influence on which sensor pathways are activated. For instance, the ω -3 polyunsaturated fatty acids have a positive effect on cardiac arrhythmia, whereas saturated stearic acid and palmitic acid do not (Brouwer *et al.* 2002; Sacks and Katan 2002). The effects of micronutrients depend on a series of physical, chemical, and physiological processes, including amount ingested, meal matrix, digestion, absorption, distribution, metabolism (biotransformation), excretion, genetic factors, and cellular mechanisms of action. Each of these processes involves a complex interaction among genes, metabolites, and environmental factors (Leung *et al.* 2009; Parker 1996). In fact, micronutrients do not act independently. The need to consider the impact of micronutrient combinations on biological processes is becoming increasingly evident, since micronutrients have overlapping or complementing actions, or even act in concert. Selenium, zinc, folate, and vitamins D, E, B2, B6, and B12 are all involved directly or indirectly in the innate immune response, oxidative stress response, and DNA metabolism (Larbi *et al.* 2008). Transcription factors are the main agents through which nutrients influence gene expression. In metabolically active organs, such as the liver, intestine, and adipose tissue, these transcription factors act as nutrient sensors by changing the level of DNA transcription of specific genes in response to nutrient changes. Therefore, it is easy to predict how nutrients, by activating these receptors, are able to influence a wide array of cellular functions. The best approach is to study the role of all actors (Chawla *et al.* 2001; Lu *et al.* 2001; Mangelsdorf *et al.* 1995).

It is not far from reality to provide clues about the mechanism that underlies the advantageous or adversary effects of a certain nutrient. Highly specific changes in gene expression might explain the beneficial or adversary effects of many nutrients. For example, the beneficial effect of poly-unsaturated fatty acids on plasma LDL levels might be linked to specific changes in the expression of genes involved in cholesterol metabolism. To carry them out requires understanding of which tissue or organ is accountable for the specific effects of a nutrient. If we do not know the function of a gene the expression of which is modified by a specific nutrient, it is difficult to clarify the mechanism essential the specific beneficial or adversary effect. Gene-expression profiling can characterize the basic molecular pathways of gene regulation by nutrients. If this vision becomes reality, it could provide a bridge between basic nutritional science and human diet-intervention studies. More specifically, it would allow us to assess the effectiveness of specific nutrients in preventing disease (Tang *et al.* 2001; Whitney *et al.* 2003a).

The challenge for the next decade is to identify nutrient-influenced molecular pathways and determine the down-stream effects of specific nutrients. Nutrigenomics can assist in this identification because it allows the genome-wide characterization of genes, the expression of which is influenced by nutrients. It is only with a complete understanding of the biochemical links between nutrition and the genome that we will be able to comprehend fully the influence of nutrition on human health.

43.5 Nutritional Transcriptomics

The transcriptome is the complete set of RNA that can be produced from the genome. Transcriptomics is the study of the transcriptome, that is, gene expression at the level of the mRNA. Using either cDNA or oligonucleotide microarray technology, it describes the approach in which gene expression (mRNA) is analyzed in a biological sample at a given time

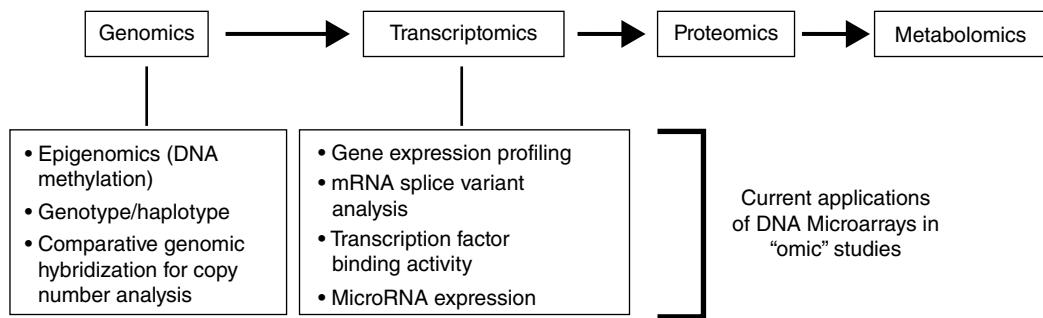


Figure 43.3 Schematic representation of the current range of applications for DNA microarray-based technologies in the broader context of structural and functional “omic” research areas. Elliot (2008). Reproduced with permission of Cambridge University Press.

under specific conditions (Figure 43.3). Regulation of the rate of transcription of genes by food components represents an intriguing site for regulation of an individual’s phenotype. A host of essential nutrients and other bioactive food components can serve as important regulators of gene expression patterns. Macronutrients, vitamins, minerals, and various phytochemicals can modify gene transcription and translation, which can alter biological responses such as metabolism, cell growth, and differentiation, all of which are important in disease process. The mRNAs are produced by a given moment and in a given tissue of a selected organism; therefore, gene expression varies according to the different circumstances and periods of time. In general terms, gene-expression profiling can be used for various purposes in nutrition research. Transcriptomics has become a central technology in the development of molecular nutrition, having the capacity to produce expression data for every gene in a given genome. Transcription factors, when activated, migrate to the nucleus and bind to a specific sequence of DNA in the promoter region of genes and, there, act by inhibiting or facilitating transcription (Dalmiel *et al.* 2012; de Lorenzo 2008). These transcription factors can be stimulated by physiological signals (triggered by nutrients/bioactive food compounds or the metabolites resulting from them), hormones, pharmacological treatments, and diseases. In nutrition research, transcriptomics can assist in providing information about the mechanisms or underlying effects of a particular nutrient or diet. It can also help identifying genes, proteins, or metabolites that change in the state of prediseases, as well as assisting on recognizing and characterizing the pathways regulated by nutrients or bioactive compounds in foods (Fialho *et al.* 2008; Mahan and Scott Stump 2005). Modulations in gene expression may be caused by epigenetic mechanisms through changes in chromosome structure such as DNA methylation and histone acetylation (Hu and Kong 2004). Overall, focusing on the analysis of RNA (the transcriptome), transcriptomics aims at measuring the level of expression of all or a selected subset of genes based on the amount of RNA present in a sample. Currently, the most powerful tool available is DNA array technology. Cases analyzing nutrient biology include the effect of leptin on mouse adipose tissue; the effect of fasting and aging in the rat hypothalamus; the effect of diets rich in polyunsaturated fatty acids on murine hepatic and hippocampal gene expression; the effects of antioxidants on muscle transcripts in rat muscle, muscle gene expression in young and old men and in Type 2 diabetic subjects; and gene expression-profiling of human visceral adipose tissue and other enormous respective studies. As such, nutrients also modulate the activity of transcription factors, or the secretion of hormones that in turn interfere with a transcription factor (transcription factors are proteins that bind to specific DNA sequences located within the promoter region of genes and can activate or inhibit their transcription). In fact, most of our knowledge on the effects of nutrients on gene expression has been acquired from animal models.

43.6 Transcriptomic Technologies

The Human Genome Project (HGP) was initiated in 1989, as a worldwide effort to clone, map, and sequence the entire 3.2-Gb human genome in a 15-year period. This enormous task was finished in 2003 with the publication of the complete human sequence. In the mainstream of the HGP, revolutionary new technologies were developed. The main distinctiveness of these innovative technologies was miniaturization, automation, high throughput, and computerization. These technical developments have now given us tools enabling high-throughput “genome-wide” approaches. These tools form the basis of the biomics era; genomics (covering DNA), transcriptomics (RNA), proteomics (protein), metabolomics (metabolites), and systems biology (integrating all of these).

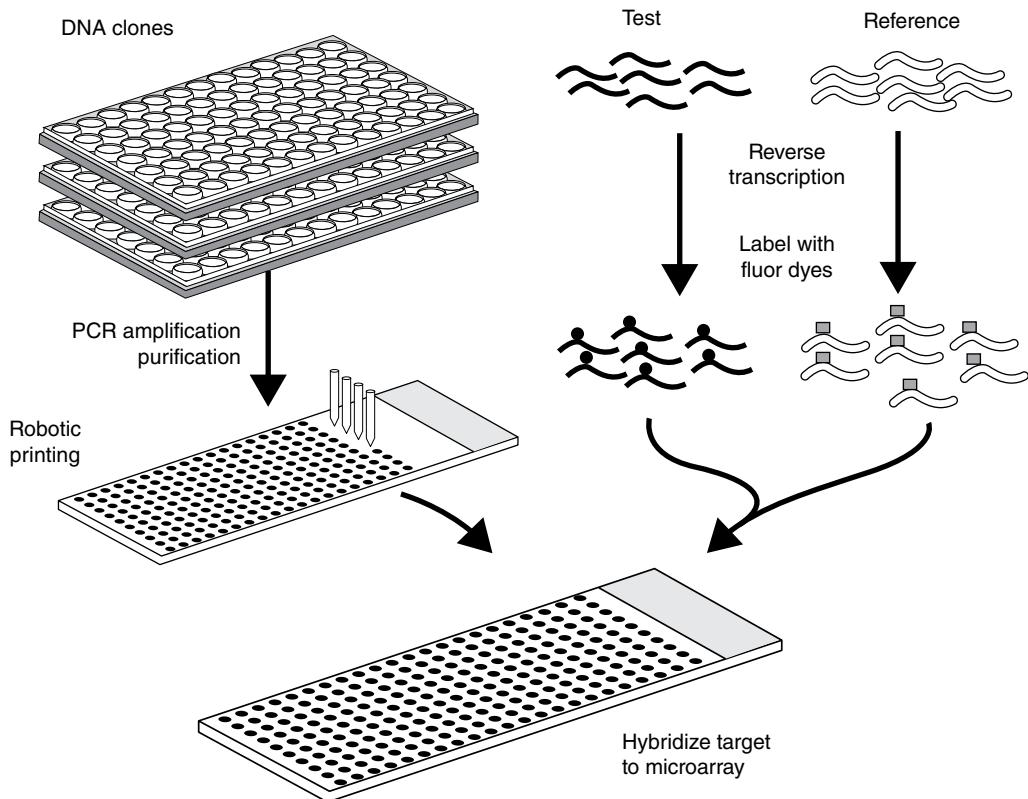


Figure 43.4(a) Gene expression microarray.

For years, the expression of individual genes has been determined by quantification of mRNA with Northern blotting. This classical technique has slowly been replaced by more sensitive techniques such as real-time PCR. Both techniques, however, can only examine gene expression for a restricted number of candidate genes at a time. This is an important restriction for their application in nutrigenomics research since the analysis of a reduced number of genes may not provide insights about the causative relationship between the bioactive micronutrient and its biological effect (Hu and Kong 2004). In contrast, the analysis of global gene expression may offer better opportunities to identify the effect of micronutrient on metabolic pathways and how this regulation is potentially altered in the progress of certain chronic diseases (Gohil and Chakraborty 2004). Two different investigative approaches have appeared to agree to quantitative and comprehensive analysis of changes in mRNA expression levels of hundreds or thousands of genes. One approach is based on microarray technology, and the other group of techniques is based on DNA sequencing (Morozova and Marra 2008). Subtle changes in gene expression, even at the single-cell level, can now be measured by quantitative techniques such as real-time PCR and high-density microarray analysis (Figure 43.4a and b). The latter allows the entire nutrition-relevant transcriptome to be studied simultaneously (Chuaqui *et al.* 2002).

During recent years, gene expression microarray has become a leading analytical technology in Nutrigenomics for the examination of the interactions between nutrients and genes. This technique is based on specific nucleic acids hybridization to measure the relative quantities of specific mRNAs in two or more samples for thousands of genes simultaneously. Gene expression microarrays are potent, but variability arising throughout the measurement process can obscure the biological signals of interest. Data acquisition and preprocessing are important post-technical steps in microarray experiments. The analysis of the vast amount of microarray data for extracting biologically meaningful information is the toughest discouraging duties (Tang *et al.* 2001). The fundamental goal of microarray expression profiling (see Figure 43.5) is to identify genes that are differentially expressed in the condition of interest (Burton and McGeehee 2004). There is a fine correlation between microarray results and other more conventional methods, indicating



Figure 43.4(b) Gene expression microarray.

the ability of the technology to create reliable results. Early applications of microarrays to nutrigenomics were related to the effects of caloric restriction on aging (Cao *et al.* 2001; Kayo *et al.* 2001; Weindruch *et al.* 2001). Shortly after, the technology was expanded to investigate other interesting aspects of nutrigenomics, including the effects of dietary protein in the gene expression (Endo *et al.* 2002), the mechanisms of nutritional long chain polyunsaturated fatty acids in molecular function cancer and normal cells (Endo *et al.* 2002; Narayanan *et al.* 2003), and the effects on transcriptome of a high- or low-carbohydrate intake (van Erk *et al.* 2006). The molecular mechanisms of certain bioactive micronutrients have been studied by microarray technology. Overall, it seems reasonable to declare that transcriptomic technology has finally achieved a level of scientific development that means it is possible for dedicated researchers to produce high quality experimental data and, with suitable statistical and bioinformatics capability, to mine vigorous biological information from that data (Guo *et al.* 2006).

Genome-wide monitoring of gene expression by means of DNA microarrays permits the simultaneous evaluation of the transcription of thousands of genes and of their comparative expression between normal cells and diseased cells or before and after exposure to different dietary components. This information should assist in the discovery of new biomarkers for disease diagnosis and prognosis prediction and of new therapeutic tools. Microarrays have been described as the hottest thing in biology and medicine. The technology emerged around 1996 and had its first high-profile uses in 1998. Microarrays represent a powerful tool for studies of diet-gene interactions. Their use, however, is still associated with a number of technical challenges and potential pitfalls. Technical variations between array platforms and analytical procedures will almost certainly lead to differences in the transcriptional responses observed. As a result, inconsistent data may be produced, significant effects may be missed and/or false leads generated. This is likely to be particularly true in the field of nutrition, in which we expect that many dietary bioactive agents at nutritionally relevant concentrations will elicit delicate changes in gene transcription. Thus, great care should always be taken in designing and executing microarray studies. A Japanese group has established a web-based, integrated database for the publications and microarray expression data in the field of nutrigenomics (<http://a-7yo5.ch.a.u-tokyo.ac.jp/index.php>). The database was designed for effective storage, management, analysis, and sharing of gene expression data to nutritional scientists involved in microarray experiments. All information in it is freely available. Currently, there are more than 400 publications and several expression data sets available for any user. The database is still under testing and construction.

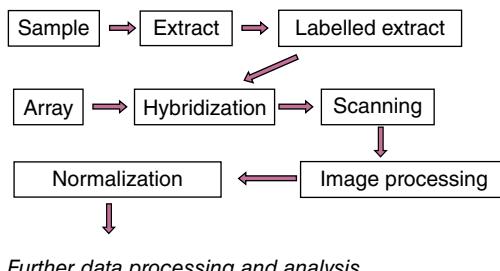
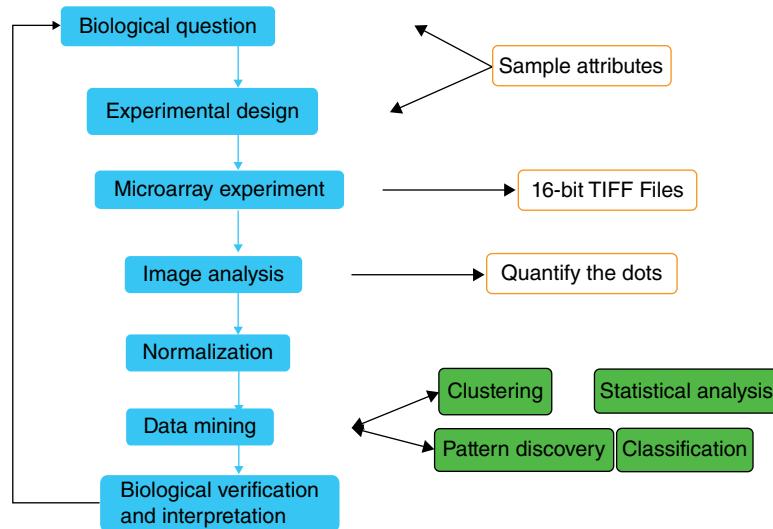


Figure 43.5 Steps of microarray experiments.

43.7 Transcriptomics and Development of New Nutritional Biomarkers

This is particularly important for nutrition research where only small changes in gene expression are generally expected to be brought about by dietary modification but, nevertheless, such changes may have profound effects in the long term on health. In terms of gene expression-based biomarker development, progress to date has been more disappointing. An interesting alternative that may help to overcome this problem is the development of markers based on expression profile "signatures" rather than single genes. Such signatures are effectively measures of cell phenotype, and can be used to look for novel biomarkers in cells that have been exposed to different levels of micronutrients. Approaches designed to identify characteristics gene expression signatures are being developed and tested widely in the context of medical research, in particular cancer research. Although they are still in comparatively early developmental stages, the applicability and power of such approaches should also be evaluated in the context of nutrition (Lyman and Kuderer 2006; Marchionni *et al.* 2008; Naderi *et al.* 2007; Xu *et al.* 2008). High-throughput genomic techniques such as gene microarrays (transcriptomics) and proteomics allow broadening of the analyses to previously unknown targets and open the way to novel hypotheses and to the discovery of new functional biomarkers. The increase in information on human genetic variation is providing a basis for investigation of the role of genetics in inter-individual variations in response to micronutrients. Genetic diseases, although often relatively rare, can provide important information on micronutrient function; Genetic diseases, although often relatively rare, can provide important information on micronutrient function. The majority of variants seem to have no impact on gene function and so only a small number of SNP actually have functional consequences. The effects of these functional variants on gene or protein function are usually more subtle than those of a mutation. Functional SNP in genes that affect micronutrient metabolism can affect responses to increased intake.

The scope for transcriptomic nutritional studies in human subjects should be considered. Limitations in access to target tissues, technical challenges of isolating sufficient high quality RNA, and the analytical problems of dealing with inter-individual variation in human subjects are all valid concerns. Nevertheless, the limited evidence that is available suggests that transcriptomic analysis in nutritional intervention studies is feasible and can be highly informative. That is why; white blood cells are commonly used for gene transcript analysis. They represent a potentially valuable and widely used tool for nutritional intervention studies with human subjects both as direct targets for studying effects on immune and inflammatory processes and as surrogates for direct sampling of less accessible tissues (Whitney *et al.* 2003b). Analyses of normal variation in leukocyte gene expression suggest that it is best to design studies in which subjects act as their own controls, since the extent of intra-individual variation, in the absence of any intervention, is markedly lower than that of inter-individual variation (Eady *et al.* 2005; Radich *et al.* 2004).

43.8 The Micronutrient Genomics Project

The MGP considers an integrative project, combining the invaluable bioinformatics tools and omics databases that are already available, such as the Human Variome Project, the National Center for Biotechnology Information (NCBI), The Human Genome Epidemiology Network (HuGENet), the European Bioinformatics Institute (EBI), and the Human Metabolome Database HMDB. An online portal was created at www.micronutrientgenomics.org. This portal allows access to all information and tools collected and structured by the Micronutrient Genomics Project. The portal presents micronutrient-related pathways and biological networks. The phenotypic expression of the gene–micronutrient interaction is visualized at the level of transcripts, proteins and metabolites. This allows an integrated view on related parameters and visualization of experimental data. The micronutrient pathway portal features pathways that combine gene product (transcriptome and proteome) and metabolome entities (Kelder *et al.* 2009; Khouri *et al.* 2007). The most interesting aspect of pathways is that they can be used in the analysis of actual study data. Gene expression Transcriptomics, gene expression regulation analyses of the mechanisms of action of micronutrients are increasing. MGP constructs micronutrient centered pathway based biological networks. Intracellular mechanisms related to micronutrient activity are linked to plasma and blood cell membrane concentrations of all relevant metabolites and proteins (van Ommen *et al.* 2008). The database will allow for the in-depth exploration of the relationship between micronutrients and chronic diseases in diverse cohorts (Table 43.1). Ultimately, the MGP online portal seeks to facilitate the kinds of research advances that will enable informed intake recommendations for specific micronutrients for both individuals and subpopulations, in order to prevent acute illness and chronic disease.

43.9 Transcriptomics in Nutrition Research

A challenge in nutrition research is the finding of relatively delicate dietary intervention on cellular function. The effects of diet on gene expression are minute and intricate so making the development of highly perceptive and precise methods fundamental. Transcriptional profiling needs perfect sampling for extraction of sufficient and high quality RNA. Access to tissues is limited particularly internal organs. To overcome such problems, it is suggested to biopsy accessible tissues including muscles and subcutaneous adipose tissue. Transcriptional profiling of these tissues is valuable; because these organs perform crucial functions playing essential role in energy metabolism. A less invasive alternative is blood cells so as disease specific gene expression patterns have been identified using blood tissue. High and low inter- and intra-individual variations, respectively, in gene expression indicates that these cells are appropriate for nutritional research. In a successful nutrigenomic study, the both a rationale supporting the study and robust hypothesis are mandatory. Of note, it should be clarified that clear research question could be resolved through whole genome transcriptomics. The type and duration of the intervention is expected to have a substantial impact on changes in transcriptional profiles. The use of biomics research to analyze the influence of nutrients on human health is thus based on two observations: (1) the nutritional environment modifies the expression of genes, and (2) depending upon the genotype of an individual, the metabolism of nutrients may vary and ultimately result in a different health status. In conducting a nutrigenomics study, a couple of parameters should be taken into account including:

- Overall good design of the study.
- Dietary variation should be limited within a study.

Table 43.1 Summary of metabolomic databases.

Database name	URL or web address	Comments
Human metabolome database	http://www.hmdb.ca	Largest and most complete of its kind. Specific to humans only
BioMagResBank (BMRB – metabolomics)	http://www.bmrb.wisc.edu/metabolomics/	Emphasis on NMR data, no biological or biochemical data
BiGG (database of biochemical, genetic and genomic metabolic network reconstructions)	http://bigg.ucsd.edu/home.pl	Specific to plants (Arabidopsis) Database of human, yeast and bacterial metabolites, pathways and reactions as well as SBML reconstructions for metabolic modeling
Fiehn metabolome database	http://fiehnlab.ucdavis.edu/compounds/	Tabular list of ID'd metabolites with images, synonyms and KEGG links
Golm metabolome database	http://csbdb.mpiimp-golm.mpg.de/csbdb/gmd/gmd.html	Emphasis on MS or GC-MS data only No biological data Few data fields
METLIN metabolite database	http://metlin.scripps.edu/	Specific to plants Human specific Mixes drugs, drug metabolites together Name, structure, ID only
NIST spectral database	http://webbook.nist.gov/chemistry/	Spectral database only (NMR, MS, IR) No biological data, little chemical data Not limited to metabolites
Spectral database for organic compounds (SDBS)	http://www.aist.go.jp/RIODB/SDBS/cgi-bin/direct.frametop.cgi?lang=eng	Spectral database only (NMR, MS, IR) No biological data, little chemical data Not limited to metabolites

- The combination of nutrients will affect digestion, uptake and transcriptional profiles.
- Performing the studies under acute nutritional conditions such as fasting and caloric restriction.
- Exercise modifies gene expression in muscle and could alter blood cell transcriptome so as levels of exercise should be similar throughout the study.
- Gene expression profiles vary throughout the day, thus sample collection should be carried out at the same time.
- Factors such as infections, antibiotics, and vaccinations influence gene expression profile.
- Adipose and muscle biopsies should be taken from the same position.
- To avoid transcription influences, samples should be taken from fasted volunteers.
- RNA quality and quantity need to be verified.

It is worth noting that with right approach, nutritional transcriptome studies produce valuable information about how nutrients affect the human body and work at cellular level.

43.10 Perspectives

We believe that in the long-term, nutrigenomics for public health has the potential to make an important contribution. We need more nutrigenomics studies by disease-oriented biomedical scientists. Furthermore, we need traditional nutritional scientists to understand the potential for molecular nutrition research in mouse models to provide insights into human nutrition. Finally, we have to convince the food industry that the time has come to use nutrigenomics to develop evidence-based nutrition. With latest advances in systems biology and genomics technologies, it is becoming possible to evaluate the biological action of a micronutrient in its whole biological context, including interactions with other nutrients in context of genetic make-up. These mechanistic and integrated approaches in the micronutrient and health relationship may complement the public health recommendation approaches and provide refinements in specific cases. For the time being, nutrigenomics will definitely discover new, tasty, readily suitable, and more proper foods. There is no doubt that investment in nutrigenomics will advance the role of nutrition in public health.

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44

Dissecting Transcriptomes of Cyanobacteria for Novel Metabolite Production

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44.1 Introduction

Cyanobacteria are the ancient organisms responsible for the formation of life on Earth. About 2.5 billion years ago when Earth began to cool down, cyanobacteria emerged that could harvest electrons from water and produce oxygen as a by-product of CO₂ fixation. This led to the “Great Oxygenic Event” (GOE). Oxygenation of Earth created an amenable environment for the creation of life. After that, some cyanobacteria were engulfed by non-photosynthetic organisms and started living as endosymbionts. Slowly, these cells got totally integrated into the host system creating plant cells where cyanobacteria metamorphosed into a specialized photosynthetic organelle called a *plastid*. The geochemical evidence suggests that oxygen reached a level such that it could inhibit nitrogen fixation and hence, differentiated specialized cells for nitrogen fixation emerged between 2.4 to 2.3 billion years ago (Tomitani *et al.* 2006).

Cyanobacteria are quite versatile photosynthetic prokaryotes offering potential applications in several sectors ranging from pharmaceuticals to fuels. They inhabit a range of terrestrial, aquatic, and marine habitats leading to their metabolic diversity and hence better industrial applications (Ruffing 2011). What makes these organisms truly unique is the ability to fix atmospheric nitrogen, produce non-ribosomal proteins, and produce a range of metabolites such as toxins, essential oils, anti-tumor agents, and so on. The toxic substances have great deal of commercial applications. They are a rich source of biologically active compounds with antiviral, antibacterial, antifungal, anticancer, and immunosuppressant properties. Recently, these organisms have attracted tremendous amounts of attention for being the candidates for third generation biofuel production. The widely accepted endo-symbiont theory owing the origin of plant cells to cyanobacteria makes it a favored organism to study among plant biologists (Figure 44.1).

Though these organisms offer enormous exciting applications, they are largely unexplored in India. Sequencing whole genomes did not seem quite feasible in the past because it was quite time consuming and expensive. Next-gen sequencers have drastically brought down the costs and have geared up the sequencing pace. In this chapter, we explore the current situation in cyanobacteria genomics and transcriptomics that can be exploited for commercial applications.

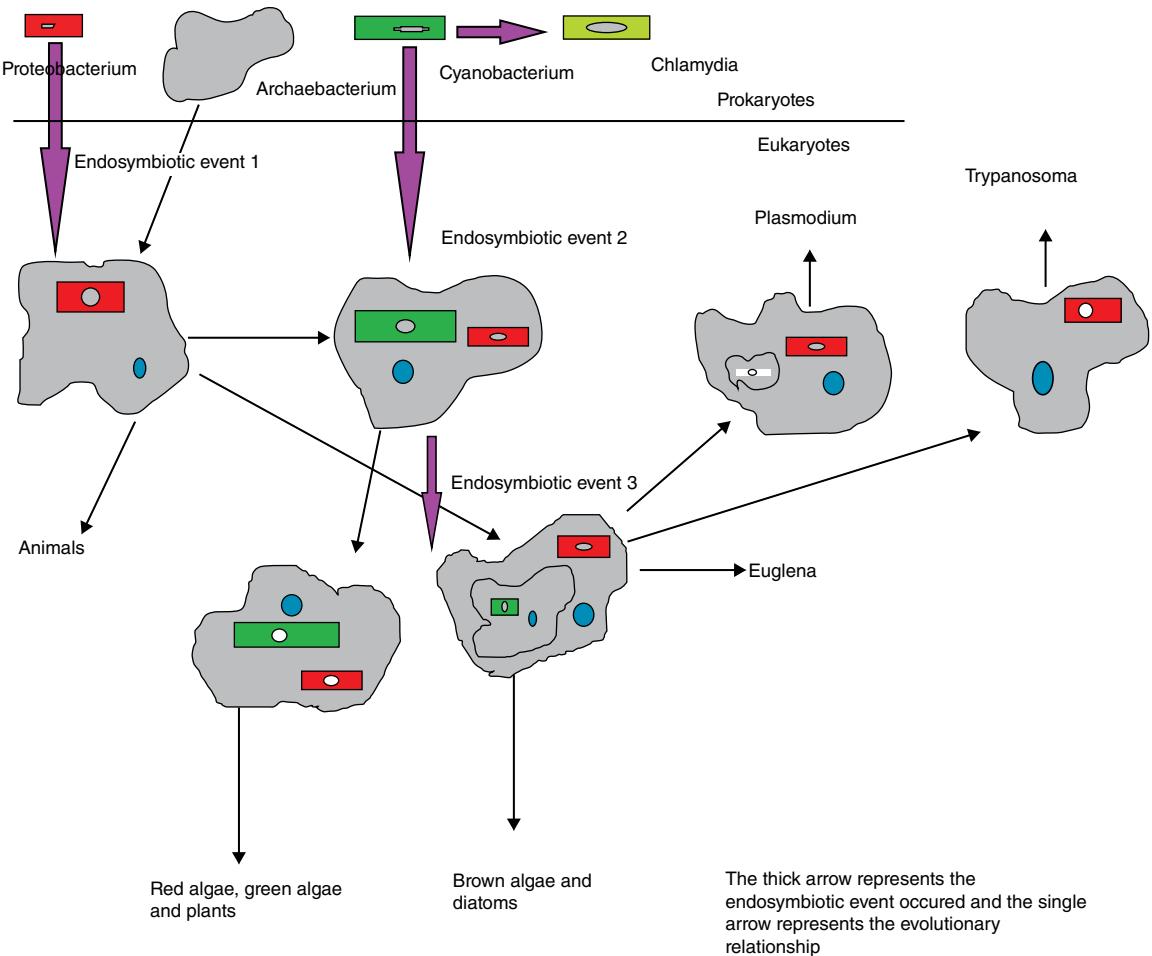


Figure 44.1 A schematic representation of evolution of plants and animal cells by endo-symbiosis. The first rectangle in first row is Proteobacteria; the second rectangle in first row indicates a cyanobacterial cell. The white rectangle inside plasmodium cell indicates loss of photosynthetic ability.

44.2 Phylogenetic Relationships in Cyanobacteria

Cyanobacteria are considered morphologically diverse but phylogenetically monophyletic. Many independent studies have been carried out that use several conserved genes such as *rbcL*, 16s, and rRNA (Tomitani *et al.* 2006) and concluded that they are monophyletic (Figure 44.2). Morphologically, five distinct groups of cyanobacteria have been identified: Group I: solitary and colonial unicellular (e.g., Chroococcales; *Synchococcus* and *Gloeocapsa*); Group II: Unicellular or pseudo-filamentous groups may form thallus like structures (e.g., Pleurocapsales; *Chroococcidiopsis*); Group III: Filamentous without cell differentiation (e.g., Oscillatoriaceae; *Oscillatoria*); Group IV: Filamentous with marked cell differentiation into specialized heterocysts and akinetes (e.g., Nostocales; *Nostoc* etc.); and Group V: Filamentous, with cell differentiation and showing pseudo branching (e.g., *Steigonematales*). However, all the phylogenetic studies of cyanobacteria indicate that filamentous forms evolved from the simpler ones and the specialized cells emerged after the filamentous forms.

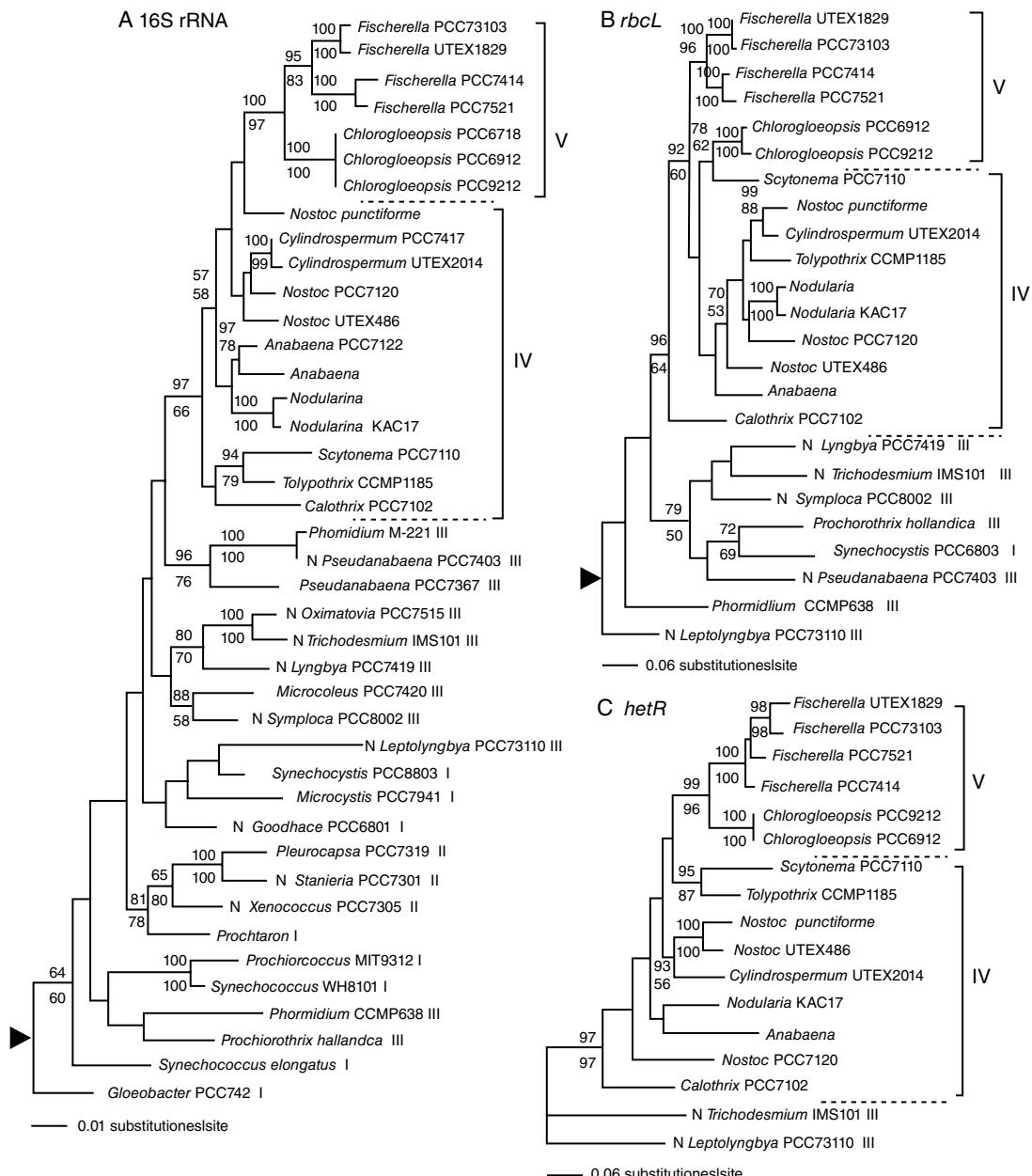


Figure 44.2 Evolutionary diagram indicating evolution in five clades of cyanobacteria. Tomitami et al. (2006). Reproduced with permission of the National Academy of Sciences of the USA.

A species called *Candidatus Gloeomargarita lithophora* was recently found by researchers. Phylogenetic analyses place this new species within the deeply divergent order *Gleobacterales*, a branch that diverged genetically from modern cyanobacteria a long, long time ago. A genetic analysis of similar bacteria in the *Gloeobacter* genus found several genes involved in photosynthesis were missing. The bacteria in this genus do not contain a thylakoid membrane, and therefore

do not have more advanced structures involved in photosynthesis that are found in other photosynthetic bacteria. This indicates that *Gloeobacter* is one of the oldest photosynthetic bacteria. The genome for *Candidatus Gloeomargarita lithophora* has not yet been studied, but a related microbe, *Gloeobacter violaceus*, has a single circular chromosome with no plasmid has been sequenced.

44.3 Genomic Studies of Cyanobacteria

Genomics in cyanobacteria is a very promising area because of the smaller genome size and high discovery rate (>95%) (Uzair *et al.* 2011). Sequencing and studying genomes and transcriptomes of cyanobacteria can pave the way for discovering novel metabolites (Baran and Gourcy 2013). The recent obvious interest of researchers in exploiting cyanobacteria have led to a rapid rise in completed genome sequences but still a lot needs to be done. The number of completely sequenced genomes for cyanobacteria is increasing rapidly and there are many ongoing and targeted genome sequencing projects (www.genomesonline.org). Currently, a Gen Bank search on available whole genome sequence lists about 296 records, out of which, 70 are gapless full assemblies, 12 full assemblies with gaps, 63 genomes at scaffold levels, and the remaining 151 assemblies at contig levels (Figure 44.3a). The number of cyanobacteria genomes available to date is overly represented by Group I and II species with very few representing Group III, IV, and V (Figure 44.3b). Out of the genomes available at the chromosome level with gaps, the genome size shows a wide variation (from 1.6 MB to ~9 MB) (Table 44.1).

Comparative genomics have revealed that genomes of some species are drastically reduced due to loss of several genes in comparison to other related genomes (Batut *et al.* 2013). It has been found that the 16S rRNA sequence of some species found in India does not match with other members belonging to same species (personal communication). This has significant evolutionary implications and gives insights on speciation. Regarding cell division, it has been found that cyanobacteria shares some genes with green algae, higher plants, and bacteria but few genes are specific to cyanobacteria only (Koksharova and Babykin 2011). All this interesting information on cyanobacteria could be obtained by studying their genomes and transcriptomes. Also, this information can help pave the way for producing desired metabolites in commercial scale through genetic engineering certain pathways.

With time, cyanobacterial genome has also changed. The smallest genome found up until now is *Prochlorococcus marinus str 9301* with a genome size of 1.6 MB and number of genes at about 1962. The advanced ones have a larger genome size compared to the primitive ones; *Nostoc punctiforme 73102* has genome size of 9.06 MB and contains 7614 genes. It is found that the large genomes are disproportionately enriched in regulation and secondary metabolism genes and depleted in protein translation, DNA replication, cell division, and nucleotide metabolism genes when compared to medium and small sized genomes. Furthermore, large genomes do not accumulate noncoding DNA or hypothetical ORFs, because the portion of the genome devoted to these functions remained constant with genome size.

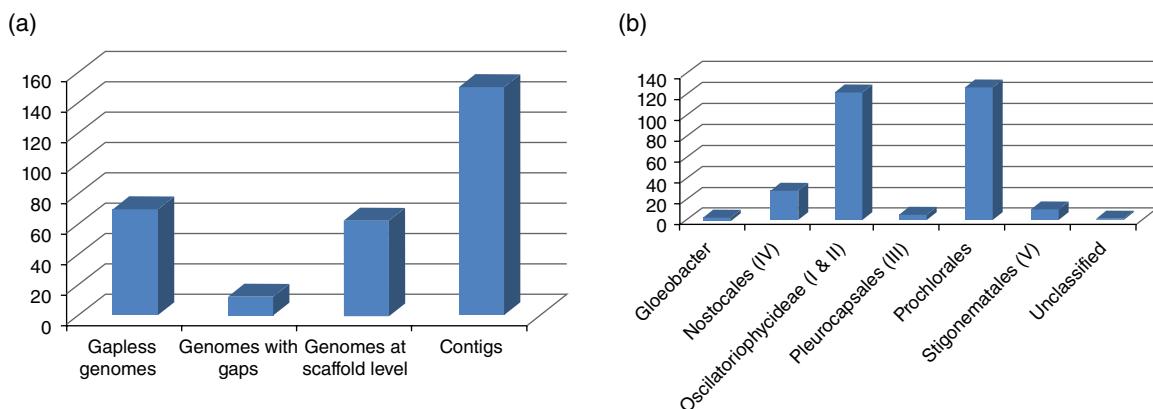


Figure 44.3 Genome sequencing statistics. (a) Availability of assembled genomes in Gen Bank. (b) Distribution of sequenced genomes in different clades of cyanobacteria.

Table 44.1 List of the completely sequenced cyanobacterial genomes in Gen Bank.

S. no	Organism Name	Genome	Transcriptome	Genome size (MB)	No. of genes
1	<i>Synechococcus</i> sp. CC9311	Yes	Yes	2.61	2944
2	<i>Synechococcus</i> sp. CC9605	Yes	Yes	2.51	2756
3	<i>Synechococcus</i> sp. CC9902	Yes	Yes	2.23	2357
4	<i>Synechococcus</i> sp. JA-3-3Ab	Yes	Yes	2.93	2897
5	<i>Synechococcus</i> sp. PCC 7002	Yes	Yes	3.41	2875
6	<i>Synechococcus</i> sp. WH 8102	Yes	Yes	2.43	2581
7	<i>Synechococcus</i> sp. WH7803	Yes	Yes	2.37	2586
8	<i>Synechococcus</i> sp. RCC307	Yes	Yes	2.22	2582
9	<i>Synechococcus</i> sp. PCC 7336	Yes	Yes	5.07	Not available
10	<i>Synechococcus</i> sp. JA-2-3B'a(2-13)	Yes	Yes	3.05	2942
11	<i>Synechococcus</i> sp. PCC 6312	Yes	Yes	3.72	3794
12	<i>Synechococcus</i> sp. PCC 7502	Yes	Yes	3.58	3666
13	<i>Chloroflexus aurantiacus</i> J-10-fl	Yes	No	5.26	3396
14	<i>Microcystis aeruginosa</i> NIES-843	Yes	Yes	5.84	6364
15	<i>Prochlorococcus marinus</i> subsp. <i>marinus</i> str. CCMP1986	Yes	Yes	1.66	1762
16	<i>Prochlorococcus marinus</i> subsp. <i>marinus</i> str. CCMP1375	Yes	Yes	1.75	1930
17	<i>Prochlorococcus marinus</i> MIT 9215	Yes	Yes	1.74	2054
18	<i>Prochlorococcus marinus</i> str. AS9601	Yes	Yes	1.67	1965
19	<i>Prochlorococcus marinus</i> str. MIT 9211	Yes	Yes	1.69	1900
20	<i>Prochlorococcus marinus</i> str. MIT 9301	Yes	Yes	1.64	1962
21	<i>Prochlorococcus marinus</i> str. MIT 9312	Yes	Yes	1.71	1856
22	<i>Prochlorococcus marinus</i> str. MIT 9313	Yes	Yes	2.41	2330
23	<i>Prochlorococcus marinus</i> str. MIT 9515	Yes	Yes	1.7	1964
24	<i>Prochlorococcus marinus</i> str. NATL1A	Yes	Yes	1.86	2250
25	<i>Prochlorococcus marinus</i> str. NATL2A	Yes	Yes	1.84	2228
26	<i>Cyanothece</i> sp. PCC 7822	Yes	Yes	6.09	5664
27	<i>Cyanothece</i> sp. ATCC 51142	Yes	Yes	4.93	4821
28	<i>Cyanothece</i> sp. PCC 7424	Yes	Yes	5.94	5410
29	<i>Cyanothece</i> sp. PCC 8801	Yes	Yes	4.68	4498
30	<i>Cyanothece</i> sp. PCC 8802	Yes	Yes	4.67	4561
31	<i>Cyanothece</i> sp. PCC 7425	Yes	Yes	5.37	5113
32	<i>Leptolyngbya</i> sp. PCC 7376	Yes	No	5.13	4654
33	<i>Acaryochloris marina</i> MBIC11017	Yes	No	8.36	8383
34	<i>Anabaena</i>	Yes	No	5.31	4797
35	<i>Anabaena cylindrica</i> PCC 7122	Yes	No	7.06	6258
36	<i>Anabaena variabilis</i> ATCC 29413	Yes	No	7.11	5813
37	<i>Arthospira platensis</i> C1	Yes	No	6.09	6153
38	<i>Arthospira platensis</i> NIES-39	Yes	No	6.79	6630
39	<i>Calothrix</i> sp. PCC 6303	Yes	No	6.96	5841
40	<i>Calothrix</i> sp. PCC 7507	Yes	No	7.02	5950
41	<i>Chamaesiphon minutus</i> PCC 6605	Yes	No	6.76	6426
42	<i>Chroococcidiopsis thermalis</i> PCC 7203	Yes	No	6.69	6033
43	<i>Crinalium epipsammum</i> PCC 9333	Yes	No	5.62	5059
44	<i>Cyanobacterium stanieri</i> PCC 7202	Yes	No	3.16	2941
45	<i>Cyanobium gracile</i> PCC 6307	Yes	No	3.34	3437
46	<i>Cylindrospermum stagnale</i> PCC 7417	Yes	No	7.61	6738
47	<i>Dactylococcopsis salina</i> PCC 8305	Yes	No	3.78	3684
48	<i>Geitlerinema</i> sp. PCC 7407	Yes	No	4.68	3912
49	<i>Geminocystis herdmanii</i> PCC 6308	Yes	No	4.26	Not available
50	<i>Gloeobacter violaceus</i> PCC 7421	Yes	No	4.66	4482

(Continued)

Table 44.1 (Continued)

S. no	Organism Name	Genome	Transcriptome	Genome size (MB)	No. of genes
51	<i>Gloeocapsa</i> sp. PCC 7428	Yes	No	5.88	5304
52	<i>Halotheca</i> sp. PCC 7418	Yes	No	4.18	3920
53	<i>Microcoleus</i> sp. PCC 7113	Yes	No	7.97	8821
54	<i>Nostoc</i> sp. PCC 7120	Yes	No	7.21	6213
55	<i>Nostoc</i> sp. PCC 7107	Yes	Yes	6.33	5538
56	<i>Nostoc</i> sp. PCC 7524	Yes	Yes	6.72	5687
57	<i>Oscillatoria acuminata</i> PCC 6304	Yes	No	7.8	6100
58	<i>Oscillatoria nigro-viridis</i> PCC 7112	Yes	No	8.27	7006
59	<i>Oscillatoriales cyanobacterium</i> JSC-12	Yes	No	5.53	5080
60	<i>Nostoc punctiforme</i> PCC 73102	Yes	No	9.06	7614
61	<i>Oscillatoria</i> sp. PCC 6506	Yes	No	8.27	7006
62	<i>Pleurocapsa</i> sp. PCC 7327	Yes	No	4.99	4665
63	<i>Pseudanabaena</i> sp. PCC 7367	Yes	No	4.89	4014
64	<i>Rivularia</i> sp. PCC 7116	Yes	No	8.73	6946
65	<i>Stanieria cyanosphaera</i> PCC 7437	Yes	No	5.54	5041
66	<i>Synechococcus elongatus</i> PCC 6301	Yes	Yes	2.7	2581
67	<i>Synechococcus elongatus</i> PCC 7942	Yes	Yes	2.74	2751
68	<i>Synechocystis</i> sp. PCC 6803	Yes	Yes	3.95	3625
69	<i>Synechocystis</i> sp. PCC 6803 substr. GT-I	Yes	Yes	3.57	3217
70	<i>Synechocystis</i> sp. PCC 6803 substr. PCC-N	Yes	Yes	3.57	3217
71	<i>Synechocystis</i> sp. PCC 6803 substr. PCC-P	Yes	Yes	3.57	3217
72	<i>Trichodesmium erythraeum</i> IMS101	Yes	No	7.75	5216
73	<i>Nostoc azollae</i> 0708	Yes	No	5.49	5380
74	<i>Thermosynechococcus elongatus</i> BP-1	Yes	Yes	2.59	2525
75	<i>Nostoc</i> sp. PCC 7107	Yes	Yes	6.33	5538
76	<i>Nostoc</i> sp. PCC 7120	Yes	Yes	7.21	6213
77	<i>Nostoc</i> sp. PCC 7524	Yes	Yes	6.72	5687
78	<i>Ruminococcus torques</i>	Yes	No	3.34	2848
79	<i>Roseburia intestinalis</i>	Yes	No	4.14	3534
80	<i>Roseburia intestinalis</i> XB6B4	Yes	No	4.29	3669
81	<i>Coprococcus catus</i> GD/7	Yes	No	3.52	3040
82	<i>Ruminococcus</i> sp.	Yes	No	3.55	3313
83	<i>Chloroflexus aurantiacus</i> J-10-fl	Yes	No	5.26	3390

As new genomes are sequenced, the need for estimating completeness of the genomes appears very important. In this effort, a number of groups have been working on finding the core conserved genes in cyanobacteria. The first such serious work was published in PNAs (Mulkidjanian, *et al.* 2006). Their work produced about 1054 protein families that were common in 14 genomes out of 15 they studied. They named this cluster as CyOG. We have listed few genes in Table 44.2 that are evolutionarily conserved among cyanobacterial species.

44.4 Plasmids in Cyanobacteria

Plasmids carry the mobile genetic elements and act as easy transporters of genes from one species to other. However, studies on evolutionary patterns and similarities within cyanobacterial species are still lacking. There are several reports to indicate that genetically diverse cyanobacteria may possess identical plasmids (Schaefer *et al.* 1993). In this regard, we have carried out extensive analysis on the similarities between the plasmids across cyanobacterial clades and also with their host and non-host genomes. The percentage of plasmid incorporation was found to be rather much smaller than earlier thought. The largest percentage of cyanobacterial plasmid incorporation into the genome is found in species *Synechocystis* (3.8%) and the smallest in *Anabaena* sp. (0.008%). Fragments of plasmids from one species were also reported in others. The plasmids from *Scytonema hoffmanni* have the largest number of hits among cyanobacterial genomes (Table 44.3).

Table 44.2 List of well conserved genes across cyanobacterial species.

Sr. No.	Gene name	Function
1	kaiABC	Controls circadian clock ¹
2	Hip1	Palindrome (GCGATCGC)
3	IS elements	Role in genome plasticity and other evolutionary aspects
4	Integrases	
5	RbcL	Gene for synthesis of RuBISCO protein and thus involved in carbon fixation pathway
6	Cb12	Present in all the photosynthetic organisms, about 80 aa in length. Regulates Calvin cycle by reversibly binding to glyceraldehydes-3P ²
6	HetR, nifH, and nif D	Nitrogenase
7	gltX gene (glutamyl-tRNA synthetase, EC 6.1.1.17)	enzyme that charges the glutamic acid tRNA
8	desC1 gene	A Δ9 acyl-lipid desaturase (EC 1.14.19.1).
9	ada gene	Responsible for correction of methylated Guanine and Thymine. Absence of this protein may lead to genome reduction in the AT rich region
10	radC	UV DNA repair protein
11	NRPSs	Synthesis of secondary metabolites
12	PKSs	Synthesis of secondary metabolites
13	ycf3	Assembly of photosystem1
14	dnaN	DNA Polymerase III beta subunit
15	argH	Fumarate lyase: Delta crystallin
16	dnaJ	DnaJ protein
17	psbN	Photosystem II reaction centre N protein
18	psbH	Photosystem II 10 kDa phosphoprotein
19	ntcA	Global nitrogen regulatory protein, CRP family of transcriptional regulators
20	glnB	nitrogen regulatory protein P-II
21	psbC	photosystem II chlorophyll-binding protein CP43
22	dnaE	DNA polymerase III, alpha subunit
23	htpG	heat shock protein HtpG
24	chlG	chlorophyll synthase 33 kD subunit
25	cbbL, rbcL	Ribulose bisphosphate carboxylase, large chain
26	pcr, por	Light dependent protochlorophyllide oido-reductase
27	PipX	PII-interacting protein X ³
28	HmpD	For polysaccharide secretion and extremely conserved in filamentous Cyanobacteria. Located in hpsA region. ⁴

Notes/References

1. Beck *et al.* (2014).
2. Stanley *et al.* (2011).
3. Espinosa *et al.* (2014).
4. Risser and Meeks (2013).

44.5 Dissecting Transcriptomes of Cyanobacteria

A Pub Med search on transcripts and cyanobacteria returns about 450 records. Most of these studies are on understanding few genes in a gene family. With the advent of next gen sequencing technologies, it has been much easier to conduct whole genome transcriptomics. Transcriptomics can help calculate the number of transcripts and thus gene activity, that is, finding activity of particular gene by knowing and comparing its expression in different cellular conditions, gene functions, and gene related diseases can be studied. Here, we discuss few of the genes that have been studied extensively from economic standpoint.

44.5.1 Biofuel Production

Among the genes that are of utmost interest to researchers; biofuel producing genes are the foremost. Of the various applications attributed to cyanobacteria, the one that seems quite exciting is biofuel production. Cyanobacterial species have been reported to produce hydrogen gas, ethanol, isobutanol, alkanes, and so on as secondary metabolites. Though the

Table 44.3 Percentage of plasmid genomes incorporated into different cyanobacterial genomes.

Plasmid	Genome	Percentage of insertion of plasmid in genome
<i>Tolyphothrix [Scytonema hofmanni]</i> UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.8	NC_013771.1	0.02746
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	NC_013771.1	2.987144
<i>Anabaena cylindrica</i> PCC 7122 plasmid pANACY.04	<i>Calothrix</i> sp. PCC 6303	0.02373
<i>Calothrix</i> sp. PCC 6303 plasmid pCAL6303.01	<i>Calothrix</i> sp. PCC 6303	0.389519
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	<i>Calothrix</i> sp. PCC 6303	0.15751
<i>Nostoc</i> sp. PCC 7120 plasmid pCC7120alpha	<i>Calothrix</i> sp. PCC 6303	0.02008
<i>Tolyphothrix [Scytonema hofmanni]</i> UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.8	<i>Calothrix</i> sp. PCC 6303	0.383387
<i>Tolyphothrix [Scytonema hofmanni]</i> UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.9	<i>Calothrix</i> sp. PCC 6303	0.053725
<i>Acaryochloris marina</i> MBIC11017 plasmid pREB1	<i>Acaryochloris marina</i> MBIC11017	1.218025
<i>Acaryochloris marina</i> MBIC11017 plasmid pREB2	<i>Acaryochloris marina</i> MBIC11017	2.062511
<i>Acaryochloris marina</i> MBIC11017 plasmid pREB3	<i>Acaryochloris marina</i> MBIC11017	1.093604
<i>Acaryochloris marina</i> MBIC11017 plasmid pREB4	<i>Acaryochloris marina</i> MBIC11017	0.801402
<i>Acaryochloris marina</i> MBIC11017 plasmid pREB5	<i>Acaryochloris marina</i> MBIC11017	0.786442
<i>Acaryochloris marina</i> MBIC11017 plasmid pREB6	<i>Acaryochloris marina</i> MBIC11017	0.889106
<i>Acaryochloris marina</i> MBIC11017 plasmid pREB7	<i>Acaryochloris marina</i> MBIC11017	1.077706
<i>Acaryochloris marina</i> MBIC11017 plasmid pREB8	<i>Acaryochloris marina</i> MBIC11017	0.691235
<i>Acaryochloris marina</i> MBIC11017 plasmid pREB9	<i>Acaryochloris marina</i> MBIC11017	0.032074
<i>Anabaena cylindrica</i> PCC 7122 plasmid pANACY.01	<i>Anabaena cylindrica</i> PCC 7122	0.484268
<i>Anabaena cylindrica</i> PCC 7122 plasmid pANACY.02	<i>Anabaena cylindrica</i> PCC 7122	0.01884
<i>Anabaena cylindrica</i> PCC 7122 plasmid pANACY.03	<i>Anabaena cylindrica</i> PCC 7122	0.708727
<i>Anabaena cylindrica</i> PCC 7122 plasmid pANACY.04	<i>Anabaena cylindrica</i> PCC 7122	0.479187
<i>Anabaena</i> sp. 90 plasmid pANA01	<i>Anabaena cylindrica</i> PCC 7122	0.008162
<i>Anabaena</i> sp. 90 plasmid pANA02	<i>Anabaena cylindrica</i> PCC 7122	0.112261
<i>Cylindrospermum stagnale</i> PCC 7417 plasmid pCYLST.01 partial sequence	<i>Anabaena cylindrica</i> PCC 7122	0.340378
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	<i>Anabaena cylindrica</i> PCC 7122	0.117076
' <i>Nostoc azollae</i> ' 0708 plasmid pAzo01	<i>Anabaena cylindrica</i> PCC 7122	0.019825
<i>Nostoc punctiforme</i> PCC 73102 plasmid pNPUN01	<i>Anabaena cylindrica</i> PCC 7122	0.058945
<i>Nostoc</i> sp. PCC 7120 plasmid pCC7120alpha	<i>Anabaena cylindrica</i> PCC 7122	0.028581
<i>Tolyphothrix [Scytonema hofmanni]</i> UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.4	<i>Anabaena cylindrica</i> PCC 7122	0.009053
<i>Tolyphothrix [Scytonema hofmanni]</i> UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.8	<i>Anabaena cylindrica</i> PCC 7122	0.394507
<i>Anabaena cylindrica</i> PCC 7122 plasmid pANACY.01	<i>Anabaena</i> sp. 90chANA01	0.062343
<i>Anabaena cylindrica</i> PCC 7122 plasmid pANACY.02	<i>Anabaena</i> sp. 90chANA01	0.031853
<i>Anabaena</i> sp. 90 plasmid pANA01	<i>Anabaena</i> sp. 90chANA01	0.081677
<i>Anabaena</i> sp. 90 plasmid pANA02	<i>Anabaena</i> sp. 90chANA01	0.351469
<i>Cylindrospermum stagnale</i> PCC 7417 plasmid pCYLST.02	<i>Anabaena</i> sp. 90chANA01	0.015753
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	<i>Anabaena</i> sp. 90chANA01	0.32047
<i>Tolyphothrix [Scytonema hofmanni]</i> UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.8	<i>Anabaena</i> sp. 90chANA01	0.595274
<i>Anabaena variabilis</i> ATCC 29413 plasmid A	<i>Anabaena variabilis</i> ATCC29413	1.419712
<i>Anabaena variabilis</i> ATCC 29413 plasmid C	<i>Anabaena variabilis</i> ATCC29413	0.544321
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	<i>Anabaena variabilis</i> ATCC29413	0.174733
<i>Nostoc punctiforme</i> PCC 73102 plasmid pNPUN03	<i>Anabaena variabilis</i> ATCC29413	0.228144
<i>Nostoc</i> sp. PCC 7120 plasmid pCC7120delta DNA	<i>Anabaena variabilis</i> ATCC29413	0.014484
<i>Nostoc</i> sp. PCC 7524 plasmid pNOST7524.01	<i>Anabaena variabilis</i> ATCC29413	0.008907
<i>Tolyphothrix [Scytonema hofmanni]</i> UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.8	<i>Anabaena variabilis</i> ATCC29413	0.386884

Table 44.3 (Continued)

Plasmid	Genome	Percentage of insertion of plasmid in genome
<i>Tolyphothrix [Scytonema hofmanni] UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.9</i>	<i>Anabaena variabilis</i> ATCC29413	0.025543
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	<i>Arthospira platensis</i> NIES-39	0.083024
<i>Tolyphothrix [Scytonema hofmanni] UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.8</i>	<i>Arthospira platensis</i> NIES-39	0.044193
<i>Crinalium epipsammum</i> PCC 9333 plasmid pCRI9333.04	<i>Calothrix</i> sp. PCC 7507	0.073855
<i>Cylindrospermum stagnale</i> PCC 7417 plasmid pCYLST.01 partial sequence	<i>Calothrix</i> sp. PCC 7507	0.021529
<i>Cylindrospermum stagnale</i> PCC 7417 plasmid pCYLST.02	<i>Calothrix</i> sp. PCC 7507	0.013584
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	<i>Calothrix</i> sp. PCC 7507	0.153576
<i>Nostoc</i> sp. PCC 7120 plasmid pCC7120alpha	<i>Calothrix</i> sp. PCC 7507	0.012487
<i>Oscillatoria nigro-viridis</i> PCC 7112 plasmid pOSC7112.02	<i>Calothrix</i> sp. PCC 7507	0.009554
<i>Tolyphothrix [Scytonema hofmanni] UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.7</i>	<i>Calothrix</i> sp. PCC 7507	0.033062
<i>Tolyphothrix [Scytonema hofmanni] UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.8</i>	<i>Calothrix</i> sp. PCC 7507	0.526696
<i>Tolyphothrix [Scytonema hofmanni] UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.9</i>	<i>Calothrix</i> sp. PCC 7507	0.125584
<i>Chamaesiphon minutus</i> PCC 6605 plasmid pCHA6605.01 partial sequence	<i>Chamaesiphon minutus</i> PCC6605	1.774862
<i>Chamaesiphon minutus</i> PCC 6605 plasmid pCHA6605.02	<i>Chamaesiphon minutus</i> PCC6605	0.122993
<i>Cylindrospermum stagnale</i> PCC 7417 plasmid pCYLST.02	<i>Chamaesiphon minutus</i> PCC6605	0.028166
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	<i>Chamaesiphon minutus</i> PCC6605	0.071323
<i>Tolyphothrix [Scytonema hofmanni] UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.8</i>	<i>Chamaesiphon minutus</i> PCC6605	0.071275
<i>Anabaena variabilis</i> ATCC 29413 plasmid C	<i>Chroococcidiopsis thermalis</i> pcc7203	0.052202
<i>Chroococcidiopsis thermalis</i> PCC 7203 plasmid pCHRO.01	<i>Chroococcidiopsis thermalis</i> pcc7203	0.032332
<i>Gloeocapsa</i> sp. PCC 7428 plasmid pGLO7428.02	<i>Chroococcidiopsis thermalis</i> pcc7203	0.012967
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	<i>Chroococcidiopsis thermalis</i> pcc7203	0.128392
<i>Tolyphothrix [Scytonema hofmanni] UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.8</i>	<i>Chroococcidiopsis thermalis</i> pcc7203	0.146316
<i>Crinalium epipsammum</i> PCC 9333 plasmid pCRI9333.01	<i>Crinalium epipsammum</i> PCC9333	0.014166
<i>Crinalium epipsammum</i> PCC 9333 plasmid pCRI9333.02	<i>Crinalium epipsammum</i> PCC9333	0.36698
<i>Crinalium epipsammum</i> PCC 9333 plasmid pCRI9333.04	<i>Crinalium epipsammum</i> PCC9333	0.062232
<i>Crinalium epipsammum</i> PCC 9333 plasmid pCRI9333.05	<i>Crinalium epipsammum</i> PCC9333	0.086219
<i>Crinalium epipsammum</i> PCC 9333 plasmid pCRI9333.07	<i>Crinalium epipsammum</i> PCC9333	0.188616
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	<i>Crinalium epipsammum</i> PCC9333	0.201766
<i>Tolyphothrix [Scytonema hofmanni] UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.8</i>	<i>Crinalium epipsammum</i> PCC9333	0.155713
<i>Cyanobacterium aponinum</i> PCC 10605 plasmid pCYAN10605.01	<i>Cyanobacterium aponinum</i> PCC10605	0.282638
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	<i>Cyanothece</i> sp. PCC 7424	0.066704
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	<i>cyanothece</i> sp. PCC7425	0.099171
<i>Tolyphothrix [Scytonema hofmanni] UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.8</i>	<i>cyanothece</i> sp. PCC7425	0.102817
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	<i>Cyanothece</i> sp. PCC7822	0.123186
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	<i>Cyanothece</i> sp. PCC8801	0.064025
<i>Tolyphothrix [Scytonema hofmanni] UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.8</i>	<i>Cyanothece</i> sp. PCC8801	0.127879
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	<i>cyanothece</i> sp. PCC8802	0.128313
<i>Tolyphothrix [Scytonema hofmanni] UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.8</i>	<i>cyanothece</i> sp. PCC8802	0.127671

(Continued)

Table 44.3 (Continued)

Plasmid	Genome	Percentage of insertion of plasmid in genome
<i>Anabaena cylindrica</i> PCC 7122 plasmid pANACY.01	<i>Cylindrospermum stagnale</i> PCC7417	0.007939
<i>Anabaena</i> sp. 90 plasmid pANA02	<i>Cylindrospermum stagnale</i> PCC7417	0.029942
<i>Cylindrospermum stagnale</i> PCC 7417 plasmid pCYLST.01 partial sequence	<i>Cylindrospermum stagnale</i> PCC7417	1.101811
<i>Cylindrospermum stagnale</i> PCC 7417 plasmid pCYLST.02	<i>Cylindrospermum stagnale</i> PCC7417	0.009167
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	<i>Cylindrospermum stagnale</i> PCC7417	0.158619
<i>Tolyphothrix</i> [<i>Scytonema hofmannii</i>] UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.8	<i>Cylindrospermum stagnale</i> PCC7417	0.374895
<i>Tolyphothrix</i> [<i>Scytonema hofmannii</i>] UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.9	<i>Cylindrospermum stagnale</i> PCC7417	0.021889
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	<i>Dactylococcopsis salina</i> PCC8305	0.039275
<i>Tolyphothrix</i> [<i>Scytonema hofmannii</i>] UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.8	<i>Dactylococcopsis salina</i> PCC8305	0.092198
<i>Anabaena variabilis</i> ATCC 29413 plasmid C	<i>Geilerinema</i> sp. PCC7407	0.01427
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	<i>Geilerinema</i> sp. PCC7407	0.160496
<i>Tolyphothrix</i> [<i>Scytonema hofmannii</i>] UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.8	<i>Geilerinema</i> sp. PCC7407	0.180171
<i>Anabaena variabilis</i> ATCC 29413 plasmid C	<i>Gloeobacter violaceus</i> PCC7421	0.018523
<i>Oscillatoria nigro-viridis</i> PCC 7112 plasmid pOSC7112.02	<i>Gloeobacter violaceus</i> PCC7421	0.018523
<i>Tolyphothrix</i> [<i>Scytonema hofmannii</i>] UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.8	<i>Gloeobacter violaceus</i> PCC7421	0.011998
<i>Gloeocapsa</i> sp. PCC 7428 plasmid pGLO7428.01	<i>Gloeocapsa</i> sp. PCC7428	0.011378
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	<i>Gloeocapsa</i> sp. PCC7428	0.102625
<i>Tolyphothrix</i> [<i>Scytonema hofmannii</i>] UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.8	<i>Gloeocapsa</i> sp. PCC7428	0.133187
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	<i>Leptolyngbya</i> sp. PCC7376	0.047679
<i>Tolyphothrix</i> [<i>Scytonema hofmannii</i>] UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.8	<i>Leptolyngbya</i> sp. PCC7376	0.022786
<i>Anabaena variabilis</i> ATCC 29413 plasmid A	<i>Microcoleus</i> sp. PCC7113	0.047588
<i>Anabaena variabilis</i> ATCC 29413 plasmid C	<i>Microcoleus</i> sp. PCC7113	0.075404
<i>Cylindrospermum stagnale</i> PCC 7417 plasmid pCYLST.02	<i>Microcoleus</i> sp. PCC7113	0.013225
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.01	<i>Microcoleus</i> sp. PCC7113	0.483761
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	<i>Microcoleus</i> sp. PCC7113	0.585562
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.03	<i>Microcoleus</i> sp. PCC7113	0.278123
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.04	<i>Microcoleus</i> sp. PCC7113	0.213696
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.06	<i>Microcoleus</i> sp. PCC7113	0.031792
<i>Oscillatoria nigro-viridis</i> PCC 7112 plasmid pOSC7112.02	<i>Microcoleus</i> sp. PCC7113	0.059394
<i>Tolyphothrix</i> [<i>Scytonema hofmannii</i>] UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.8	<i>Microcoleus</i> sp. PCC7113	0.082231
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	<i>Microcystis aeruginosa</i> NIES-843	0.138085
<i>Anabaena cylindrica</i> PCC 7122 plasmid pANACY.01	<i>Microcystis aeruginosa</i> NIES-843	
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	<i>Nostoc azollae</i> 0708	0.015127
<i>Tolyphothrix</i> [<i>Scytonema hofmannii</i>] UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.4	<i>Nostoc azollae</i> 0708	0.207388
<i>Tolyphothrix</i> [<i>Scytonema hofmannii</i>] UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.8	<i>Nostoc azollae</i> 0708	0.029189
<i>Anabaena variabilis</i> ATCC 29413 plasmid A	<i>Nostoc punctiforme</i> sp. PCC73102	0.371505
<i>Anabaena variabilis</i> ATCC 29413 plasmid C	<i>Nostoc punctiforme</i> sp. PCC73102	0.041182
<i>Cylindrospermum stagnale</i> PCC 7417 plasmid pCYLST.01 partial sequence	<i>Nostoc punctiforme</i> sp. PCC73102	0.404348
<i>Cylindrospermum stagnale</i> PCC 7417 plasmid pCYLST.02	<i>Nostoc punctiforme</i> sp. PCC73102	0.341962
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	<i>Nostoc punctiforme</i> sp. PCC73102	0.042542

Table 44.3 (Continued)

Plasmid	Genome	Percentage of insertion of plasmid in genome
' <i>Nostoc azollae</i> ' 0708 plasmid pAzo01	<i>Nostoc punctiforme</i> sp. <i>PCC73102</i>	0.069721
<i>Nostoc punctiforme</i> PCC 73102 plasmid pNPUN01	<i>Nostoc punctiforme</i> sp. <i>PCC73102</i>	1.07722
<i>Nostoc punctiforme</i> PCC 73102 plasmid pNPUN02	<i>Nostoc punctiforme</i> sp. <i>PCC73102</i>	1.123369
<i>Nostoc punctiforme</i> PCC 73102 plasmid pNPUN03	<i>Nostoc punctiforme</i> sp. <i>PCC73102</i>	0.261384
<i>Nostoc punctiforme</i> PCC 73102 plasmid pNPUN04	<i>Nostoc punctiforme</i> sp. <i>PCC73102</i>	0.072405
<i>Nostoc punctiforme</i> PCC 73102 plasmid pNPUN05	<i>Nostoc punctiforme</i> sp. <i>PCC73102</i>	0.016249
<i>Nostoc</i> sp. PCC 7120 plasmid pCC7120alpha	<i>Nostoc punctiforme</i> sp. <i>PCC73102</i>	0.027714
<i>Nostoc</i> sp. PCC 7120 plasmid pCC7120beta DNA	<i>Nostoc punctiforme</i> sp. <i>PCC73102</i>	0.162225
<i>Nostoc</i> sp. PCC 7120 plasmid pCC7120gamma DNA	<i>Nostoc punctiforme</i> sp. <i>PCC73102</i>	0.162492
<i>Tolyphothrix</i> [<i>Scytonema hofmanni</i>] UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.6	<i>Nostoc punctiforme</i> sp. <i>PCC73102</i>	0.18332
<i>Tolyphothrix</i> [<i>Scytonema hofmanni</i>] UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.8	<i>Nostoc punctiforme</i> sp. <i>PCC73102</i>	0.53411
<i>Tolyphothrix</i> [<i>Scytonema hofmanni</i>] UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.9	<i>Nostoc punctiforme</i> sp. <i>PCC73102</i>	0.078173
<i>Anabaena variabilis</i> ATCC 29413 plasmid C	<i>Nostoc</i> sp. <i>pCC7107</i>	0.123495
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	<i>Nostoc</i> sp. <i>pCC7107</i>	0.175487
<i>Nostoc punctiforme</i> PCC 73102 plasmid pNPUN03	<i>Nostoc</i> sp. <i>pCC7107</i>	0.031059
<i>Nostoc</i> sp. PCC 7120 plasmid pCC7120alpha	<i>Nostoc</i> sp. <i>pCC7107</i>	0.511136
<i>Nostoc</i> sp. PCC 7120 plasmid pCC7120beta DNA	<i>Nostoc</i> sp. <i>pCC7107</i>	0.134601
<i>Nostoc</i> sp. PCC 7120 plasmid pCC7120delta DNA	<i>Nostoc</i> sp. <i>pCC7107</i>	0.093747
<i>Nostoc</i> sp. PCC 7120 plasmid pCC7120gamma DNA	<i>Nostoc</i> sp. <i>pCC7107</i>	0.01177
<i>Tolyphothrix</i> [<i>Scytonema hofmanni</i>] UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.6	<i>Nostoc</i> sp. <i>pCC7107</i>	0.05057
<i>Tolyphothrix</i> [<i>Scytonema hofmanni</i>] UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.8	<i>Nostoc</i> sp. <i>pCC7107</i>	0.404008
<i>Tolyphothrix</i> [<i>Scytonema hofmanni</i>] UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.9	<i>Nostoc</i> sp. <i>pCC7107</i>	0.026383
<i>Anabaena cylindrica</i> PCC 7122 plasmid pANACY.03	<i>Nostoc</i> sp. <i>pCC7120</i>	0.020113
<i>Anabaena variabilis</i> ATCC 29413 plasmid A	<i>Nostoc</i> sp. <i>pCC7120</i>	0.16912
<i>Anabaena variabilis</i> ATCC 29413 plasmid C	<i>Nostoc</i> sp. <i>pCC7120</i>	0.059887
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	<i>Nostoc</i> sp. <i>pCC7120</i>	0.173424
<i>Nostoc punctiforme</i> PCC 73102 plasmid pNPUN01	<i>Nostoc</i> sp. <i>pCC7120</i>	0.030403
<i>Nostoc</i> sp. PCC 7120 plasmid pCC7120alpha	<i>Nostoc</i> sp. <i>pCC7120</i>	0.192305
<i>Nostoc</i> sp. PCC 7120 plasmid pCC7120beta DNA	<i>Nostoc</i> sp. <i>pCC7120</i>	0.013331
<i>Nostoc</i> sp. PCC 7120 plasmid pCC7120gamma DNA	<i>Nostoc</i> sp. <i>pCC7120</i>	0.057766
<i>Tolyphothrix</i> [<i>Scytonema hofmanni</i>] UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.8	<i>Nostoc</i> sp. <i>pCC7120</i>	0.550565
<i>Tolyphothrix</i> [<i>Scytonema hofmanni</i>] UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.9	<i>Nostoc</i> sp. <i>pCC7120</i>	0.020737
<i>Anabaena variabilis</i> ATCC 29413 plasmid A	<i>Nostoc</i> sp. <i>Pcc7524</i>	0.257226
<i>Cylindrospermum stagnale</i> PCC 7417 plasmid pCYLST.01 partial sequence	<i>Nostoc</i> sp. <i>Pcc7524</i>	0.021808
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	<i>Nostoc</i> sp. <i>Pcc7524</i>	0.209072
<i>Nostoc</i> sp. PCC 7120 plasmid pCC7120alpha	<i>Nostoc</i> sp. <i>Pcc7524</i>	0.344158
<i>Nostoc</i> sp. PCC 7120 plasmid pCC7120gamma DNA	<i>Nostoc</i> sp. <i>Pcc7524</i>	0.27923
<i>Nostoc</i> sp. PCC 7524 plasmid pNOS7524.01	<i>Nostoc</i> sp. <i>Pcc7524</i>	0.143722
<i>Tolyphothrix</i> [<i>Scytonema hofmanni</i>] UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.8	<i>Nostoc</i> sp. <i>Pcc7524</i>	0.444248
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	<i>Oscillatoria accuminata</i> PCC6304	0.108447
<i>Oscillatoria accuminata</i> PCC 6304 plasmid pOSCIL6304.01	<i>Oscillatoria accuminata</i> PCC6304	0.178543
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	<i>Oscillatoria nigro-viridis</i> PCC7112	0.072603

(Continued)

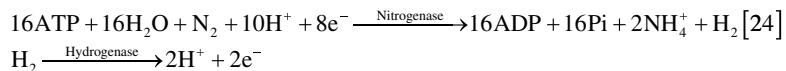
Table 44.3 (Continued)

Plasmid	Genome	Percentage of insertion of plasmid in genome
<i>Oscillatoria acuminata</i> PCC 6304 plasmid pOSCL6304.01	<i>Oscillatoria nigro-viridis</i> PCC7112	0.009079
<i>Oscillatoria nigro-viridis</i> PCC 7112 plasmid pOSC7112.01	<i>Oscillatoria nigro-viridis</i> PCC7112	0.881105
<i>Oscillatoria nigro-viridis</i> PCC 7112 plasmid pOSC7112.02	<i>Oscillatoria nigro-viridis</i> PCC7112	2.676837
<i>Oscillatoria nigro-viridis</i> PCC 7112 plasmid pOSC7112.03	<i>Oscillatoria nigro-viridis</i> PCC7112	0.736982
<i>Oscillatoria nigro-viridis</i> PCC 7112 plasmid pOSC7112.04	<i>Oscillatoria nigro-viridis</i> PCC7112	0.235646
<i>Tolyphothrix [Scytonema hofmannii]</i> UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.8	<i>Oscillatoria nigro-viridis</i> PCC7112	0.100601
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	<i>Pleurocapsa</i> sp. PCC7327	0.16702
<i>Stanieria cyanosphaera</i> PCC 7437 plasmid pSTA7437.02	<i>Pleurocapsa</i> sp. PCC7327	0.015782
<i>Tolyphothrix [Scytonema hofmannii]</i> UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.8	<i>Pleurocapsa</i> sp. PCC7327	0.078988
<i>Pseudanabaena</i> sp. PCC 7367 plasmid pPSE7367.01	<i>Pseudanabaena</i> sp. PCC7367	2.013344
<i>Tolyphothrix [Scytonema hofmannii]</i> UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.8	<i>Pseudanabaena</i> sp. PCC7367	0.113538
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	<i>Rivularia</i> sp. PCC7116	0.095982
<i>Tolyphothrix [Scytonema hofmannii]</i> UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.8	<i>Rivularia</i> sp. PCC7116	0.095982
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	<i>Stanieria cyanosphaera</i> PCC7437	0.104737
<i>Stanieria cyanosphaera</i> PCC 7437 plasmid pSTA7437.01	<i>Stanieria cyanosphaera</i> PCC7437	0.419879
<i>Stanieria cyanosphaera</i> PCC 7437 plasmid pSTA7437.02	<i>Stanieria cyanosphaera</i> PCC7437	0.154031
<i>Tolyphothrix [Scytonema hofmannii]</i> UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.8	<i>Stanieria cyanosphaera</i> PCC7437	0.078929
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	<i>Synechococcus elongatus</i> sp. PCC 6301	0.209624
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	<i>Synechococcus elongatus</i> sp. PCC 7942	0.381913
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	<i>Synechococcus</i> sp. JA-2-3B'a(2-13)	0.076214
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	<i>Synechococcus</i> sp. JA-3-3Ab	0.158349
<i>Tolyphothrix [Scytonema hofmannii]</i> UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.8	<i>Synechococcus</i> sp. JA-3-3Ab	0.079174
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	<i>Synechococcus</i> sp. PCC 6312	0.16285
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	<i>Synechococcus</i> sp. PCC 7002	0.081315
<i>Synechococcus</i> sp. PCC 7002 plasmid pAQ7	<i>Synechococcus</i> sp. PCC 7002	0.148369
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	<i>Synechococcus</i> sp. PCC 7502	0.072701
<i>Synechococcus</i> sp. PCC 7502 plasmid pSYN7502.01	<i>Synechococcus</i> sp. PCC 7502	3.802319
<i>Tolyphothrix [Scytonema hofmannii]</i> UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.8	<i>Synechococcus</i> sp. PCC 7502	0.072302
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	<i>Synechococcus</i> sp. RCC307	0.05398
<i>Anabaena variabilis</i> ATCC 29413 plasmid C	<i>Synechocystis</i> sp. PCC6803	0.014412
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	<i>Synechocystis</i> sp. PCC6803	0.155647
<i>Synechocystis</i> sp. PCC 6803 plasmid pSYSA	<i>Synechocystis</i> sp. PCC6803	0.819763
<i>Synechocystis</i> sp. PCC 6803 plasmid pSYSA_M	<i>Synechocystis</i> sp. PCC6803	0.819763
<i>Synechocystis</i> sp. PCC 6803 plasmid pSYSG	<i>Synechocystis</i> sp. PCC6803	0.829194
<i>Synechocystis</i> sp. PCC 6803 plasmid pSYSG_M	<i>Synechocystis</i> sp. PCC6803	0.829194
<i>Synechocystis</i> sp. PCC 6803 plasmid pSYSM	<i>Synechocystis</i> sp. PCC6803	0.823849
<i>Synechocystis</i> sp. PCC 6803 plasmid pSYSM_M	<i>Synechocystis</i> sp. PCC6803	0.527834
<i>Synechocystis</i> sp. PCC 6803 plasmid pSYSX DNA	<i>Synechocystis</i> sp. PCC6803	0.48583
<i>Synechocystis</i> sp. PCC 6803 plasmid pSYSX_M	<i>Synechocystis</i> sp. PCC6803	0.48583
<i>Tolyphothrix [Scytonema hofmannii]</i> UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.8	<i>Synechocystis</i> sp. PCC6803	0.151337
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	<i>Thermosynechococcus elongatus</i> BP-1	0.095032
<i>Microcoleus</i> sp. PCC 7113 plasmid pMIC7113.02	<i>Trichodesmium erythraeum</i> IMS101	0.088167
<i>Tolyphothrix [Scytonema hofmannii]</i> UTEX 2349 genomic scaffold Tol9009DRAFT_TPD.8	<i>Trichodesmium erythraeum</i> IMS101	0.038554

amount is low, it can be scaled up to reach commercial levels through genetic engineering. Entrepreneurs are eyeing on the biofuel industry and companies like Joule Unlimited, Algenol, USA (www.algenol.com), and Synthetic Genomics, Inc., USA (www.syntheticgenomics.com) are already involved in the same (Ruffing 2011).

Hydrogen production has been reported by many strains of cyanobacteria. Among the many strains tested, *Anabaena variabilis* displayed highest hydrogen production (Ruffing 2011). Hydrogen biosynthesis and metabolism involves three enzymes; nitrogenase, uptake hydrogenase, and bidirectional hydrogenase. Nitrogenase is found only in heterocystous Cyanobacteria and it produces more hydrogen than bidirectional hydrogenase. Apart from nitrogenase, all heterocystous Cyanobacteria also contain uptake hydrogenase, which re-oxidizes the released hydrogen. The enzyme transfers the electrons from hydrogen for the reduction of oxygen via the respiratory chain in a reaction known as oxyhydrogenation or Knallgasreaction (Hallenbeck *et al.* 2012). Experiments involving deletion of uptake hydrogenase gene (*hupL*) increased hydrogen gas production 3–7-fold. On the other hand, deleting bidirectional hydrogenase gene (*hoxH*) decreased hydrogen gas production in heterocystous cyanobacteria (Ruffing 2011). A list of genes involved in production of economically important metabolites are listed in Table 44.4.

Enzymatic reactions associated with hydrogen production in cyanobacteria:



Ethanol is today the most common biofuel worldwide because it can be mixed with diesel and used unaltered. Unlike other traditional crops, cyanobacteria produce ethanol naturally by fermentation but the yield is very low (Quintana *et al.* 2011). Recombinant ethanol production has been obtained by introducing pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase II (*adh*) genes from the ethanol producing *Zymomonas mobilis* into *S. elongatus* PCC 7942 and *Synechocystis* sp. PCC 6803 (Figure 44.4). When these genes were expressed under the control of *rbcLS* operon promoter, alone and in combination with the *E. coli* lac promoter, the reported yields in the engineered strain *S. elongatus* PCC 7942 were 54 nmol OD730 unit⁻¹ Liter⁻¹.day⁻¹ (Deng and Coleman 1999). When the same genes were expressed under the control of a strong light driven promoter, *psbAII*, there was substantial increase in ethanol production to 5.2 mmol OD730 unit⁻¹ Liter⁻¹ day⁻¹ (Dexter and Fu 2009).

Cyanobacteria can also produce ethanol from cellulosic material. Cellulose is found as extracellular deposits of up to 25%. *Synechococcus* sp. PCC 7942 transformed with cellulose synthase genes from *Gluconobacter xylinus* produced extracellular non-crystalline cellulose that is ideal for ethanol production (Quintana *et al.* 2011). However, ethanol production by a living organism is coupled with a stress response. Recently transcriptomics studies have been carried out to silence genes involved in stress response, so that ethanol production can be scaled up (Wang *et al.* 2012).

Table 44.4 List of genes involved in the production of commercially important products.

Genes	Occurrence	Role
Aar(acyl-acyl carrier protein reductase) and ado (Aldehyde deforming oxygenase)	90% of cyanobacterial genomes	Fatty Acyl ACP -> Alkane
Nitrogenase, uptake hydrogenase (<i>hupL</i>) and bi-directional hydrogenase (<i>hoxH</i>)	Mostly heterocystous bacteria	Hydrogen gas production by oxyhydrogenation or Knallgasreaction ¹
pyruvate decarboxylase (<i>pdc</i>) and alcohol dehydrogenase II (<i>adh</i>)	<i>Zymomonas mobilis</i>	
Bara-Bark cluster	<i>Lyngbya majuscula</i>	Non-ribosomal protein synthetic pathway
McyS gene cluster	<i>Microcystis</i> , <i>Anabaena</i> , and <i>Planktothrix</i>	Non-ribosomal protein – toxic to humans
CS-505 and CS-509 gene cluster ²	<i>Cylindrospermum</i> sp.	Cylindrospermopsin a toxic substance.

Notes/References

1. Hallenbeck *et al.* (2012).
2. Sinha *et al.* (2014).

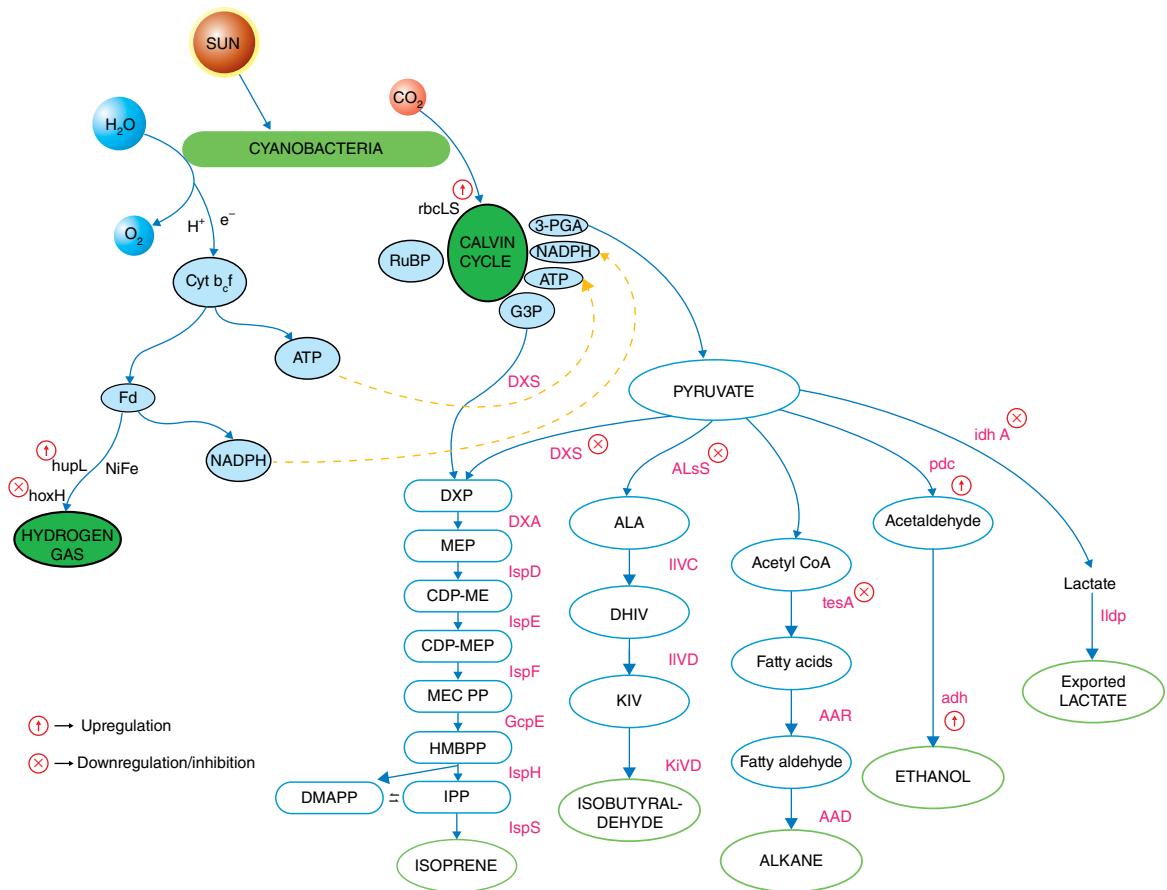


Figure 44.4 A hand-drawn metabolic pathway diagram combining several organisms to indicate the path for biofuel production. The enzymes marked X indicate knocking those genes will upregulate biofuel production. The ones marked with upward arrows indicate over-expression of those genes have significant improvement in biofuel production.

Organizations like The Federal Ministry of Education and Research(Germany) and the US Department of Energy are investing on the field of bioethanol production (Quintana *et al.* 2011). The company Joule Unlimited has recently engineered a cyanobacteria and claimed ethanol production at the rate of ~1 mg/l/h; much higher than other reported strains (Ducat *et al.* 2011). Longer chain alcohols are catching attention of researchers as they possess higher energy content and their storage and transport is easier than ethanol (Quintana *et al.* 2011).

Recently, researchers have gained interest in isobutanol as a better replacement for gasoline than ethanol because of its properties like low hygroscopicity, higher energy density, and high compatibility with the current infrastructure (Machado and Atsumi 2012). The 2-ketoacid pathway found in cyanobacteria for amino acid synthesis has been engineered for isobutyraldehyde production.

Several recent reports elucidate upon alkane production in cyanobacteria. Acyl-acyl carrier protein reductase (aar) and aldehyde deformylating oxygenase (ado) are known to synthesize alkanes from fatty acyl-ACP. The regulatory mechanisms and organization of these genes are complex indicating a tight regulation (Klahn *et al.* 2014).

Free fatty acid production has been reported as a result of knocking out acyl-ACP synthetase in *Synechocystis elongatus* PCC 7942 (Ruffing 2013).

44.5.2 Novel Metabolite Producing Genes in Cyanobacteria

Cyanobacteria are known to harbor a range of metabolic pathways producing many economically important products. Most of the metabolites are produced via a non-ribosomal protein synthesis and polyketide pathways involving clusters of genes (Gupta and Prasanna 2012). Barbamide produced by *Lyngbya* sp. has molluscicidal activity – is encoded by a cluster of 12 open reading frames *barA–barK* (Chang *et al.* 2002). Apratoxin A, an anticancer compound found from *Moorea bouillonii*, is synthesized by a gene cluster (Grindberg *et al.* 2011). Dolastatin 10 and curacin A are anticancer agents produced by marine cyanobacteria (Gerwick *et al.* 2001) and are coded by a polyketide synthase-non-ribosomal peptide synthase cluster. Gallinamide A, is a known inhibitor of human *Cathepsin* L, a cysteine protease is produced by marine cyanobacteria (Miller *et al.* 2014).

An UV absorbant scytonemin and mycosporin that has great potential as a sunscreen is synthesized by *Nostoc* sp. (Gao and Garcia-Pichel 2011) and is produced by a cluster of 18 ORFs. Microcystin is a hepato-toxin, produced by a range of cyanobacterial species such as *Microcystis*, *Anabaena*, and *Planktothrix* is encoded by a gene cluster *mcyS* (Pearson *et al.* 2010). Recent transcriptomics studies reveal that most of the metabolites are produced under a light phase indicating their involvement in the diurnal pattern of cellular metabolism (Straub *et al.* 2011). Gamma linolenic acids (GLA), which have health benefits, are synthesized by *Spirulina platensis* and *Arthrosira* sp. (Euler and Eliassen 1967). Endoglucanases/chitinases/chitosanases are some of the antifungal protein products produced by ayanobacteria species, and those very useful for agriculture are produced by *Anabaena* sp. and *Synechocysis* sp. (Adams 2004).

44.6 Conclusion

The availability of sequencing technologies has now opened up a new opportunity for researchers to sequence the genomes and transcriptomes and quickly discover transcript coding for economically important compounds. In many mega diverse countries including India, genomics may prove the next big research tool for understanding the mechanisms of expression and regulation of such transcripts using powerful bioinformatics tools.

Acknowledgment

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45

Inflammation, Nutrition, and Transcriptomics

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45.1 Introduction

With advances in medicine, the fight against infectious disease has been won. However, the decline in infections has seen a corresponding increase in chronic disease. Chronic diseases are the biggest health threat in Western societies and are an escalating problem globally. The causes of these chronic diseases are multifactorial but inflammation seems to be a common underlying theme.

This chapter aims to tackle the nature of low-grade chronic inflammation and its causes with a focus on the role of diet and nutrition in its development. We will also discuss how nutrigenomics and, in particular, the technique of transcriptomics can be applied to study the effects of dietary changes on inflammation.

45.2 Inflammation

Rogers (2008) said of inflammation “inflammation may be useful when controlled, but deadly when it is not.” Inflammation is the body’s first response to protect itself from further damage due to physical, chemical, or biological injury. Regardless of the cause, inflammation evolved as an adaptive response to restore homeostasis. A controlled inflammatory response is beneficial, but if dysregulated then it becomes detrimental (Medzhitov 2008). Any inflammatory reaction that has started needs to be subsequently ended otherwise it can lead to disease (Ruiz-Nunez *et al.* 2013).

Traditionally, there have been two types of inflammation described, acute or chronic. Acute inflammation is the short term response that results in healing. Chronic inflammation in contrast is a prolonged response that usually results in damage. Progress has been made into defining the mechanism of acute inflammation; however, much less is known about chronic inflammation (Medzhitov 2008). A subtype of inflammation has been more recently described “metaflammation”, (metabolically triggered inflammation). This condition is principally triggered by nutrients and/or metabolic surplus, and engages similar signaling pathways to those involved in classical inflammation (Hotamisligil 2006). Metaflammation is a persistent low-grade systemic inflammation, and has been associated with many if not all chronic human diseases including; cardiovascular disease (CVD), arthritis, Type 2 diabetes, autoimmune diseases, and cancer (Egger and Dixon 2011; Weiss 2008).

Metaflammation differs from classical inflammation in that it is; (1) low-grade, resulting in a small increase in immune system markers, (2) persistent, (3) has systemic effects, (4) has antigens that are less apparent as foreign agents and thus are called “inducers”, (5) perpetuates disease, and (6) reduces metabolic rate. Therefore, traditional inflammation caused by pathogen or

injury can be thought of has “hot” and metaflammation, caused by nutrition or metabolism as “cold” (Calay and Hotamisligil 2013). Figure 45.1 and Table 45.1 highlight the differences between classical inflammation and metaflammation.

Classical inflammation, triggered by an injury stimulus, first identified by the Roman physician Aurelius Celsus, is characterized by redness and heat caused by increased blood flow, swelling caused by fluid accumulation, and pain resulting from the swelling (Calay and Hotamisligil 2013, Egger 2012; Hakansson and Molin 2011).

There are four stages in the process of inflammation:

1. Blood vessels widen, increasing blood flow
2. Blood vessel permeability increases
3. White blood cells recruited to the site of injury
4. Adjustments to the metabolism.

Once inflammation has been initiated it will proceed until the cause of inflammation has been resolved and healing can start. However, if the source of inflammation is not eliminated then the inflammation continues and develops into chronic inflammation (Hakansson and Molin 2011).

It is becoming apparent that most chronic typically Western diseases, including cardiovascular disease, Type 2 diabetes, arthritis, and many types of cancer, have as their primary cause an unhealthy lifestyle and that systemic low-grade inflammation is a common denominator (Galland 2010; Ruiz-Nunez *et al.* 2013).

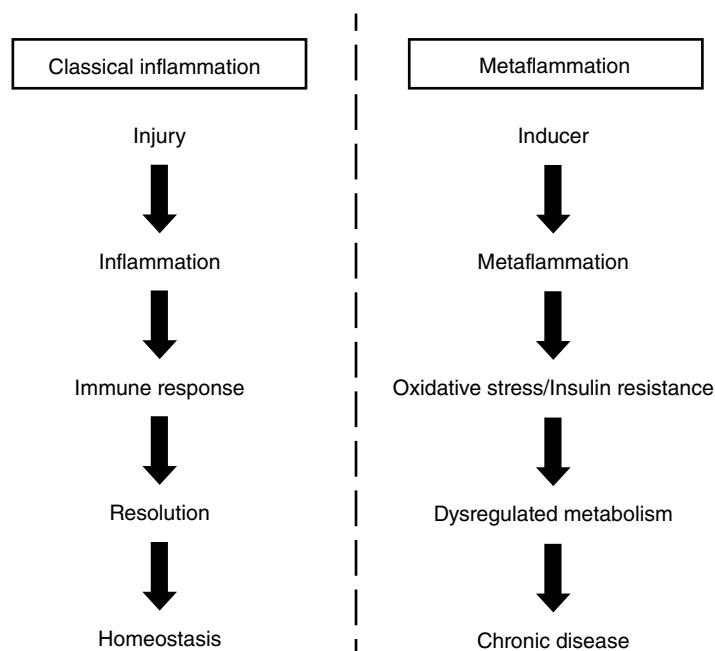


Figure 45.1 A simple representation showing the differences between classical inflammation and metaflammation.

Table 45.1 Differences between acute inflammation, chronic inflammation and metaflammation, adapted from (Calder *et al.* 2013).

	Acute Inflammation	Chronic inflammation	Metaflammation
Trigger	Pathogens, injury	Failure to resolve acute inflammation	Lifestyle or environmental “inducer”
Onset	Immediate	Delayed	Delayed
Duration	Days	Unlimited	Unlimited
Outcome	Resolution or progression to chronic inflammation	Tissue damage	Perpetuates disease, reduces metabolism

There are many factors in the Western lifestyle that can cause chronic systemic low grade inflammation. These factors can be subdivided into two main categories; an unbalanced diet (Anand 2008; Egger and Dixon 2010; Galland 2010) and non-food related factors, which could exert effects due to obesity (Egger and Dixon 2010, 2011).

Any attempt to create an optimal healthy diet must take into account the impact of diet on inflammation. The impact can be direct by eliciting an effect on the inflammatory response itself alternatively the impact can be observed as a result of becoming overweight and the accompanying associated inflammatory effects (Galland 2010).

45.3 Nutrition

The last 100 years was a time of enormous progress in human nutrition, essential vitamins and minerals were identified and the task of identifying genetic variants that affect metabolism was started. Also, nutrition related improvements resulted in increased life expectancy in almost every country (Willett 2008).

During the last 20–30 years, we have increased our understanding of how diet and lifestyle can affect chronic diseases. Although there is still much to learn, it has been shown that cardiovascular disease, diabetes and certain cancers can be prevented or at least delayed by simple and modest changes to lifestyle and diet (Ruiz-Nunez *et al.* 2013; Willett, 2008).

Traditionally, diet studies have examined the effect of an individual food or nutrient (Calder *et al.* 2011). It has now been recognized that this has limitations as foods are very rarely consumed individually, but rather in combination and so recent studies now look at dietary patterns in relation to health and disease (Moeller *et al.* 2007; Newby *et al.* 2003). Many studies have proven that a diet high in long-chain omega-3 fatty acids, fruits, vegetables, nuts, and whole grains, and low in refined grains, saturated fats, and sugars can help in reducing inflammation (Luciano *et al.* 2012). For the Western world, a Mediterranean diet may be the most suitable (Galland 2010).

45.3.1 Mediterranean Diet

The *Mediterranean diet* term is used to refer to a dietary pattern observed in olive-growing regions of southern Europe, particularly Greece, southern Italy, and southern Spain. The traditional Mediterranean diet consists of olive oil (as the major source of fat), is rich in fruit, vegetables, legumes, whole grains, and fish, with a moderate intake of wine, usually red, with meals and a lower intake of red meat (Chrysohou *et al.* 2004; Dai *et al.* 2008; Esposito *et al.* 2004; Galland 2010; Sofi *et al.* 2008; Trichopoulou *et al.* 2003).

The value of the Mediterranean diet in protecting against chronic disease, especially cardiovascular disease, is well established (Bakker *et al.* 2010; Bouwens *et al.* 2009; de Lorgeril and Salen, 2006; Esposito *et al.* 2011a, b; Kastorini *et al.* 2011; Salas-Salvado *et al.* 2008; Sola *et al.* 2011).

Studying nutrition is inherently complex. We eat approximately 1.5 kg of food and drink 2 l of fluid a day, this contains numerous essential and non-essential nutrients, and thousands of chemical compounds, some with known functions, but many without. These interact with each other, different cells/organs in the body, and the microbiota. On top of that there is human individual variation, with genes, SNPs, and epigenetics all having an effect, as well as the effect of the physiological environment at the time. Age and sex also have an effect, this complexity demands advanced approaches to understand the relationship between diet and health (Norheim *et al.* 2012).

All of these factors exhibit interaction and so studying them individually or in isolation is difficult. This has led in part to the new field of *nutrigenomics*.

45.4 Nutrigenomics

Nutrition research has undergone a shift in focus towards molecular biology and genetics; this is due to the realization that the effects of nutrition on health and disease cannot be understood without the knowledge of how nutrients work at the molecular level. This new approach has come about due to the new tools available to study the molecular effects of nutrition. Gene expression changes can be measured at the single-cell level by RT-PCR or microarray. Microarrays in particular are a very important tool in the study of nutrigenomics (Muller and Kersten 2003).

Nutrigenomics is the study of genome-wide influences of nutrition, to identify dietary signatures in response to a specific nutrient and to understand how this influences homeostasis. Further, nutrigenomics also aims to identify the genes that confer a risk for diet-related diseases (Muller and Kersten 2003).

45.5 Dietary Factors and Inflammation

Foods can be anti-inflammatory or pro-inflammatory, and an ideal diet would contain a balance. Some examples of foods with anti-inflammatory properties are oily fish, fruit, and vegetables, whereas saturated fatty acids and trans fatty acids are pro-inflammatory. Other pro-inflammatory factors in the diet are a high $\omega 6:\omega 3$ ratio, low intake of long-chain polyunsaturated fatty acids from fish (EPA, DHA), low vitamin D, vitamin K, and magnesium, high GI carbohydrates, disbalance in micronutrients that make up anti/pro-oxidant network, and a low intake of fruit and veg (Ruiz-Nunez *et al.* 2013).

Evidence indicates that consumption of Mg, fiber, omega-3 PUFA, MUFA, flavonoids and carotenoids, and fiber from fresh fruit and vegetables results in decreased levels of inflammation. Whereas refined starches, sugar, saturated and trans fats, high-GI carbohydrates, and a high 6:3 PUFA ratio result in increased levels of inflammation (Galland 2010; Ruiz-Nunez *et al.* 2013). A summary is shown in Table 45.2.

A number of intervention studies have investigated the impact of fruit and vegetables as a food group or as an individual. Three intervention studies focusing on fruit and vegetables as a group reported a reduction in blood biomarkers of inflammation (Esposito *et al.* 2003; Sanchez-Moreno *et al.* 2003; Watzl *et al.* 2005). However, when the focus was on a single fruit or vegetable, the results were inconsistent (Basu *et al.* 2010; Castilla *et al.* 2006; Curtis *et al.* 2009; Dalgard *et al.* 2009; Karlsen *et al.* 2007, 2010; Kelley *et al.* 2006; Larmo *et al.* 2008; Zern *et al.* 2005). The evidence is convincing that a high overall intake of fruit and vegetables is associated with a lower state of inflammation.

Results regarding fish consumption and inflammation are also inconsistent with studies showing fish consumption results in reduced inflammatory markers (Lopez-Garcia *et al.* 2004; Zampelas *et al.* 2005) but other studies show no association (Hickling *et al.* 2008; King *et al.* 2003; Madsen *et al.* 2001). However, as mentioned earlier, there are numerous factors that interact that can have an effect on the outcome of a dietary intervention trial, including population studied, biomarker examined, specific food, and other dietary components.

Observational studies have reported that whole grains are associated with lower inflammatory markers. An intervention study found that the bioprocessing of whole wheat in bread affected the anti-inflammatory properties (Mateo Anson *et al.* 2011).

Healthy eating patterns, such as the Mediterranean diet or vegetarian diets, are associated with lower levels of inflammatory markers with the strongest evidence available for whole grains, fruits and vegetables, and fish as components of these diets.

Both over-nutrition and deficiencies in specific nutrients can cause inflammation. Nutrition quality, with excess sugar, salt, alcohol, and saturated fats along with reduced levels of fiber, fruit, vegetables, and grains have been associated with increased metaflammation (Calder *et al.* 2011; Egger and Dixon 2010, 2014). Processed foods have also been found to cause more inflammation than whole foods.

Table 45.2 Summary of pro/anti-inflammatory foods and lifestyle factors, adapted from (Egger and Dixon, 2010).

Pro-inflammatory	Anti-inflammatory
Nutrition	
Excessive alcohol	Fruits/vegetables
Excessive energy intake	Restricted energy intake
Western style diet	Mediterranean style diet
Saturated fat	Mono-unsaturated fats
Trans fatty acids	Olive oil
High N6:N3 ratio	low N6:N3 ratio
Low fiber	High fiber
High GI food	Low GI foods
Salt	Nuts
Lifestyle	
Obesity	Weight loss
Smoking	Smoking cessation
Exercise (too much and too little)	Fitness/physical activity
Sleep deprivation	
Stress	
Environment	
Age	
Pollution	

Dietary advice changes over time and there is no current agreement on what is an ideal diet. However, Michael Pollan answers the age-old question of “what should we eat?” with seven simple words “Eat food. Not too much. Mostly plants.” (Pollan 2008) and is something to bear in mind when formulating your personal long-term nutritional goal (Egger and Dixon 2014).

As mentioned earlier, there are also indirect diet-related factors; these include abnormal microbiota composition, chronic stress, smoking, insufficient exercise, and insufficient sleep. These factors can all contribute to eating patterns and obesity, which is linked to metaflammation (Table 45.2).

As can be seen from Table 45.2, the foods causing a pro-inflammatory response are those that are new to the diet and have been modified or processed in some way. Whereas the anti-inflammatory foods generally contain elements that humans have a long evolutionary association with. Likewise the lifestyle causing elements and environmental pollution are associated with a modern industrialized lifestyle (Egger and Dixon 2010). Aging is also pro-inflammatory but is something we are powerless to prevent.

Genomic tools can be used in two complementary strategies. The first is the traditional hypothesis-driven approach the second is the systems biology approach.

45.6 Transcriptomics

Transcriptomics can be defined as: the study of mRNA within a specific cell or tissue at a specific time.

Understanding the transcriptome is essential for interpreting the functional genome and the molecular constituents of cells/tissues. It is also necessary for understanding the development of diseases (Wang *et al.* 2009).

The key aims of transcriptomics are:

1. To identify all species of transcript at a given point
2. To determine transcriptional structure of genes
3. To quantify the changing levels of transcripts under different conditions and during development.

Transcriptomic studies have greatly improved the understanding of the complex interactions between genes and nutrition, and enable the assessment of nutritional interventions on a global scale (Wang *et al.* 2009). Microarrays provide the technology to simultaneously quantify the abundance of thousands of mRNA transcripts (Wong 2012). The first microarrays were developed nearly 20 years ago (Schena *et al.* 1995); however, issues with regard to data quality, reproducibility, and analysis coupled with concerns over genome coverage have delayed the realization of the full potential of transcriptomics until more recently.

The principle behind microarrays is quite simple: RNA is reverse transcribed to cDNA with the inclusion of labeling molecule. The labelled cDNA is applied to a chip that contains probes of defined genes. The cDNA and probes hybridize via nucleic acid interactions and the amount of hybridization reflects the amount of each specific RNA species present. One limitation of transcriptomics is it only provides a snapshot of steady-state mRNA abundance, which can be influenced by multiple factors (Wong 2012).

Analysis of microarray data is a complex process, which is constantly evolving. It involves normalization followed by statistical testing taking into account multiple correction comparisons. This will generate a list of differently expressed genes, which then have to be further analyzed to generate relevant biological meaning. A common approach to data interpretation is to generate “heat-maps”, which visually cluster samples and genes together based on similarity (Wong 2012). This is a broad approach, a more specific approach is to use of databases, such as Gatter, Gorilla, Panther, or commercially, IPA, to examine if a gene list is enriched for specific genes or pathways that are biologically related based on the existing literature (Wong 2012).

In general, gene-expression profiling can be used for three distinct purposes in nutrition research and is reviewed in detail in (Muller and Kersten 2003). The three purposes are:

1. Provide information about the mechanism underlying the effects of a specific nutrient or diet.
2. Help to identify genes, proteins, or metabolites that are altered in pre-disease states and can therefore act as molecular biomarkers.
3. Help to identify and characterize pathways regulated by specific nutrients (Norheim *et al.* 2012).

The main challenge in human studies is access or lack thereof to human tissue samples for analysis. This is why blood, specifically peripheral blood mononuclear cells (PBMCs), is commonly accepted as a surrogate. mRNA taken from blood

Table 45.3 Comparison of microarray with RNA-seq adapted from (Wang et al. 2009).

Technology	Microarray	RNA-seq
Principle	Hybridization	Sequencing
Resolution	5–100 bp	1 bp
Background	High	Low
RNA amount required	High	Low
Relative cost	High	Low

has its advantages in that it is easy to obtain and provides a broad picture of systemic response. However, it also has its disadvantages in that it has limitations regarding organ specific clinical manifestations (Wong 2012).

Human dietary intervention trials have successfully used transcriptomics to show diet can induce changes in gene expression (Crujeiras *et al.* 2008; Kallio *et al.* 2007; Marlow *et al.* 2013).

45.6.1 RNA-seq

Microarray technology has achieved its technical limits (Mutz *et al.* 2013) and is being replaced by next-generation sequencing. Next-generation sequencing (NGS) is rapid and inexpensive and offers high throughput gene expression profiling, genome annotation, and discovery of non-coding RNA. It is based on the sequencing by synthesis method called pyrosequencing and is often referred to as RNA-seq (Mutz *et al.* 2013). Table 45.3 shows a comparison between the microarray and RNA-seq.

RNA-seq has now become the technology of choice for gene expression analysis, however, the order of magnitude difference in the data generated from RNA-seq has resulted in challenges in relation to analysis and storage (McGettigan 2013). NGS generates millions of short (25–450 bp) DNA fragments per sequencing run, which creates raw data sets up to 50 Gb in size, which subsequently has to be analyzed (Mutz *et al.* 2013).

RNA-seq has enabled the profiling of samples from very small starting amounts of RNA including facilitating the application of single-cell transcriptomics (McGettigan 2013).

The real advantage of RNA-seq has been to open up new applications, including studying the transcriptome of non-model organisms and exposing the true complexity of the mammalian transcriptome (McGettigan 2013). Pyrosequencing can also be used to analyze SNPs as well as whole-genome sequencing (Mutz *et al.* 2013). RNA-seq can evaluate absolute transcript levels, detect novel transcripts and isoforms, map intron/exon boundaries, and reveal SNPs and splice variants. Epigenetic modification and other gene regulation events can be analyzed by NGS technologies (Mutz *et al.* 2013).

45.7 Conclusions

So what is the best diet to reduce chronic disease? This is not an easy question to answer, as there are many factors at work and our understanding of the role nutrition plays is incomplete. However, by applying the techniques of transcriptomics and RNA-seq, among others, to well-designed dietary intervention studies we can increase our knowledge and understanding, which should result in more specific dietary advice. This process will be gradual, but over time we can develop what should be an optimal health diet. But the diet is only half the solution, lifestyle changes would also have to be made. It is very encouraging to note that with moderate changes to lifestyle and diet analysis showed that 80% of coronary heart disease, 90% of T2DM, 70% of colon cancer, and 70% of stroke could be avoided (Willett 2008).

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46

Transcriptomics and Nutrition in Mammals

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46.1 Introduction

Nutrition, due to its characteristics of universality and essentiality, is undoubtedly the most important environmental factor that living beings are faced with. Therefore, a complex process of adaptation from a genomic point of view is expected. In this way, dietary nutrients may influence gene expression at different levels such as transcription, maturing, and stability of RNAs, their translation into proteins, and post-translational modifications. The first three steps make the amount of RNA up; being the messenger RNA, particularly more studied compared to other RNAs such as microRNA (miRNA) and long non-coding RNA (lncRNA). In addition, transcriptional studies have been more intensively addressed due to their constant chemical properties of water-solubility and ability to recognize complementary molecules of RNAs in contrast to proteins where such uniform patterns do not exist. When transcriptional studies are performed on a whole-genome scale, that is, analyzing all the transcripts present, we usually refer to them as transcriptomic analyses or the *transcriptome*. In contrast to DNA, there is not a single transcriptome for an organism but one for each cell. In addition, it may change in different environmental circumstances. So far, the simultaneous analysis of thousands of transcripts has been carried out by specific hybridization to miniaturized, ordered arrangement of nucleic acid fragments from individual genes located at defined positions on a solid support, named DNA microarrays. These are powerful tools to detect transcriptomic changes in multiple samples obtained from biological models subjected to the effect of different nutrients. Recently, next-generation DNA sequencing technology has allowed us to sequence all the transcripts from a cell and to obtain their distribution (Han *et al.* 2011; Wang *et al.* 2009b). Despite its enormous potential, its application to this field has been limited because of its novelty,

price, and computing requirements; see Table 46.1 for a comparison of advantages and disadvantages of DNA microarrays versus DNA sequencing in transcriptomic profiling.

In this chapter, we have reviewed the influence of macronutrients on transcriptomes from the main organs and tissues involved in metabolism and that are interconnected (Figure 46.1): adipose, intestine, liver, and muscle, and the data that have been almost utterly gathered using DNA microarrays. Our endeavor tries to combine two different approaches; on one hand, the nutrigenomics field, in the sense of how dietary components interact with genes and their products to alter phenotype; and on the other, a systems biology focus to look for a comprehensive integration of different organ responses. The ultimate goal of these studies would be the possibility of delivering personalized nutrition to prevent metabolic diseases such as insulin resistance, obesity, or metabolic syndrome (Mathur *et al.* 2011).

Table 46.1 Comparison of DNA microarrays vs DNA sequencing in transcriptomic profiling.

Properties/approach	DNA microarrays	DNA sequencing
Gene expression profiling in un-annotated genomes	No	Yes
LncRNA and miRNA	Not simultaneously	Yes
Identifies known and unknown transcripts isoforms (splicing, polyadenylation, initiation sites)	No	Yes
High dynamic range of detection	No	Yes
Extremely accurate quantification of transcripts	No	Yes
Reveals transcriptional boundaries down to single-base resolution	No	Yes
Computer management	Affordable	Difficult
Costs	Affordable	High

Adapted from Wang *et al.* (2009b) and Han *et al.* (2011).

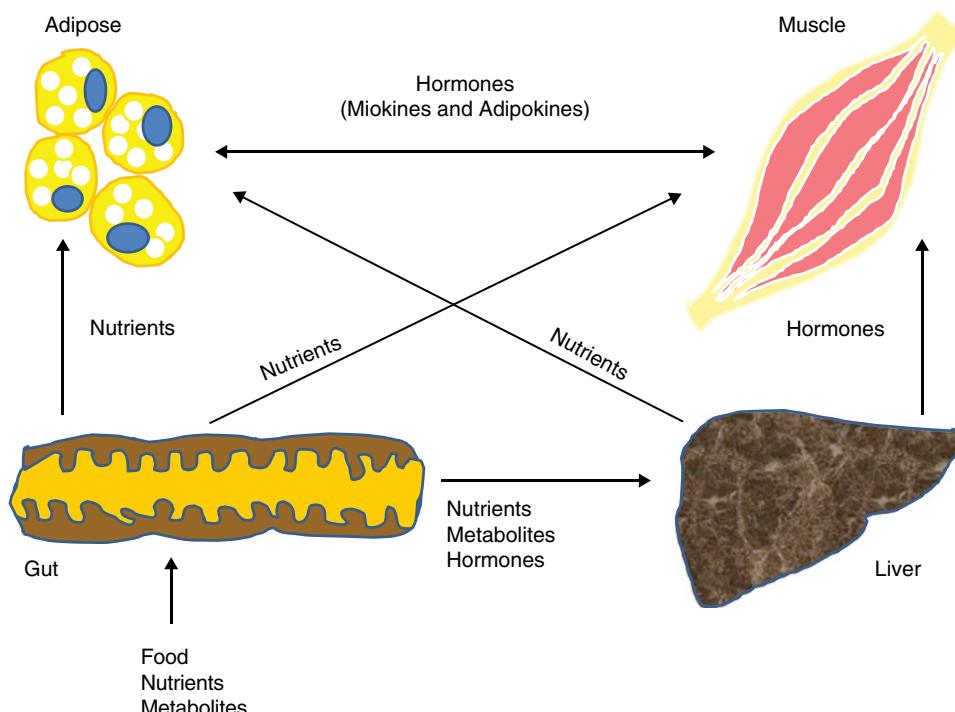


Figure 46.1 Main organs involved in metabolism. Arrows indicate the flux of metabolites and hormones among them.

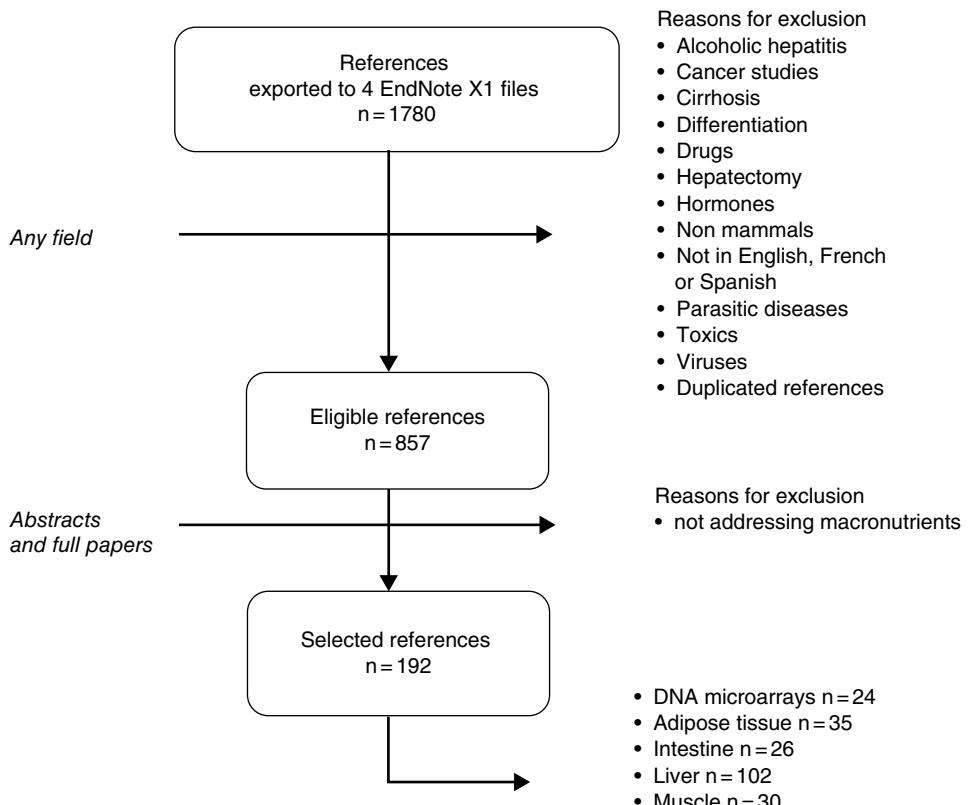


Figure 46.2 Flow chart displaying the stages used to select the references considered. EndNote X1 (Thomson Reuters, New York, NY). Note that the sum of different tissues does not match with the total number of references since several references analyze more than one tissue.

The present review has adhered to systematic review guidelines (Moher *et al.* 2009). As displayed in Figure 46.2, a search in Pub Med (www.ncbi.nlm.nih.gov/pubmed/) using the keywords (adipocyte, adipose tissue, intestine, intestinal tissue, liver, muscle combined with DNA microarray, microarray, transcriptomic, transcriptional profile, and diet or nutrients) identified 1780 hits from November 1945 to February 12, 2014. The search was refined by removing documents related to cancer, viruses, and so on, and the resulting data base was purged by eliminating duplicate documents. The 857 papers obtained were critically reviewed to verify that high-throughput analyses were carried out and that macronutrients were studied in mammals. Documents that failed to meet any of these criteria were also discarded. Thus, this review covers the works related to the effects of macronutrients on adipose, intestinal, hepatic, and muscular transcriptomes from 192 papers. The aim is to analyze the interactions among organs in order to profile the molecular landscape of nutrigenomics in complex organisms.

46.1.1 DNA Chips or Microarrays

DNA chips and microarrays are a widely used technology for generating high-throughput data at affordable costs. Their use provides semiquantitative, genome-wide gene expression results due to a lack of lineal response to cover all transcript concentrations (saturation and the lack of sensitivity in the high- and low-abundant transcripts, respectively). Conceptual limitations are given by the number of probes on the matrix to interrogate for the presence of a certain transcript and the selection of the capturing fragment that may pose a challenge in genes with different splicing or confusion in multigene families (Osada 2013).

Most widely used microarrays (www.ncbi.nlm.nih.gov/geo/) are those offered by companies: Affymetrix, and Agilent. In both cases, oligonucleotides are synthesized on a solid matrix. While Affymetrix GeneChip arrays use a set of 11–20 nucleotides in pairs of probes for a region of a transcript: one with perfect match and the other with a mismatch (www.affymetrix.com/estore/index.jsp), Agilent arrays use a set of 60 nucleotides (www.genomics.agilent.com/literature.jsp?contentType=Brochure). Labeling and sample processing are equally different. Therefore and due to these differences, comparison may be only indicative at the present time since it is required standardized platforms, internal and/or external controls, similar normalizations (Gotoh *et al.* 2011; Kakuhata *et al.* 2007) and analysis implementations (Osada 2013). Despite these limitations, a good agreement across different platforms has been observed and lists of platform-independent tissue-specific genes have been obtained (Russ and Futschik 2010).

Biological analyses of RNA *per se* pose interesting challenges as well, elicited by the nature and extent of RNA half-life or cellular location (Jung *et al.* 2014) across tissues in one individual or among individuals in response to circadian rhythms, growth hormone signaling, immune response, androgen regulation, lipid metabolism, social stress, extracellular matrix, epigenetic programming, or other micro-environmental variables. These sources of variability may explain the observed differences between genetically identical mice, and can influence the experimental design and the interpretation of data (Vedell *et al.* 2011), particularly in studies addressing immune response, stress, amine metabolism, cell growth, ubiquitination, or hormonally regulated genes (Pritchard *et al.* 2001, 2006). For these reasons, and despite the lost information regarding individual variation, RNA samples from different subjects are often pooled in microarray experiments to reduce the cost and complexity of analyses, and this approach was found statistically valid and efficient. In this regard, appropriate RNA pooling can provide equivalent power, and improve efficiency and cost-effectiveness for microarray experiments with a modest increase in total number of subjects and correction for the technical difficulty in getting sufficient RNA from a single subject (Peng *et al.* 2003). Furthermore, properly constructed pools provide nearly identical measures of transcription response to individual RNA samples (Do *et al.* 2010). In our experience, before pooling it is critical to verify that all samples have the same histological pattern (Guillen *et al.* 2009a, b). Using an inbred strain of mice, many animals may experience subtle or profound hepatic alterations of cell distribution due to biliary atresia, for instance, or other congenital defects. Including those animals into a pool can profoundly bias the obtained results.

All these limitations should be taken into consideration to evaluate microarray results in the most efficient manner (Wang *et al.* 2006). Due to these caveats, validation of observed changes is a common practice. Real-time quantitative polymerase chain reaction (qPCR) has become the preferred option due to its high sensitivity, accuracy, high-throughput format, and relatively low costs (Dahl *et al.* 2007). Nonetheless, it is endorsed with its own limitations as well, namely non-specific amplification of non-target genes, amplification of fragments of gene families, need of reference genes, and so on (Martinez-Beamonte *et al.* 2011b; Wang and Seed, 2003).

The deluge of data also requires integration in friendly and efficient ways to facilitate a simple use in functional genomics (Battke and Nieselt, 2011; Le Bechec *et al.* 2008) and to advance in gene annotation for gene clustering, genotype/phenotype correlation studies, or tissue classification, when only 10% of genes have a known function so far (Andersson *et al.* 2014; Marquet *et al.* 2003). The open initiatives of the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/) and Geneatlas (www.ebi.ac.uk/gxa/home), together with minimum information about microarray experiments (MIME) (Brazma *et al.* 2001) are crucial steps toward standardization, comparison, and new analyses by different users in the unprecedented availability of gathered information.

46.2 Adipocyte Transcriptome

Adipose tissue plays a central role in energy balance regulating both energy storage and expenditure. Furthermore, it is an endocrine tissue that produces and secretes a large number of molecules of great importance for lipid and glucose metabolism, among them multiple adipokines. The two types of adipose tissue, white (WAT) and brown (BAT), do not have the same distribution and functions in the organism, but this functional balance is important for metabolic homeostasis (Nakai *et al.* 2008). Biochemistry and physiology of the adipose tissue is intensely influenced by lifestyle (Brattbakk *et al.* 2013), and diet composition (as Table 46.2 summarizes) and an imbalance between high dietary energy intake and low physical activity lead to an increase in adipose tissue mass and obesity, at present considered as an epidemic in the Western societies (Frigolet *et al.* 2011). The expansion of WAT mass produced during the development of obesity in form of adipose tissue depots is closely associated with insulin resistance, and a large visceral fat mass is commonly referred to as its main risk factor (Sjoholm *et al.* 2006). This expansion is mediated in part through an increase in adipocyte size, and these enlarged adipose cells produce increased levels of adipokines such as interleukin 1, leptin, angiotensinogen, and MCP-1 (Morine *et al.* 2013; Okada *et al.* 2009; Takahashi *et al.* 2003; Turban *et al.* 2002). These adipokines have

Table 46.2 Adipocyte transcriptomic changes in several nutritional conditions.

Condition	Model	Finding	References
Caloric restriction	Mouse	Length of restriction is important	(Park and Prolla 2005)
	Rat	Decreased oxytocin expression and involvement of SREBP-1	(Chujo <i>et al.</i> 2013; Pohjanvirta <i>et al.</i> 2008).
	Goat	Different responses among fatty depots	(Faulconnier <i>et al.</i> 2011)
	Humans	Complex transcriptional response influencing lipogenesis, protein synthesis, beta-oxidation, insulin resistance, and inflammation	(Bouchard <i>et al.</i> 2010; Capel <i>et al.</i> 2008, 2009; Franck <i>et al.</i> 2011; Johansson <i>et al.</i> 2012; Olofsson <i>et al.</i> 2008)
Carbohydrates	Rat	<i>Adiponutrin</i> , <i>leptin</i> , and <i>Ppargc1a</i> are influenced by sucrose	(Paternain <i>et al.</i> 2012)
	Human	Low carbohydrate diets decrease inflammation	(Brattbakk <i>et al.</i> 2013)
High fat diets	Mouse	A high fat meal decreases adiponectin Chronic consumption influences signal transduction, inflammation, oxidative stress, and lipid metabolism	(Bolduc <i>et al.</i> 2010)
		The response to high fat diet depends on the genetic background	(Choi <i>et al.</i> 2013; Lee <i>et al.</i> 2012; Okada <i>et al.</i> 2009)
Nature of fat	Cow	Omental adipose tissue was particularly sensitive to polyunsaturated fatty acids	(Luo <i>et al.</i> 2003; Morton <i>et al.</i> 2011; Sawada <i>et al.</i> 2010)
	Pig	Docosahexaenoic acid-supplemented diets reduced FoxO1 and FoxO3	(Hiller <i>et al.</i> 2013)
	Mouse	CLA isomers influence lipid catabolism and adipogenesis	(Chen 2012)
Dietary protein	Rat	Protein restriction upregulates genes involved in carbohydrate, lipid, and protein metabolism and adipocyte differentiation	(House <i>et al.</i> 2005)
		Soy protein prevents adipocyte hypertrophy	(Guan <i>et al.</i> 2005)
			(Frigolet <i>et al.</i> 2011)

CLA, conjugated linoleic acid.

clear pro-inflammatory effects, and participate in chronic inflammation developed in obesity, which also generates changes in adipocyte response to different stimuli. On the contrary, other adipokines such as adiponectin or Acrp30 play a critical role in basal metabolism and its decrease in serum is associated with increased risk for Type 2 diabetes (Combs *et al.* 2003; Yamauchi *et al.* 2003).

46.2.1 Influence of Caloric Restriction

Due to the interesting open debate regarding lifespan and caloric restriction, this issue has deserved great attention in several animal models, and different strategies regarding this topic have been studied. When comparing two periods of caloric restriction (CR) on gene expression in WAT of 10–11-month-old male C57BL/6 mice, Park *et al.* found that the transcriptional patterns of a short-term CR were similar to those derived from non-fasted control mice, but they differed greatly after a long time CR (Park and Prolla, 2005). These animals showed a complete or partial prevention of the majority of transcriptional profiles observed in aging, and it was suggested that CR retards the aging process by reducing endogenous damage. An acute progressive feed restriction in rats also revealed decreased expression of oxytocin, a hormone that enhances glucose oxidation in the WAT (Pohjanvirta *et al.* 2008). Using Wistar and transgenic dwarf rats fed *ad libitum* or subjected to caloric restriction, Chujo *et al.* showed that caloric restriction-associated remodeling of white adipose tissue was executed through SREBP-1 and independent of growth hormone (Chujo *et al.* 2013). Thus, long-term CR could be a highly effective means of extending lifespan and postponing age-related diseases, at least in rodents.

In lactating goats (*Capra hircus*), the effect of two extreme nutritional conditions (control diet vs 48-h feed deprivation) on gene expression profiling of omental and perirenal adipose tissues showed that 456 genes were differentially expressed in the omental and 199 in the perirenal adipose tissue. Transcriptional changes involved in lipid metabolism (decreased lipid synthesis and triglyceride storage capacity as well as increased fatty acid oxidation) were consistent with reduced energy deposition in adipose tissues in response to a 48-h fast (Faulconnier *et al.* 2011). A different response was observed among fatty depots.

During caloric restriction in obese subjects with the metabolic syndrome the mRNA levels of adipose tissue C/EBPalpha, a transcription factor involved in glucose and lipid metabolism, were reduced (Olofsson *et al.* 2008). In an intervention in obese women following a low-fat (high carbohydrate) or a moderate-fat (low carbohydrate) hypoenergetic diets (Capel *et al.* 2008), both diets induced similar weight loss and regulated the gene expression of 1000 genes in adipose tissue. It was seen as an effect of the fat-to-carbohydrate ratio on five genes (*FABP4*, *NR3C1*, *SIRT3*, *FNTA*, and *GABARAPL2*) with increased expression during the moderate-fat diet. Continuing their investigations in obese woman, Capel *et al.* (2009) established a dietary weight loss program composed of an energy restriction phase with a very-low-calorie diet and a weight stabilization period composed of low-calorie diet followed by a weight maintenance diet. Body weight and fat mass decreased during the program. The regulation of adipose tissue gene expression changed along the different phases, and 464 adipocyte genes, the majority of them involved in metabolism, were downregulated during energy restriction, upregulated during weight stabilization, and unchanged during the maintenance diet. To identify adipocyte genes regulated by changes in caloric intake independent of alterations in body weight, Franck *et al.* (Franck *et al.* 2011) investigated obese subjects given a very low-caloric diet followed by gradual reintroduction of ordinary food, and healthy subjects undergo overfeeding. They saw that 52 genes were downregulated, and 50 were upregulated by caloric restriction and regulated in the opposite direction by refeeding and overfeeding. Among these, there were genes involved in lipogenesis (*ACLY*, *ACACA*, *FASN*, *SCD*), control of protein synthesis (*4EBP1*, *4EBP2*), beta-oxidation (*CPT1B*), and insulin resistance (*PEDF*, *SPARC*). A later study (Johansson *et al.* 2012) in subcutaneous abdominal adipose tissue during weight loss from obese human subjects on a low-calorie diet and the posterior weight maintenance demonstrated that the majority of differentially expressed genes showed to be either upregulated (1104) or downregulated (1059) during weight loss, and then oppositely regulated during weight maintenance, before returning toward baseline when subjects remained weight stable after weight loss. After the whole process 750 genes were differentially expressed, 483 of which were downregulated and 267 upregulated. Some of the upregulated genes participate in lipid mobilization and tissue rearrangement during the weight-reduction process, whereas several genes involved in inflammation were downregulated, reinforcing the evidence that weight loss leads to a reversal of the proinflammatory capacity of obesity. When abdominal subcutaneous adipose tissue was studied in overweight/obese postmenopausal women with high and low response to caloric restriction intervention (Bouchard *et al.* 2010), there were significant DNA methylation differences between the high and low responders, affecting genes known to be involved in weight control and insulin secretion, and 644 genes were differentially expressed between the two groups after diets. Overall, adipocyte response to caloric intake is executed through a complex transcriptional response.

46.2.2 Effect of Dietary Carbohydrate Content

Diet composition is an important factor that can modify the biochemistry and physiology of the adipose tissue. When the effects of a high-fat sucrose diet were study in periovaric adipose tissue of adult female rats that had experienced 180 min daily of maternal separation during lactancy (Paternain *et al.* 2012), it was shown that early-life stress affects the response to this diet later in life by modifying expression of *adiponutrin*, *leptin*, and *Ppargc1a*. The opposite was addressed in obese men after a 28-day diet with a decreased energy percentage from carbohydrates and an increased energy percentage from proteins; a diet intervention that induced a gene expression response similar to that produced by caloric restriction (Brattbakk *et al.* 2013). Among the downregulated genes that responded to the diet intervention there were a high number of genes involved in the regulation of immunological processes, which suggests that diet composition plays an important role.

46.2.3 Effect of Dietary Fat Content

As previously mentioned, adipocyte function is under regulation by food intake and dietary nutrients (He *et al.* 2004), and disorders in this aspect can lead to obesity. These responses may be as early as 3 h, when Bolduc *et al.* noted that lipid catabolism and adiponectin gene expression were repressed in the mesenteric adipose tissue of mice following a high-fat meal (Bolduc *et al.* 2010). In longer periods (20 weeks), mice consuming a high-fat diet (HFD) become obese and insulin resistant, and WAT gene expression showed large number of genes differentially expressed between diets, including those involved in signal transduction, inflammation, oxidative stress, and lipid metabolism (Choi *et al.* 2013, Lee *et al.* 2012, Okada *et al.* 2009). These results indicate that length and HFD elicit important transcriptional changes.

Involvement of epigenetics in the development of obesity has also been proposed (Kamei *et al.* 2010). Recent evidence seems to confirm this issue. In a study of three generations of C57BL/6 mice fed normal chow and HFD (Ding *et al.* 2014), body and parametrial adipose tissue weight increased in female mice across generations under continuous HFD stress. Genes involved in inflammatory response showed increased expression, accompanied by increased macrophage infiltration. The offspring of obese mothers are shown to prefer HFD, creating a “feed-forward cycle” in female mice across generations as demonstrated by increased adiposity and progressive inflammation in adipose tissue. Furthermore, exposure to a HFD during the periconceptional/gestation/lactation period to obese, diabetic females conveyed that their female offspring showed expression changes of genes involved in metabolism and in global DNA methylation (Attig *et al.* 2013).

The response to HFD varies depending on the genetic background. Comparing the response of polygenic Fat mouse strain from a comparator Lean strain to these diets, differential gene expressions were found, some previously linked to obesity, such as *C1qrl1* and *Np3r*, and others such as *Thbs1*, *Ppp1r3d*, *Tmepai*, *Trp53inp2*, *Ttc7b*, *Tuba1a*, *Fgf13*, and *Fmr* were elevated in Fat mouse adipose tissue (Morton *et al.* 2011). In this regard, estrogen-related receptor alpha-deficient and perilipin A overexpressing transgenic mice, showed resistance to diet-induced obesity, specifically in adipocytes (Luo *et al.* 2003, Sawada *et al.* 2010) by an increase in the expression of genes associated with fatty acid beta-oxidation and heat production, and a decrease in the genes associated with lipid synthesis. These studies emphasize that obesity results from a complex interaction between genetic and environmental factors.

46.2.4 Nature of Fat

Effects of supplementation of diet for 10-week with polyunsaturated fatty acids (PUFA) versus saturated fatty acids (SAT) in lactating German Holstein dairy cows (Hiller *et al.* 2013) was studied in subcutaneous, perirenal and omental adipose tissue. The main transcriptomic responses to dietary PUFA were obtained in omental adipose tissue, with upregulated ACACA, ADFP, CEBPA, FASN, LPL, PPARG, SCD, and SREBF1 expression. The contribution of specific PUFA has been equally investigated. In this regard, docosahexaenoic acid-supplemented diets reduced the expression of FoxO1 and FoxO3 in pig adipose tissue, and also decreased the expression of FoxO1 and FoxO3 in human and porcine primary adipocytes (Chen *et al.* 2012). The use of conjugated linoleic acid (CLA) isomer, t10c12-CLA, reduced epididymal, mesenteric, and brown adipose tissues in polygenic obese (M16-selected) and nonobese (ICR-control) mice (House *et al.* 2005). These changes were accompanied by increased expression of *Ucp* (1 and 2), *Cpt*, *Tnfa*, and *Cas3*, but decreased expression of *Pparg*, *Glut4*, perilipin, caveolin-1, adiponectin, resistin, and *Bcl-2*.

46.2.5 Effects of Quality and Protein Content

The type of dietary protein may contribute to obesity development as proved a study on the effect of soy protein versus casein (Frigolet *et al.* 2011) on white adipose tissue genome profiling in rats with diet-induced obesity. Rats were fed with soy or casein control diets and with a HFD containing soy or casein. Rats fed soy diets gained less weight and had significantly smaller adipocytes irrespective of diet. Soy protein consumption increased adipocytary expression of genes involved in fatty acid oxidation and reduced leptin mRNA. Thus, soy protein consumption modifies the gene expression profile of adipose tissue and prevents adipocyte hypertrophy and obesity, despite the consumption of a high-fat diet.

When protein was restricted in obese rats throughout pregnancy and lactation (Guan *et al.* 2005), offspring were born smaller than controls and in adulthood developed visceral adiposity. Comparing the pattern of gene expression in visceral adipose tissue, 650 transcripts were identified as differentially expressed. There was a global upregulation of genes involved in carbohydrate, lipid, and protein metabolism and genes involved in adipocyte differentiation. However, in marked contrast to other rodent models of obesity, the expression of a large number of genes associated with inflammation was reduced in this rat model. These data suggest that early-life undernutrition exerts permanent effects on the structure and function of key metabolic organs that will condition adulthood responses.

46.3 Intestinal Transcriptome

Gut epithelium forms a selective barrier that has evolved by developing complex mechanisms not only to allow nutrient absorption, but also to protect against entry of infectious agents and foreign antigens into the body. Regarding the nutrition field, absorption of nutrients – sugars, amino acids, fatty acids, ions, minerals, and vitamins – results of paramount importance, and both transcellular and paracellular transport mechanisms may participate. The former usually involve specific cell-surface transporters or acceptor molecules that allow for selective uptake of molecules that are transported

across the cell, while paracellular transport involves the movement of molecules, across an electro-chemical gradient, between cells (Kotler *et al.* 2013). In contrast with other organs involved in nutrition and analyzed in this chapter, enterocytes are subjected to an amazing rhythm of renovation and differentiation from crypt to villus cells. During differentiation of jejunal enterocytes, the expression of many genes related to cell growth rapidly decreased, while expression of genes related to digestion and absorption of nutrients and ions increased. In this way, the expression of a subset of genes related to the digestion and absorption of starch and sucrose was highest at the middle of the villi, whereas expression of genes related to dietary fat absorption was highest at the top of the villi. Several transcriptional factors such as *Pdx1*, *Foxa2*, and *Thra* were expressed in the crypt, whereas *Klf15* was highly expressed during the crypt-villus transition. Expression of *Klf4* and *Pparg* was highest at the top of the villi. Therefore, different subsets of gene expressions are switched on according to the differentiation state of these cells during their trajectory along the crypt-villus axis (Suzuki *et al.* 2009). Likewise, enhanced expressions of a large number of genes involved in immune response and inflammation in the colon contribute to the functional decline associated with aging in old mice (Steegenga *et al.* 2012). These facts make nutritional regulation more complex in this organ, and all considered aspects are summarized in Table 46.3.

46.3.1 Influence of Caloric Restriction

Fasting ends when nutrition starts. Accordingly, fasting may pose interesting avenues and will be firstly addressed, and, as expected, liver and intestines were involved. Starvation of FVB mice revealed that the biliary lipids could be mobilized to supply the enterocytes with luminal fuel and to stabilize transport systems in the intestine for ensuring a rapid recovery when food supply resumes (Sokolovic *et al.* 2010).

Table 46.3 Intestinal transcriptomic changes in several nutritional conditions.

Condition	Model	Finding	References
Fasting	Mouse	Utilizes bile as source of energy	(Sokolovic <i>et al.</i> 2010)
High-glucose diet	Mouse	Modifies digestive enzyme, gastrointestinal peptide and nutrient transporters	(Du <i>et al.</i> 2010).
Resistant starch	Rat	Maintenance of genomic integrity	(Conlon 2012)
Hypoallergenic wheat flour	Rat	Oral immunotolerance	(Narasaka <i>et al.</i> 2006)
High-fat meal	Mouse	Decreased cell defense and <i>Apoa4</i>	(Yoshioka <i>et al.</i> 2008)
Long-term HFD	Mouse	Increased <i>Gip</i> , <i>Pdx1</i> , and <i>Rfx6</i>	(Suzuki <i>et al.</i> 2013)
<i>Trans-10,cis-12</i> conjugated linoleic acid	Caco-2 cells	Increased transepithelial Ca^{2+} transport	(Murphy <i>et al.</i> 2007)
Threonine restriction	Pig	Immune and defense responses, energy metabolism, protein synthesis, and regulation of paracellular permeability	(Hamard <i>et al.</i> 2010)
Red meat	Human	DNA damage repair, cell cycle, apoptosis, WNT signaling, and nucleosome remodeling	(Hebels <i>et al.</i> 2012)
Heme	Rat	Increased pentraxin	(Van Der Meer-Van Kraaij <i>et al.</i> 2003)
Casein peptide	Caco-2 cells	Up-regulated expression of occludin	(Tanabe, 2012)
Microbiota and fatty acids	Mouse	Polyunsaturated fatty acids modulated microbiota-induced inflammation	(Cooney <i>et al.</i> 2012; Roy <i>et al.</i> 2007)

46.3.2 Effects of Carbohydrate Content of Diets

The long term intake of high-glucose diet was explored in C57BL/6J mice and it was found that the expression of genes related to digestive enzymes, gastrointestinal peptides, and nutrient transporters were significantly changed. This may indicate a suppression of digestive enzyme gene expression, attenuation of alimentary tract movements and nutrient transportation by administration of this monosaccharide (Du *et al.* 2010).

The source of complex carbohydrates or polysaccharides has been explored as well. Resistant starches coming from high amylose wheat, high amylose maizes or butyrylated starch reversed dietary protein-induced colonocyte DNA damage in rats by modifying the expression of colonic genes associated with the maintenance of genomic integrity (notably *Mdm2*, *Top1*, *Msh3*, *Ung*, *Rere*, *Cebpa*, *Gmn*, and *Parg*). The use of high amylose wheat elicited gene changes not observed with other sources of amylose, which could be due to the presence of other fiber components (Conlon *et al.* 2012). In this animal, consumption of hypoallergenic wheat flour showed less than 30 genes exhibited up- or downregulation of two-fold or more. Upregulation of some of the genes responding to the interferon-gamma signal may be related to a possible oral immunotolerance (Narasaka *et al.* 2006).

46.3.3 Effect of Dietary Fat Content

Despite the fact that intestinal fat absorption is a crucial step in energy balance, not many high-throughput studies have explored the influence of fat in this organ. A postprandial study using a high fat meal showed delayed expressions of peptidases, decreased cell defense and *Apoa4* transcripts despite the absence of changes in plasma triglyceride. In contrast, this diet induced transcripts related to cell growth and organization (Yoshioka *et al.* 2008). Since *Apoa4* is a direct target of estrogen-related receptor alpha (Carrier *et al.* 2004), and APOA4 plays an important role in fat absorption, this receptor may play a role in intestinal fat transport as well.

Long term administration of HFD for 8 weeks induced obesity and gastric inhibitory polypeptide (GIP) hypersecretion in heterozygous mice expressing GIP coupled with green fluorescent protein *in vivo*, by increasing *Gip*, *Pdx1*, and *Rfx6* mRNA expressions in enteroendocrine K-cells. Knockdown of *Rfx6* and *Pdx1* decreased *Gip*, which indicates that *Rfx6* and *Pdx1* mediate the response of GIP to HFD (Suzuki *et al.* 2013). Thus, transcriptional regulatory factor X6 (*Rfx6*) and pancreatic and duodenal homeobox 1 (*Pdx1*) are transducers of HFD in this organ.

Quality of fat has been analyzed in human intestinal-like Caco-2 cells. In this sense, *trans*-10, *cis*-12 CLA modified the expression of 918 genes which modulated a number of processes related to carcinogenesis, and increased transepithelial Ca^{2+} transport with changes in molecular mediators of paracellular pathways (Murphy *et al.* 2007).

46.3.4 Effects of Quality and Protein Content

In piglets, a low threonine diet significantly modified the expression of ileum genes involved in immune and defense responses, energy metabolism, protein synthesis, and regulation of paracellular permeability (Hamard *et al.* 2010). These results show that threonine contributes to maintain gut physiology.

In humans consuming diets enriched in red meat, the increase in fecal water genotoxicity was significantly correlated with gene expressions involved in biological pathways indicative of genotoxic effects such as DNA damage repair, cell cycle, apoptosis, WNT signaling, and nucleosome remodeling pathways. This may explain the increased colorectal cancer risk associated with this type of diets (Hebels *et al.* 2012). Furthermore, this effect has been attributed to the heme content, since dietary heme increased colonic cytotoxicity, epithelial cell turnover, and carcinogenesis biomarkers in rats. Using high-throughput analysis of RNA in this model, a novel gene named mucosal pentraxin was identified in colon and this seems to play a role in cellular death (Van Der Meer-Van Kraaij *et al.* 2003).

A peptide obtained by the hydrolysis of casein, a major milk protein, Asn-Pro-Trp-Asp-Gln (NPWDQ) inhibits the permeability to ovalbumin, one of the food allergens, in Caco-2 cells. In this action, up-regulated expression of the occludin gene was observed whereas the levels of other genes involved in maintaining cell interactions remained unchanged (Tanabe 2012). These findings suggest that milk-derived peptide(s) enhance intestinal barrier and may provide a new significance to lactation due to the role in promoting the maturation of the intestinal barrier. Therefore, the modulation of this barrier function should be envisioned as a positive aspect to avoid entry of infectious agents and foreign antigens.

46.3.5 Environmental Conditions of Intestine

These represent a new challenge for its study since the gastrointestinal tract is the largest reservoir of commensal microorganisms (microbiota) in the mammals, providing nutrients and space for the survival of microbes while concurrently operating mucosal barriers to confine the microbial population. Accumulating evidence indicates that these are involved in various physiological functions in the gut and their imbalances (dysbiosis) may cause pathology (Yu *et al.* 2012). This complex biological systems is also modulated by composition of food. Studies carried out in subjects with metabolic syndrome have shown that the quality of grains has a minor effect on the intestinal microbiota composition, and suggest that the dietary influence on the microbiota involves other dietary components such as fat (Lappi *et al.* 2013). In addition, composition of intestinal microflora has also been observed to modulate colonic gene expression in mice (Roy *et al.* 2007). Specifically, bacterially induced inflammation was associated with reduced expression of proteins from pathways of metabolism and digestion/absorption/excretion of nutrients/ions, and increased expression of cellular stress and immune response proteins. To treat this inflammation, polyunsaturated fatty acids such as n-3 eicosapentaenoic acid (EPA) and n-6 arachidonic acid (AA) have been effectively used. Interestingly, the involved mechanisms were different, while EPA acted via the PPAR α , AA increased energy metabolism and cytoskeletal organization, and reduced cellular stress responses (Cooney *et al.* 2012, Roy *et al.* 2007). In dextran sodium sulfate-induced intestinal inflammation in rats, a diet enriched in goat's milk oligosaccharides and cellulose was also efficient to control the process (Lara-Villoslada *et al.* 2006). Due to the consequences of the microbiome, increased interest in its manipulation is emerging and different nutritional combinations are expected to be assayed.

46.4 Hepatic Transcriptome

The liver is an organ primarily responding to diet and controlling plasma carbohydrate, protein and lipid levels. In addition, it is responsible for metabolism and excretion of xenobiotics. For these reasons, it has been profusely studied in experimental models as the number of references considered for this issue clearly reflects (Figure 46.2). Before setting out to analyze the influence of different macronutrients, the transition of fasting to feeding and the influence of calorie restrictions will be explored and a summary of these findings provided in Table 46.4.

46.4.1 Influence of Fasting and Feeding

In fasting FVB mice, the transcriptional profiles suggested increased cholesterol trafficking in the liver and a decreased in the small intestine. These were accompanied by increased the bile salt and lipid output rates, hepatic and intestinal lipid turnover, and enhanced trans-intestinal cholesterol excretion. In contrast, fecal sterol loss declined sharply (Sokolovic *et al.* 2010). Genes involved in amino-acid, lipid, carbohydrate, and energy metabolism showed significant responses to fasting in mouse liver, a response that peaked at 24 h, and was largely abated by 72 h. The strong induction of the urea cycle, in combination with increased expression of enzymes of the tricarboxylic-acid cycle and oxidative phosphorylation, indicated a strong stimulation of fatty-acid and amino-acid oxidations and ketone-body formation peaking at 24 h. The induction of genes involved in the unfolded-protein response underscored the cell stress due to enhanced energy metabolism. The continuous high expression of enzymes of the urea cycle, malate-aspartate shuttle, and the gluconeogenic enzyme *Pepck*, and the re-appearance of glycogen in the pericentral hepatocytes indicate that amino-acid oxidation yields to glucose and

Table 46.4 Hepatic transcriptomic changes in different nutritional conditions.

Condition	Model	Finding	References
Fasting	Mouse	Energy generation in early and glucose and glycogen synthesis in prolonged fasting	(Sokolovic <i>et al.</i> 2008)
Fasting/feeding	Mouse	Modulation of PPAR α	(Oka <i>et al.</i> 2009)
	Rat	Increased abundance of protein in polysomes	(Reiter <i>et al.</i> 2005)
Caloric restriction	Mouse, Rat	Changes in stress response, xenobiotic metabolism, and lipid metabolism mediated by PPAR α	(Corton <i>et al.</i> 2004; Higami <i>et al.</i> 2006)

glycogen synthesis during prolonged fasting (Sokolovic *et al.* 2008). Upregulation of hepatic *Txnip* expression was observed in wild-type mice when driven into torpor by prolonged fasting. The elevated *Txnip* expression, as a molecular nutrient sensor, may be regulating energy expenditure and fuel use during the extreme hypometabolic state of torpor (Hand *et al.* 2013). Thus, the changes in liver gene expression during fasting indicate that, in the mouse, energy production predominates during early fasting and that glucose production and glycogen synthesis become predominant during prolonged fasting and new molecules may participate in this balance.

The transition between fasting and refeeding represents a real challenge to a cell, not surprisingly 6000 genes were differentially expressed in mouse liver (Jun *et al.* 2011). Thioredoxin binding protein-2(TBP-2) appears to be a key regulator of peroxisome proliferator activated receptor α (PPAR α), and its coordinated regulation of PPAR α and insulin secretion is crucial in the feeding-fasting nutritional transition. Indeed, fasting-induced reduction in the expression of lipogenic genes targeted by insulin via SREBP1, such as *Fasn* and *Thrsp*, was abolished in *Tbp-2*-deficient mice, and the expression of lipoprotein lipase was downregulated, which was consistent with the lipoprotein profile. In fed *Tbp-2*-deficient mice, there were elevated expressions of PPAR α and PPAR γ coactivator-1 α (PGC-1 α) proteins and their target genes *Cd36*, *Fabp2*, *Acot1*, and *Fgf21*, whereas the fasting-induced up-regulation of PPAR α was attenuated (Oka *et al.* 2009). In *Clock* mutant mice bred under constant light to attenuate the endogenous circadian rhythm, hepatic *Per1*, *Per2*, and *Dec1* expressions were significantly increased while that of *Rev-erba* decreased within 1 h of feeding following 24-h fasting (Oike *et al.* 2011). Thus, fasting is inducing a complex transcriptional response where PPAR α and TBP-2 are important agents.

Similar studies have been carried out in rats and expression profiles of hepatic energy metabolism-related genes were different in rats allowed to feed for 6 h after either 18-h or 24-h fasting. In addition, refeeding induced upregulation of the genes encoding immunoproteasome components (Ushijima *et al.* 2010). Following feeding, 42 proteins involved in protein synthesis increased their abundance in polysomes (Reiter *et al.* 2005). These results indicate that changes at the protein level may exist in this situation either by proteasomal degradation or enhanced translation.

46.4.2 Influence of Caloric Restriction

As mentioned in other sections of this chapter, this issue has deserved great attention in several animal models. In rats, a 30%-caloric restriction (CR) modified hepatic expression of genes involved in the stress response, xenobiotic metabolism, and lipid metabolism. While genes involved in lipid metabolism were independent of the growth hormone/insulin growth factor-1-signaling, those involved in stress response and xenobiotic metabolism were dependent on this cascade. Moreover, CR enhanced the gene expression involved in fatty acid synthesis after feeding and those encoding mitochondrial beta-oxidation enzymes during food shortage, probably via transcriptional regulation by PPAR α . Thus, caloric restriction promotes lipid utilization through hepatic transcriptional alteration and may prevent hepatic steatosis (Higami *et al.* 2006). In this sense, upregulation of the three *Nr4a* receptors was observed in the livers of Brown Norway rats. Based on the proposed roles of the NR4A nuclear receptors in sensing and responding to changes in the nutritional environment, and in regulating glucose, lipid metabolism, and insulin sensitivity, it was hypothesized that these proteins may participate in caloric restriction adaptation (Oita *et al.* 2009). Different levels of restriction (5–30% of calories) had an important impact on genes of lipid metabolism, something that was observed with the lowest used level and despite the absence of changes in body weight. These results that changes at the transcriptomic level may be really early (Saito *et al.* 2010).

Not only did the level of CR seem to be important, but also length of intervention. Both just 4-week and long-term-caloric restriction reversed the majority of aging-induced changes in hepatic gene expression, mainly increased inflammation, cellular stress, fibrosis, and reduced capacity for apoptosis, xenobiotic metabolism, normal cell-cycling or DNA replication (Cao *et al.* 2001). With a more prolonged regimen (6 months), male C57BL/6 mice fed 75% of a normal diet showed that antioxidant and phase II enzymes, as well as metallothionein 1 and autophagy gene expressions, were increased (Giller *et al.* 2013). The benefits of caloric restriction were established rapidly, but they were also lost within 2 weeks and disappeared after 6 months of refeeding (Giller *et al.* 2013). This indicates that dietary restriction has to be maintained continuously to keep its beneficial effects.

The influence of experimental design may influence results as well. In fact, two acute progressive feeding restriction regimens causing identical diminution of body weight (19%) but differing in duration (4 vs 10 days) led to distinct patterns of differentially expressed genes in liver. Albeit some major pathways of energy metabolism were similarly affected (particularly fatty acid and amino acid catabolism), the longer regimen also induced deregulation of circadian rhythms (Pohjanvirta *et al.* 2008). Increased hepatic expressions of *Cpt1a* and *Star* decreased *Fasn* in offspring were found in 20% calorie-restricted lactating dam rats (Koniczna *et al.* 2014). In mid-lactation Holstein cows with dietary restriction, 312 hepatic genes (155 downregulated, 157 upregulated) were differentially expressed. Expression of GPX3 and of genes

associated with gluconeogenesis (PC, PDK4), inflammation (SAA3), and signaling (ADIPOR2) increased, while BBOX, a key for L-carnitine biosynthesis, and the transcription factor HNF4A were downregulated. Biosynthesis of cholesterol and energy generation by mitochondrial respiration were the most relevant, inhibited functions, and flux toward gluconeogenesis, the most enhanced (Akbar *et al.* 2013). Increased mRNA coding for interleukin 1 β , serum amyloid A, C-reactive protein and haptoglobin, and decreased glucocorticoid receptor were also observed in the liver of mid-lactating goats receiving a 60%-restricted diet for 9 weeks (Dong *et al.* 2013). Thus, inflammatory responses and the metabolism of lipids, protein, and carbohydrate were significantly altered. This hepatic metabolic adaptation is suggesting re-distribution of energy in the liver to provide glucose for the lactating mammary gland during feed deprivation and may ultimately lead to a decline in milk quality.

The hepatic transcript profile in wild-type mice undergoing caloric restriction presents some overlaps with those of agonists of lipid-activated nuclear receptors, including PPAR α , liver X receptors, and their obligate heterodimer partner, retinoid X receptors. In fact, 19% of all gene expression changes were dependent on PPAR α , including *Cyp4a10* and *Cyp4a14*, those involved in fatty acid omega-oxidation, acute phase response genes, and epidermal growth factor receptor but not on PGC-1 α . Based on these observations, it is hypothesized that some effects of caloric restriction are mediated by PPAR α (Corton *et al.* 2004) in agreement with the data observed in rats. Transcriptomic analysis was used to identify genes commonly upregulated in the liver of CR-mice and Ames dwarf mice, which are deficient in growth hormone, prolactin, and thyroid-stimulating hormone, and live significantly longer than their normal siblings. A search for sequence similarity among those found genes revealed the presence of consensus sequence motifs named dwarfism and caloric restriction-responsive elements. This has been exploited to prepare a highly sensitive bioassay to identify agents mimicking the anti-aging effects of caloric restriction (Chiba *et al.* 2010).

Large animals have equally been subject of this intervention. Over 2900 hepatic genes were differentially expressed in response to the 60%-energy restricted Holstein-Friesian and Holstein-Friesian \times Jersey cows. Following 3 weeks of energy restriction, glucose-sparing pathways, gluconeogenesis, genes involved in hepatic stress, and cytoskeletal remodeling were increased (Grala *et al.* 2013). Beef cattle genetically selected for feed efficiency showed 161 hepatic genes that were differentially expressed. These genes were involved in seven gene networks affecting cellular growth and proliferation, cellular assembly and organization, cell signaling, drug metabolism, protein synthesis, lipid metabolism, and carbohydrate metabolism (Chen *et al.* 2011). Overall, food restriction and efficiency are complex processes that are highly regulated.

In the following section, the influence of different macronutrients on the hepatic transcriptome will be reviewed and a summary is shown in Table 46.5.

46.4.3 Effects of Carbohydrate Content of Diets

Despite the assumed statement that carbohydrates should be the main source of calories in most diets, not many studies have been published regarding their influence and a few of them have dealt with the complex ones. In C57BL/6J mice, a high glucose content diet modified expressions of genes related to thiol redox, peroxisomal fatty acid oxidation and cytochrome P450, and enhanced oxidative stress (Du *et al.* 2010). A high sucrose diet in Nagoya-Shibata-Yasuda, but not in C3H mice, increased hepatic expression levels of *Pparg2*, as well as *G0s2*, a target of *Pparg*, which are known to be adipocyte-specific genes. In contrast, hepatic levels of *Kat2b* (transcriptional regulation), *Hsd3b5* (steroid hormone metabolism) and *Cyp7b1* (bile acid metabolism), initially lower in Nagoya mice, were further decreased in this mouse strain receiving high sucrose diet (Nojima *et al.* 2013). When complex mixtures such as sweet corn were given to mice, 1600 genes modified their expression some of them related to cell proliferation and programmed cell death. In the Wnt signaling pathway, which is involved in cell proliferation, the levels of Jun and beta-catenin expression were downregulated, while those of *Rb* and *p53*, negative regulators of the cell cycle, or those of *Bok*, *Bid*, and *Casp4* involved in apoptosis were increased (Tokaji *et al.* 2009). These results point out to heterogeneity in response to these diets and additive implication of genes when complex mixtures were administered.

In rats, a 11-day administration of diet containing 20% maple syrup significantly lowered hepatic function markers, and the hepatic expression of genes coding for the enzymes of ammonia formation were downregulated as well compared to a group receiving 20% sugar mix syrup diet (Watanabe *et al.* 2011). Short-chain fructooligosaccharide increased the expression of PPAR α , phytanoyl-CoA 2-hydroxylase 2, lipoprotein lipase and tyrosine aminotransferase, and farnesoid X receptor (FXR) target genes in the rat liver (Fukasawa *et al.* 2010). The activation of lipoprotein lipase and FXR-target genes may participate in the lipid lowering effects of those compounds (Fukasawa *et al.* 2009). Hypoallergenic wheat flour, when fed to rats, upregulated genes known to respond to the interferon-gamma signal, which may be related to possible oral immunotolerance following its feeding (Narasaka *et al.* 2006). Therefore, carbohydrates elicit a wide range of responses.

46.4.4 Effect of Dietary Fat Content

This is an aspect that in liver has been widely analyzed in terms of amount and type of fat. Surprisingly, the influence of a fat-free diet has been scarcely studied. One study in rat liver consuming this type of diets showed that emerin, an integral protein of the inner nuclear membrane, was highly expressed independently of the sterol regulatory element binding regulation pathway (Mziaut *et al.* 2001).

46.4.4.1 Studies on High Fat Diet

As fat intake should be the second source of calories, it is not surprising that these aspects have been profusely addressed by different studies varying the approaches in terms of time-period, amount, and source of fat.

At postprandial evaluation of hepatic gene expression, our group carried out a study in male rats using a bolus of 5 ml of extra-virgin olive oil. Under these experimental conditions, *A2m*, *Slc13a5*, and *Nrep* mRNA expressions were induced and their changes associated with those observed in plasma triglycerides (Martinez-Beamonte *et al.* 2011a).

At sustained consumption, different time-periods have been tested ranging from 1 week to 24 weeks. A high-fat diet administration for 1 week to obese mice decreased hepatic *Cyp3a* expression without modifying the expression of nuclear receptors involved in its transcriptional regulation. Changes in the hepatic expression of this cytochrome have been also observed in mice fed this type of diet and that eventually became obese (Yoshinari *et al.* 2006).

Table 46.5 Effect of macronutrients on hepatic transcriptomic changes.

Condition	Component	Model	Finding	References
Carbohydrates	Glucose	Mouse	↑ Oxidative stress	(Du <i>et al.</i> 2010)
	Sucrose	Mouse	↑ <i>Pparg2</i>	(Nojima <i>et al.</i> 2013)
	Sweet corn	Mouse	↑ Cell proliferation	(Tokaji <i>et al.</i> 2009)
	Maple syrup	Rat	↓ Ammonium	(Watanabe <i>et al.</i> 2011)
	Fructooligosaccharide	Rat	↑ FXR	(Fukasawa <i>et al.</i> 2010)
Amount of fat	Bolus of fat	Rat	↑ <i>A2m</i> , <i>Slc13a5</i> , and <i>Nrep</i>	(Martinez-Beamonte <i>et al.</i> 2011a)
	High fat diet	Mouse	↓ <i>Cyp3a</i> , <i>Scd1</i>	(Toye <i>et al.</i> 2007; Yoshinari <i>et al.</i> 2006)
Nature of fat	High fat diet	Rat	↑ Inflammation	(Swindell <i>et al.</i> 2010)
	MUFA, PUFA	Rat	↓ beta-oxidation	(Xie <i>et al.</i> 2010)
	n-3 PUFA	Mouse, rat	↓ Lipogenesis	(Deng <i>et al.</i> 2004)
			Modified cellular regulators	(Berger <i>et al.</i> 2006; Eletto <i>et al.</i> 2005; Lapillonne <i>et al.</i> 2004)
Cholesterol	CLA	Hamster, mouse, rat	Variable on the genetic background	(Guillen <i>et al.</i> 2009b; Miranda <i>et al.</i> 2009; Reynolds <i>et al.</i> 2013)
	High level	Mouse	↓ CREP	(Inoue <i>et al.</i> 2005)
	Modest level	Mouse	↑ Inflammatory response	(Maxwell <i>et al.</i> 2003; Obama <i>et al.</i> 2011; Rezen <i>et al.</i> 2008; Vergnes <i>et al.</i> 2003)
Protein	Soy vs casein	Rat	↓ Lipogenesis	(Tachibana <i>et al.</i> 2005; Takamatsu <i>et al.</i> 2004)
	Branched AA	Rat	↓ Ammonium, FA uptake	(Jia <i>et al.</i> 2013)
	Low methionine + choline	Mouse	↓ Oxidative stress	(Zhou <i>et al.</i> 2008)
	Low methionine + choline + folate	Rat	Alteration of DNA methylation	(Ghoshal <i>et al.</i> 2006)

Key: AA, amino acids; CLA, conjugated linoleic acid; CREB, cAMP response element-binding protein; FA, fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

Prolonged administration of HFD has been used to develop non-alcoholic fatty liver disease (NAFLD) or insulin resistance (Toye *et al.* 2005) and to study both intertwined processes (Musso *et al.* 2009). In 129S6 male mice fed diets containing 40% fat for 15 weeks, this insult increased hepatic transcription of genes involved in uptake, intracellular transport, modification, and elongation of fatty acids. Unexpectedly, genes involved in beta-oxidation and lipoprotein secretion were upregulated. NAFLD was present, despite a downregulation of stearoyl-coenzyme A desaturase 1 (*Scd1*) and uncoordinated regulation of *Scd1* and sterol regulatory element binding protein 1c (*Srebp1c*) transcriptions (Toye *et al.* 2007). The latter, SREBP-1c, has been shown to play a crucial role in HFD-induced changes in lipid metabolism (Capel *et al.* 2013). *Scd1* was associated with the expression of patatin-like phospholipase domain containing 3 (PNPLA3). Increased expression of the latter gene in Western-type diet-fed C57BL/6 and LDLR-deficient mice was found to be associated with the changes of other lipogenic genes (*Me1*, *Spot14*) (Hoekstra *et al.* 2010). In the development of NAFLD in obese rodents, overexpression of the genes related to lipid metabolism, adipocyte differentiation, defense, and stress responses was also noticeable. These livers are supplied with large amounts of free fatty acids either through increased fatty acid biosynthesis or through decreased fatty acid oxidation, which may lead to increased mitochondrial respiratory activity (Do *et al.* 2010; Kim and Park, 2010) or better coupling of the respiratory chain (Franko *et al.* 2014). This could represent an adaptation to fuel overload and the high energy-requirement of an unsuppressed gluconeogenesis. In addition to the involvement of SREBP-1c, NR1H3 (LXR α) or transcription factor early growth response 1 (EGR1) may coordinate cell signaling under HFD. In this regard, mice lacking endogenous miR-155 fed HFD for 6 months developed increased hepatic steatosis compared to controls. This finding was associated with increased hepatic expression of genes involved in glucose regulation (*Pck1*, *Cebpa*), fatty acid uptake (*Cd36*), and lipid metabolism (*Fasn*, *Fabp4*, *Lpl*, *Abcd2*, *Pla2g7*). This pattern of gene expression and miRNA target prediction indicated that *Nr1h3* (LXR α) was a direct miR-155 target gene and responsible for the liver phenotype (Miller *et al.* 2013). Regarding EGR1, this transcription factor was identified as a putative regulator of hepatic diet-related differential gene expression using mice fed human and chimpanzee diets. However, 90% of the dietary response to the primate diet found in mice was not observed in primates, which may underscore potential limitations of mouse models in dietary studies (Weng *et al.* 2012). When NAFLD progresses to the more severe stage of non-alcoholic steatohepatitis (NASH), activation of c-Jun/AP-1 was observed (Dorn *et al.* 2014), and this cascade was found correlated with inflammation. These results emphasize the involvement of those cascades, but their fine tuning remains to be established. Equally, the contribution of different sources of fat may be important since C57BL/6 mice fed a high-fat lard diet showed decreased expression of lipogenic genes and upregulation of those involved in fatty acid oxidation (Kirpich *et al.* 2011). Furthermore, the different fatty components may be important. Indeed, while a 15%-fat containing diet caused differential regulation of 200 genes, a further enrichment of this diet in 10% cholesterol and 0.5% cholate modified the expression of 788 in C57BL/6 and 1010 genes in APOE3Leiden mice, respectively. Genes involved in lipid metabolism and inflammation were strongly affected by genotype and diet. This diet enriched in fat, cholesterol, and cholate reduced the expression of genes involved in bile acid, sterol, steroid, fatty acid, and detoxification metabolism. This common regulation of genes underlying lipid and detoxification processes has been proposed as a mechanism to protect against the accumulation of toxic endogenous lipids and bile acids (Kreeft *et al.* 2005). These results indicate that amount and nature of fat component may influence the changes in gene expression and reflect a wide genomic versatility to cope with different circumstances.

As indicated, HFD seems to stimulate inflammatory mechanisms in liver (Kirpich *et al.* 2011; Toye *et al.* 2007). Indeed, infiltration of hepatic tissue by leukocytes has been observed. This fact leads to elevated expression of immune-associated transcripts, particularly of genes encoding components of the toll-like receptor signaling pathway (e.g., *Irf5* and *Myd88*). In some strains (e.g., NZB/BINJ, B6), 50–60% of transcripts elevated by HFD might correspond to hepatic infiltration. In 4-week-old female BALB/c mice, HFD-influenced activation of acute inflammatory responses (*Orm1*, *Lbp*, *Hp*, and *Cfb*), disordered lipid metabolism and deregulated cell cycle progression. These alterations may have led to favorable conditions for the formation of both pro-inflammatory and pro-mitotic microenvironments in the target organs that promote immune cell infiltration and differentiation (Kim *et al.* 2013). In this regard, mice overexpressing aortic monocyte chemoattractant protein-3 on a high-fat, high-cholesterol diet for 12 weeks showed an increased lipid accumulation in their livers and increased activities of antioxidant enzymes (An *et al.* 2013). Interestingly, DBA mice appeared to exhibit resistance to localized hepatic inflammation associated with atherogenic diet. This emphasizes that the effect is genetically controlled and sensitive to both strain and sex (Swindell *et al.* 2010). A further evidence of genetic background was given by a more exacerbated transcriptional response observed in HFD-fed apolipoprotein E2 mice, where genes encoding chromatin-remodeling enzymes, such as jumonji C-domain-containing histone demethylases that regulate histone H3K9 and H3K4 trimethylation (*H3K9me3*, *H3K4me3*), were significantly altered in steatotic livers. The global methylation status in lipid-accumulated mouse primary hepatocytes showed that hepatic lipid accumulation induced aberrant *H3K9me3* and *H3K4me3*

status in PPAR α and hepatic lipid catabolism network genes, reducing their mRNA expression compared with non-treated control hepatocytes (Jun *et al.* 2012). Aging is also an important variable since hepatic gene expression changes were more pronounced in the context of aged C57BL/6J mice (Capel *et al.* 2011), and the molecular mechanisms underlying high-fat feeding or aging, which mediated insulin resistance, were not identical. These pieces of evidences point out that HFD promote the recruitment of inflammatory cells by hepatocytes through cascades influenced by genetic background, sex, and aging.

As mentioned earlier, the response to diets differs among mouse strains. In this sense, the C57BL/6J-fed mice exhibited signs of insulin resistance after a long-term consumption of a HFD, while the A/J mice did not (Alevizos *et al.* 2007; Misra *et al.* 2007). When gene expression of both strains was analyzed, resistance to steatosis in A/J mice fed a HFD was associated with a coordinated upregulation of genes controlling peroxisome biogenesis and beta-oxidation, and an increased expression of the elongase *Elovl5* and desaturases *Fads1* and *Fads2*. In agreement with these observations, peroxisomal beta-oxidation was increased, and lipidomic analysis showed increased concentrations of long chain fatty acid-containing triglycerides, arachidonic acid-containing lysophosphatidylcholine, and 2-arachidonoylglycerol, a cannabinoid receptor agonist. The anti-inflammatory CB2 receptor was the main hepatic cannabinoid receptor and was highly expressed in Kupffer cells. A/J mice had a lower pro-inflammatory state as determined by lower plasma levels of IL-1 β and granulocyte-CSF, and reduced hepatic expression of their mRNAs, which were found only in Kupffer cells. This suggests that increased 2-arachidonoylglycerol production may limit Kupffer cell activity (Hall *et al.* 2010). Moreover, HFD feeding in the A/J, but not in the C57BL/6, mouse livers upregulated 13 oxidative phosphorylation genes without changes in ATP production, which indicated increased uncoupling of the A/J mitochondria (Poussin *et al.* 2011). Thus, variations in the expression of peroxisomal beta-oxidation-, of anti-inflammatory lipid-, and oxidative phosphorylation activity-involved genes may protect A/J mouse livers against the initial damages induced by HFD that may lead to hepatosteatosis. Using a different approach by exaggerating Fat and Lean mouse strain differences with chronic high fat feeding, a distinct gene expression profile of line, fat depot and diet-responsive inflammatory, angiogenic and metabolic pathways was observed (Morton *et al.* 2011). Even within a strain, different phenotypes of genetically homogenous C57BL/6 mice fed a HFD for 9 months were observed. While most become obese and diabetic, a significant fraction remains lean and diabetic or lean and non-diabetic. Diabetic tendency was associated with preserved hepatic lipogenic gene expression and increased plasma levels of very low density lipoprotein. In contrast, the lean mice showed a strong reduction in the expression of hepatic lipogenic genes, in particular of *Scd1*, a gene linked to sensitivity to diet-induced obesity; the lean and non-diabetic mice presented an additional increased expression of eNos in liver. There was a progressive establishment of the different phenotypes, and development of the obese phenotype involved re-expression of *Scd1* and other lipogenic genes (de Fourmestraux *et al.* 2004). These facts indicate an enormous complexity among genetic variants responsible for the observed phenotypes of the different mouse strains and a dynamic adaptive response to new challenges.

The characterization of chronic response to HFD has been addressed in rats as well. Feeding a high-fat-sucrose diet for 8 weeks in male Wistar rats resulted in liver fat accumulation and modifications in genes involved in obesity and lipid metabolism (*Lepr*, *Srebf2*, *Agpat3*, and *Esr1*) (Cordero *et al.* 2013a, b). In other study, *Srebf1* and *Scd1* had upregulation, whereas others like *Ppara*, carnitine palmitoyltransferase 1, and 3-hydroxy-3-methylglutaryl-coenzyme A reductase had repressed expression. Metabolomic analysis showed that tetradecanoic acid, hexadecanoic acid, and oleic acid were elevated while arachidonic acid and eicosapentaenoic acid were decreased in HFD-fed livers. Glycine, alanine, aspartic acid, glutamic acid, and proline contents were also decreased. In obesity-prone and obesity-resistant rats receiving HFD, fatty acid metabolism, Krebs cycle, and amino acid metabolism were also the origin of metabolites differing between the two phenotypes (Li *et al.* 2008). These results revealed that, in this model, fatty acid utilization through beta-oxidation was inhibited and lipogenesis was enhanced by this type of diets (Xie *et al.* 2010).

Besides the differences observed depending on genotypes, epigenetics may play a role in HFD response as imprinted gene studies carried out in C57BL/6J mice and in Japanese macaques have unveiled (Vige *et al.* 2006). In this regard, consumption of a maternal high-fat (35% fat) diet resulted in increased fetal liver triglycerides and histologic findings of NAFLD, accompanied by a hyperacetylation at K14 of Histone 3 and depletion of histone deacetylase 1. Using chromatin immunoprecipitation differential display PCR to link fetal modifications of Histone 3 acetylation with alterations in gene-specific expression, a 40% increase in the expression of *Gpt2*, *Dnaja2*, and *Rdh12* was found, while *Npas2*, a peripheral circadian regulator, was downregulated (Aagaard-Tillery *et al.* 2008). On the contrary, feeding a control diet to DIO mice during the periconceptional/gestation/lactation period led to a pronounced sex-specific shift (17–43%) from susceptibility to resistance to HFD, in the female offspring. This fact was associated with an enhancement of hepatic pathways protecting against steatosis, the unexpected upregulation of neurotransmission-related genes and the modulation of a vast imprinted gene network (Attig *et al.* 2013). Methylation levels of specific CpG sites from *Srebf2*, *Agpat3*, *Esr1*, and *Fsn* promoter

regions showed changes due to the obesogenic diet in rats as well (Cordero *et al.* 2013a, b). Thus, the response to HFD depends on the feeding of the mother in those crucial periods.

Moreover, the combination of social stress and a Western diet resulted in significant perturbations in lipid regulation, including two key features of the metabolic syndrome: increased plasma levels of non-HDL cholesterol and intrahepatic accumulation of triglycerides. These effects were accompanied by a number of changes in the expression of hepatic genes involved in lipid regulation and transcriptional activity of LXR, SREBP1c, and ChREBP (Chuang *et al.* 2010). Another interesting aspect regarding experimental design has been proposed in pigs where two regimens of administration were analyzed: either continuous feeding or alternate supply of a HFD for several weeks. Interestingly, two different molecular responses were observed in liver, while the former group showed post-transcriptional induction of CYP2E1 activity, the second presented a transcriptional induction of CYP3As and better adaptive response with no sign of effective tissue inflammation (Puccinelli *et al.* 2013). In both cases, these approaches better reflect the human feeding.

46.4.4.2 Influence of the Nature of Fat

This represents an important issue, not surprisingly a growing number of studies are addressing the influences of monounsaturated (MUFA) or polyunsaturated fatty acid (PUFA)-containing diets. In this way, substitution of dietary monounsaturated or polyunsaturated fatty acid (olive oil and menhaden oil) for carbohydrate reduced hepatic expression of SREBP-1c, with concomitant reductions in hepatic triglyceride content, lipogenesis, and expression of enzymes related to lipid synthesis in corpulent James C. Russell (JCR:LA-cp) rats. Unexpectedly, this substitution increased expression of many PPAR-dependent enzymes mediating fatty acid oxidation (Deng *et al.* 2004). Olive oil substitution for butter (10% of total energy) for 2 weeks modulated several genes related to lipolysis or lipogenesis in normal rats (Eletto *et al.* 2005), and allowed to identify genes from other metabolisms (*Fsp27* and *Syt1*) in apoE-deficient mice (Guillen *et al.* 2009b). In Sprague-Dawley rats, an intake of 70% as olive oil induced antioxidant genes and decreased those involved in inflammation and fibrosis (Ronis *et al.* 2012). The fish oil diet further increased expression of lypolitic and lipogenetic enzymes, and induction of SREBP-1c by insulin was dependent on LXR α . Expression of mRNA encoding fatty acid translocase and ATP-binding cassette subfamily DALD member 3 was also increased in livers of corpulent JCR rats, indicating a potential role for these fatty acid transporters in the pathogenesis of disordered lipid metabolism in obesity (Deng *et al.* 2004).

Among long chain PUFA, different transcriptomic responses were observed. Indeed, C57Bl/6J mice fed an n-3 PUFA-depleted diet for 3 months showed hepatic alterations similar to those observed in NAFLD patients and could be involved the gut microbiota composition since fructo-oligosaccharide supplementation reduced the hepatic triglyceride accumulation through a PPAR α -stimulation of fatty acid oxidation and lessened cholesterol accumulation by inhibiting SREBP2-dependent cholesterol synthesis. Fructo-oligosaccharide effects were related to a decreased hepatic miRNA33 expression and to an increased colonic glucagon-like peptide 1 production (Pachikian *et al.* 2013). On the contrary, the n-3 PUFA-containing diets, provided as fish oil, regulated lipolytic, particularly peroxisomal lipolysis, and lipogenic gene expression, the tissue specificity of this regulation in mice (Lapillonne *et al.* 2004), rats (Eletto *et al.* 2005), and in JCR:LA-cp rats (Deng *et al.* 2004), and suppressed secretion of very-low-density lipoprotein in obesity-resistant Slc:Wistar/ST male rats (Hashimoto *et al.* 2013). These diets also increased bile and cholesterol excretion controlling cholesterol metabolism by inducing cholesterol 7alpha-hydroxylase and its upstream transcription factors: D-site binding protein (DBP) and LXR α in mice (Berard *et al.* 2004), and independently of LXR transcription in JCR:LA-cp rats (Deng *et al.* 2004). These fatty acids regulated the expression of genes involved in many other pathways such as oxidative stress response and antioxidant capacity, cell proliferation, cell growth and apoptosis, cell signaling, and cell transduction. They act as cellular regulators (Lapillonne *et al.* 2004), but they require SREBP1 since spontaneously hypertensive (SH) rats, which present a naturally occurring variation in the gene, showed different mechanisms of response to n-3 long-chain PUFA-enriched diet (Boschetti *et al.* 2013).

The balance of dietary n-6 and n-3 PUFA exerts a profound influence on metabolism and cell signaling as a work studying the consumption of n-6 (rich in 20:4n-6), n-3 (rich in 20:5n-3, 22:5n-3, and 22:6n-3) and a combination of both unveiled. The mixture had unique effects on murine hepatic transcripts involved in cytoskeletal and carbohydrate metabolism, whereas n-6 affected amino acid metabolism via β -catenin signaling. All three diets affected transcripts linked to apoptosis and cell proliferation, with the evidence that n-3 increased apoptosis and decreased cell proliferation via various transcription factors, kinases, and phosphatases. They also influenced lipid transport, lipoprotein metabolism, and bile acid metabolism through diverse pathways. N-3 activated cytochromes P450 form hydroxylated fatty acids known to affect vascular tone and ion channel activity. Fatty acid synthesis and delta 9 desaturation were down regulated by the combination, implying that a mixture of 20:4n-6, 20:5n-3, and 22:6n-3 is most effective at down regulating synthesis, via INS1, SREBP, PPAR α , and TNF signaling. Heme synthesis and the utilization of heme for hemoglobin production were affected by n-6

and n-3. Relative to other groups, n-3 increased numerous transcripts linked to combating oxidative stress such as peroxidases, an aldehyde dehydrogenase, and heat shock proteins, consistent with the major LC-PUFA in n-3 (20:5n-3, 22:5n-3, 22:6n-3) being more oxidizable than the major fatty acids in n-6 (20:4n-6) (Berger *et al.* 2006). Likewise, a soybean oil diet promoted hepatic lipid accumulation by suppressed lipolysis in the peroxisomes and normal levels of VLDL secretion (Hashimoto *et al.* 2013).

Differences may arise among isomers of linoleic acid. In female mice, the supply of 0.5% of t10, c12-conjugated linoleic acid (CLA) isomer reduced expression of fatty acid oxidation genes including flavin monooxygenase (FMO)-3, cytochrome P450, carnitine palmitoyl transferase 1a, acetyl CoA oxidase and PPAR α , and increased expression of fatty acid synthase. In this way, both the decrease of fatty acid oxidation and their increased biosynthesis contribute to the CLA-induced fatty liver (Rasooly *et al.* 2007). Other mechanisms may also be involved, since when this isomer was provided in Western-type diet to *Apoe*-deficient mice, significant associations among nine-gene (*Fsp27*, *Aqp4*, *Cd36*, *Ly6d*, *Scd1*, *Hsd3b5*, *Syt1*, *Cyp7b1*, and *Tff3*) expressions and the degree of hepatic steatosis were described (Guillen *et al.* 2009b). These effects were not observed in Syrian Golden hamsters giving this isomer (Miranda *et al.* 2009). Likewise, different responses to the c9, t11-CLA isomer were observed between *Apoe*- and *Leptin*-deficient mice on insulin signaling and lipogenic pathways, which were adversely affected in the former, but improved in the latter mice (Reynolds *et al.* 2013). Even for CLA isomer responses, the genetic background plays an important role on the observed outcome.

In an attempt to explain the mechanisms whereby dietary trans fatty acids modificate plasma cholesterol levels, incubation of human HepG2 cell line with elaidic acid was carried out and it was observed up-regulated many of the proteins responsible for synthesis, esterification, and hepatic import/export of cholesterol. Furthermore, a profound remodeling of the cellular membrane occurred at the phospholipid level (Vendel Nielsen *et al.* 2013). These findings may contribute to explain the reported changes.

In addition to the reported differences mentioned previously, feeding regimen may play a significant role. Indeed, hepatic *Elov13* expression followed a diurnal rhythm in mature male mice that was inverted when those animals were exclusively fed during the day. Thus, the diurnal *Elov13* expression in mouse liver is under strict control by glucocorticoids and androgens. This expression was equally coordinated with peroxisomal fatty acid oxidation since *Elov13* mRNA was increased and decreased in peroxisomal transporter ATP-binding cassette, subfamily D, member 2 knock-out, and transgenic mice, respectively (Brolinson *et al.* 2008).

The hepatic differential response to HFD may be also regulated by miRNA. In this regard, *miR-27b* levels were increased in mice on a HFD (42% calories from fat) and this miRNA regulated the expression of several key lipid-metabolism genes, including *Angptl3* and *Gpam* (Vickers *et al.* 2013). Eighteen miRNAs exhibited differential expression in response to a high-cholesterol, high-fat diet in responder baboons compared to 10 in non-responder baboons (Karere *et al.* 2012). Based on these observations, miRNA may play an important role in diet response.

46.4.4.3 Influence of Dietary Cholesterol Content

The enrichment of cholesterol into mouse diets has been a strategy to induce NAFLD as well, but the used percentages have been highly variable. Using high percentages ranging from 10 to 80%, Inoue *et al.* (2005) observed an open fatty liver phenotype after a 12-week intervention. In these conditions, protein expression of cAMP response element-binding protein (CREB) was suppressed, while gene expressions involved in lipid metabolism, adipogenesis-related genes, PPAR γ , and its targeted gene, *Cd36*, were upregulated in the liver. With rather low dietary cholesterol contents (0.0% vs 0.5% cholesterol, w/w), 69 unique hepatic clusters were modified (37 downregulated and 32 upregulated). When six downregulated genes were analyzed in transgenic mice overexpressing truncated nuclear and active forms of SREBP-1a and SREBP-2, all were induced. In mice treated with the LXR agonist TO901317, 13 out the 32 cholesterol-upregulated genes were dependent on LXR activation. In this way, six novel dietary cholesterol-regulated genes were identified, three putative SREBP target genes (calcium/calmodulin-dependent protein kinase 1D, fatty acid binding protein 5, and proprotein convertase subtilisin/kexin 9), and three putative LXR target genes (a disintegrin and metalloprotease domain 11, apoptosis-inhibitory 6, and F-box-only protein 3) (Maxwell *et al.* 2003).

In C57BL/6J mice, dietary cholesterol also induced hepatic genes involved in drug metabolism and acute inflammation (including three genes of the serum amyloid A family, three major histocompatibility class II antigen genes, and several cytokine-related genes). It downregulated cholesterol biosynthesis genes as expected (Vergnes *et al.* 2003, Rezen *et al.* 2008). In *Apoe*-deficient mice, an 1.25% cholesterol dietary content also regulated hepatic triglyceride metabolism through a suppression of *Lipin1* and *Lipin2* and by decreasing PGC-1 α , which upregulates the transcription of *Lipin1* (Obama *et al.* 2011). Therefore, cholesterol may induce an inflammatory response and modulate hepatic triglyceride.

The Diet1 genetic locus was found to be responsible for the resistance to diet-induced hypercholesterolemia and atherosclerosis phenotype in B6By compared to B6J mice. Analyzing hepatic expression profiles from both strains, B6By mice showed elevated levels for key bile acid synthesis proteins, including cholesterol 7 α -hydroxylase and sterol-27-hydroxylase, and the oxysterol nuclear receptor, LXR α , FXR, 7alpha-hydroxylase, sterol-12alpha-hydroxylase, and hepatic bile acid transporters. Thus, B6By strain owns a higher rate of bile acid synthesis and transport (Phan *et al.* 2002). A different response to dietary cholesterol among rats allowed mapping a sensitive locus (Dihc2) to chromosome 14. Dihc2 was linked to a region including 33 genes and predicted transcripts and identified RGD1309450_predicted, a homologous gene of SMEK2, as a strong candidate for responsiveness to dietary cholesterol (Asahina *et al.* 2009). These results evidence that cholesterol metabolism is intertwined with many other metabolisms, and the global controllers are not well-known yet.

46.4.5 Effects of Quality and Protein Content

This aspect has received much less attention compared to the studies addressing influence of fat. In one work, the hepatic gene expression profiles of rats fed 12% casein, 12% gluten, and protein-free diets for 1 week revealed that a few hundred genes were up- or downregulated by more than two-fold after feeding the gluten or the protein-free diet. Gluten feeding increased expression of genes for synthesis and catabolism of cholesterol (Endo *et al.* 2002). The effect of consumption of enzymatically produced, hypoallergenic wheat flour on gene expression profiles in rats confirmed the safety of this novel food product (Kato and Kimura, 2003, Kato *et al.* 2004). The source of protein either coming from soy or casein modified hepatic expressions of genes related to lipid metabolism, transcription factor, and antioxidant enzymes (Takamatsu *et al.* 2004). Especially in lipid metabolism, the downregulated genes were related to fatty acid synthesis and the up-regulated genes are related to cholesterol synthesis and steroid catabolism (Tachibana *et al.* 2005). Similar findings of amino acid impact were observed when studying that consumption of diets containing more than 4% Leu in 6% protein content resulted in growth retardation and reduced liver weight, whereas the administration of the same dose of Leu with 12 or 40% protein did not affect Sprague-Dawley rats, and six candidate gene markers were identified in liver (Imamura *et al.* 2013). Using obese Sprague-Dawley rats, different sources of protein (casein, skim milk or casein enriched with leucine) and different levels of Ca²⁺ differentially regulated hepatic genes associated with insulin, PPAR and mammalian target of rapamycin pathways. Specifically, hepatic insulin receptor substrate (*Irs*) and *Akt* were altered in hepatic tissue in response to leucine and it may be involved in the improved insulin sensitivity observed in the group receiving this amino acid (Eller *et al.* 2013).

The supply of specific branched-chain amino acids reversed the observed gene expression changes in cirrhotic Wistar rats. Among these, downregulation of fatty acid translocase (*Fat/Cd36*), glutamine synthetase, and pyruvate dehydrogenase kinase isoenzyme 4 was observed, believed to promote lower uptake of fatty acids, lower ammonia incorporation, and higher uptake of glucose, and thus to provide an energy source without using the branched amino acids. In this way, their catabolism and skeletal muscle protein would be slowed, maintaining their concentrations in blood (Jia *et al.* 2013).

The amount of dietary protein was also explored in pregnant mammals, to test the influence on their offspring. A low-protein diet during pregnancy induced an age-associated increase in hepatic *Cidea* expression of offspring together with increased lipid accumulation and oxidative stress (Carr *et al.* 2014). In this regard, they were fed either a gestational low (LP, 6%) or an adequate (AP, 12%) protein diets, and their offspring was nursed by foster sows receiving standard diets and after weaning, they receive these diets. Differential expression of genes related to lipid metabolism (e.g., fatty acid metabolism, biosynthesis of steroids, synthesis and degradation of ketone bodies, fatty acid elongation, and bile acid synthesis) and cell cycle regulation (e.g., mitotic roles of PLK, G₁/S checkpoint regulation, and G₂/M DNA damage checkpoint regulation) were observed. The transcriptomic modulations point to persistent functional demand on the liver towards cell proliferation in the LP group but not in the AP group at identical nutritional conditions during postnatal life due to divergent “programming” of the genome (Oster *et al.* 2012). Also in pigs, this type of experimental approach was translated into modification of 318 genes’ expressions in the liver female offspring. Particularly, those of *de novo* fatty acid synthesis were downregulated whereas several genes of lipolysis and phospholipid biosynthesis were upregulated (Doring *et al.* 2013).

More severe amino acid deprivations such methionine- and choline-deficient diets have been used for 6 weeks in C57BL/6 mice to induce NASH. In these conditions, the GPx family, Fmo2, and peroxiredoxins were significantly upregulated, whereas *Scd1*, *Catalase*, and *Serpib1b* were significantly downregulated. Thus, oxidative stress-related genes were differentially expressed in the livers of mice with diet-induced NASH (Gornicka *et al.* 2011). These kinds of diets also downregulated the expression of *Riz1*, an activity associated with greater H3 lysine 9 methylation in RIZ1 target genes. Thereby, RIZ1 is a critical target of methyl donors as represented by methionine and choline (Zhou *et al.* 2008).

An even more severe regimen was used in male F344 rats, by feeding a L-methionine-deficient diet, deprived of folic acid and choline to induce hepatocellular carcinomas. In this setting, alterations of components of the DNA methylation machinery namely, *de novo* DNA methyltransferases (*Dnmt3a* and *3b*), maintenance DNA methyltransferase (*Dnmt1*), and methyl CpG binding proteins were observed, and in these changes both transcriptional and post-transcriptional mechanisms took place. This could represent early stages of hepatocarcinogenesis (Ghoshal *et al.* 2006).

In the different responses to protein diets, miRNAs may participate. Using Lieber-De Carli or methionine-choline-deficient (MCD) diets to induce liver steatosis resulted in increased miRNA levels, being more severe those seen in animals receiving the MCD diet. Some of them such as *miR-182*, *miR-183*, and *miR-199a-3p* showed a different pattern: downregulated in Lieber-deCarli diet and upregulated with the MCD diet (Dolganiuc *et al.* 2009). In another approach, using an amino acid-defined and choline-deficient diet to induce hepatocarcinogenesis, oncogenic *miR-155*, *miR-221/222*, and *miR-21* were upregulated and the most abundant liver-specific *miR-122* was downregulated in C57BL/6J mice. Targets of *miR-21* and *miR-155* such as phosphatase and tensin homolog (*Pten*) and CCAAT/enhancer binding protein beta (*C/ebp-beta*) showed reduced hepatic expression, and nuclear factor- κ B, an activator of *miR-155* gene, was elevated (Wang *et al.* 2009a). Therefore, miRNA may participate in regulating multiple gene expressions arisen by protein changes and in this way, contribute to the development of liver pathology.

46.5 Muscular Transcriptome

Skeletal muscle is not a mere component of locomotor system. In fact, recent evidences have proposed a secretory role for it by liberating different “myokines” (Pedersen 2013). In addition, its high throughput analyses cover a wide range of interests, varying from the study of physiopathology of metabolic diseases or lifespan mechanisms to animal production studies. Not surprisingly, nutritional interventions have been carried out in humans, rodents, or different livestock species with different aspects which will be discussed in this section and are summarized in Table 46.6.

46.5.1 Influence of Caloric Restriction

The addressed experiments can be classified in four different categories according to their focus: first, those exploring the influence of an acute caloric restriction represented by an overnight fast; second, nutritional interventions pursuing the role of genes in diverse pathological processes, third, chronic caloric restriction used to understand its role in lifespan

Table 46.6 Muscular transcriptomic changes in several nutritional conditions.

Condition	Model	Finding	References
Fasting Caloric restriction	Rat	Increased utilization of fatty acids	(Robertson <i>et al.</i> 2011)
	Human	Delayed aging process by causing a metabolic shift toward increased protein	(Byrne <i>et al.</i> 2005; Mercken <i>et al.</i> 2013; Oita <i>et al.</i> 2009; Sreekumar <i>et al.</i> 2002a; Weindruch <i>et al.</i> 2001)
	Mouse	turnover and decreased macromolecular damage	
	Rat		
	Steer		
High-fat feeding	Mouse	Activation of pathways involved in signal transduction, inflammation, cancer, oxidative stress, substrate transport, and metabolism	(Choi <i>et al.</i> 2013; Lee <i>et al.</i> 2012; Meugnier <i>et al.</i> 2007; Morine <i>et al.</i> 2010; Sparks <i>et al.</i> 2005)
Protein restriction	Pig Steers	Downregulation of protein synthesis	(Hamill <i>et al.</i> 2013; Reverter <i>et al.</i> 2003)
Protein source	Rat	Gluten feeding induced of the expression of genes for synthesis and catabolism of cholesterol and BCAA, slowed the catabolism of skeletal muscle protein	(Kato <i>et al.</i> 2004)
			(Jia <i>et al.</i> 2013)

BCAA, branched amino acids.

or in sarcopenia; and finally, the effect of caloric restriction in flesh quality in animal production. Regarding the first category, Robertson *et al.* showed decreased expression of genes involved in glycolytic pathways and a shift toward increased utilization of fatty acids following an overnight (16-h) fast in rats. In fact, several genes within these metabolic pathways, including key rate limiting genes, changed simultaneously, raising unanticipated interactions and new mechanisms of control (Robertson *et al.* 2011). In an experiment in search of a gene function, Karlsson *et al.* observed that *Hsd11b1* mRNA levels were associated with metabolic and anthropometric parameters in subjects with normal glucose homeostasis but not in subjects with impaired glucose homeostasis that underwent a very low calorie diet for 16 weeks (Karlsson *et al.* 2010). The influence of chronic caloric restriction addressed to understand their role in lifespan has been widely explored. Mercken *et al.* showed that caloric restriction (CR), in both rats and humans, induces dramatic changes of the transcriptional profile that resemble those of younger individuals. This common signature consisted of three key pathways typically associated with longevity: IGF-1/insulin signaling, mitochondrial biogenesis, and inflammation (Mercken *et al.* 2013). In rats, a 36-week caloric restriction (60% of control diet) enhanced the transcripts involved in reactive oxygen radical scavenging, tissue development, and energy metabolism while decreasing expression of those involved in signal transduction, stress response, and structural and contractile proteins (Sreekumar *et al.* 2002a). Transcriptional patterns of gastrocnemius muscle from calorie-restricted male C57BL/6 mice evidenced that CR retards the aging process by causing a metabolic shift toward increased protein turnover and decreased macromolecular damage (Weindruch *et al.* 2001). Using mRNA-Seq of skeletal muscle of old mice subjected to CR, Dhahbi *et al.* found complex CR-associated changes in expression of mRNA isoforms, many of which occur without changes in total message abundance and thus would not be detected by methods other than this technique (Dhahbi *et al.* 2012). In an attempt to identify a global controller of these changes in skeletal muscle of CR-Brown Norway rats, Oita *et al.* found upregulation of several NR4A transcriptional targets (*Ucp3*, *Ampk3*, *Pgc1a*, and *Pgc1b*) and hypothesized that NR4A proteins may contribute to CR-induced metabolic adaptation (Oita *et al.* 2009). As mentioned, this aspect has been also studied in animal production. In this regard, nutritional restriction on bovine skeletal muscle from 10 Brahman steers allowed identifying co-regulated gene clusters related to protein turnover and cytoskeletal metabolism that may explain remodeling of muscle tissue (Byrne *et al.* 2005). These works reveal that the use of high-throughput approaches provides a new tool to measure muscular biological age and to evaluate at the molecular level the efficacy of calorie restriction and its contribution to aging.

46.5.2 Effect of Dietary Fat Content

The comparison of dietary fat content has been the aspect more frequently studied in muscle. In this regard, in response to high-fat feeding (60% kcal) for 20 weeks in mice, skeletal muscle showed a large number of differentially expressed genes (191) with activation of pathways involved in signal transduction, inflammation, oxidative stress, substrate transport, and metabolism (Lee *et al.* 2012). In C57BL/6N mice fed a high-fat diet for 2, 4, 8, and 12 weeks, muscular induction of olfactory receptors and cancer-related genes were observed (Choi *et al.* 2013). Regulation of signaling pathways, cancer, cell transport, and metabolism of nucleotides, amino acids, energy and vitamins was found when the HFD provided high levels of CLA for 28 days to leptin-deficient mice on C57BL/6J genetic background (Morine *et al.* 2010). In muscle of male C57BL/6J mice fed a HFD for 3 and 21 days, OXPHOS, PGC1 mRNAs, cytochrome C and PGC1 α protein were downregulated (Sparks *et al.* 2005). In skeletal muscle of healthy volunteers during fat overfeeding for 28 days, a coordinated stimulation of triacylglycerol synthesis, inhibition of lipolysis, and reduction of fatty acid oxidation were observed (Meugnier *et al.* 2007). Combined, these results suggest muscle is highly sensitive to changes in fat diet with independence of species. In fact, the increase in lipoprotein lipase gene expression in hindlimb muscle 9 months after consuming these diets was associated with the obesity/diabetes status of mice (de Fourmestraux *et al.* 2004). In muscle, adiponectin activated AMP kinase and PPARalpha pathways thereby increasing beta-oxidation of lipids, leading to decreased triglycerides content, which ameliorated insulin resistance under a HFD (Yamauchi *et al.* 2003). Thus, increased muscle fatty acid uptake not accompanied by beta-oxidation may favor insulin resistance and in this way muscle being an important metabolic gatekeeper.

It is interesting to note that these HFD diets did not modify the differential gene expression pattern among gastrocnemius, quadriceps, and soleus muscles (de Wilde *et al.* 2010). In contrast, a HFD provided to rats elicited sex-dependent gene expressions in the soleus muscle. Specifically, genes encoding myofibrillar proteins and glycolytic proteins were expressed higher in males than females, reflecting greater muscular activity and higher capacity for utilizing glucose as an energy fuel. However, a series of genes involved in oxidative metabolism and cellular defenses were more upregulated in females than males (Oh and Yun 2012). This is in agreement with the results of Sreekumar *et al.* who observed reduced

expression of genes involved in free-radical scavenging and tissue development and increased expression of stress response and signal transduction genes in skeletal muscle from male rats fed on high-fat diet for 36 weeks (Sreekumar *et al.* 2002b). The individual variability of intramuscular fat content in pig longissimus muscles was related to differences in gene expressions involved in early adipogenesis, such as architectural transcription factor high-motility hook A1, mitogen activated protein-kinase14, and cyclin D1 (Liu *et al.* 2009). All these works raise new aspects of the biology of muscular tissue, such as different muscle, the influence of sex, and individual differences.

Few studies have analyzed the influence of different sources of fat. In this sense, replacement of saturated fatty acids by polyunsaturated fatty acids from sunflower or linseed and supplemented with DHA in lactating German Holstein dairy cows for 10 weeks had little impact in longissimus muscle (Hiller *et al.* 2013). Clearly, this topic needs to be addressed in more depth.

46.5.3 Effects of Quality and Protein Content

Regarding the protein content, protein dietary restriction in an isocaloric diet differentially expressed genes participating in functions related to cell cycle, muscle growth, extracellular matrix organization, collagen development, lipogenesis, and lipolysis of porcine semimembranosus muscle. In addition, expression of adipokines including leptin, TNF α and HIF1 α were increased and the hypoxic stress response was induced (Hamill *et al.* 2013). Reverter *et al.* also identified 27 differentially expressed genes in muscle responding to varying levels of energy and protein in the diet of 11 steers (Reverter *et al.* 2003). Therefore, this dietary manipulation negatively influences protein synthesis pathways, with potential consequences for growth.

The source of protein was explored by Kato *et al.* in rats fed on 12% of casein, gluten, and protein-free diets for one week. Gluten feeding induced the expression of genes for synthesis and catabolism of cholesterol (Kato *et al.* 2004). In this animal model with decompensated cirrhosis, the effects of branched-chain amino acid-enriched diet was translated in downregulation of fatty acid translocase, glutamine synthetase, and pyruvate dehydrogenase kinase isoenzyme 4. These changes seem to promote lower uptake of fatty acids, lower ammonia incorporation, and higher uptake of glucose, and thus to provide an energy source without using BCAA, slowing the catabolism of skeletal muscle protein, and maintaining BCAA concentrations in blood (Jia *et al.* 2013). Muscle is also responsive to quality of proteins.

46.6 Conclusion

This chapter covers the use of high-throughput gene expression at the transcriptomic level to unveil the changes experienced by different organs when dietary macronutrients are changed. In particular, faced to stimuli such as fasting/feeding, caloric restriction, different carbohydrates, amount, and nature of fats and proteins, these organs show a wide repertoire of responses, as revealed by this powerful technology, which evidences the metabolic flexibility of these organs to tackle these conditions.

A profound elaboration of data is required yet to establish patterns of dietary response in the today fragmented information puzzle. This can be envisioned in terms of standardized arrays to systematically test the same genes in all studies, well-controlled diets to track changes accordingly, rigorous selection of strain and animal models, enough sample size and tissue controls to have real changes. In addition, analyzing data and the emergence of new, even more powerful technology, such as RNA sequencing, may provide a new twist on the degree of information obtained. Using this, the analysis of miRNA and lncRNA will bring a new perspective to the field. Despite these limitations, transcriptomic analysis is a powerful tool to discover gene function – as roughly 10% of genes have an assigned function – and explore cellular changes in the varied nutritional field because of its relative simplicity of handling. These analyses should bear in mind that RNA is an intermediate between the genome and the proteome that will always require proteomic and metabolomic confirmations of suggested changes.

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Part IX

Nutriethics

Nutritional Sciences at the Intersection of Omics Disciplines and Ethics: A Focus on Nutritional Doping

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47.1 Introduction

Food, and broadly speaking, nutrition, is a particularly complex topic and for this reason should be addressed using a multidisciplinary, holistic approach (Bordoni and Capozzi, 2014). Food, besides being a primary and basic need to sustain life, has indeed many profound societal, economic, medical, and ethical implications. As Tom Standage wrote, food does more than merely give sustenance, rather more it acts “as a catalyst of social transformation, societal organization, geopolitical competition, industrial development, military conflict, and economic expansion. From prehistory to the present, the stories of these transformations form a narrative that encompasses the whole of human history.” (Standage, 2009). Food-related disciplines should, therefore, take into account this vast array of implications.

As a social factor, food has deeply influenced societies, enabling their establishment and fostering their development, as well as structuring their histories and ideologies. Jean Anthelme Brillat-Savarin, a French politician and philosopher of gastronomy, has authoritatively claimed that “the fate of the nations hangs upon their choice of food.” Since food has been viewed as wealth, long before the discovery and introduction of money, it has been exploited to create hierarchies and tensions and to exert power by unequally supplying food resources and by providing a limited access to food. The nutritional chain, being the continuum from the production step (harvesting, manipulating, supplying, allocating, and distributing) to the consumption level, has been the source of divisions and distinctions among the social classes, and between men and women, since the dawn of human history. History of food is, at least in part, also the history of social positions and iniquities. Food has contributed to gender dynamics, by delegating different tasks of foraging, food preparation, and allocation between males and females: the old paradigm of the hunter-gatherer dichotomy has been the origin of the sexual/familial division of labor. Not only has food influenced gender dynamics, but many a social crisis has been due to food shortages, as well as famines such as the Great Irish Famine or Stalin’s famine (Standage, 2009). Many wars have been fought for the sake of food supplies and the outcomes of many conflicts from the Revolutionary War of 1770–1780 and Civil War of 1860 in the USA to the Cold War (Standage, 2009) have been influenced by the possibility to adequately feed the soldiers. As Vegetius wrote in his *Epitoma rei militaris*,

...armies are more often destroyed by starvation than by battle, and hunger is more savage than the sword ... other misfortunes can in time be alleviated: fodder and grain supply have no remedy in a crisis except storage in advance.
(trans. Milner, 1996)

History of humanity and mankind is also an “edible history”. Food preparation and production has been the first real industrial process, giving birth to the Industrial Revolution and characterizing all the industrial age (Standage, 2009). Historical changes, such as modernization, urbanization, migration flows, and globalization, have deeply modified nutritional patterns and eating habits, leading to a drastic shift from home-made to industrial food – the so-called McDonalization (Holmboe, 2000; McDonald, 2012). In the latter scenario, food is considered and treated as a mere marketable commodity or utility, often losing its symbolic and cultural meaning (Azétsop and Joy, 2013).

As a cultural factor, food has been considered a medium of exchange, as a gift, since it has been used to shape social relationships by sharing and distributing it during feasts, rituals, and ceremonies, contributing to cohesiveness, but also as a way of defining human identity by including/excluding in one’s own diet certain nutrients (*food avoidance*, such as in kosher and halal cuisine) (Farouk *et al.*, 2014; Velarde *et al.*, 2014). Eating or food restriction/fasting have a valuable religious meaning. Particular culinary beliefs, prohibitions, and taboos, as well as practices and consumption patterns, characterize the different cultures. On the other hand, food taboos also have an evolutionary meaning, perhaps protecting from the intake of harmful or likely to be contaminated food (Meyer-Rochow, 2009; Henrich and Henrich, 2010; Brito Júnior and Estácio, 2013;).

In medicine, it is noteworthy that many diseases are in part caused by poor nutrition habits – from eating disorders and obesity to metabolic, cardiovascular, neurodegenerative pathologies, and cancers; and therefore nutritional education is of high importance since, according to the World Health Organization (WHO) and other public health related agencies, most diseases could be prevented with a proper healthy dietary lifestyle.

Also concerning these last aspects, food has important political and societal implications, since food-related pathologies impose a relevant burden and represent a priority in many policy agendas: from healthy food/drink promotion (Puhl *et al.*, 2014) to unhealthy food/beverage prohibition policy, such as the ban of alcohol or taxes applied to the sale of junk foods.

Moreover, nutrition has always had an exquisite anthropological value, filling the gap between nature and culture, representing an important aspect of one’s own identity and being a fascinating universe of symbols, metaphors, rituals, and myths (Lévi-Strauss, 1964, 1965; Mintz and Du Bois, 2002; Jones, 2007). On the basis of these myths, some cultures attribute a strong prophylactic and therapeutic power to certain foods (Richards, 1893; Tavakkoli-Kakhki *et al.*, 2014) and in some cases this has been supported by scientific evidence.

In ethics, food has a wide range of implications, from healthy food accessibility (Mishra, 2012), origin, safety, security, quality, and traceability (Darnton-Hill *et al.*, 2004) to environmental sustainability and ecosystem preservation (Wahlqvist, 2014).

Public health nutrigenomics and related allied health sciences, as we will see, could play a major role towards the ambitious effort of understanding and exploiting the enormous potential of nutritional disciplines in a global and societal perspective (Chávez and Muñoz de Chávez, 2003; Omenn, 2010).

Food, indeed, cannot be treated using a reductionist approach; instead it is only through systematic thinking that we can dissect the profound impact of nutrition on humans and population health. This is becoming particularly the case in the current postgenomics era.

47.2 Nutrigenomics and Nutriproteomics

Throughout time, nutritional science has benefited from different disciplines and contributions, ranging from analytical chemistry to biology, with important discoveries and insights from Lavoisier and Liebig (Sales *et al.*, 2014). However, these fields have not merged and led to a convergent vision of what food is. Nutrigenomics represents the natural evolution of nutrigenetics as a specialty in the field of genomics disciplines. The history of nutrigenetics began with the discovery of the mutation of the gene encoding the enzyme phenylalanine hydroxylase (PAH), which converts the aminoacid phenylalanine to other essential compounds in the body and whose impairment causes phenylketonuria (PKU). Another important step in the history of nutrigenetics was the discovery made by Sir Archibald Garrod of the mutation of gene encoding the hepatic enzyme homogentisate 1,2-dioxygenase (HGD), which causes alkaptonuria. Later, nutrigenetics had devoted itself to the systematic study of the effect of genetic variation on dietary response, remaining focused on the single gene level. Nutrigenomics has broadened this perspective, taking into account gene-gene interactions and networks, studying the gene expression given by the interaction of food and genes at a pathway level. In recent years, nutrigenomics has further increased the complexity of its topic of study, integrating the study of gene with the food-induced chromosomal changes and perturbations.

Also nutriproteomics has benefited from the advances in the field of proteomics technologies (Barnes and Kim, 2004), enabling the study of protein-protein interactions and pathways at an unprecedented level.

To this end, nutrigenomics and nutriproteomics have begun to emerge as a unique convergence of nutritional, genomics and proteomics knowledge strands since the completion of the Human Genome Project (HGP) more than a decade ago. These new fields present an opportunity for the design of customized diets potentially able to counterbalance the extant obesity epidemic and remedy metabolic diseases; fields such as nutrigenomics (or nutritional genomics) (Mutch *et al.*, 2005) and nutriproteomics (or nutritional proteomics) (Kussmann, 2010; Ozdemir *et al.*, 2010) have garnered attention as distinct and highly specialized branches of postgenomics personalized medicine (Bragazzi *et al.*, 2011; Nicolini *et al.*, 2012; Racapé *et al.*, 2012; Orlando *et al.*, 2013). Nutrigenomics examines the food-genome intersection both in health and disease, while nutriproteomics encompasses the interactions between the nutrients and protein translation, expression, and modification on a scale of the human proteome. Together, they offer the advantages of genomics such as understanding the role of hereditary factors in relation to food effects while employing proteomics so as to study gene products at the protein-protein interaction/proteome level. It is anticipated that other branches will arise (Ibáñez *et al.*, 2013), such as nutriepigenetics/nutriepigenomics (Garhäuser, 2013) and nutrimeabomics (Swann and Claus, 2014; Collino *et al.*, 2009; Rezzi *et al.*, 2007; Claus and Swann, 2013). As a postgenomics discipline, these aim to overcome a reductionist approach by using multidisciplinary science, characterized by the integration of the biological, social, and environmental dimensions. Foodomics is emerging as a broad discipline that studies food and nutrition through the coherent and highly integrated application of advanced omics approaches, such as genomics, transcriptomics, epigenetics/epigenomics, proteomics/peptidomics, and metabolomics (Bordoni and Capozzi, 2014; Cifuentes, 2014).

The interaction between food components and genes/genes products is rather complex and multifaceted, being mutual and reciprocal: nutrients act on the human genome either directly or indirectly via epigenetic mechanisms subtly modulating and tuning the expression of the genome (e.g., hypo- or hyper-methylating it). On the other hand, genes are responsible for food absorption, biotransformation, and utilization. Food and genes interact with each other in a dynamic way. The outcome of a diet is indeed the non-linear result derived from the complex interaction between health/disease condition and one's own genetic make-up, as well as the environment.

Food components can be involved in different cellular and biological events: from cell signaling, growth, and apoptosis, as well as in each step of carcinogenesis (from initiation, to promotion, and progression) (Dang *et al.*, 2014; Fenech, 2014; Kang, 2013).

As such, the tasks of nutrigeno/proteomics are various: from the study of particular gene signatures in response to dietary components, either healthy ones or contaminated by food-borne pathogens, to the discovery of biomarkers to detect unsafe products or foods of doubtful safety (such as genetically modified organisms, GMOs) or the underpinning of the molecular basis of crops growth, fruit ripening, and breeding for enhancing the resistance of plants and livestock to pathogens and diseases (agriogenomics) (Santoni *et al.*, 2014).

The definition of a nutrient is complex: nutritional compounds can be divided into different chemical classes, according to the definition by Trujillo and collaborators: phytochemical, fungochemical, bacteriochemical, and zoochemical, among the others (Trujillo *et al.*, 2006).

The ambitious and shared goal of nutrigenomics and nutriproteomics is to provide insights for diets that can impact gene and protein networks with a view to improving population health (a new field called Public Health Nutrigenomics/ Nutriproteomics).

They are also noteworthy in sports medicine, since they could provide athletes with crucial information for personalized training and nutrition in order to achieve the best result possible and express one's own potential. The convergence of food science with omics sciences (genomics, proteomics, metabolomics, or metabonomics, etc.) is the broader overarching tenet under which nutrigenomics and nutriproteomics are emerging, be they in drug therapy, nutritional sciences, or sports medicine. If omics sciences call for a broader understanding of health as a complex dynamic concept situated in a social and ethical context, the application of a deterministic and reductionist approach to nascent fields of such as nutrigenomics/proteomics may lead to ethical issues and concerns. The study of ethics issues embedded in nutrigenomics and various intersections of food science with omics have been termed *nutri-ethics*, and discussed by various authors recently, together with analyses of responses coping with the uncertainties of emerging postgenomics health technologies (Bergmann *et al.*, 2006; Chadwick, 2004; Castle *et al.*, 2006; Godard and Ozdemir, 2008; Ozdemir and Knoppers, 2013; Slamet-Loedin and Jenie, 2007; Trujillo *et al.*, 2006). Nutri-ethics can be seen as an evolution of the classical concept of nutritional ethics, with which it has some features in common, but has also unique characteristics due to the unprecedented innovations brought along by omics disciplines. According to one etymological analysis, the suffix "ome" present in various data-intensive omics fields is derived from the Sanskrit *OM* (meaning "completeness and fullness") (Bragazzi, 2013a; Yadav, 2007).

The main idea behind the data-intensive omics disciplines is that the high-throughput biomarker data obtained in parallel from successive hierarchies of cell biology can take into account the built-in molecular redundancies preserved in biology during the course of human evolution. As stated before, the interactions between the human omics variation at the level of the genome, proteome, metabolome, and food are dynamic and bidirectional: in the specific cases of nutrigenomics/proteomics, they study both the network of influences of micronutrients and macronutrients over the human genome and the proteome and in effect, the complex responses of the human organism to food in the form of effectiveness and/or toxicity (Schwartz, 2014). Consequently, nutrigeno/proteomics can help modulate cellular and molecular pathways (Fenech, 2008; Mathers, 2006), and foster the design and development of strategies for obesity (Rudkowska and Pérusse, 2012), for metabolic pathologies (such as phenylketonuria) (Levy, 1999) or chronic diseases. Some encouraging and promising studies have shown results in the context of cancer (Low *et al.*, 2006; Riscuta and Dumitrescu, 2012).

The potential of nutrigeno/proteomics is considerable (Liu and Qian, 2011) and includes impacts on design and development of new drugs (Astley, 2007; Lundstrom, 2013) but a broad consensus is still lacking about safety and risk assessment using such new approaches in postgenomics medicine (Arab, 2004; Bouwman *et al.*, 2008; Castle and Ries, 2007).

47.3 Sports Nutriproteogenomics

In the case of sports medicine, nutrigeno/proteomics has so far been applied to select proper macronutrients for treating and preventing heavy exercise-induced immunodepression, for assessing and monitoring the athlete's nutritional status, and other few examples using *in vitro* and animal models. Against this scientific background, ethical issues of nutrigenoproteomics are discussed in the subsequent paragraphs, with emphasis on the current limitations and the dizzying potentials of the omics data-intensive research for science and society. Additionally, I discuss the need to communicate the uncertainty as a fundamental intrinsic part of nutrigeno/proteomics, the gaps regarding the lack of adequate governance in this nascent postgenomics field, and issues over providing a proper nutritional education to the athletes as an onus of the international sports organizations. It is true that nutrigeno/proteomics is a promising emerging field paving the way for personalized medicine and dietetics, even though tangible results are not likely to come along in the very near future. This calls for many ethical issues: some of these are in common with the classical ethics of nutrition/sports ethics or can be seen under a new light and perspective (like food accessibility, safety, security and traceability, food medicalization, human enhancement, nutritional supplementation-based doping, and sport medicalization), others are absolutely novel (such as personalized nutrition, gene-nutrients interactions, and personalized nutritional doping).

In the last year the progresses in biology, sport physiology, exercise science, and related specialties have given rise to new ethical issues. They have undoubtedly contributed to a more sophisticated training program (Applegate and Grivetti, 1997). Manipulating and cheating behaviors previously relying on magic, primeval rituals, or strange beliefs, such as the consumption of deer liver or lion hearts (Applegate and Grivetti, 1997), are now sustained by accurate scientific evidences and are based on advanced knowledge in the field of molecular nutrition. Doping has always existed since the dawn of sport (Applegate and Grivetti, 1997) but has changed face today. The discovery of vitamins represents a turning point for the history of sport (Applegate and Grivetti, 1997). While in the past, protein supplementation consisted of the exaggerated consumption of high-protein meals, it is now moving on a scientific footing, strictly relying on protein isolation, purification, and delivery of single aminoacides or a complex mixture of them, such as branched-chain aminoacids or BCAAs (that is to say, leucine, isoleucine, and valine that represent a third of muscle proteins), citrulline, and beta-alanine, which is a non-proteogenic limiting substrate to carnosine biosynthesis, or carnosine itself (Applegate and Grivetti, 1997; Liddle and Connor, 2013).

All these issues have to be elucidated and steered with anticipatory governance and fully addressed within a coherent framework, even though some aspects of my discussion concern the immediate future more than the actual and urgent present. Several issues, including food safety, deserve particular emphasis. Manipulated and manufactured meals such as the engineered metabolic byproducts of essential nutrients (like beta-hydroxy-beta-methylbutyrate, or HMB, derived from leucine), novel foods like GMOs, together with herbal preparations, phytochemical products, and other kinds of enhanced/fortified meals (Cardenas, 2013), have met with public resistance due to fears for alleged health risks, although public attitudes towards nutrigenomics/proteomics will likely vary in different global regions. Nutrigeno/proteomics could help ascertain food safety but also lead to production of functional foods, which would clearly blur the distinction between food and therapeutics.

This distinction was clear for Hippocrates, to whom the following saying is traditionally attributed; "Let food be your medicine, and medicine be your food". Even though it could not be of Hippocrates or could be of one of his disciples (Datta and Vitolins, 2014), this statement underlines the importance of a mutual relationship between these two factors, without establishing a priority of one over the other. In other words, seen through the lens of Hippocrates, food is for well-being but

not specifically only for health. The “medicalization of food” could have negative consequences, compressing the multidimensionality of food values into a more narrow perspective and mechanistic, biomedical paradigm, seeing the nutrients only as “molecules” (molecular nutrition) rather than underpinning the complexity of the nutritional behavior, in all its components (ranging from social determinants to biological make-up).

Medicalization of food is, indeed, often declined at the individual level, rather than situated within the frame of public health. It is a part of contemporary society, together with an excessive medicalization of the body and culture (Atkinson, 1985; Chadwick, 2004; Vertinsky, 1998), as well as the medicalization of sports.

Food is not merely a medicine or a vehicle for drug delivery, a meal is made up of both non-essential and essential compounds, of both non-functional and functional components and the act of eating has, as already stated, different functions – from building up one’s own identity and sharing and communicating with others to satisfying a basic need. By blurring the boundary between food and therapeutics, all these functions could conceivably erode. Another important concern – indeed, more technical – is about the statistical reliability and robustness of the acquired nutrigeno/proteomics data that could be potentially misleading if used passively with a deterministic idea of the relationship between genes, proteins, and nutrients whilst, as we maintained before, the relationship is more complex, dynamic, and non-linear. This is typical of nutrigeno/proteomics research, however, as the number of variables far exceeds the number of biological samples available in a given study. Uncertainty is not an accidental property of postgenomics science, but it is integral to it and must be taken into account using an anticipatory policy, and this must be communicated as such (Ozdemir *et al.*, 2011, 2013).

The situation with nutrigeno/proteomics and its attendant ethical dimensions are further complicated by direct-to-consumer (DTC) tests that bypass the traditional doctors’ office; they can be ordered directly by the consumer without the involvement of a health-provider (Goddard *et al.*, 2007; Gulisano, 2013; Ries and Castle, 2008). The clinical utility of these DTC tests remain uncertain and dubious, also in part because uncertainty is often not communicated adequately. Enchanted by hype, users of DTC could utilize these tests with negative impact on their health. The Internet has largely contributed to spreading of sometimes misleading information about nutraceuticals and ergogenics aids, making them more easily available for sale and consumption (Adams, 2010; Pirola *et al.*, 2010; Thevis *et al.*, 2011). Development of a regulatory environment in the near future can help safeguard consumers’ interest, as well as educating both the health providers and DTC users (Bragazzi, 2013b; Pipe and Ayotte, 2002). On the other hand, the Internet has the potential to properly educate young adult and adolescent athletes, with the design and development of *ad hoc* websites, such as anti-doping hotlines (Böttiger *et al.*, 2013).

47.4 Nutritional and Sports Ethics

Since the time of Aristotle’s “Nicomachean Ethics”, it is well known that a good, correct, and balanced diet is a fundamental part of athlete’s training (the so-called “nutritional supplementation-based training”). Moreover, this diet can be differentiated according to the competing discipline, depending whether the sport is aerobic or anaerobic, which degree of energetic expenditure, power, strength, and endurance is required, and so on (Helms *et al.*, 2014; Mugham and Burke, 2011; Ranchordas, 2012; Ranchordas *et al.*, 2013; Sousa *et al.*, 2013; Stellingwerff, 2013). But even if a “sport-specific diet” exists, this is limited to some guidelines and anyway, it is not tailored to the specific needs of the individual. On the other hand, it is known that the consumption of certain food and substances (like caffeine and carbohydrates) could, at least in the short term, modify and alter the result of a sports performance (Ayotte, 1999; Gleeson, 2013; Hawley, 2013). However, the precise effects and mechanisms of these substances are often criticized, being controversial. The exact definition of “nutritional doping” is challenging, since it has raised a lot of doubts and objections (Miah, 2011). Some scholars claim that athletes naturally use food to enhance their sports performances, different to common reasons and motivations like suppressing cravings (Aoi *et al.*, 2001). For this reason, nutritional enhancement is just “breeding” (like Andy Miah has stated, 2006), as the specification of “functional food” is superfluous for sportsmen (Miah, 2006; Radak *et al.*, 2001; Yfanti *et al.*, 2010).

A point that should not be forgotten in the discussion is that sports training and exercise could result in changes in immune system, leading to immunosuppression and to an increased susceptibility to infectious diseases (Calabrese and Nieman, 1996; Harper Smith *et al.*, 2011; Martin *et al.*, 2009). Heavy training could imply the production of some reactive oxygen and nitrogen species (RONS) or other oxidant molecules, which lead to plasma lipid peroxidation and DNA damage at the level of muscular tissues (Kobayashi *et al.*, 2014; Vezzoli *et al.*, 2014), even though it seems that practicing regular sport would result into an adaptive response to exercise-induced oxidation. On the other hand, moderate physical activity can contrast the effects of aging, specifically mobilizing senescent T-cells from the peripheral tissues into the blood, while after apoptotic events, newly functional T-cells occupy and expand the naïve T-cell repertoire (Phillips *et al.*, 2007). However, some experimental findings have not replicated exercise-induced immunosuppression (Neves Sda *et al.*, 2009).

Anyway, doses of antioxidant supplements could restore the proper immune system, and the marketing of antioxidant products is based on this very claiming, notwithstanding some controversial experimental findings (Strobush *et al.*, 2011) or the complete lack of evidence for some of these putative immune-boosting compounds (Gleeson *et al.*, 2004).

More generally speaking, it is accepted that doing sport leads to some associated para-physiological conditions, like dehydration, and fluid and ion imbalance (Goulet, 2012; Zoorob *et al.*, 2013). Bearing in mind these criticisms, and considering sports physiology as also underpinned by sports genomics, we propose to differentiate “nutritional training” – which is the use of foods normally present in diet to foster better sports performances and to restore a proper physiological status – from “nutritional doping”, which is the malicious manipulation of nutrients. Nutritional doping is when athletes use nutraceuticals, phytochemical products, high doses of essential macronutrients and micronutrients, supplements and stimulants such as creatine, RNA, or a mixture of nucleosides such as inosine, caffeine, antioxidants such as smilax, n-3 fatty acids, sodium bicarbonate, glucose polymers, and glycerol to improve and enhance their strength (Chadwick, 2005; King *et al.*, 2012), or eat voluntarily meals contaminated by drugs (Applegate and Grivetti, 1997; Green *et al.*, 2001). Thus, they may manipulate important performance parameters and indicators, like energy supplies (by controlling the muscle contractions and energy-releasing metabolic processes), time to exhaustion and fatigue threshold (by decreasing the production and accumulation of lactate), oxygen uptake and oxygen consumption in the muscle and other tissues, respiratory quotient, and so on. All this gives nutritionally doped athletes an unfair advantage in respect to the others, violating the fundamental principle of sports ethics. One must underline the fact that this “nutritional enhancement” does not represent the augmentation or the fulfillment of a “(genetic and biological) potential” – which should indeed require hard work on oneself, practice, exercise, fatigue, and training – but it can be seen as a short cut and as the consequence of a vision of sport downgraded to mere business and entertainment. So far, nutritional doping is a no-man’s land: a gray area in which there are no governing bodies and authorities that control and regulate the nutritional supplements industry. If this is true for elite athletes, it is even truer for recreational athletes, as we will see in the following paragraphs (Diehl *et al.*, 2012).

Differences in regulation between drugs and foods may lead to the false idea that nutritional supplement – and nutritional doping – may be considered legal. If the distinction between a drug and a dopant may be clearer, the barrier between food and food enhancer is blurred and undermined.

Moreover, epidemiological surveys have shown that athletes have little proper and adequate knowledge about nutrition, despite the numerous sources of information available and consult very few nutritional professionals, despite their access to them (Heaney *et al.*, 2011). Athletes, females in particular, are particularly vulnerable to eating disorders. Coelho and collaborators claim that educational programs are the best method for targeted prevention of eating disorders, together with pre-participation exams, the routine use of dietary markers, and the administration of validated self-report questionnaires or clinical interviews among athletes for an early recognition of symptoms (Coelho *et al.* 2010, 2014).

Response to food and nutrients is different among the population (Yaktine and Pool, 2007), but if a personalized diet is the ambitious goal of nutrigenoproteomics, athletes could exploit omics-based information to change consequently their diet or make use of special gene-based and engineered meals (Chadwick, 2005). People with a certain set of alleles can metabolize some types of food in a distinctly faster fashion (including doping preparations) and so nutrigenoproteomics in sport entails both the control of quantity and quality of food in order to achieve desired results and possibly Olympic laurels (Chadwick, 2005).

If a functional food is being supplemented with particular nutrients and/or enriched by dopants, it might not be effective in some athletes because of their genetic makeup; the athlete could then make use of another nutritional dopant or could modify/ engineer it on the basis of omics-provided information. In this sense, nutritional doping has passed beyond the “one-size-fits-it-all” first-generation doping, in which the same dopant was used by many athletes in an ineffective way to the second- generation nutritional doping, with the introduction and promises of postgenomics biotechnologies. Moreover, this could make the detection of doping even more tricky and challenging, increasing the cost and the burden of anti-doping policies (Kayser *et al.*, 2007). New anti-doping technologies are becoming more and more complex, exploiting the same omics sciences (Pitsiladis *et al.*, 2014; Pottgiesser and Schumacher, 2013; Reichel, 2011), enabling the detection in abnormal traces of compounds such as human growth hormone (hGH), recombinant erythropoietin (rhEpo), or the misuse of autologous blood transfusion (blood doping) (Pitsiladis *et al.*, 2014; Pottgiesser and Schumacher, 2013; Reichel, 2011), also by using biofluids that currently have been little explored or used for anti-doping controls, like sweat (Mena-Bravo and Luque de Castro, 2014).

The peculiar aspect of this nutritional doping is that it is a *personalized* doping, since it is tailored according to the specific needs of an athlete and not just generic as the first generation or classical doping. The role of nutri-ethics then appears central in sports ethics: due to the growing number of sports scandals, the more and more widespread and increasing doping attitude and behaviors, an ethical framework over food and nutrition, not to mention novel biotechnologies related to nutrition, are timely and crucial (D’Arcy *et al.*, 1997; Kirka and Colquhoun, 1989).

Nutri-ethics should guide nutrigenoproteomics applications and uses, according to ethical values and ideals but prescription of the values that govern nutri-ethics in the face of nutrigenoproteomics should call for public and stakeholder deliberation. Future laboratories could help athletes in choosing healthy and dope-free foods, because some foods could be contaminated by doping agents and athletes may be not aware of this. Deciding to take (or not) a supplement even if legal *per se* is not an easy choice: athletes and above all, elite athletes, are constantly under pressure. For this reason, they should be supported by nutritional experts. Moreover, educational efforts should be made in advising athletes against an unrestricted and indiscriminate use of nutritional supplements. The wrong, inadequate nutrition can in fact lead to sports underperformances, due to the imbalance of nutrient concentration, a negative energy balance, and alterations to biochemical and metabolic pathways. On the other hand, nutrigenomics coupled with sports genomics may inform a better understanding of the expression of the genes related to oxidative stress and other parapathophysiological conditions, being particularly helpful for vulnerable athletes (Bamman *et al.*, 2014) and suggesting *ad hoc* diets.

The different omics specialties converge in a unique approach, termed *sportomics* and defined as a “holistic and top-down”, “non-hypothesis-driven research on an individual’s metabolite changes during sports and exercise” (Bassini and Cameron, 2014). Not only does sportomics include metabonomics, but it also relies on the athlete’s biological passport (Suagy *et al.*, 2014; Verne, 2014) or profile (Zorzoli *et al.*, 2014), and would enable the systematic study of sport-induced perturbation at any level (genome, transcriptome, proteome, etc.).

This would be of particular importance in adolescent athletes, who are particularly under pressure, being biologically and psychologically vulnerable and are prone to doping use in a critical transition developmental phase, in which the metabolic, endocrine apparatus are not mature yet, as well as their coping resources and resilience strategies. Among the different psychological predictors of attitude toward sports doping (Hodge *et al.*, 2013), dissatisfaction with one’s own body among adolescent males constitutes an important driver in desiring to lose weight and gaining more muscle, therefore is associated with excessive exercises, consumption of supplements and ergogenic aids, and attitudes towards doping (in particular, use of protein powders and anabolic steroids) (Yager and O’Dea, 2014). Another vulnerable category is given by recreational athletes: in that setting, nutraceuticals and doping agents are not well regulated as in the environment of elite athletes (Lippi *et al.*, 2008).

47.5 Conclusions

In this book chapter, I explained how the changes and progresses in molecular biology justify the birth of a new discipline, termed *nutri-ethics*, and situated at the junction of nutrigenomics and nutriproteomics within the current postgenomics medicine (Bragazzi, 2013c). In particular, I emphasized how nutrigenoproteomics is important for sport and personalized medicine since it could provide important information for personalized training in order to achieve the best result possible and to express one’s own potential, since nutrition plays an important role for athletes. But nutrigenoproteomics could also be used as a platform for personalized doping, thus constituting an advancement of the “classical nutrition-based doping” (i.e., the use of nutraceuticals, stimulants, and supplements). First-generation nutritional doping being “one-size-fits-it-all” may not be effective for all athletes and, by exploiting new biomarker technologies (a sort of “molecular and evidence-based doping”), an athlete could seek the best attainable result.

This instrumental use of nutritional science (that I define nutrigenoproteomics-based doping) would contravene sports ethics, being a potential enhancer and representing a tension between individual and public needs (Chadwick, 2004, 2005), giving athletes advantages in the competitions not coming from their fatigue, training, and motivation. Guidance from World Anti-Doping Agency (WADA) to fill in the gap by providing clear criteria to distinguish between “nutritional training” and “nutritional doping” would be useful in the age of postgenomics biotechnologies, since between them there is a “moral difference”. Each food component should be clearly identified and its potential benefit and risk clearly stated and explained on the basis of their evidence-based effectiveness and bioactivity (Brouns *et al.*, 2002; Deldicque and Francaux, 2008). Athletes should be advised and counseled of the risks of sports medicalization (Cristani *et al.*, 2007; McNamee, 2012). This also calls for proper communication of the attendant uncertainty of diagnostics tests emerging in the future from nutrigenomics and nutriproteomics. Moreover, since some kinds of sports, particularly those carried out in extremely stressful, highly competitive surroundings, can lead to a decrease in immunocompetence (Gleeson and Bishop, 2000), whose multifactorial condition can be worsened by improper nutrition, and given the actual status of knowledge of correct and adequate eating behaviors among athletes, it is imperative to educate them. An adequate level of knowledge is indeed protective against doping behaviors (Kondric *et al.*, 2013).

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Part X

Nanotechnology

48

Current Relevant Nanotechnologies for the Food Industry

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48.1 Introduction

Currently, new frontier technology is essential to keep leadership in the food and food processing industry. Consumers demand fresh, authentic, convenient, and flavorful food products. The future belongs to new products and new processes, with the goal of enhancing the performance of the product, prolonging shelf life, freshness, and improving the safety and quality of food product. Nanotechnology has the potential to revolutionize the future food industry.

Research in the nanotechnology field has skyrocketed over the last decade, and already there are numerous companies specializing in the fabrication of new forms of nanosized matter, with anticipated applications that include medical therapeutics and diagnostics, energy production, molecular computing, and structural materials (Bagchi *et al.* 2010; German *et al.* 2006). Up to now, nanoscale devices are often manufactured with the view to imitate the nanodevices found in nature and include proteins, DNA, membranes, and other natural biomolecules.

Despite the excitement surrounding nanotechnology and the abundance of funding dollars being poured into it, however, one industry that has been slow to catch on is the food industry. This is not so surprising, as public preference for “natural” food products has historically inhibited the implementation of emergent food technologies, and nanotechnology has been no exception. Indeed, while public opinion about general nanotechnology applications has ranged from neutral to slightly positive, some studies suggest that consumers remain wary about “nanofoods” (Castellini *et al.* 2007; Cobb and Macoubrie 2004; Currall *et al.* 2006; Satterfield *et al.* 2009; Siegrist *et al.* 2007).

Nevertheless, scientists and industry stakeholders have already identified potential uses of nanotechnology in virtually every segment of the food industry, from agriculture (e.g., pesticide, fertilizer or vaccine delivery; animal and plant pathogen detection; and targeted genetic engineering) to food processing (e.g., encapsulation of flavor or odor enhancers; food textural or quality improvement; new gelation or viscosifying agents) to food packaging (e.g., pathogen, gas or abuse sensors; anticounterfeiting devices, UV-protection, and stronger, more impermeable polymer films) to nutrient supplements (e.g., nutraceuticals with higher stability and bioavailability). Undeniably, the most active area of food nanoscience research and development is packaging. This is likely connected to the fact that the public has been shown in some studies to be more willing to embrace nanotechnology in “out of food” applications than those where nanoparticles are directly added to foods (Satterfield *et al.* 2009; Siegrist *et al.* 2007, 2008).

Table 48.1 Range of sizes of nanomaterials in the food sector.

Structures	Diameter or length (nm)
DNA	12
Glucose	21–75
Liposome	30–10,000
LDH	40–300
Amylopectin	44–200
Casein micelle	60–100
PLA nanosphere	100–300
Zein	200
Cubosome	500
Nanosensors	<1000

For food applications, two approaches to attain nanomaterials are the top-down approach and bottom-up approach (Table 48.1). The “top-down” approach involves physically machining materials to the nanometer size range by employing processes such as grinding, milling, etching, and lithography. For example, a high water binding capacity wheat flour of a fine size can be obtained by dry-milling technology. By contrast, self-assembly and self-organization are concepts derived from biology that have inspired a bottom-up food nanotechnology. Bottom-up techniques build or grow larger structures atom by atom or molecule by molecule. These techniques include chemical synthesis, self-assembly, and positional assembly (Acosta 2008; Meetoo 2011; Sanguansri and Augustin 2006; Sozer and Kokini 2009).

Owing to the greater surface area of nanoparticles per mass unit, they are expected to be more biologically active than larger sized particles of the same chemical composition. This offers several perspectives for food applications. Nanoparticles can, for instance, be used as bioactive compounds in functional foods (Chau *et al.* 2007).

In the food industry, several novel applications of nanotechnologies have become apparent, including the use of nanoparticles, such as micelles, liposomes, nanoemulsions, biopolymeric nanoparticles, and cubosomes, as well as the development of nanosensors, which are aimed at ensuring food safety (Esposito *et al.* 2005; Ligler *et al.* 2003; Nasongkla *et al.* 2006; Yih and Al-Fandi 2006). Worldwide sales of nanotechnology products to the food and beverage packaging sector increased year by year.

However, despite the increased marketing efforts in the nanotechnology sector, research into nanotechnology of food and food-related products is only just beginning to develop (Chau *et al.* 2007). Food nanotechnology is still a lesser-known subfield of the greater nanotechnology spectrum, even among professional nanotechnologists. Some examples of the use of nanotechnology in food products are cooking oils that contain nutraceuticals within nanocapsules, nanoencapsulated flavor enhancers, and nanoparticles that have the ability to selectively bind and remove chemicals from food. The main reasons for the late incorporation of food into the nanotechnology sector are issues associated with the possible labeling of the food products and consumer-health aspects.

To date, most of the research on nanotechnology focused on the electronics, medicine, and automation sector. The knowledge gained from these sectors could be adapted for the use of food and agriculture products, such as for applications in food safety (e.g. detecting pesticides and microorganisms), in environmental protection (e.g. water purification), and in delivery of nutrients (Roco 2003; Ulijn *et al.* 2007).

The current nanotechnologies relevant to food industry are present. The current issues in nanotechnology in view of the potential risks of nanomaterials for health and the environment are also discussed.

48.2 Nanotechnology in Food Industry

Nanofoods, according to the Helmut Kaiser Consultancy (Helmut Kaiser Consultancy Group 2009), are estimated to experience increasing growth in the development of food and dairy related nanoproducts and patent applications. Nanotechnology can be applied to develop nanoscale materials, controlled delivery systems, contaminant detection, and to create nano devices for molecular and cellular biology from how food is grown to how it is packaged. The application of nanotechnology with respect to the food industry will be covered under two major directions; food additives (nano inside) and food packaging (nano outside). For nano inside, this mainly includes nanoparticles, nanodispersions, nanocapsules, nanocolloids, nano emulsions, nanofibers, nanotubes, and so on. For nano outside, nanoparticles, nanocoatings, nanolaminates, nanosensors, and so on are introduced.

48.2.1 Nanoparticles (NPs)

Nanomaterials have shown great potential in facilitating the development of new technologies. Nanoparticles (NPs), because of their large surface area and special characteristics, have been used in the development of solar cells (Lamberti *et al.* 2013), sensors (Alvares *et al.* 2012), catalysts (Michalak *et al.* 2014), drug delivery systems (Probst *et al.* 2013), imaging techniques (Jarzyna *et al.* 2009), and food chemistry (Sahraei *et al.* 2013; Tikekar *et al.* 2013).

Nanosize metallic particles, such as gold, silver, zinc, and copper, are not only more stable but also possess a high surface area to volume ratio with increased surface reactivity. Therefore, metallic nanoparticles (NPs) have been widely used industrially for the past two decades. Among the metal NPs, silver nanoparticles (AgNPs) have attracted especial attention in the food packing sector because of their remarkable and broad spectrum of antimicrobial effects against food-borne pathogens. The incorporation of AgNPs into the food packaging system could effectively inhibit the growth of pathogenic microorganisms (Costa *et al.* 2011). Various physical and chemical methods have been utilized for the production of nanoparticles. Generally, chemical methods have been widely used for the preparation of nano-scale metallic particles. Recently, more eco-friendly, biocompatible, and safe biological methods have been developed (Guo 2013).

48.2.2 Nanodispersion

Nanodispersion is an alternative and promising approach to overcome bioavailability problems. The increase in bioavailability is due to the special characteristics of nanodispersions. It has been shown that nanosized-dispersion increases the surface areas and dissolution velocities of poorly soluble compounds, thus increasing their saturation solubility.

Food industry is constantly exploring efficient and cost effective intervention strategies to control the growth of pathogenic and spoilage microorganisms to ensure microbial quality and safety of consumer foods (Sanguansri and Augustin 2006). For minimally-processed foods, naturally occurring antimicrobial compounds are a viable option. Essential oils from aromatic plants, generally herbs or spices, are one group of naturally occurring antimicrobials. Traditionally used as flavoring agents, they are gaining popularity due to their preservative effects such as antifungal, antibacterial, and antioxidant properties.

However, the poor water-solubility of essential oils makes them difficult to incorporate into foods, and their tendency to bind with hydrophobic food constituents reduces availability for antimicrobial action. In order to overcome these challenges, essential oils may be encapsulated to enhance solubility and dispersibility in aqueous media, reduce losses due to binding with food constituents, and increase antimicrobial efficacy by promoting contact with bacterial cell components.

In the literature (Shah *et al.* 2013), to prepare conjugates, solutions containing whey protein isolate (WPI) and maltodextrin (MD) at a mass ratio of 1:2, 1:1, and 2:1 were spray-dried, and the collected powder was heated in an oven at 90°C for 2 h for conjugation (via the Maillard reaction). To encapsulate eugenol by emulsion–evaporation, emulsions were prepared with an oil phase (with 20%w/v eugenol in hexane) at 10%v/v volume and an aqueous phase with conjugates dissolved at a net protein concentration of 3.7%w/v by using a high-speed homogenizer at 15,000 rpm for 3 min. Emulsions were then spray dried. All spray drying experiments were performed at an inlet temperature of 150°C, 35 m³/h air flow rate, 600 kPa compressed air pressure, feed rate of 6.67 ml/min and the recorded outlet temperature of 80–90°C. The spray dried capsules were collected and stored in a freezer at -18°C. Eugenol was dispersed in nanocapsules prepared with conjugates of WPI and MD (of various chain lengths). Results show that when eugenol was encapsulated in the conjugate made with MD40 at a WPI: MD mass ratio of 1:2, the nanodispersion was transparent and was characterized for antimicrobial efficacy against *E. coli* O157:H7 strains ATCC 43889 and 43894, and *Lm* strains Scott A and 101 in tryptic soy broth (TSB) and milk with different fat levels (whole, 2% reduced fat, and skim) at 35 or 32°C, with comparison to the same levels of free eugenol. In TSB, antimicrobial efficacy of nanodispersed eugenol against *E. coli* O157:H7 and *Lm* was not improved, with minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values being 0.25 g/l higher than those of free eugenol. Free eugenol performed better in TSB because there was no interfering compound and the MIC and MBC were below the solubility of eugenol. In milk, nanodispersed eugenol was consistently observed to be more effective than free eugenol, with MIC and MBC values above the solubility limit of eugenol. The nanodispersed eugenol was speculated to be evenly distributed in food matrices at concentrations above the solubility limit and supplied the antimicrobial locally when the binding caused eugenol level below the inhibition requirement. Nanodispersed eugenol thus provides a novel approach for incorporation in foods to improve antimicrobial efficacy without changing turbidity.

48.2.3 Nanocapsules

Encapsulation improves the stability, solubility, and bioavailability of encapsulated species and promotes its controlled release. Nano-encapsulation is a process by which one compound is covered by another, producing particulate dispersions or solid particles, with sizes ranging from 10 nm to 1 μm . Depending upon the method of preparation of nanoparticles, nanospheres, or nanocapsules can be obtained. Nanocapsules are systems in which the bioactive compound is soluble in the core, confined to a cavity surrounded by a polymer membrane, while nanospheres are matrix systems in which the drug is physically and uniformly dispersed.

Nanocapsule systems are used for the delivery of drugs, peptides, proteins, genes, and so on, and several compounds have been encapsulated. In the literature a number of methods are cited; most nanoparticles have been mainly prepared by dispersion of preformed polymers, polymerization of monomers, and ionic gelation or coacervation of hydrophilic polymers (Mohanraj and Chen 2006).

Kleidson Brito de Sousa Lobato *et al.* (2013) investigated the characterization and stability evaluation of bixin nanocapsules. It is well known that bixin is an antioxidant and the predominant pigment found in fat-soluble preparations that are used to colorize butter, cheese, bakery products, oils, ice creams, sausages, cereals, and extruded products. Consequently, bixin important to human health and bixin's prevalence in the food industry as a colorant and antioxidant motivates the study of nanoencapsulation as a suitable technique for increasing the solubility of bixin in aqueous media.

Bixin nanocapsules were produced by the interfacial deposition of preformed poly- ϵ -caprolactone (PCL). PCL (250 mg), capric/caprylic triglyceride (400 μl), sorbitan monostearate (95 mg), and bixin were dissolved in a mixture of acetone (60 ml) and ethanol (7.5 ml) under stirring (40°C). This organic solution was added to the aqueous solution (130 ml) containing Tween 80 (195 mg).

Results show that no significant changes ($p < 0.05$) were observed in the particle diameter over 119 days of storage when evaluated using both laser diffraction (LD) and Dynamic light scattering (DLS). The solubilization of bixin in aqueous media enhances the future possibility of using bixin in low-fat foods and in studies performed to evaluate its effects *in vivo*, which may expand the breadth of bixin's industrial application.

48.2.4 Nanocolloids

Bioavailability and bioaccessibility have received growing interest in food and pharmaceutical areas over the past few decades. Since the solubility of bioactive compounds determines their bioavailability, the slow dissolution or solubilization of nutrient functional lipid compounds in the aqueous based systems causes their low absorption rate and consequently, their low bioavailability. Several researches have been showed that the colloidal delivery systems can be used as an efficient alternative for low bioavailability problems of water insoluble bioactive compounds (Rossi *et al.* 2010; Tan and Nakajima 2005). In these delivery systems the small particle size leads to an increase in the available specific surface area for dissolution or solubilization, or in different enzyme activities (Yuan *et al.* 2008). Different surface-active biopolymers, such as polysaccharides and proteins, or emulsifiers, such as lecithin, polysorbates, sugar esters, and monoglycerides, can be used to stabilize various nanosized systems. Dissimilarities in molecular characteristics of these stabilizer compounds (molecular weights, structures, functional groups, polarities, and charge) lead to wide differences in their solubilities, physical states, rheological properties, optical properties, chemical stabilities, surface activities, bioactivities, and other physico-chemical and physiological properties.

Navideh Anarjan *et al.* (2012) produced Astaxanthin colloidal particles using a solvent-diffusion technique in the presence of different food grade surface active compounds, namely, Polysorbate 20 (PS20), sodium caseinate (SC), gum Arabic (GA), and the optimum combination of them (OPT).

Astaxanthin (0.08% (w/w)) powder was dissolved in a mixture of 38% (w/w) dichloromethane and 62% (w/w) acetone as organic phase at room temperature. The organic to aqueous phase ration was set at 11.5% (w/w). The aqueous phase, which was composed of 2.5% (w/w) stabilizer and 0.02% (w/w) sodium azide in 0.05 M phosphate buffer with pH = 7.0, was magnetically stirred for overnight to get completely hydrated. The organic phase was gradually dispersed into the water phase afterwards, and the coarse emulsion was formed by homogenization in a conventional homogenizer at 5000 rpm for 5 min. It was then passed three times through a high-pressure homogenizer at 30 MPa to produce a fine emulsion. The solvent was subsequently removed from the emulsion by rotary evaporation. The selected stabilizer systems for preparation of astaxanthin colloidal particles were PS20, SC, and GA, individually, as well as a mixture of 29% (w/w) PS20, 65% (w/w) SC, and 6% (w/w) GA, coded as OPT, which

was found through a mixture design optimization procedure that led to production of astaxanthin colloidal particles with the most desired physicochemical and stability characteristics.

Results indicate that the three component stabilizer system composed of PS20, SC, and GA could produce the astaxanthin colloidal particles with smaller particle size, PDI, conductivity, and higher zeta potential, mobility, cellular uptake, color intensity, and *in vitro* antioxidant activity. In addition, all prepared astaxanthin colloidal particles had significantly ($p < 0.05$) higher cellular uptake than pure astaxanthin powder.

48.2.5 Nanoemulsions

The interest in nanoemulsions has experienced a continuous increase in recent years, as evidenced by the numerous publications and comprehensive reviews (Fryd and Mason 2012; Gutiérrez *et al.* 2008; Maali and Hamed Mosavian 2012; McClements 2011) on the subject. This enormous interest is triggered by the wide range of applications, namely in the pharmaceutical (Machado *et al.* 2012; Shakeel *et al.* 2010), cosmetic (Al-Edresi and Baie 2009), food (Rao and McClements 2011; Silva *et al.* 2012), chemical Ragupathy *et al.* 2011), and so on, industries. Nanoemulsions (submicrometer size droplets) have advantages over conventional emulsions (micrometer size droplets) due to their small droplet size which confers them stability against sedimentation or creaming and a transparent or translucent optical aspect (similar to that of microemulsions). However, nanoemulsions, in contrast to microemulsions, which are thermodynamically stable, are nonequilibrium systems that may undergo flocculation, coalescence, and/or Ostwald ripening. Nevertheless, with an appropriate selection of system components, composition, and preparation method, nanoemulsions with a high kinetic stability can be achieved. It is generally accepted that nanoemulsion main breakdown process is Ostwald ripening (diffusion of molecules of the disperse phase from small to big droplets, through the continuous phase, as a consequence of their different Laplace pressures) (Taylor 1998).

Nanoemulsions have been commonly prepared by high-energy methods using mechanical devices able to produce intense disruptive forces, namely, high-shear stirrers, high pressure homogenizers, and ultrasound generators. Nanoemulsion formation by these methods is quite straightforward as the higher the energy input the smaller the droplet size.

However, the level of energy required to obtain nanometer-scaled droplets is very high and therefore cost-inefficient, especially considering that only a small amount (around 0.1%) of the energy produced is used for emulsification (Tadros *et al.* 2004). In contrast, low-energy emulsification methods, making use of the internal chemical energy of the system, are often more energy efficient as only simple stirring is needed, and generally allow producing smaller droplet size than high-energy methods. Nevertheless, depending on the system and composition variables, similar droplet sizes can be achieved by both types of methods (Solé *et al.* 2006; Yang *et al.* 2012).

The emulsion droplet size is known to impact crystallization properties of dispersed fat phase. Owing to the emulsified state in which each droplet needs a nucleus or impurity to initiate nucleation, extensive supercooling is required for lipid droplets in a dispersed system to induce crystallization compared to its bulk counterpart. Variations in emulsion droplet size may alter the crystallization and structural behavior of emulsified fats, particularly with "nanoemulsions" (droplet diameter below 0.2 μm) (Bugeat *et al.* 2011; Bunjes *et al.* 2000). It was reported that submicron-size droplets were exhibited at lower crystallization temperatures than micron-size ones in *n*-hexadecane oil-in-water emulsions, whereas crystallization temperatures of tripalmitin, tristearin, and triauroylglycerol nanoparticles just slightly changed with decreasing average size in the nanometer-size range (Bunjes *et al.* 2000; Dickinson *et al.* 1991).

48.2.6 Nanofibers/Tubes

Nanofibers with diameters from 10 to 1000 nm are ideal for serving as platforms for bacterial cultures as well as a structural matrix for artificial foods. Since nanofibers are usually not composed of food grade substances, they have only a few potential applications in the food industry (Weiss *et al.* 2006). The food industry can use electrospun microfibers in several ways; as building elements of the food matrix for imitation/artificial foods and as nanostructured and micro structured scaffolding for bacterial cultures (Bikiaris and Triantafyllidis 2013).

For tubes, the use of nanotubes has predominantly been for non-food applications. Carbon nanotubes are popularly used as low resistance conductors, catalytic reaction vessels, and detections, especially for carbon nanotubes (CNTs). CNT have attracted considerable interest because of their unique mechanical and electronic properties combined with a large specific surface area, and been used extensively in the many common detection systems that are based on optical detection, biodection including a large specific surface area for antibody immobilization, and immunosensors for the detection of toxins in food Dias *et al.* 2013; Palaniappan *et al.* 2013; Yang *et al.* 2007).

48.3 Natural Biopolymers

Starch and its derivatives are other important natural polymers that could be further improved through nanotechnology approaches. After extrusion, the starch is typically converted to a thermoplastic material with low mechanical resistance and poor protection against oxygen and moisture (Chen and Evans 2005; de Carvalho *et al.* 2001; Lopez-Rubio *et al.* 2006; McGlashan and Halley 2003). Park *et al* prepared hybrids of thermoplastic starch (TPS) with nanoclay and investigated the resulting properties. They found that the strong interaction between the TPS and the nanoclay improved tensile strength and lowered water-vapor permeability compared with the native TPS matrix (Park *et al.* 2002).

Biopolymers are another important class of biodegradable polymers formed from biological monomers, including polylactic acid (PLA), polyhydroxybutyrate (PHB), and polycaprolacton (PCL). Biopolymers are biodegradable and biocompatible and can be formed into films or molded into objects. However, biopolymer applications in the food-packaging industry have some important limitations that are caused by their relatively poor gas-barrier properties and brittleness. In an attempt to overcome these limitations, nanoclays have been used as supportive filling agents in a biopolymer matrix where they formed nanocomposite structures (Cabedo *et al.* 2006; Chen *et al.* 2003). Cabedo *et al.* showed that the addition of nanoclays, in this case kaolinite nanofillers, to PLA films improved both thermal stability and mechanical properties without decreasing barrier properties. These positive results are expected to increase the potential food-packaging applications of these polymers (Cabedo *et al.* 2006).

48.4 Nanotechnology for Food Packaging

48.4.1 Silver Nanoparticles and Nanocomposites as Antimicrobial Food Packaging Materials

Silver has a long history of being used as an antimicrobial agent in food and beverage storage applications. Numerous ancient societies stored wine and water in silver vessels. Web searches on the historic uses of silver reveal anecdotal reports of early settlers placing silver dollars or silver spoons at the bottom of milk and water bottles to prolong shelf life, and of seafaring ships or airliners lining their water tanks with silver to keep water potable for long periods of time. Silver was the sterilization agent for water on the Russian MIR space station and on NASA space shuttles (Silver 2003), and silver's broad-spectrum antimicrobial activity and relative low cost have made it a candidate as the active disinfecting agent for water in developing countries (Solsana and Méndez 2003). In 2009, the FDA modified the food additive regulations to permit the direct addition of silver nitrate as a disinfectant to commercially bottled water at concentrations not to exceed 17 µg/kg (US Food and Drug Administration 2009).

Silver has numerous advantages over other antimicrobial agents. Compared to molecular antimicrobials, which are generally targeted to specific organism classes, silver is broad spectrum and toxic (to varying degrees) to numerous strains of bacteria, fungi, algae, and possibly some viruses. Being an element, silver is shelf stable for long periods of time. Conventional wisdom regards silver as safe to humans and other higher order organisms when used responsibly, and silver-based pharmaceuticals have few if any acute or chronic known side-effects at FDA-permitted doses. Silver is reasonably effective at penetrating biofilms, which has been a drawback to many molecular antimicrobials. Furthermore, though bacterial strains which manifest silver-resistance are known and these mechanisms have been studied, some researchers have suggested that silver may be less susceptible to the buildup of resistance than molecular antimicrobials (Chopra 2007; Montiero *et al.* 2009). This remains an area of some uncertainty. Even so, however, the explosion of interest in silver as a broad-spectrum antimicrobial agent during the last two decades may be in part due to the proliferation of resistance to strong molecular antimicrobials; in that respect, silver has been shown (Echague *et al.* 2010; Jones *et al.* 2004) to be an effective bactericide against antimicrobial-resistant bacterial strains (e.g., MRSA), which have become a concern (Gemmell *et al.* 2006) in hospitals.

Despite the long history of silver as an antimicrobial, the mechanism of this activity remains a matter of active research. The general explanation offered is that silver kills by at least one of the following mechanisms: (1) interference with vital cellular processes by binding to sulfhydryl or disulfide functional groups on the surfaces of membrane proteins and other enzymes; (2) disruption of DNA replication; and (3) oxidative stress through the catalysis of reactive oxygen species (ROS) formation. However, controversy exists regarding which of these mechanisms is most important. For instance, one study (Dibrov *et al.* 2002) presented evidence that showed that silver binding specifically to membrane proteins disrupts ion and proton transport across the membrane, while another found that Ag ions permeate to the cellular interior, where they interfere with ribosomal activity and disrupt the production of several key enzymes responsible for energy production (Yamanaka *et al.* 2005). With respect to interference of DNA replication, cell wall damage resulting from silver binding to membrane proteins and DNA

condensation in *Escherichia coli* and *Staphylococcus aureus* has been observed; the condensation of DNA in response to the presence of Ag ions has been cited as a defense mechanism, which, while protecting the DNA from harm, limits the ability of cells to self-replicate (Feng *et al.* 2000). In contrast, a separate report asserted that Ag complexes of glutamic and tartaric acids actively interfere with DNA unwinding, and suggested that Ag ion binding to enzymes and membrane proteins is a comparatively minor contributor to silver's antimicrobial effect (Batarseh 2004). Gram-negative bacteria (e.g., *E. coli*) are generally more susceptible to silver treatment than Gram-positive bacteria (e.g., *S. aureus*) because transport of positively charged silver ions across the thicker, peptidoglycan-rich outer membranes of Gram-positive bacteria is slow relative to transport across the thinner membranes of Gram-negative specimens (Feng *et al.* 2000). Finally, there is evidence that the antibacterial activity of silver zeolites derives from silver's ability to catalyze the production of reactive oxygen species, which causes cell death by creating oxidative stress (Inoue *et al.* 2002); in support of this idea, antioxidant rich *Bacillus* spores are highly resistant to silver antimicrobials, whereas vegetative and relatively anti-oxidant poor *Bacillus* cells are quite vulnerable (Galeano *et al.* 2003). It is certainly possible that all of these mechanisms contribute to the antimicrobial activity of silver, which would explain its broad effectiveness as well as the infrequent reports of silver-resistant bacterial strains.

48.4.2 Nanolaminates/Coating

Nanotechnology provides food scientists with a number of ways to create novel laminate films suitable for use in the food industry. A nanolaminate consists of two or more layers of material with nanometer dimensions that are physically or chemically bonded to each other. Nanolaminates can give food scientists some advantages for the preparation of edible coatings and films over conventional technologies and may thus have a number of important applications within the food industry. Edible coatings and films are currently used on a wide variety of foods, including fruits, vegetables, meats, chocolate, candies, bakery products, and French fries (Azeredo *et al.* 2009; Silvestre *et al.* 2011) to prevent moisture and gas exchange, act as a vehicle to deliver colors, flavors, antioxidants, enzymes, and antibrowning agents, and could also increase the shelf life of manufactured foods, even after the packaging is opened.

These coatings or films could serve as moisture, lipid, and gas barriers. Alternatively, they could improve the textural properties of foods or serve as carriers of functional agents such as colors, flavors, antioxidants, nutrients, and antimicrobials. The basic functional properties of edible coatings and films depend on the characteristics of the film-forming materials used for their preparation. The composition, thickness, structure, and properties of the multilayered laminate formed around the object could be controlled in a number of ways, including changing of the type of adsorbing substances in the dipping solutions, the total number of dipping steps used, the order that the object is introduced into the various dipping solutions, the solution and environmental conditions used (pH, ionic strength, dielectric constant, temperature, etc.). The driving force for adsorption of a substance to a surface would depend also on the nature of the surface and the nature of the adsorbing substance and it could be: electrostatic, hydrogen bonding, hydrophobic interactive, thermodynamically incompatible, and so on.

48.4.3 Nanosensors

Packaging equipped with nanosensors is also designed to track either the internal or external conditions of food products, pellets, and containers throughout the supply chain. For example, such packaging can monitor temperature or humidity over time and then provide relevant information of these conditions, for example by changing color.

Fresh produce or meats (which are either spoiled or unpalatable) exhibit odors, colors, or other sensory characteristics that can be easily discerned by consumers. When packaging materials prevent extensive sensory exposure, however, consumers must rely on sell-by dates, which are determined by producers based on a set of idealized assumptions about the way that the food is stored or transported. While the sell-by date for a carton of milk may indicate to a consumer that the product should be good for a period of 2 weeks, this date may no longer be applicable if that milk was stored above its optimal temperature for an hour, either in a delivery truck or in a warm automobile.

The unique chemical and electro-optical properties of nanoscale particles offer solutions to this problem. Through bottom-up engineering, nanomaterials can be devised that are able to detect the presence of gasses, aromas, chemical contaminants, and pathogens, or respond to changes in environmental conditions. This not only is useful for quality control to ensure that consumers are able to purchase products which are at their peak of freshness and flavor, but it also has the potential to improve food safety and reduce the frequency of food-borne illnesses. Such technology would obviously benefit consumers, industry stakeholders, and food regulators. Some companies and some already market nanotechnology products that help consumers determine whether certain foods are likely to be palatable, but most of the work on nanosensors or assays for food-related analytes is still in the early stages of development (Ai *et al.* 2009; Cao *et al.* 2010; Kuang *et al.* 2011; Staiano *et al.* 2009).

The researchers studied the effects of parameters that affect size, size distribution, and surface properties of nanoparticles. The produced nanoparticles can be used in novel drug and food delivery systems. The aim of the research was to design a drug nano-carrier with fine size and distribution particle size and appropriate surface properties, which is ideal for use in novel drug delivery systems (Taheri *et al.* 2012). It expresses that egg albumin nanoparticles were produced through simple coacervation method so they can be used as an appropriate drug nanocarrier in novel drug delivery systems. The analyses showed that the produced nanoparticles have almost spherical shape, and they are formed with desirable surface morphology (they have very smooth surface). In addition, it was turned out that egg albumin nanoparticles had a mean size of less than 100 nm. The simple coacervation method was considered an appropriate method for the production of this type of nanoparticles. Therefore, egg albumin nanoparticles can be considered very good candidates to be used as drug and food nanocarriers.

Iranian researchers measured very tiny amounts of some of toxic heavy metals in water and foodstuff by using SBA-15 nanoporous compound functionalized with guanidin groups as perfect sorbent for metals (Leila *et al.* 2013). In the first stage of the research, SBA-15 nanoporous compound functionalized with guanidin was prepared and its structural properties were investigated. In the next stage, the compound was used for the extraction and preconcentration of some of heavy metals that are classified among toxic and hazardous elements for the living creatures and environment, and as a result, it made possible the measurement of very small amounts of the elements. Finally, the concentration of lead, copper, cadmium, and zinc ions were measured in various water and food samples, and promising results were obtained.

Nanopores in the structure of SBA-15 increase the interface of sorbent with the solution, and therefore, ion sorption capacity increases on this compound. Besides, the high regularity and homogeneity of the nanopores result in excellent repeatability of the extraction process. In addition, nanoporous SBA-15 functionalized with guanidin was used as a new extraction agent to concurrently extract lead, copper, cadmium, and zinc ions. The extraction was carried out in 25 ml of solution containing 2 mg/l of each ion in 10 min and with very small amount of sorbent (10 mg).

A recent paper in *Trends in Food Science & Technology* provides a summary of the kind of applications industry and universities are working on (Chaudhry and Castle 2011), which is listed in Table 48.2.

Table 48.2 Summary of the food applications and status of nanotechnologies.

Application	Status
Processed nanostructured or -textured food (e.g., less use of fat and emulsifiers, better taste)	A number of nanostructured food ingredients and additives understood to be in the R&D pipeline; e.g., mayonnaise
Nanocarrier systems for delivery of nutrients and supplements in the form of liposomes or biopolymer-based nanoencapsulated substances	A number are commercially available in some countries and over the Internet
Organic nanosized additives for food, supplements, and animal feed	Materials range from colors, preservatives, flavorings to supplements and antimicrobials
Inorganic nanosized additives for food, health food, and animal feed	A range of inorganic additives (silver, iron, silica, titanium dioxide, selenium, platinum, calcium, magnesium) is available for supplements, nutraceuticals, and food and feed applications
Food packaging applications; e.g., plastic polymers containing or coated with nanomaterials for improved mechanical or functional properties (see for instance: www.nanowerk.com/news/newsid=20901.php)	This area makes up the largest share of the current/short-term market for nanotech applications in the food sector (e.g., plastic polymers with nanoclay as gas barrier; nanosilver, and nanozinc oxide for antimicrobial action; nanotitanium nitride for strength)
Nanocoatings on food contact surfaces for barrier or antimicrobial properties	A number of nanomaterial-based coatings are available for food preparation surfaces and for coating food preparation machinery
Surface-functionalized nanomaterials	Main uses are currently in food packaging; possible uses emerging in animal feed
Nanosized agrochemicals	R&D stage
Nanosensors for food labeling (see: www.nanowerk.com/news/newsid=6667.php)	R&D stage
Water decontamination	Nano iron is already available in industrial-scale quantities. A number of companies thought to be using the technology in developing countries
Animal feed applications	Nanosized additives specifically developed or are under development for feed include nanomaterials that can bind and remove toxins or pathogens

48.5 Outstanding State-of-the-Art Issues

One hundred years ago, if you wanted the best paint for your house you bought lead paint. Eighty years ago if you wanted to insulate your boiler, the best technology was asbestos. Today, if you want to produce the healthiest, tastiest, safest, longest-lasting food, you can use nanotechnology.

Nanotechnology—the science of dealing with matter on a minuscule, or molecular, scale—is, in a somewhat ironic twist, becoming a bigger industry. On top of that, the USDA (United States Department of Agriculture) has announced that it plans to use nanotechnology to improve food safety, Americans' health and bioactives in functional foods.

But we are probably consuming and coming into contact with more nanotechnology than we think we are. It is already used widely in diet and sports drinks (i.e., to encapsulate vitamins and minerals, and also to aid metabolism), nutraceuticals and non-foods (e.g., shoe liners, coffee makers, drugs, cosmetics, and water purifiers).

In foods, nanotechnology is largely being used, to date, to make them more functional—encapsulating nutraceuticals and eliminating harmful fats—and to make them safer—sprays containing antimicrobials can be used to coat foods to extend their shelf-life.

There are also new uses for nanotechnology in food on the horizon. Nanotechnology is being used in packaging, too. Nanoclay materials have been placed between the two layers of a Miller Light bottle to prevent the ultra violet rays from getting into the beer and spoiling the hops. Nanoclays can also help keep carbon dioxide in the bottle (and for plastic juice bottles can keep the oxygen out). Nano packaging can also keep food safer. For example, oxygen sensors composed of ink that contains nanoparticles of titanium dioxide can be incorporated into packaging. These nanoparticles become sensitive to oxygen levels once they are exposed to UV, and then change color, indicating that the product has been exposed to oxygen and may be—or is—spoiled.

Soon we will see fresh food such as broccoli wrapped in a nano saran wrap that prevents oxidation from taking place so the shelf-life of the vegetable extends from 3–4 days to 3–4 weeks. And the foods themselves are starting to be affected in many steps of the food cycle as nanotechnology is being used on plants, in processing, packaging, and storage.

It all sounds very futuristic and good for us, doesn't it? So did lead paint and asbestos, remember (Sahoo *et al.* 2007).

Despite all these possible—and potentially life-threatening—downsides, the food industry is embracing nanotechnology because of the competitive advantages it brings to the food manufacturers themselves—not because of advantages to consumers.

But if tests show nanotechnology is safe for humans, we could be in for some exciting times.

Nanotechnology could also be used in agriculture—to enhance the ability of plants to absorb nutrients, for example, or in the form of nanosensors that could be used to monitor soil conditions and crop growth and to decrease waste and cost while increasing production. However, these agricultural nanomaterials could be blown onto other crops, onto our clothes, or our bodies.

You may be able to harvest more food using nanotechnology but the problem is what the unintended application of that technology is? It is a question of, which risk do I choose?

Because of all the uncertainty surrounding what nanotechnology foods are going to do for us, and to us, the clamor of the debate is rising to a level similar to that over genetically modified foods. So if it follows in the path of the GMO (genetically modified organism) furor, the questions about nanotechnology will not be answered for quite some time.

Below are some of the lessons that can be learned from the GMO debate:

Do not mess with food;

If you do, do not try to pretend that it is just like traditional food;

Label all products that contain nano materials;

Develop a pre-market approval process;

Require approvals BEFORE products go on the market and monitoring AFTER;

Give the public a voice in deciding what to put on the market;

Conduct further research to address the knowledge gaps that currently exist and develop specific risk assessment methodologies. Fund this adequately;

Apply the precautionary principle. Do not wait for illnesses and death before regulating.

48.6 Conclusion

Nanotechnology is becoming increasingly important for food industry. Promising results and applications are already being developed in the areas of food packaging and safety. The incorporation of nanomaterials into food packaging is expected to improve the barrier properties of packaging materials and should thereby help to reduce the use of valuable raw materials

and the generation of waste. Meanwhile, there are social and ethical issues of using nanotechnology in food industry that must be considered. Currently, the potential risks of nanomaterials to human health and to the environment are unknown. Special attention should also be given to consumer attitudes towards food nanotechnology. It is crucial to discuss the benefits and risks of this highly promising technology. Governments should consider appropriate labeling and should also set down regulations that will help to increase consumer acceptability.

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