Metabolomics of cancer cell cultures to assess the effects of dietary phytochemicals

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Cancer is a multifactorial disease and is a major cause of morbidity and mortality worldwide. Dietary phytochemicals have been used for the treatment of cancer throughout history due to their safety, low toxicity, and general availability. Several studies have been performed to elucidate the effects of the dietary phytochemicals on cancer metabolism and many molecular targets of phytochemicals have been discovered. Despite remarkable progresses, their effects on cancer metabolism have not yet been fully clarified. The recent development of metabolomics allowed to probe much further into the metabolism of cancer highlighting altered metabolic pathways and offering a new powerful tool to investigate the cancer disease. In this Review, we discuss the main metabolic alterations of cancer cell and the potentiality of phytochemicals as promising modulators of cancer metabolism. We will focus on the application of NMR-based

metabolomics on breast and hepatocellular cancer cell lines to evaluate the impact of curcumin and resveratrol on cancer metabolome with the aims to demonstrate the premise of this approach to provide useful information for a better understanding of impact of diet components on cancer disease.

Keywords

Metabolomics, NMR metabolic profiling, cancer cell lines cultures, curcumin, resveratrol

INTRODUCTION

Cancer is a complex process involving multiple changes in cell physiology, which lead to an abnormal cell growth (neoplasia) as the biological endpoint of the disease (Seyfried and Shelton, 2010). Cancer development is a growing health problem around the world particularly with the steady rise in life expectancy, increasing urbanization and the subsequent changes in environmental conditions, including lifestyle and diet. Increasing evidences suggest an association between the type of food people eat, their health and their life expectancy. It is estimated that one third of all cancer deaths could be avoided through appropriate dietary modification (Liu, 2004; Kushi et al., 2012). In this context, a high dietary intake of fruits and vegetables as well as whole grains is strongly associated with a reduced risk to developing chronic disease, such as cardiovascular diseases, neurodegenerative disorders and cancer (Woodside et al., 2013; Liu, 2013 a; Liu, 2013 b; Slavin and Lloyd, 2012). In effect, plant-based food, such as fruits, vegetables and whole grains contain significant amounts of ñbioactive phytochemicalsò with various biological properties including antioxidant, antiproliferative and DNA repair that may provide desiderable health benefits (Surh, 2003; Choi et al., 2013; Lee et al., 2013). Several *in vitro* studies have been performed to elucidate the chemopreventive effects of the components of fruit and vegetables on cancer-associated signal-transduction pathways and many molecular targets of phytochemicals have been discovered. Dietary phytochemicals are able to target a plethora of cellular molecules and molecular pathways including reactive oxygen species, inflammation, cell cycle, apoptosis, invasion, angiogenesis, transcription factors, and protein kinases (Thakur et al., 2014). Despite remarkable progresses, the identification of cellular and molecular targets of phytochemicals is still incomplete and their effects on cancer

metabolism have not yet been fully discovered or clarified. Cancer cell lines have been widely used to investigate the cancer associated molecular mechanism and represent an excellent model to evaluate the effect of dietary phytochemical in cancer. To understand the molecular biological events contributing to the cancer development and the effects of phytochemicals on cancer metabolic pathways, the OMICS-tools including genomics, transcriptomics and proteomics, have been largely adopted (Kok et al., 2012). However, gene and protein analysis are unable to define the chemical and metabolic events happening in cancer process. Recent development of metabolomics allowed detecting altered metabolic pathways in cancer cells offering a new powerful tool to investigate the cancer metabolism (Vermeersch and Styczynski, 2013). In this context, NMR-based metabolomics particularly lends to the study of cancer metabolome allowing an ease of quantification, straightforward metabolite identification, and ability to determine unexpected metabolites (Nicholson et al., 1999). Remarkable advances in NMR-based metabolomics such as analytical technologies and statistical approach have allowed to probe much further into the metabolism of cancer, discovering new associations between metabolites and cancer phenotypes as well as the alterations in the cellular concentrations of metabolites that can act as novel diagnostic biomarkers in cancer disease. However, many questions remain to be answered about how dietary phytochemicals interfere with multiple signaling pathways aberrant in cancer. For this reason, studying the metabolic events associated with phytochemical exposure can provide mechanistic insights on phytochemicals-exerted chemopreventive activities and useful information to development personalized therapeutic intervention. In this Review, we discuss the main metabolic alterations of cancer cell and the potentiality of phytochemicals compounds as promising and clinically useful modulators of cancer metabolism. We will focus

on the application of NMR-based metabolomics on breast and hepatocellular cancer cell to evaluate the impact of curcumin and resveratrol on cancer metabolome. Our intention is to provide an overview of how NMR-based metabolomics can be used to monitoring the effect of dietary phytochemicals on altered cancer metabolome and thus demonstrate the premise of this approach to provide useful information for a better understanding of impact of diet components on cancer disease**.**

A FRESH LOOK AT CANCER METABOLISM

The main physiological alterations in cancer cell include: self-sufficiency in growth signals, insensitivity to growth inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained vascularity (angiogenesis), tissue invasion and metastasis (Hanahan and Weinberg, 2000). Underlying these hallmarks acquired during the multistep development of cancer there are genome instability, which generates the genetic diversity hastening their acquisition, and inflammation, which fosters multiple hallmark functions. Advanced progress in the last decade has added two emerging hallmarks of cancer cells: reprogramming of respiration and energy metabolism and evading immune destruction that lead to define cancer as a ñmetabolic diseaseò (Seyfried and Shelton, 2010; Hanahan and Weinberg, 2011). Although no specific gene mutation or chromosomal abnormality is common to all cancers, nearly all cancers express metabolic dysregulation. It is emerging interest in metabolites, termed ñoncometabolitesò, whose abnormal accumulation causes metabolic and potential transformation to malignancy (Yang et al., 2013). The cancer metabolism is characterized by high glycolytic and glutaminolytic capacities, high phosphometabolite levels

and a high channelling of glucose carbons to synthetic processes. These hallmarks allow tumor cells to proliferate under strong variations in oxygen and glucose supply (Mazurek and Eigenbrodt, 2003). In contrast to normal differentiated cells, which derive most of their energy needed for cellular processes from mitochondrial oxidative phosphorylation, most cancer cells reprogram energy metabolism becoming heavily dependent on substrate level phosphorylation to meet energy demands. This phenomenon, known as the ñWarburg effectò consists of using aerobic glycolysis to produce energy, causing an increase in the flux of glucose through glycolysis and a down-regulation of flux through the tricarboxylic acid (TCA) cycle (Vander Heiden et al., 2009). On the other hand, unlike most normal cell, cancer cell preferentially ófermentô glucose to lactate even in presence of sufficient oxygen to support mitochondrial oxidative phosphorilation. It has now become clear that the Warburg effect represents only the tip of the iceberg with regard to the metabolic rearrangements that characterize cancer cells, which involve not only aerobic glycolysis but also changes in mitochondrial respiration and glutaminolysis, an increased flux through the pentose phosphate pathway (PPP), elevated rates of lipid biosynthesis, altered mevalonate pathway, and limited levels of autophagy (Vander Heiden et al., 2009; Schulze and Harris, 2012; White, 2012). It is well known that the coordination of metabolic activity with cell-cycle progression is crucial to support the proliferation of normal cells. In cancer cells, the deregulation of cell-cycle controls some of the metabolic adaptations observed in cancer. The group of Karen Vousden has identified the trascription factor TIGAR and its importance in regulating glycolytic flux in cancer cells. TIGAR functions as a fructose-2,6-bisphosphatase (FBPase), reducing the levels of fructose-2,6-bisphosphate, which result in decreased glycolytic activity and increased flux through the pentose-phosphate pathway. The

activity of TIGAR is important to maintain cellular redox balance and its depletion results in increased levels of reactive oxygen species (Lee et al., 2014). An important role in cancer metabolism is played by AMP-activated protein kinase (AMPK), a master regulator of cell metabolism and activity of many catabolic and anabolic processes. Rapid proliferation and limited nutrient supply are likely to cause chronic activation of AMPK in cancer cells, thereby inducing a strong selective pressure to inactivate this pathway. Several types of cancer show inactivation of the AMPK pathway through downregulation of AMPK expression (Hardie, 2013). Another important change in cancer cell regards the mitochondria which are structurally and functionally abnormal and incapable of generating normal levels of energy, having an altered lipid composition of membranes and electron transport chain function (Seyfried et al., 2014). In particular, the main alteration in mitochondrial membrane lipids regards the content or composition of cardiolipin, an inner membrane enriched lipid, whose abnormality disrupts the mitochondrial proton motive gradient, inducing electron transport abnormalities, and thus a reduction in respiratory energy production. Changes in cardiolipin content and composition can also inhibit uptake of ADP through the adenine nucleotide transporter thus altering the efficiency of oxidative phosphorylation. It is well known that mitochondria are essential for the synthesis of citrate by the tricarboxylic acid (TCA) cycle aims to produce cytoplasmatic acetyl-coenyme A (CoA), a central source of acetyl groups for lipid synthesis and protein acetylation. To supply their energetic requirements by aerobic glycolysis, cancer cells rapidly convert glucose into lactate or pyruvate, which enters in mitochondria, where it is introduced into the TCA and oxidized to acetyl-Coenzyme A. However, oxidative mitochondrial metabolism can be impaired in cancer cells as a result of mutations in components of the TCA cycle or electron transport

chain. For example, the loss of function mutations in genes encoding the Krebs cycle enzymes fumarate hydratase (FH) and succinate dehydrogenase (SDH) cause the accumulation of fumarate and succinate, respectively, whereas the gain of function mutations in isocitrate dehydrogenase (IDH) genes increase levels of Dï 2-hydroxyglutarate (Pollard et al., 2005; Dang et al., 2010). These metabolites have been implicated in the dysregulation of cellular processes including the competitive inhibition of Ŭ-ketoglutarateï dependent (Ŭ-KGï dependent) dioxygenase enzymes (also known as 2-oxoglutarateï dependent dioxgenases) and posttranslational modification of proteins (Thompson, 2009; DeBerardinis and Thompson, 2012; Zhang et al., 2010). As resulted of hypoxia, the entry of pyruvate into the TCA cycle can be inhibited as well as the synthesis of citrate through this route. Under these conditions citrate need for lipid synthesis can be produced by the NADPH-dependent isoforms of isocitrate dehydrogenase (IDH1 and IDH2), through the reductive carboxylation of glutamine derived from Ŭ-ketoglutarate (Cardaci and Ciriolo, 2012). Consequently, the export of acetyl-CoA in the cytosol where can act as a building block for anabolic reactions that promote cell growth and proliferation is enhanced. In this manner, acetyl-CoA is also increasingly made available for mevalonate metabolism. It is well known that mevalonate pathway is involved in cholesterol biosynthesis as well as in protein prenylation and hyperactivity of this pathway is implicated in various aspects of cancer development and progression (Gruenbacher and Thurnher, 2014). In order to accumulate biomass during cell growth and proliferation, cancer cells not only increase glucose uptake need to produce intermediates for the synthesis of lipids, proteins and nucleic acids, but also enhance anaplerosis process through glutamine uptake and glutaminolysis, replenishing intermediates of the tricarboxylic acid (TCA) cycle which are redirected into

biosynthetic reactions (Miccheli et al., 2006). Not surprisingly that in addition to glucose, cancer cells are able to use other substrates to obtain energy such as glutamine that can be metabolized to nucleic acids, amino acids and lipids needed for cell proliferation. Using glutamine as a substrate, cancer cell can maintain viability and growth from energy generated through substrate level phosphorylation in the TCA cycle (Hensley et al., 2013). Energy obtained through this pathway could give the false impression of normal oxidative phosphorylation, as oxygen consumption and CO_2 production can arise from glutaminolysis and uncoupled oxidative phosphorylation. Increased glutaminolysis occurs in proliferating cancer cells when glycolysis energy production is not sufficient (Anso et al., 2013). This is the reason for which glutamine is often present in high concentrations in cancer cell culture media. With the emergence of modern molecular biology, research on cancer metabolism has mainly focused on the identification of oncogenes and tumor suppressors whose mutations have a significant role in cancer cellular metabolism. These researches have revealed that several oncogenic signalling drive the pathways responsible for the metabolic response of normal cells to tumor growth-promoting signals. Carcinogenesis process is characterized by a plethora of mutations affecting AKT1 (a serine/threonine kinase), p53 (a tumor suppressor protein), HIF, as well as mitochondrial enzymes succinate dehydrogenase (SDH) and fumarate hydratase (FH) that are undergo a loss-of-function with the consequent accumulation of succinate and fumarate. The accumulation of these metabolites inhibits enzymes required to degrade HIF and in turn cause HIF overespression (Upadhyay et al., 2013). Another oncogenic transcription factor is MYC, a regulator of several enzymes in glucose metabolism that plays an important role in glutaminolysis. In particular MYC, stimulates the glutamine transporter, indirectly regulating glutaminase (GLS), a

mitochondrial enzyme that converts glutamine to glutamate (Gao et al., 2009). Moreover, the activation of oncogene MYC also promote the alternative splicing of the pyruvate kinase gene PKM resulting in a change in expression of pyruvate kinase (PK) isoforms, from pyruvate kinase isozyme M1 towards isozyme M2. PK catalyzes phosphoenolpyruvate (PEP) conversion into pyruvate, a limiting step in glycolysis. In cancer cells, the glycolytic isoenzyme pyruvate kinase type M2 (M2-PK) is one key regulator of the tumor metabolome and it is generally over-expressed. M2-PK can occur in a highly active tetrameric form and in a nearly inactive dimeric form. In cancer cells, the dimeric form of M2-PK always predominates inducing the accumulation of glycolytic intermediates for biosynthetic processes and thus enhancing glutaminolysis process to produce energy (Cairns et al., 2011). The mutation of p53 is also involved in metabolic reprogramming of cancer cells. The tumor suppressor p53 is involved into maintain mitochondrial activity through the expression of cytochrome c oxidase 2 (Sui et al., 2011). The increase of its expression inhibits glycolytic activity and increases the availability of glucose-6-phosphate that entries into the oxidative via of the pentose phosphate pathway. Consequently, the production of ribose and NADPH for nucleotide biosynthesis is supported. During carcinogenesis, increased levels of fatty acids have been observed indicating that fatty acid synthesis is stimulated (Yoshii et al., 2014). In many tumors, lipids are involved in cell dislodgement, invasion, migration, and proliferation process. For example, choline, phosphocholine, phosphatidylcholine, and glycerophosphocholine are needed for cell wall synthesis and are increased in brain, breast, prostate and liver cancers (Ackerstaff et al., 2001; Glunde et al., 2004). Finally, sphingolipids have been also reported as tissue biomarkers of

cancer for their role in tumor growth and proliferation and they have been also used in anti-cancer therapy (Saddoughi et al., 2008).

"METABOLOMICS" AS POWERFUL TOOL TO STUDYING CANCER METABOLISM

The OMICS-tools, including genomics, transcriptomics and proteomics, have been largely adopted to understand molecular biological events contributing to the cancer development. However, gene and protein analysis are unable to define the chemical and metabolic events happening in cancer process. Recent development of metabolomics allowed detecting altered metabolic pathways in cancer cells offering a new powerful tool to investigate the cancer metabolism. Metabolomics is defined as the ñquantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modificationò (Nicholson et al., 1999). Since metabolites represent end products of gene expression and enzyme activities of organisms, analysing the metabolites can reveal the consequences of altered gene expression and enzymes activities, protein expression and signalling pathways. The metabolome is defined as the set of metabolites in a cell, tissue, organ or organism and reflects the dynamic and continuous fluxes of metabolic pathways. Through the study of metabolome, the simultaneous assessment of thousands of metabolites, the identification of the presence, concentration and fluxes of specific metabolites, and recognition of the critical metabolic pathways recruited in carcinogenesis can be evaluated. The elucidation of these metabolites and pathways may provide essential insights into both the intercellular environment and host/tumour interaction, allowing recognition of new biomarkers for diagnosis and prediction of outcome, new therapy targets and novel approaches for monitoring response and

toxicity (Bu et al., 2012). Dependent on instruments and experimental design, a metabolomic profile can be generated through targeted analysis of metabolites associated with a particular metabolic pathway or untargeted analysis of all detectable metabolite. Nevertheless the enormous complexity of data obtaining from metabolomics studies, a major advantage in the application of a metabolomics to study of cancer metabolism, consist in an improved capability to detect up to many hundreds of metabolites in parallel, providing an efficient method for monitoring altered cancer biochemistry. The main technologies or platforms, which have been developed to detect metabolites, are based on NMR spectroscopy and mass spectrometry. In particular, NMR spectroscopy is an analytical method commonly used to analyze the small molecule composition of cells, tissues or biofluids like urine, blood serum and saliva (McGhie and Rowan, 2012). Although its low sensitivity, it has the advantage of being a non-invasive and non-destructive technique relatively rapid, that generates spectra from a biological sample within about several minutes. Moreover, thought two-dimensional (2D) NMR spectroscopy it is possible improve metabolite specificity and solving overlapping signals. A particular subset of the metabolomics field focuses on 13C-NMR spectroscopy that consists in use of labeled substrates (e.g., ^{13}C labelled glucose) to define metabolic fluxes or biomarkers in disease states (Yang et al., 2014). This approach allows obtaining information on the metabolic processes involved in utilization of the 13C labeling metabolites and analysing isotopomer distribution of the different metabolite. For example, glucose can undergo glycolysis to lactate or be shunted through the pentose phosphate pathway to form ribose, and the 13C labelled carbons in glucose can reveal how much goes into each pathway. This information provides a better understanding of the pathways that are upregulated or downregulated and can define metabolic phenotypes in

disease states. The first step of a NMR-based metabolomics analysis consists in identifying and quantifying the largest set of metabolites, with relatively low molecular weight (up to ~1000 Da or less) based on their NMR chemical shifts. Successively, the spectral data are analyzed by pattern recognition methods. Generally, pattern recognition methods can be used for reducing the dimensionality of complex data sets, for example by 2D or 3D mapping procedures, thereby facilitating the visualization of inherent patterns in the data set. Alternatively, unsupervised and supervised algorithms can be used. Unsupervised methods find patterns in the data without any biases. An example of unsupervised method is principal components analysis (PCA), which allows the expression of most of the variance within a data set using a smaller number of factors or principal components. This method is useful for comparing pathological samples with control samples, but supervised algorithms that model each class individually are preferred when the number of classes is large. A common supervised method is partial least squares (PLS), which link a data matrix containing independent variables from samples to a matrix containing dependent variables (or measurements of response) for those samples. A relatively novel approach for the analysis of multivariate data is ANOVA-simultaneous component analysis (ASCA). In this method the parameter estimation aspect of ANOVA is merged with PCA or Simultaneous Component Analysis (SCA), such that the drawbacks of both methods are removed (Jansen et al., 2005). As a matter of fact, ANOVA is a univariate method used to determine the effect of different experimental factors on the variation in a dataset but cannot take the covariance between different variables into account. SCA can be seen as PCA for multiple matrices and is a widely used method that models the relationships between the different variables in a multivariate dataset by analyzing its covariance or correlation matrix. Unlike PCA

or SCA which does not take the experimental design into account, ASCA takes both the covariance between the multiple variables and the design of the experiment into account and is particularly useful when the significance of the effect of one or more factors on the experimental data can be evaluated (Smilde et al., 2005). In field of metabolomics applied on cancer studies, this approach may be useful when the effect of a substance such as natural compounds, drug or chemoagent on cancer metabolism can be evaluated. To date, nuclear magnetic resonance (NMR) metabolomics has already been successfully applied to study various cancers such as ovarian, breast, pancreatic, oral, esophageal, lung, prostate, bladder, and colorectal malignancies, playing a prominent role in early detection and diagnosis of cancer as well as in prediction of aggressiveness of a tumor and thus in the evaluation of medical interventions and therapies to cancer (Ben Sellem et al., 2011; Weljie et al., 2011; Bathe et al., 2011; Tiziani et al., 2009; Hasim et al., 2012; Carrola et al., 2011; Teahan et al., 2011; Cao et al., 2012; Chun et al., 2009; Farshidfar et al., 2012). Despite these evidences, there are several gaps in knowledge about the cancer metabolome. For example, among distinct tumor types, profiles vary with respect to many metabolites. Moreover, as early diagnosis and prevention are the key factors in reducing the mortality of cancer, the most scientific efforts in chemoprevention are devoted to identified bioactive compounds such as phytochemicals and overall to characterizing underlying mechanisms of their anticarcinogenic activities. However, the biotransformation pathways and metabolic effects of chemopreventive phytochemicals on cancer metabolism have been further elucidated.

DIETARY PHYTOCHEMICALS IN CANCER: FROM HORMETIC BEHAVIOUR TO MOLECULAR TARGETS

Throughout the history, nature, especially higher plants, has provided a source of medicines for the treatment of a wide spectrum of diseases. In the cancer field, plant-derived agents, such as vinblastine and vincristine, etoposide, paclitaxel (Taxol), docetaxel, topotecan, and irinotecan, are among the most effective cancer chemotherapeutics currently available demonstrating that plants are the forefront of natural product drug discovery (Cragg et al., 2014). In the food area, a plethora of population-based studies, indicate that people who eat about five servings of fruit and vegetables a day have approximately half the risk of developing cancer of those who eat fewer than two servings. Vegetables and fruit are excellent sources of cancer preventive phytochemicals and about numerous plant-based foods that possess cancer-preventive properties have been identified. Phytochemicals are defined as bioactive non-nutrient plant compounds in fruits, vegetables, grains and other plant foods that possess substantial therapeutic potential, including anticarcinogenic and antimutagenic properties linked to reducing the risk of major chronic diseases, including cancer (Liu, 2004). The representative chemopreventive phytochemicals and their dietary sources include curcumin (turmeric), resveratrol (grapes), diallyl sulphide (garlic), genistein (soybeans), [6]-Gingerol (ginger), lycopene (tomatoes), epigallocatechin-3-gallate (green tea), sulphoraphane (broccoli), indole-3-carbinol (cabbage), caffeic acid phenethyl ester (honey) and many others carotenoids (Ŭ-Carotene, ɓ-Carotene, Lutein, Zeaxanthin), phenolic compounds such as phenolic acids (gallic, ferulic, sinapic, p-coumaric, protocatechuic), flavonoids (quercetin, kaempferol, luteolin, apigenin, naringenin, genistein), stilbens, coumarins and tannins, alkaloids, nitrogen-containing compounds and organo-sulfur compounds. Although > 5000 phytochemicals have been identified, the action mechanisms by which exert their protective function against cancer have not yet fully discovered

or clarified. Moreover, it should be considered that many plant food derived phytochemicals show a hormetic behavior. The term ñhormesisò has been largely used by toxicologists to describe the phenomenon in which a specific chemical compound is able to induce a biphasic response at different doses. Generally, there is a stimulatory or beneficial effect at low doses and an inhibitory or toxic effect at high doses (Son et al., 2008). Recently, the concept of hormesis has been adopted in the fields of biology, medicine and plant food science to describe the stimulatory response of cells and organisms to a compound, or physical agent, at low doses, and the inhibitory response to the same agent at higher doses. In context of plant food, several phytochemicals show a hormetic behavior but the majority of these have not yet been investigated for their ability to exert a biphasic action in cancer cells. An example of phytochemical with hormetic behavior is retinoic acid (vitamin A) which in low amounts is essential for normal development and eye function, but in high amounts can cause anorexia, headache, drowsiness, altered mental states (Hayes, 2007). In colon cell lines HCT-116 and HT-29, the flavonol quercetin shows a biphasic modulation of cell proliferation, while on adenocarcinoma cell lines MCF-7 it promotes cell proliferation in the whole concentration range used (Van der Woude et al., 2003). All plant food derived phytochemicals should be systematically investigated *in vitro* to evaluate a hormetic like doseï response effect in relation to carcinogenesis, thus their safety and effectiveness in regard to cancer prevention. During the last years, there has been substantial progress in identifying the biochemical events that are associated with the multistage process of carcinogenesis, and now we are better aware of how certain dietary phytochemicals are able to alter this process. Many of the cellular and molecular events regulated by phytochemicals occur in cell-signaling

pathways that regulate cell-cycle proliferation, differentiation as well as apoptosis. It is well known that some phytochemicals ―switch on‖ or ―turn off‖ the specific signaling molecules, depending on the nature of the signaling cascade they target, preventing abnormal cell proliferation and growth. Among the most common phytochemicals derived from plant food, curcumin (diferuloylmethane or 1,7-bis-(4-hydroxy-3-methoxyphenol)- 1,6-heptadiene-3,5-dione, CUR), the major active compound present in extracts from the rhizome of *Curcuma longa* (Zingiberaceae), is the best known (Figure 1).

Curcumin has been used for centuries in traditional medicines and as a spice it provides curry with its distinctive colour and flavour. Extensive researches have shown that it plays an important role in the prevention and treatment of various pro-inflammatory chronic diseases, including neurodegenerative, cardiovascular, metabolic, autoimmune disease and cancer (Chattopadhyay et al., 2004). In the oncology field, curcumin has been studied for its anti-proliferative, anti-inflammatory, anti-angiogenic and pro-apoptotic effects in various tumor types *in vitro* (Wilken et al., 2011). In order to enhance the therapeutic potential of curcumin, this agent has been modified or used in combination with other agents in *in vitro* cancer models (Zhou et al., 2014; Malhotra et al., 2014). It is a highly pleiotropic molecule with the potential to modulate the biological activity of a number of signaling molecules. In cancer, a central role in cell proliferation and survival against anticancer treatment is played by nuclear factor-kB (NF-kB), Akt, and their downstream cascades that can lead to the upregulation of antiapoptotic Bcl-2 proteins. Curcumin can modulate these signals by inhibiting the NFkB pathways at multiple levels (Shishodia et al., 2007). For example, it has been demonstrated inhibit NF-kB in a human myeloid leukaemia cell line by blocking phosphorylation and subsequent degradation of IəB

(Han et al., 2002). Curcumin has also a role in reactive oxidative stress (ROS). ROS has opposing effects on cancer: it can be an insult causing DNA mutations in carcinogenesis, and it can also drive mitochondrial apoptosis. Reducing DNA insult by scavenging ROS is important for the prevention of cancer, while generating ROS to drive mitochondrial apoptosis is more important when treating malignancies. Curcumin was shown to induce an important ROS scavenging enzyme hemeoxygenase-1, a redoxsensitive inducible enzyme, via nuclear factor 2-related factor (Nrf-2) regulation, a master regulator of cellular antioxidant defense systems. Curcumin is a ROS scavenging enzyme inducer but, it also uses ROS to kill cancer cells. ROS generated by curcumin in JURKAT cells induce apoptosis disrupting the redox balance, and in human renal Caki cell downregulating Bcl-xl and inhibitors of apoptosis proteins (IAP) (Woo et al., 2003). The activation of Nrf2 cell-signaling pathways leads to the down regulation of adipokines, including alpha-tumor necrosis factor (TNF-Ŭ), interleukin (IL)-6, resistin, leptin, and monocyte chemotactic protein-1, and the upregulation of adiponectin. Other molecular targets of curcumin include antioxidant enzymes (glutamate cysteine ligase, GCL), cyclooxygenase-2 (COX-2) and phospholipase A2 (PLA2). Curcumin induce an increase of the level of cellular GSH and induces de novo synthesis of GSH in quiescent hepatic stellate cells (HSC), the most relevant cell type for hepatic fibrogenesis, by stimulating the activity and gene expression of glutamate-cysteine ligase (GCL), a key rate-limiting enzyme in GSH synthesis (Zheng et al., 2007). The enzyme PLA2 is responsible for the hydrolysis of membrane phospholipids that release arachidonic acid, which serves as a substrate for pro-inflammatory mediators, such as prostaglandins and leucotriens. Aberrant arachidonic acid metabolism is involved in the inflammatory and carcinogenic processes. In HT-29 human colon cancer cells,

curcumin blocks the release of arachidonic acid and its metabolites, inhibiting the phosphorylation of PLA2 and thus the activation process of this enzyme as well as decreasing the expression of COX-2 that catalyzes the conversion of arachidonic acid to prostaglandins (Hong et al., 2004). Curcumin was shown to upregulate p53 expression followed by an increase in p21 resulting in cell-cycle arrest at G0/G1 and/or G2/Mitosis. This is followed by the upregulation of Bax expression, which induces apoptosis (Choudhuri et al., 2002). In p53 mutant or knockout ovarian cancer cell lines, curcumin induced p53-independent apoptosis which involved p38 mitogen-activated protein kinase (MAPK) activation and inhibited Akt, resulting in decreased expression of Bcl-2 and survivin (Watson et al., 2010). Generally, the beneficial effects of curcumin in cancer are challenged by the fact that many drugs kill tumor cells through induction of oxidative stress and the activation of the anti-oxidant response of curcumin is considered to be at the origin of most beneficial effects of this phytochemical in the prevention and relief of oxidative stress-associated diseases. In this sense, curcumin show a hormetic behavior exerting anti- and pro-oxidant effects in response to a number of cell stressing agents. Because there is much expectation on the use of curcumin in cancer treatment, there is a need for identifying metabolic biomarkers of the response and improving knowledge on the action mechanisms. Another common phytochemical derived from plant food is resveratrol (3,4ô,5-trihydroxy-*trans*-stilbene), a phytoalexin present in grapes, red wine, peanuts and certain berries (Figure 2).

Resveratrol was first isolated and identified in 1940 by Michio Takaoka from the dried root of white hellebore (*Veratrum grandiflorum*) but the interest for this phytocemical burst in 1990 when it was suggested that resveratrol was associated with the ñFrench paradoxò a term coined

to describe the observation that French people had a relatively low incidence of coronary heart disease mortality despite a diet rich in satured fat. This unexpected low mortality has been attributed to the regular consumption of red wine, in line with epidemiological studies suggesting that a moderate intake of red wine reduced the risk of cardiovascular disease (Renaud and De Lorgeril, 1992). In recent years, the interest in this molecule has increased nearly exponentially following the major findings that is chemopreventive in different type of cancer such as breast, colorectal, liver, prostate and pancreatic cancer (Widlund et al., 2013). However, therapeutic application of these beneficial effects remains very limited due to its short biological half-life, labile properties, rapid metabolism and elimination. For this reason, different studies were undertaken to obtain synthetic analogs of resveratrol with major bioavailability and anticancer activity (Chimento et al., 2013). Moreover, the exact mechanisms through which resveratrol exerts a wide range of effects is currently still unclear. Indeed, the effects of resveratrol represent a ótwo-edged swordô in that it has contrasting effects at low and high doses. At low doses, it appears to stimulate the proliferation of healthy and cancer cells and has therapeutic effects on healthy cells. High doses, were, however, found to be able to inhibit proliferation of cells, healthy or not, so highlighting the potential of resveratrol as a new treatment for cancer. Many different molecular mechanisms of action of resveratrol, strictly dependent on cell type or organ and on experimental conditions, as well as on dose and treatment, have been shown to influence cell metabolism with a long list of involved up or down regulated target genes and activated or suppressed factors /cofactors in cancer. It has been proposed that resveratrol modulates the phosphatidylinositol-3 kinase (PI3K) pathway, which in turn mediates cell proliferation and apoptotic pathways. PI3K activity is enhanced at low

concentrations of resveratrol (10 mM) while being inhibited at concentrations >50 mM. Resveratrol affects many different targets related to mTOR. mTOR is an conserved serine/threonine kinase that integrates signals from growth factors, nutrients and stress factors and controls multiple downstream processes, including mRNA translation, lipid and nucleotide synthesis, cell-cycle progression, autophagy and the shape and survival of cells (Sarbassov et al., 2005). It represents one of the major growth and survival pathways that is dysregulated in many human cancers and contributes to cancer a central controller of cell growth, proliferation, metabolism and angiogenesis. It is found that resveratrol inhibit the activity of the mTOR pathway proteins, and to activate AMP, which can suppress mTOR signalling (Widlund et al., 2013). It is known that the expression of matrix metalloproteases (MMPs) correlates with tumor invasion and metastasis. In this context, resveratrol reduced the expression of MMPï 2 and MMPï 9 in mammary cancer and decreased the level of vascular epithelial growth factor (VEGF), a protein crucial for angiogenesis and maintaining tumor growth, thus inhibiting angiogenesis (Banerjee et al., 2002; Zhang et al., 2005). Resveratrol can inhibits key proteins in signal transduction pathways such as mitogen-activated protein kinases, activator protein-1 (AP-1), and NFəB. Kuo et al. (2002) examined the anti-proliferative effects of resveratrol in two human liver cancer cell lines, namely HepG2 and Hep3B. The results showed that resveratrol inhibited cell growth only in p53-positive HepG2 cells, which was a result of cellular apoptotic death via p53-dependent pathway. It was also shown that resveratrol-treated cells were arrested in G1 phase and were associated with an increase in p21 and Bax expression. Overexpression of anti-apoptotic Bcl-2 has been associated with elevated cyclooxygenase-2 (COX-2) expression and resveratrol was shown to suppress COX activity (Khanduja et al., 2004). In particular,

resveratrol binds to COX-2 directly inhibiting its enzyme activity, and the COX-2-mediated PGE2 production, in human colon adenocarcinoma cells *in vitro* and *ex vivo* (Bishayee, 2009). More recent research on the molecular mechanisms by which resveratrol might exert many of its biological effects has emphasized the importance of its interaction with sirtuins. In this context, resveratrol increase the deacetylase activity of Sirtuin 1 (SIRT1) and enhance SIRT-1 dependent metabolic processes both *in vivo* and *in vitro*. SIRT1 is a genetic modulator that is part of the health-promoting pathway that is activated by calorie restriction. Increased SIRT1, in turn, inhibits expression and/or activity of several oncogenes, leading to reduced cell proliferation, increased apoptosis, and tumor suppression (Deng, 2009). Resveratrol also activates MAPK at low concentrations (1 pM to 10 ε M), but higher concentrations (50ï 100 ε M) of resveratrol can inhibit this signal transducing kinase in cancer cells (Miloso et al., 1999). It has been shown that resveratrol induces ERK1/2 activation in prostate, breast, head and neck cancer cells (Lin et al., 2002; Tang et al., 2006; Nguyen et al., 2008; Lin et al., 2008). Although gene and protein expression in cancer cells after resveratrol treatment have been extensively studied, the knowledge about metabolic alterations caused by resveratrol is still limited. To date, only a few studies by NMR-based metabolomics approach have been performed to evaluate the ability of dietary phytochemicals to modulate cancer metabolism. However, a better understanding of the cellular effects exerted by dietary phytochemicals is vital to property utilize such compounds as promising agents that promote health and that can be used as biomarker to prevent cancer.

NMR-BASED METABOLOMICS OF CANCER CELLS: UNDERSTANDING THE ROLE OF PHYTOCHEMICALS

The importance of cancer cell lines as in vitro models in cancer metabolism analysis

Cancer cell lines have been widely used for research purposes to investigate genetic, epigenetic and cellular pathways involved in cancer process, for the study of proliferation deregulation, apoptosis and cancer progression. Moreover, they have been used to define potential molecular markers and for the screening and characterization of cancer therapeutics. There are numerous advantages for the use of cancer cell lines as an experimental model for the study of cancer. In fact, cancer cell lines are pure populations of tumor cells and they represent these cells without the complexity of the *in vivo* environment due to the stromal and inflammatory cells. They maintain the tumor-specific chromosome abnormalities, show the same morphologic and molecular characteristics of the primary tumor and, in general, maintain the expression of the ñhallmarks of cancerò. Moreover, they are easy to handle and manipulate, own a high homogeneity and a high degree of similarity with the initial tumor as well as an unlimited auto-replicative ability. To date, there is a high variety available of cancer cell lines that have performed their largely use. For this reason, cancer cell lines emerge as an excellent model for the study of the biological mechanisms involved in cancer (Vargo-Gogola and Rosen, 2007; Van Staveren et al., 2009). Among various cancer types, breast cancer is the most frequent cancer of women (23% of all cancers), with an estimated 1.15 million new cases. The incidence of the disease is increasing in industrialized countries due to the increased presence of estrogenic compounds in the environment, often associated to co-morbidities such as diabetes and obesity and in particular, the incorrect diet. A considerable part of our knowledge on breast carcinomas is based on *in vivo* and *in vitro* studies of breast cancer cell lines. Comparative metabolic profiling of breast cancer and normal cells has improved our understanding of the fundamental

mechanisms of carcinogenesis and opens new opportunities in target and drug discovery. Recently, ^{1}H-NMR based metabolomics was used to examine the effects of hypoxia in the MDA-MB-231 human breast cancer cell line (Weljie et al., 2011). In particular, by means orthogonal partial least squares discriminant analysis (OPLS-DA) a set of metabolites responsive to hypoxia has been identified. These included primarily energy metabolites and amino acids, indicative of known alterations in energy metabolism, and protein synthesis or catabolism. Two of the more interesting findings from the study were that pyruvate level was significantly altered and lactate level was not significantly different in the cell culture media under hypoxic conditions. A metabolite whose concentration was reduced was 2-oxoglutarate. It is a key metabolite in the citric acid cycle of oxidative phosphorylation in mitochondria, a process that is reduced under hypoxic conditions, but also as a result of altered HIF-1-regulation during aerobic glycolysis. Another common human cancer worldwide is hepatocellular carcinoma. The liver is one of the most important organs associated with digestion, detoxification, production and storage, so the liver has a high metabolic rate, and therefore liver diseases including hepatocellular carcinoma are associated with metabolic disorders (Smith, 2013). The exact molecular mechanisms of hepatocellular carcinoma and effective prevention and treatment are still unclear. Immortalized liver-derived cell lines were proposed as an ideal model to study cancer liver metabolism because of their unlimited availability and phenotypic stability. Recently, a quantitative two-dimensional high resolution magic angle spinning (HRMAS) proton-NMR spectroscopy-based metabolite profiling has been applied in HepG2 cancer cell. HepG2 phenotype was characterized by high levels of glutathione, high levels of creatine, low levels of free amino acids, increased levels of phospholipid derivatives (mostly phosphocholine),

and lower lactate content in cell lines with the higher proliferation rate (Bayet-Robert et al., 2010).

Effect of dietary phytochemicals in breast cancer cell lines

To date, only a few attempts have been performed to study the effect of phytochemicals on the breast cancer cell metabolism by NMR-based metabolomics approach. Interestingly, Bayet-Robert and Morvan (2013) have applied ^{1}H-NMR based metabolomics to investigate the response of MCF7 and MDAMB-231 breast cancer cells to increasing doses of curcumin. MCF7 and MDAMB-231 breast cancer cells differ by their expression of hormonal (estrogen and progesterone) receptors (only MCF7 cells express these receptors and are sensitive to hormonotherapy). In both cell types, the major metabolic targets of curcumin at low and high dose are glutathione (GSH) and lipid metabolism. By multivariate statistical analyses such as PCA and PLS-DA, metabolic transitions between low and high doses of curcumin treatment have been revealed evidencing a biphasic or hormetic behavior of metabolites involved in GSH and lipid metabolism. In particular, GSH accumulates at low doses (0.5 to 10 mg/l) and decrease at high doses (25 and 50 mg/l). As is well known, GSH plays a central role in cell protection against oxidative stress as a cofactor of GSH peroxidases and glutamate-cysteine ligase (GCL), the main regulatory enzyme of GSH synthesis. At low dose, curcumin treatment induces an upregulation of GSH biosynthesis and related metabolites including homocystein (Hcy), creatine and taurine, through activation of GCL and these data suggest an activation of transsulfuration process, as confirmed by the decreased levels of Hcy (Bayet-Robert et al., 2013). Trans-sulfuration process plays an important role in glutathione homeostasis and is regulated by

cystathionine beta-synthase (CBS). In response to dramatic requirement for GSH, CBS pathway is recruited. At high dose, GSH levels decrease probably due to an exhaustion in GSH precursors and to the reactivation of glutathione S-transferase (GST). GST enzymes belong to a family of multifunctional detoxification proteins that protect cells from electrophilic compounds. Generally, in cancer cells GSTs are normally overexpressed and involved in multidrug resistance whereas in breast cancer cells, GST activity has a biphasic behavior decreasing at low doses and increasing at higher doses. Also free fatty acids accumulate at high curcumin dose only, in both MCF7 and MDA-MB-231 cells, demonstrating that lipid metabolism is another target of curcumin in breast cancer cell. At high dose (25 and 50 mg/l) total fatty acid and polyunsatured fatty acids dramatically increase while glycerophosphoethanolamine (GPE) and glycerophosphocholine (GPC). GPE and GPC are two byproducts of phospholipid metabolism originating from activity of phospholipase A2 (PLA2). In this way, PLA2 is submitted to a downregulation probably as a means for the cell to limit release in the cytosol of membrane-originating fatty acids and thus propagation of oxidative stress. The accumulation of PUFA and total FA at high dose curcumin is associated to the inhibition of cyclooxygenase-2 (COX-2) whose activity is downregulated by curcumin also in HT-29 colon cancer cells (Lee et al., 2009). Another metabolic biomarker of the response of breast cancer cells to curcumin is gluconate (Gna), a product of glucose oxidation and metabolic biomarker of that ROS production crush tumor cell defences arousing the drop in DNA content, cell cycle arrest and cell death (Morvan, 2013). In Figure 3, a schematic representation of the main effects of curcumin on cancer cell metabolism is reported.

With the aim to discover novel biomarkers for the response of breast cancer cell to phytochemicals, a targeted metabolomics approach based on high-throughput liquid chromatography coupled to mass spectrometry has been used to investigate the effect of resveratrol on human breast cancer cell lines MCF-7 and MDA-MB-231 metabolism. Jäger W et al. (2011) have observed that in both cell lines, resveratrol strongly interacts with metabolism of biogenic amines metabolism, increasing the synthesis of serotonin, kynurenine, and spermindine in both cell lines up to 61-fold. Moreover resveratrol significantly stimulates the aminoacid metabolism increasing their levels more than 100-fold at a resveratrol dose of 100 µM corresponding to its daily intake as beverage (red wine) or as dietary supplement. In particular, resveratrol induce the synthesis of branched chain amino acids (BCAA), sulfur-containing amino acids such as methionine, glycogenic amino acids, urea cycle amino acids and others that are released from the cytoplasm into the medium. The changes in tryptophan, serotonin and kynurenine levels indicate that enzymatic conversion of tryptophan to the bioactive metabolite serotonin through tryptophanhydroxylase (TPH) and kynurenine through tryptophan-2,3-dioxygenase (TDO) and kynurenine monooxygenase (KMO) is stimulated. In various diseases that are associated with cellular immune activation, decreased tryptophan levels together with increased kynurenine to tryptophan ratio (Kyn/Trp) ratio are found. In brain tumors, the kynurenine pathway is the principal route of tryptophan catabolism leading to the formation of the essential pyridine nucleotide, nicotinamide adenine dinucleotide, and important neuroactive metabolites, including the neurotoxin, quinolinic acid, the neuroprotective agent, picolinic acid, the TH17/Treg balance modulator, 3-hydroxyanthranilic acid (3-HAA), and the immunosuppressive agent, kynurenine (Adams et al., 2012). Treatment of breast cancer cell lines

with resveratrol has also an effect on polyamine metabolism stimulating the synthesis of putrescine and its conversion to the spermidine and spermine suggesting thus an activation of ornithine decarboxylase (ODC) and spermidine synthase. Putrescine, sperimidine and spermine are metabolically active polyamines essential for a variety of cellular processes related to signal transduction such as the induction of cell death (Takao et al., 2006). The conversion of putrescine to the metabolically active polyamines spermidine and spermine occurs early during cell proliferation and it is mediated by S-adenosylmethionine decarboxylase, the rate-limiting enzymes of polyamine biosynthesis. Polyamines are mainly involved in the regulation of gene expression by altering DNA structure and by modulating signal transduction pathways (Igarashi and Kashiwagi, 2010). These metabolites regulate important cellular processes including cell proliferation and viability (Gerner and Meyskens, 2004). Hence, intracellular polyamine levels are homeostatically maintained by processes involving biosynthesis, catabolism and transport (Linsalata and Russo, 2008). Alterations in polyamine homeostasis lead to changes in intracellular polyamine pools that have important implications for cell growth. As previously mentioned, cancer cells metabolism is characterized by changes in fatty acid levels. High doses of resveratrol induce an increase in extracellular arachidonic acid and its metabolite 12S-HETE through the action of 12-lipoxygenase, suggesting the release of arachidonic acid from cell membrane phospholipids upon activation of phospholipase A2. Increased levels of 12S-HETE may therefore indicate oxidative stress in tumor cells under resveratrol treatment (Nazarewicz et al., 2007). Finally, resveratrol also reduces prostaglandin E2 (PGE2) levels, confirming that this polyphenol is an inhibitor of cyclooxygenase 2 (Murias et al., 2004). In Figure 4, a schematic representation of the main effects of resveratrol on cancer cell metabolism is reported.

Effect of dietary phytochemicals in hepatocellular cancer cell lines

To date, the alone metabolomic study based on ^{1}H-NMR spectroscopy and multivariate analysis used to evaluate the effect of resveratrol on hepatic cancer cells belongs to Massimi et al. (Massimi et al., 2012). In this work, the global metabolic effect of resveratrol has been studied on exometabolome of HepG2 cells, an actively proliferating human hepatoblastoma line that exhibits many features specific to human differentiated hepatocytes. Exometabolome reflects the cell physiological state and thus its metabolome in terms of substrate utilization and production, as well as substrate flux distribution. By means PLS multivariate analysis, a discrimination between control and resveratrol treated cancer cell cultures characterized in the latter by a decreased glucose and amino acid utilisation for energy production, with succinate replenishing the Krebs cycle as an anaplerotic substrate has been revealed. In particular, resveratrol mimics an energy-restricted state characterized by a decreased glucose uptake and lactate production with suppression of AKT and mTOR signaling as recently observed in human ovarian cancer cells (Kueck et al., 2007). AMPK acts as a cellular energy and glucose sensor, phosphorylating and inactivating acetylcoenzyme A carboxylase-2 (ACC2) which produces malonyl-CoA, an inhibitor of fatty acid uptake by mitochondria via the carnitine palmitoyltrabsferase system. Using labelling substrates such as [U-$^{13}C_{18}$] oleate, fatty acid utilization by cancer cell can be evaluated. From higher percentage fractional enrichment of C4 and C5 carbon atoms of glutamate, Massimi et al. have evaluated by ^{13}C-NMR the contribution of acetyl-CoA, formed thought the mitochondrial beta oxidation of oleate, to the synthesis of ketoglutarate in the krebs cycle and suggested a shift from glycolisis to fatty acid ϐ-oxidation to produce energy. The results have shown that after resveratrol treatment, HepG2 cells utilize less

valine, isoleucine, lactate, succinate, glutamine, ornithine, glucose, tyrosine, and consume more succinate, hystidine and phenylalanine. Moreover, it has been demonstrated that resveratrol treatment induces an increase of anaplerotic role of succinate which is utilised in TCA cycle. It has been proposed that significant correlation between succinate and lactate that is released through glycolysis can be associated to an activation of Sirt3 in the mitochondrial subcellular compartment in resveratrol treated cancer cells.

CONCLUSIONS AND FUTURE PERSPECTIVE

NMR-based metabolomics applied to cancer cell lines studies have yielded novel insights on the impacts of dietary phytochemicals exposures on cancer metabolism, revealing their ability to modulate intracellular targets and signalling pathways. Up to date, the effects of curcumin and resveratrol have been tested only on metabolism of breast and hepatocellular cancer cell lines by NMR-based metabolomics approach. It would be desirable to extend these analyses to other cancer cell lines. Given the great deal of attention for dietary phytochemicals from the scientific community due to their demonstrated ability to affect the mechanisms of cancer development, further investigations should be performed to test the effects of a wider range of phytochemicals derived from plant food on cancer metabolome.

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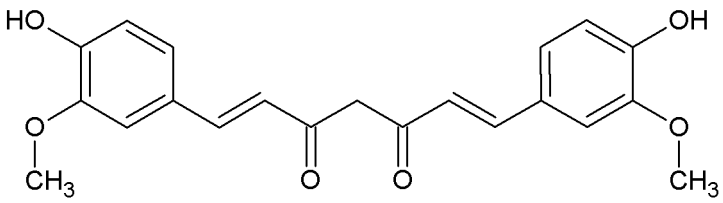

Figure 1 Chemical structure of curcumin.

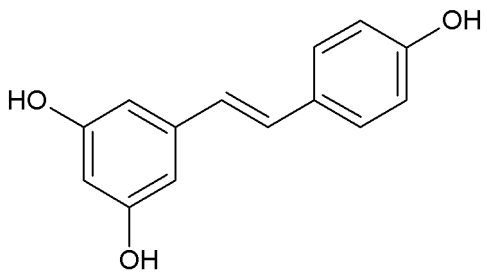

Figure 2 Chemical structure of resveratrol.

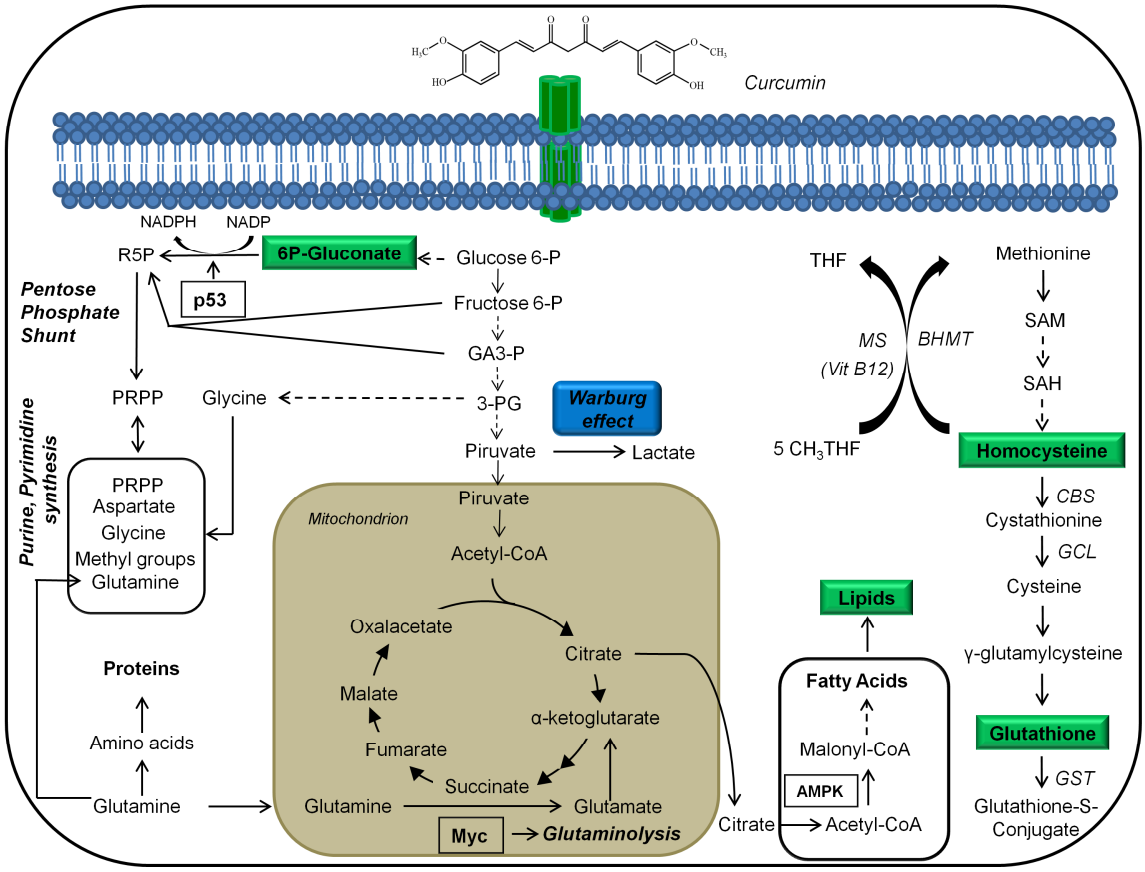

Figure 3 Schematic representation of effects of curcumin on cancer cell metabolism. The changed metabolites after curcumin treatment are represented by green boxes. Solid arrows in the network diagram specify a single step connecting two metabolites; dashed arrows indicate at least two steps; dashed arrows indicate at least two steps. R5P (Ribose 5-posphate), PRPP (Phosphoribosyl pyrophosphate), GA3P (glyceraldehyde 3- phosphate), 3PG (3-phosphoglycerate), THF (tetrahydrofolate) *MS* (methionine synthase), *BHMT* (betaine-homocysteine methyltransferase), SAM (S-adenosylmethionine), SAH (S-adenosylhomocysteine), *CBS* (cystathionine-ɓ-synthase) *GCL* (glutamate cysteine ligase), *GST* (glutatione S-transferase).

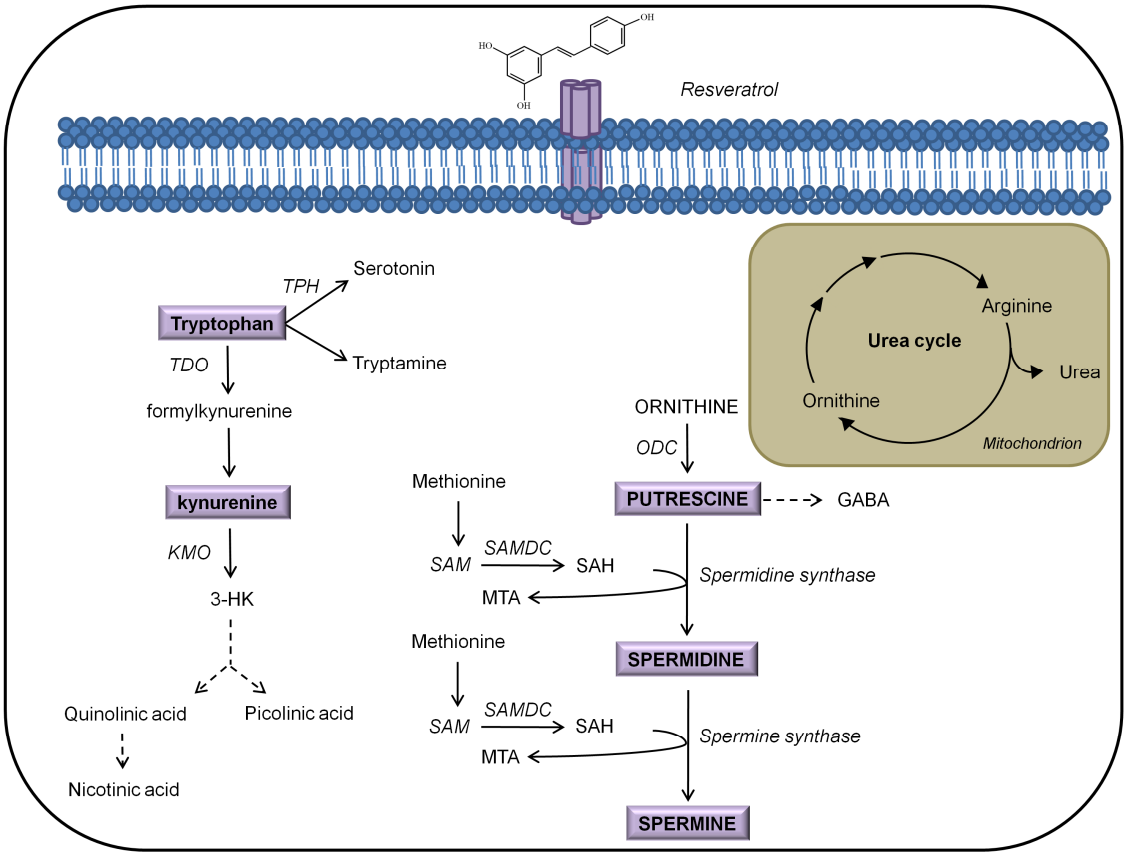

Figure 4 Schematic representation of effects of resveratrol on cancer metabolism. The changed metabolites after resveratrol treatment are represented by violet boxes. Solid arrows in the network diagram specify a single step connecting two metabolites; dashed arrows indicate at least two steps; dashed arrows indicate at least two steps. TDO (tryptophan 2,3-dioxygenase), TPH (tryptophan hydroxylase), KMO (kynurenine 3-monooxygenase), 3-HK (3-hydroxykynurenine), SAM (S-adenosylmethionine), *SAMDC* (S-Adenosylmehtionine decarboxylase), SAH (S-adenosylhomocysteine), MTA (5'-methylthioadenosine), ODC (ornithine decarboxylase), GABA (gamma-aminobutyric acid).