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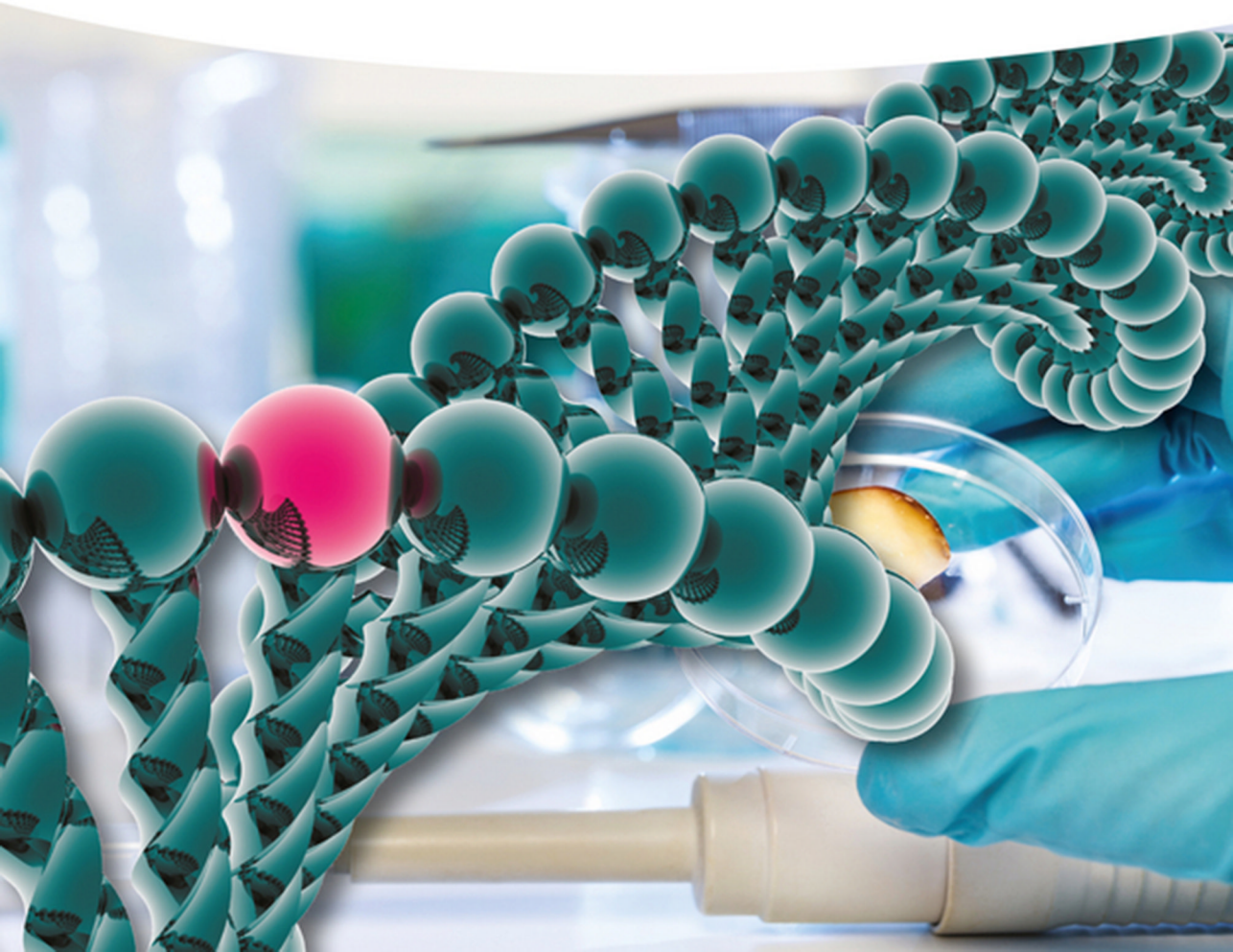
Biomarker Validation

Technological, Clinical and Commercial Aspects



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Preface

The term biomarker is a very general one and covers a wide range that includes cells, genes, proteins, hormones, or lipids. The official definition of a biomarker by the NIH (National Institutes of Health, USA) is "A characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention."

Complex organ function or characteristic changes in biological structures can be used as medical indicators/biomarkers, for example, for patient stratification and prediction of the response to a treatment. In recent years, the interest in the pharmaceutical industry in the exploration of biomarkers was increased, and many small and medium enterprises were founded. This is reflected by an over 10-fold increased number of clinical trials with biomarkers in the recent years. Despite intensive research efforts and huge amounts of money that have been invested by research institutes and pharmaceutical industry as well as by governments, many complex diseases, such as AIDS or cancer, are not or (too) late diagnosable or even treatable.

The process of biomarker validation and development includes the discovery phase, clinical study design, qualification, verification, assay optimization, clinical validation, and commercialization of the biomarker(s). The present book deals with all these different aspects and examples of current methods and concepts are given. A complete overview of all methods used today to identify biomarkers and the different kinds of biomarkers that might contribute to better diagnosis and treatment is not the focus of the book. The book rather sheds some light on how biomarkers are validated today, what the common hurdles are, and what can be learned from many years/decades of biomarker research and validation. The book is divided into different sections, addressing among others, technologies for biomarker discovery, clinical trials, and bioinformatics. Finally, future opportunities and trends of biomarkers and the challenges in the future are discussed.

We think these different sections deal with the essential aspects of biomarker validation and provide trustworthy data.

This book contains contributions from experts in different fields and from universities as well as research institutes to make sure that varying viewpoints are presented.

We want to thank Wiley-VCH-Verlag for giving us the opportunity to write this book. Furthermore, the editors thank the project coordinators Dr. Frank Weinreich and Dr. Gregor Cicchetti.

We also want to acknowledge the authors for distributing their chapters and spending their time in preparing interesting articles.

Finally, we want to thank all colleagues who made this book possible.

In conclusion, we hope that you will find this book inspiring literature and gain useful knowledge about biomarkers.

Sarah Schumacher
Harald Seitz

1
Biomarkers – Past and Future

Siegfried Neumann

1.1
Introduction

Biomarker is a term used for a characteristic property that can be precisely measured and objectively validated. Biomarkers serve as indicators of biological processes in health, disease or disease stages, or in the body's response to a therapeutic intervention. "Biomarker" is relatively new as a buzz word as it was recently coined in conjunction with the advent of molecular analysis in research programs and in exploratory medical diagnosis. However, quantitative data on the characteristics of physiological reactions in relation to functional changes and on molecular analytes in blood, serum, or other body fluids have been used in research and laboratory medicine for long, for example, some since the beginning of the preceding century.

Key advances in analytical instrumentation and its development for analytical precision and sensitivity revolutionized our knowledge of molecular and cellular biology of body functions. On the other hand, discoveries in basic biology science continuously pushed the demands on increasing the resolution power of technologies. This is true for science and technology in the high-speed sequencing of whole genomes and their transcription profiles, for the differentiation of protein patterns in a biological sample toward unprecedented borders of resolution, and for high-resolution submicroscopic cellular imaging. The convergence of many disruptive developments in instrument-based analytical precision with scientific discoveries in the molecular universe of genomic DNA, various types of RNA molecules as their transcripts, and proteins as the translation products led to the elucidation of crucial molecular interactions and allowed modeling of regulatory networks. In the field of malignant diseases, new paradigms for the molecular biology of pathogenetic processes lead to a deeper understanding of pathophysiological mechanisms working in the onset and spread of cancer.

As a consequence, one can expect that in medical practice the classification of diseases and the rules and decisions on their treatment will see dramatic changes in the near future. In a variety of diseases, the canonical definitions based on clinical symptoms, for example, a descriptional classification, and treatment based on

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an afflicted organ will both change toward molecular diagnosis, altered molecular markers, and a targeted intervention directed toward causative mechanisms that drive a pathological process. In the best case, identification of those molecular drivers can lead to early intervention and also provide options for prophylaxis.

In biomedicine, there are an ever-increasing number of new molecular markers for metabolic disorders, cardiovascular diseases, neurological disorders, chronic inflammatory diseases, and cancer. Accumulating data from molecular diagnostics offer physicians various complex marker panels to stratify patients for subtypes of a disease, evaluate the disease stage, and adjust their therapy regimen accordingly. This direction is now overarchingly termed as "*precision medicine*." Such a research-driven direction in medicine is nourished by activities in laboratory medicine, *in vivo* diagnostics for body/tissue imaging and cytology, and histology research by pathologists. This short overview illuminates the technologic status of biomarkers through selected examples, for example, with a main focus on oncology, and tries to anticipate where the developments on biomarkers might go in the coming years.

1.2 Definitions of Biomarkers

In 2001 the Biomarkers Definitions Working group termed "*biomarker*" as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention [1].

In a similar understanding the term "*cancer biomarker*" was described as a "biomarker that is present in tumor tissue or serum and includes many different molecules, such as DNA, mRNA, or proteins: Tumor biomarkers are measured in tumor tissue and, tumor DNA biomarkers are measured from tumor tissue" [2]. In addition, there is now evidence that a tumor biomarker may also become delineated from the titer and genotype of circulating tumor cells [3] and from the circulating tumor cell DNA.

Biomarkers signal differences in biological states. The many potential applications of using biomarkers also include detection of pharmacologic profiles in a given group of probands, for instance, for detecting kidney toxicity after drug exposure in clinical trials with novel drugs or after the approval of drugs [4]. The term biomarker is not restricted by how the marker is measured. Thus, a valuable quantitative data profile may be obtained by means of a physical, optical, or enzyme assay, immunochemistry, mass spectrometry, cytological and histochemical analyses or by *in vivo* imaging techniques such as magnetic resonance tomography or positron emission tomography. In addition, a combination of data from various analytical levels is frequently in use.

In preclinical drug research and development biomarkers can address different key questions. They can contribute selectively to either one of the following research aims:

- *Pharmacodynamic biomarkers*: are related to the mechanisms of the action of a drug and give answers on whether the drug reaches and interacts with its target and whether this creates downstream effects.
- *Disease biomarkers*: are related to a disease type or subtype and answer questions such as whether the drug affects a disease-relevant or a disease-relevant intermittent phenotype, which can be measured with a biomarker or biomarker panel.
- *Predictive biomarkers*: are associated with the response or lack of response to a particular therapy.
- *Surrogate biomarkers*: substitute a clinical endpoint. They are expected to predict a clinical benefit or harm or – on the contrary – a lack of benefit or harm. They inform on whether the treatment can reduce the disease burden as measured by clinical endpoints.

For the use of biomarkers in clinical practice a solid understanding and knowledge of their role in molecular and cellular biology of disease processes is a prerequisite in order to demonstrate a benefit in reduction of disease burden. Response monitoring by using *imaging biomarkers* in clinical trials will support identification of effective versus ineffective drugs and dose finding, and might correlate with the overall clinical efficacy. In conclusion, biomarkers can serve for a variety of crucial medical objectives, both in preclinical research and clinical development of drugs as well as in diagnosis, treatment decisions, and prognosis in clinical practice [5].

1.3 Biomarkers in the Past

Biomarkers of various molecular classes have been in medical use since the beginning of the twentieth century, for instance, since the impact of inborn errors of metabolism on neurological development was recognized by Garrod (1908) [6]. In the middle of the twentieth century the development accelerated when filter-based and spectral photometers became available to the laboratories and when the analytical portfolio for enzyme assays was continuously expanding. This step allowed measuring the presence and levels of many enzymes in samples from healthy and diseased individuals by clinical chemistry. The introduction of protein separation by electrophoresis and the quantitation of proteins and peptide hormones by radioimmunoassays and later by enzyme-linked immunoassays helped discover the presence of various tissue-derived oligo- and polypeptides as well as glycoproteins as disease markers in circulating blood, cerebrospinal fluid, and other body fluids as well as in tissue extracts. This then lead to the introduction of many enzymes, proteins, peptide markers, steroids, and metabolite-like lipids and cholesterol as measurable entities in panels of diagnostic parameters.

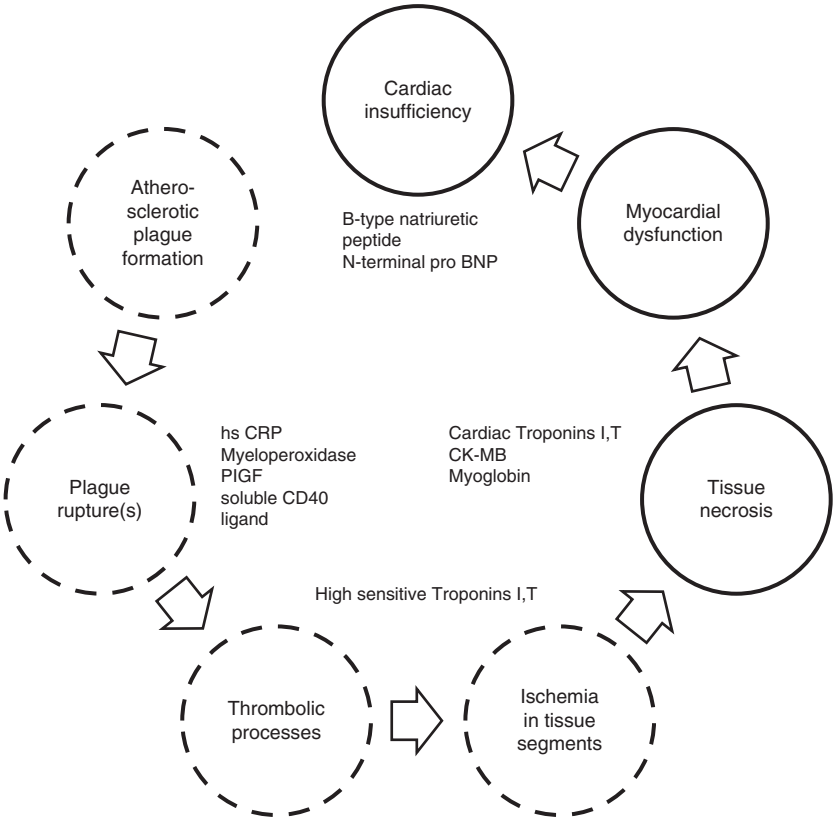
Table 1.1 Established diagnostic measurements by clinical chemistry on serum, selected by organ, or systemic disease.

Disease	Serum analyte	
Liver	Enzymes	Glutamate-oxalacetate-transaminase (EC 2.6.1.1)
		Glutamate-pyruvate-transaminase (EC 2.6.1.2)
		Glutamate-dehydrogenase (EC 1.4.1.3)
		Alkaline phosphatase (EC 3.1.3.1)
		Gamma-glutamyl-transferase (EC 2.3.2.2)
	Protein	Carbohydrate deficient transferrin
	ECM compounds	Collagen propeptide
	Metabolites	Galactose tolerance
		Ammonia
Pancreas	Enzymes	α-Amylase (EC 3.2.1.1)
		Lipase (EC 3.1.1.3)
Kidney	Metabolites	Creatinin
		Urea
	Function tests	Creatinin clearance
Heart, cardiovascular diseases	Enzymes	Creatinkinase (EC 2.7.3.2)
		Creatinkinase MB isoenzyme (EC 2.7.3.2)
		Lactatdehydronase (EC 1.1.1.27)
	Structural proteins	Cardiac troponins cTnT, cTnI
	Proteins	Myoglobin
		C-reactive protein
		Pregnancy-associated plasma protein-A (PAPP-A)
	Peptides	B-type natriuretic peptide (BNP)
		N-terminal pro BNP (NT-pro BNP)
Bone disease	Enzyme	Alkaline phosphatase (EC 3.1.3.1)
	Hormone	Parathormone
	Metabolites	Phosphate
		Calcium

Table 1.1 as a selected listing makes a case on how the detection of tissue-derived markers, in the form of enzyme activities, protein concentrations, and metabolite levels, helped in the detection and staging of organ damage in a variety of classes of human disease.

Clinical Chemistry/Laboratory Medicine departments were the main drivers to exploit the wealth of information from the occurrence and levels of these analytes for early detection, differential diagnosis, and therapy control in clinical practice, and for outdoor patients. For more detailed information, the reader is referred to textbooks on clinical chemistry [7–10].

Many advances have been achieved in the discovery of biomarkers as sensors for different stages in long-lasting disease processes. Immunochemical assays in a practical performance format and characterized by high analytical accuracy were a major breakthrough here. For instance, such progress has been achieved for a



hs CRP – high sensitive C-reactive protein, PIGF –Phosphatidylinositol-glycan biosynthesis class f protein,
CK-MB – Creatin phosphokinase, isoenzyme MB (EC 2.7.8.1), BNP – B type natriuretic peptide, NT-pro BNP – N-terminal-pro BNP

Figure 1.1 Biomarkers in the diagnosis of cardiovascular disease processes. (Adapted from 11, modified.)

routine utilization of biomarkers in cardiovascular diseases. This also includes emergency situations. Immunoassays for cardiac markers allow a longitudinal follow-up from the early onset of atherosclerosis to the final damage in myocardial tissue due to acute myocardial infarction (Figure 1.1).

In an acute disease attack solid phase immunoassay techniques for point of care (PoC) testing allow the determination of a selection of these biomarkers (cTnI, creatine kinase isoenzyme MB, myoglobin) and enable a rapid detection of acute myocardial infarction in the emergency room. Thereby, the use of PoC tests on biomarkers is crucial for differential diagnosis and for decision making on early interventions through an appropriate anti-clotting therapy [12].

In oncology, technology in laboratory medicine reached a boom by the introduction of immunoassays for tumor markers. In advanced stages of a malignant

disease tumor marker molecules are produced or secreted by malignant cells. They appear in the blood or other body fluids. Their absolute specificity for tumor has been under discussion for long and this restriction definitely has prevented their broad utilization in screening for early tumor detection in patients with no clinical symptoms [13, 14]. Tumor marker tests have to guarantee high accuracy in terms of specificity and sensitivity. When they reach these requirements they are frequently useful for *differential diagnosis and staging of tumor-bearing patients.* Table 1.2 lists tumor marker assays, which until now have been frequently used in medical practice. In most indications their main clinical application is for therapy monitoring and recognition of tumor progression, for example, by longitudinal observation of blood levels of tumor marker following the initial therapeutic intervention.

The next phase in the state-of-the-art of tumor markers started with the introduction of the therapeutic antibody trastuzumab (Herceptin®) against

Table 1.2 Frequently used established tumor markers (excerpted from Ref. [7]).

Analyte	Assay technology	Major applications
Carcinoembryonic antigen (CEA)	Immunoassays (EIA, FIA, CIA)	Carcinoma of pancreas, lung, colorectum, stomach, breast
Cancer antigen 19-9 (CA 19-9)	Immunoassays (RIA, EIA)	Carcinoma of pancreas, bile ductus, liver, stomach, colon
Mucins of mamma carcinoma (CA 15-3, CA 549, MCA, BCM)	Immunoassays (RIA, FIA, EIA)	Carcinoma of breast, ovarium, bile ductus, and others
Alpha fetoprotein (AFP)	Immunoassays (RIA, FIA, EIA)	Hepatocellular carcinoma, germ cell carcinoma
Human chorion-gonado trophin (HCG)	Immunoassays (RIA, FIA, EIA)	Chorioncarcinoma, mole, germ cell carcinoma
Prostate-specific antigen (PSA)	Immunoassays (RIA, EIA)	Prostate carcinoma
Minimal residual disease (MRD) in leukemia or lymphoma	PCR	Evidence for residual leukemia cells, chronic myeloid leukemia, and others
Erb-B2	IHC, FISH	Carcinoma of breast, gastric cancer
Erb-B1	IHC, FISH	Colorectal carcinoma. head- and neck-carcinoma, lung carcinoma, and others

EIA – enzyme linked immunoassay; FIA – fluorescence immunoassay; RIA – radio immunoassay; PCR – polymerase chain reaction technique; IHC – immunohistochemistry; FISH – fluorescence *in-situ* hybridization; MCA – mucin-like carcinoma associated antigen; and BCM – breast cancer mucin .

the tumor-associated antigen erbB-2. This molecule is amplified both in copy numbers of its gene and by the expression of the protein on the surface of tumor cells in a large fraction of patients with mammary tumor. These modalities of amplification can be detected by immunohistology (for increased erbB-2 expression in the tissue from a tumor biopsy) and/or by proof of erbB-2 gene amplification by fluorescence in situ hybridization in the tissue sample; see Table 1.2. A combination of chemotherapy with the trastuzumab antibody has led to a significant increase in overall survival time in a fraction of those treated patients who showed overexpression of this tumor marker. By the end of the 1990s, this instant success initiated the broader awareness that appropriate biomarkers can provide a valuable stratification of patients into a class of potential responders for a special immunological treatment versus non-responders. This is nowadays cited as the "*personalized medicine*" paradigm.

A similar situation was matched some years later with another therapeutic antibody, for example, cetuximab (Erbitux®), which is directed to epidermal growth factor (EGF) receptor erbB-1, another physiologically important member of the erb-B family of surface receptors for the growth factors EGF, TGF alpha, and some others (Table 1.2).

Both of these fields of therapeutic application subsequently met significant limitations insofar as a durable curative efficacy of this personalized approach could not be achieved. Obviously, the ubiquitous heterogeneity of tumor cells within a tumor and in its metastases as well as the robustness of tumors by means of clonal selection dynamics in the course of a treatment continuously demand additional therapeutic options.

1.4 Novel Molecules and Structural Classes of Biomarkers by New Technologies

Molecular biology revolutionizes biomedicine in ongoing activities [15]. It helps understand biologic defects, elaborate new drug targets and candidates for new biological entities as candidate drugs, and elucidates molecular diversity as a source for novel biomarkers. On the cellular level, all stages in the regulatory hierarchy of molecules can serve for discovery of biomarkers.

- *DNA* on the genomic level by copy number variations, gene amplification and mutations, genetic polymorphisms, and disease-related methylation patterns
- *RNA transcripts* by their expression levels and alternative splicing products, also by the expression of noncoding transcripts as RNAi or microRNA molecules
- *Proteins* by expression levels, altered activities, and localizations, and by a variety of post-translational modifications
- *Lipids and other metabolites* by their molecular identity and concentration.

Functional genomics is the most advanced technical approach for the detection of biomarkers in research and clinical diagnostics, using a highly developed set of technologies for analytics of nucleic acid molecules as frequently used in

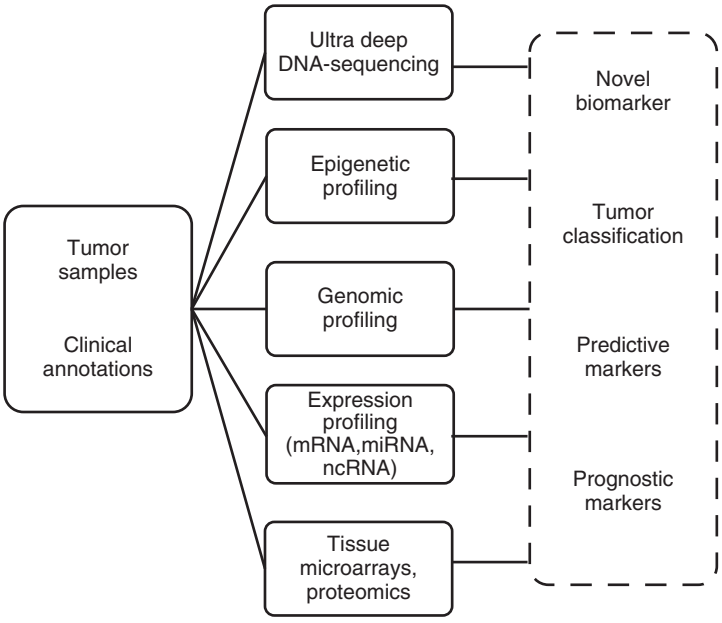


Figure 1.2 Impact of genomics on identification of novel biomarker candidates.

oncology research (Figure 1.2). This development started in the 1990s (see for instance 13, 14).

Genomic sequences can now be retrieved from comprehensive genome data bases such as ENSEMBL [16], which collects whole genome sequences, single nucleotide polymorphisms, and data on gene expression in different tissues. Other genome data bases such as the NCBI Genome [17] or CODIS [18] organize information on sequences, maps, chromosomes, assemblies, and annotations. The broad range of established applications for molecular diagnostics is given in appropriate details in modern textbooks (such as in 8, 9, 19). Gene expression profiles are now also developing in clinical research in cardiovascular diseases [20]. *Network-based stratifications* of tumor mutations were recently demonstrated in ovarian and uterine cancer cohorts [21] from the Cancer Genome Atlas [22].

Following the worldwide active collection of genomic data, similar activities came up in protein data collection and their asservation for data mining and biomarker search in data bases such as the Kyoto Encyclopedia of Genes and Genomes (KEGG) [23], Gene Ontology (GO) [24], and STRING [25], among others, all of which help predict protein functions and functional interaction networks of proteins. In May 2014 two seminal publications [26, 27] presented catalogs on large human proteomes. This opens a deep search for biomarkers of proteins in health and disease in a similar manner to the earlier search of data bases for human genome(s) and transcriptome(s). The mass-spectroscopy-based *Proteomics DB* [27] contains proteomic data from 60 human tissues, 13 body

fluids, and 147 cancer cell lines. Altogether both data bases cover some 18 000 proteins of the estimated 20 000 proteins in the human proteome. *Clinical proteomics* will now closely follow these presentations by associating distinct protein patterns with the onset or progression of a disease. Clinical proteomics will seek biomarkers and biomarker signatures, assign those biomarkers by appearance and levels to clinical subtypes in order to support differential diagnosis, and correlate biomarker levels with clinical outcome, such as those reported in [28] on proteomic risk markers for coronary heart disease and stroke.

Last but not least, metabolites also enter the spotlight. In the past, metabolites such as glucose or circulating lipids have long been used in diagnostics as risk biomarkers for atherosclerosis, coronary heart disease, or stroke. Recently, large epidemiologic studies using mass spectroscopic differential analyses discovered associations between patterns of small molecular weight molecules and metabolic diseases. Some novel fingerprints of small molecules in serum become apparent and these may step in as potential biomarkers for predictive and prognostic diagnostics [28, 29]. In addition, a new epigenome-wide study on this population revealed some correlations between DNA methylation and metabolic traits (called *metabotypes* by these authors) in human blood [31].

The direct correlations between aberrant DNA sequences, transcriptome profiles, protein patterns, and metabolic profiles within a cell or a tissue have yet to be more tightly elucidated – a demanding task for future work by bioinformatics.

1.5 Biomarkers in Drug Research

Biomarkers are now established as important tools both in preclinical research and clinical development of a candidate drug and this extends to the marketing phase of a drug (Table 1.3). In preclinical research biomarkers can serve as target molecules for drug activity screening and for elucidation of their mode of action in a molecular or cellular test setting. On the organismal level, biomarkers can signal both efficacy and toxicological effects in an animal model. In drug discovery work, biomarkers can play a major role in studies on pharmacodynamics:

- In *proximal pharmacodynamics,* reactions of the biomarker can correlate with target modulation independent of the overall response of the model; thus it helps optimize a drug lead candidate and is important information for dose finding for first-in-man studies.
- In a more *distal phase in pharmacodynamics,* biomarkers help
  - demonstrate the modulatory effects along a signaling pathway,
  - provide information on dose finding,
  - show efficacy of a drug and
  - thus contribute to demonstrate the proof of principle of a drug candidate.

Table 1.3 Relevance of biomarkers in the drug research process.

Targets	Preclinical stage	Clinical trial: early phases	Clinical trial: late phases	Post-marketing
Identification	HTS for lead discovery	Bioavailability	Stratification of patient populations for disease subtypes	Patient stratification
Validation	SAR of targets for lead compounds	Bioequivalence		Monitoring of therapy response
Analyses of disease causes and for key factors for disease progression	Pharmacokinetics	Dose–response ratio	Dosage finding	Recognition of adverse drug responses
	Pharmacodynamics		Data submission for approval	Competitive effectiveness studies
	Toxicity testing Mode of action	Toxicity in man Proof of concept in man		

HTS – high-throughput screening and SARs – structure–activity relationships.

In the *clinical development* of new drugs biomarkers serve different objectives. Table 1.3 defines some objectives for biomarker monitoring. In the early clinical phases, for example, *phase I and II,* the focus is on pharmacological criteria; in late phase II, dosage optimization and patient stratification are in focus, which then is extended in multicentric studies by *phase III.* The Boston Consulting Group (BCG) provided data on the use of biomarkers in clinical trials through a study that was based on data from the data base clinicaltrials.gov over the period from 2005 to 2011 [32]. In all these trials the share of studies that used biomarkers was

- 21% in phase I,
- 27% in phase II,
- 17% in phase III, and
- 18% in phase IV.

By now, it is a commonplace statement by major drug companies that they involve accompanying biomarker studies in more than half of their trials, especially for the clinical development of a novel drug. Another survey from 2010 found that at least 50% of clinical trials are collecting DNA from study participants to aid in the discovery of drug-related safety and efficacy biomarkers, and 30% of the companies in the survey require all drug compounds in development to have a biomarker [33].

How should biomarkers be clinically evaluated? For validation of biomarkers in clinical oncology the European Expert Group proposed a four-phase model for biomarker monitoring [34]. Following this proposal will imply that in clinical

Table 1.4 Sources and types of biomarkers.

	Type of biomarkers	Difficulty to discover	Difficulty to implement in clinic routine
Pharmacodynamic biomarker (PDγ)	Plasma marker imaging protein expression (IHC) expression profiles		
Disease biomarker	Imaging, plasma marker, protein expression, expression profiles		
Predictive biomarkers	Plasma marker, imaging, protein expression, (IHC), genetic/genomic alterations, expression profiles		
Surrogate biomarker	Plasma marker protein expression (IHC), expression profiles		

The size within an arrow indicates the level of problem.
IHC – immunohistochemistry.

phase I biomarker kinetics and correlation with tumor burden in the patient population are assessed. Phase II evaluates whether the biomarker is able to identify, exclude, and/or predict changes in the patients' disease status. Phase III evaluates the effectiveness of the intervention, which is guided by the tumor biomarker by measuring the patient outcome in randomized trials. Phase IV will monitor the long-term effects when monitoring of the tumor biomarker has become the standard in patient care.

Which molecular entity should a biomarker or a marker use in order to become translated to use in routine laboratory medicine? Table 1.4 presents a practical view regarding this issue.

These analytical approaches differ distinctly in terms of assessment of samples, needs for appropriate instrumentation and demands on laboratory and technical expertise: Plasma markers in the blood sample as easily accessible biological specimen are technically feasible, imaging markers need expensive instrumental investments and require that for *in vivo* use technological facilities are available on call, protein pattern analysis by immunohistochemistry needs provision and storage of suitable cell or tissue samples, and transcriptome analysis on a cellular extract and by polynucleotide enrichment demands availability of an experienced molecular biology facility. Research laboratories and routine clinical practice, such as that under the conditions of a hospital outside of a university medicine environment, will differ in their views on how to establish proper implementation of testing for molecular biomarkers.

1.6 Current Development and Future Trends for Biomarkers in Laboratory Diagnostics

1.6.1 Biomarker Test Validation

Where does clinical validity of biomarkers stand now? The early phase in biomedicine in the years after the human genome has been sequenced started from a gray zone: "Even now, disappointment might be expected, in part because rules of evidence to assess the validity of studies about diagnosis and prognosis are both underdeveloped and not routinely applied" [35]. What then is needed to overcome this situation? In an OECD workshop in 2008 on biomarkers the discussions went around a concept that separated the development of biomarkers or a multivariate panel of them into two phases [36]:

- *Assay development*, which covers the development of the assay technology, with work to prove its analytical validity and collection of data to prove the association of the biomarker(s) with a disease or with distinct disease stages to reach scientific validity.
  Criteria for the analytical accuracy of an assay are well defined for developmental projects in laboratory medicine. Such criteria are sensitivity, specificity, positive and negative predictive values, receiver operator characteristics, likelihood ratios, and odds ratios [7–10, 19, 37, 38]; see also Chapter *XX* in this book.
- Next, the developed assay will be translated to a *test version* for work on clinical validation. This involves measurement of the test performance under clinical real-life conditions. These differ from those in a research laboratory in many aspects. Clinical scientists or pathologists will then validate the technical maturity of the test under clinical conditions and clinicians will review and report on its clinical utility.

The accuracy of a biomarker assay or a combination of biomarkers in a diagnostic model is proved by their ability to

- identify a target disease in a patient,
- differentiate between diseases with similar clinical symptoms, or
- predict a patient's response to treatment or monitor effects of therapy on the patient's disease burden.

For validation of a pattern recognition model in discovery-based research the biostatistics of overfitting can become a problem [35]. It can be reduced by splitting the tested population into two groups, that is, a training set and an independent validation set, both endowed with biostatistically sufficient group sizes. This separation step can be organized by randomly splitting the original test population, which should be present in sufficient size, into two cohorts (split-sample validation) [35].

In a well-planned design for development of a diagnostic molecular biomarker, the overall sequence chain for studies should extend from a discovery phase to

Table 1.5 Phases in the development of diagnostic biomarkers.

Phase	Description	Objectives
I	Discovery	Identification of promising biomarker candidates
	Assay development	Define and optimize the analytical process into robust, reproducible, and valid device
	Retrospective validation	Clinical assay detects disease, develop a first algorithm for combination test
II	Retrospective refinement	Validate early detection properties of biomarker (set), development/refinement of algorithm for combination tests
III	Prospective investigation	Determine diagnostic accuracy (sensitivity, specificity) in situation of clinical practice
IV	Randomized controlled trial	Quantify effect of making the biomarker information available to the physician to optimize treatment
V	Health economics study	Quantify cost-effectiveness, evaluate clinical utility from a societal perspective

Adapted from Ref. [2], modified.

clinical validation and proof for clinical utility through steps with various objectives (Table 1.5) [2].

These studies cover both work on analytical development, analytical and scientific validity, clinical validity, and finally utility in clinical decision making. However, by now only in a few cases biomarker studies have made it up to presenting results for the final phase, that is, data provision for a benefit–cost ratio. Clinical utility has to be proved to reach acceptance by the medical community, regulatory authorities, and willingness of healthcare payers for reimbursement. This is written even though by common sense the benefits of biomarker testing for patients and physician may be obvious to the developers.

When industrial providers are going to introduce a biomarker product to the market a variety of information will be needed in order to verify the clinical relevance of testing and to provide its evidence in order to reach a broad clinical exploitation [36, 39].

- A conclusive analysis of the available data from studies before and after its market introduction
- The clinical claims for handling, appropriate labeling, and product information
- Definition of patient groups with special restrictions for use of the measurement
- Information on comparable measurements that support safety and/or mode of application
- Indications, clinical confounding factors, stage and severity of a disease, criteria for a suitable sample, patient populations.

1.6.2 Companion Diagnostics in Clinical Pharmacology

Patients in a population almost differ by an *intrinsic variability* in pharmacokinetics and pharmacodynamics, for example, variation is seen in delivery and input rate, drug metabolism and transport, drug access to the site(s) of action, type of transduction, drug–target interaction, disease stage, and homeostasis. In addition, there are issues with *extrinsic variability,* such as drug–drug interactions, drug–food interactions, and interactions with endogenous substances. One drug with one dosage does not fit all patients within a given disease class. It was reported that the percentage of a patient population for which a particular drug in a disease entity is ineffective ranges between 38 and 75% on the average [40].

In many indications clinical prediction on how a patient will react to a treatment can be supported by biomarker measurements for efficacy and safety. *Pharmacogenomics* (genomics to study how a drug acts) and *pharmacogenetics* (scientific information on how genes affect a person's drug response) create a large potential to optimize drug selection and drug dosing for therapy. Novel low molecular weight drugs in the kinase inhibitor class as new medical entities have recently been clinically developed and successfully submitted to the regulatory authorities in the United States and Europe. In these cases the application of a measurement for efficacy by an appropriate biomarker has become indispensable to check the patient's ability to respond to that drug. Regulatory authorities combined the approval of these drugs with the need to determine the putative responsiveness by a suitable biomarker [33, 41].

In each of the enlisted drugs from Table 1.6 the biomarker technology applies a molecular biology test to affirm that the patient carries the crucial genetically determined aberration that is addressed by the singular drug for a protein target-selective mode of action. In this way, the patient population will be stratified.

There are ongoing activities in academic research and by research and development departments in drug companies to extend this companion diagnostics approach, be it in a combination of new drugs with evidence for a disease-driving function of the target or by addressing such targets by existing drugs that have not been used for that disease, for example, in a new medical use case. In a report from 2013 genetic classification has been successfully used to classify patients in a population of more than 6000 patients for lung tumor subtypes [42]. The results of this consortial study have led to a reclassification of one cancer subtype, that is, large cell lung cancers. The results also initiated a wider clinical use of stratified patient management and prognostic information. Various patients experienced a distinct survival benefit when their therapy has been adapted according to the information from genetic testing on a selected set of candidate genetic markers (ERB-B2amp, ALKfusion, B-RAFmut, EGFR mut, K-RASmut, PIK3CAmut, FGFr1amp, DDR2mut).

When therapeutic antibodies were introduced to tumor treatment (Section 1.3), it became apparent that a therapeutic response was observed in just a share of the patient groups. As a consequence, patients will be analyzed for a strong expression

Table 1.6 Recent advances in companion diagnostics for cancer treatment by kinase inhibitors.

Drug	Indication	Company	Companion diagnostic device	Year of approval of CDx	Clinical relevance
Vemurafenib (Zelboraf®)	Metastatic melanoma	Roche, Daiichi-Sankyo	Cobas®4800 BRAF V600E mutation test (Roche)	PMA (FDA) in August 2011	Patient stratification for treatment inclusion
			THxID®-BRAF test (bio Merieux)	PMA (FDA) In May 2013	
Crizotinib (Xalkori®)	Advanced non-small-cell lung cancer, subpopulation	Pfizer	Vysis ALK Break Apart FISH Probe Kit (Abbott Molecular, Inc.)	PMA (FDA) in September 2011	Selection of NSCLC subgroup with translocation of ALK for Crizotinib therapy
Erlotinib (Tarceva®)	Metastatic non-small-cell lung cancer, subpopulation, for first-line monotherapy	Roche	Cobas EGFR-mutation test (Roche)	Cobas EGFR-mutation test CE marked by second half of 2011	Selection of NSCLC subgroup with EGFR-activating mutation subtype

CDx – companion diagnostic.

of the antibody target before treatment with these biologicals is taken into clinical consideration. The established immunochemical or molecular biology tests help stratify the patients. Patients who are considered not to develop a response do not get this costly treatment, which besides its therapeutic benefits in a share of the responder patients may also introduce adverse effects to many of the recipients of the biopharmaceutical.

In the case of the therapeutic antibodies *cetuximab* and *panitumumab,* which both bind to the tumor-associated target EGF receptor, a response testing for responsiveness does not only look at whether the tumor tissue overexpresses the target on the tumor cells. In addition, a molecular biology test looks for the *mutational status of the gene for K-RAS*, which is a main mediator of signal transduction downstream of the EGF receptor (Figure 1.3).

In the course of clinical trials with these antibodies it became clear that tumor cells with mutants of the putative oncogene K-RAS show a constitutive activation for cell cycle progression, survival, and proliferation irrespective of the antibody binding to its EGF receptor target. This evidence led the FDA and European Medicines Agency (EMA) to the approval of cetuximab, which is given in combination with chemotherapy combined with obligatory testing for

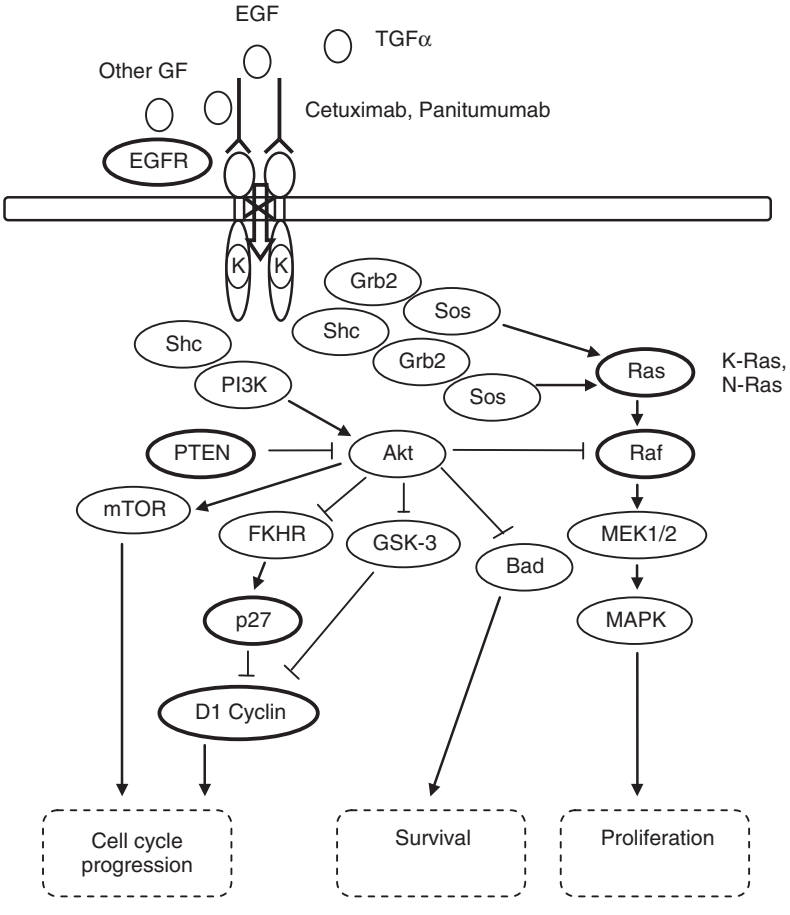


Figure 1.3 *Relevance of tumor biomarkers for antibody therapeutics.* Cetuximab or panitumumab are antibody drugs that block the EGF-receptor-mediated cell activation. Mutants in pathways for signal transduction, which have to be considered for therapy resistance, are marked by bold letters.

the presence of wild type K-RAS. Approval is valid for first-line treatment of metastatic colorectal tumor [43]. There is upcoming preliminary clinical evidence that some mutations in other genes for mediators of the signal transduction network, which all are downstream of the EGF receptor, may also be related to resistance to the antibody treatment (indicated by bold circles in Figure 1.3).

1.6.3 Biomarker Multivariate Index Assays

Variation of both the occurrence of biomarkers and their levels reflects variability, which is a common observation in biology, for example, on the level of a human population, between tissues and cells of an individual, and/or between different

persons with an apparently similar disease. For instance, cells in a tissue sample differ by type, their stage in the cell growth cycle, and by their being embedded in a cellular environment. In tumor samples there are many reasons that the tumor cells in a given biopsy may individually differ in their genetic constitution such as in the presence and activity of tumor driver and circumstantial passenger genes. Biomarker signatures were developed, which in parallel sense the presence and expression levels of multiple molecular players, for example, those which by the current clinical science are considered as tumor drivers or as accessible drug targets, respectively. Clinical research in oncology had to run a long phase of validation to establish such diagnostic or prognostic biomarker panels by *in vitro diagnostic multivariate index assays (IVDMIAs*). Some of these multivariate index assays reached approval for clinical use (Table 1.8). Their main indication is for the prediction of risk for tumor progression or for response to the drugs in the therapeutic portfolio, respectively.

Many more biomarker multivariate index assay sets are to follow. Research on such novel marker panels is ongoing worldwide. At present, a series of them are offered as *laboratory-developed test***s (***LDT's).* Establishing reliable algorithms for a final marker combination from a broader set of candidate markers needs dedicated heavy expert work by bioinformatics. The finally fixed combination shall be validated extensively in order to safeguard its discriminatory power and its reliability for risk prediction or therapy responsiveness, respectively. There are ongoing discussions in working groups of diagnostics providers with regulatory authorities on the necessary validation work on data for biomarker panels and suitable algorithmic calculations, which all shall be submitted for approval for clinical use [33, 44–46].

1.6.4 Regulatory Policies on Biomarker Tests

In the United States novel *in vitro* diagnostic tests (IVDs) fall into main categories as *medical devices* [33, 47]:

- Diagnostic tests by *Class I or II,* which need a *510(k) premarketing clearance* by FDA. These are tests where an analogous test with FDA approval is already on the market. The submission contains data on the intended use and classification, data sets in comparison to the established test peer, and the analytical profile for validation: precision, linearity, specificity, and sensitivity in patient groups, compared to the established peer test.
- Diagnostics test by *Class III,* which need a *Premarket Approval* (*PMA*) by FDA. These are tests that result in information with a high risk profile, such as in diagnostics or therapy for cancer or when the clinical use of the marker/technology is novel and no analogous test is available. Data such as in the 510(k) are needed; additionally, information must be submitted on clinical results, and on correlation of the test results with the disease stage and with the clinical information.

Table 1.7 Clinical practice for use of companion diagnostics together with biopharmaceuticals.

Drug	Main indications	CDx	Approval status	Clinical relevance
Trastuzumab (Herceptin®)	Breast cancer, gastric cancer	IHC	PMA (FDA)	Patient stratification for inclusion into treatment
		FISH CISH two-color CISH	PMA (FDA)	
Pertuzumab (Perjeta®)	Breast cancer	IHC	PMA (FDA)	Patient stratification for inclusion into treatment
Adotrastuzumab emtansine (Kadcyla®)	Gastric cancer	FISH	PMA (FDA)	
Cetuximab (Erbitux®);	Colorectal cancer	IHC	PMA (FDA)	Patient stratification for cancer-associated antigen expression
Panitumumab (Vectibix®)	Head and neck cancer	qRT-PCR	PMA (FDA)	Patient selection for K-RAS wild-type expression

Excerpted from Ref. [40].
CDx – companion diagnostic; IHC – immunohistochemistry; FISH – fluorescence in-situ hybridization; CISH – chromogenic in-situ hybridization; qRT-PCR – quantative real time polymerase chain reaction.

It is quite obvious that novel biomarker and multivariate index assays fall under the second category. Thus, their path to reaching approval needs extensive analytical and clinical contributions and adequate documentation. FDA-cleared tests exist for a limited number of markers, such as those listed in Tables 1.6 – 1.8.

However, most of the recently developed innovative molecular diagnostics on biomarkers or signatures have reached a status as *LDTs,* which presently are not overseen by the FDA. Laboratories performing LDTs fall under the rules of the *Clinical Laboratory Improvement Amendment (CLIA)* [47 – 48]. Clinical laboratories obtain CLIA certifications from Centers for Medicare and Medicaid Services (CMS). FDA is currently considering its responsibility for clearing LDTs [49]. To date, the majority of molecular tests have not been submitted for FDA approval [33]. In 2013 a review reported that CLIA-certified laboratories developed LDTs that are used for over 2000 genetic tests [48]. As that review discusses, LDTs are developed quickly and fill a void where no FDA-approved test is available, but LDTs may lack adequate validation. Therefore, risk-aware validation of LDTs and proof for a reliable discriminatory power remain a constant challenge [2].

In Europe, diagnostics are regulated by rules for medical products under the EC Directive IVD 98/79/EC on *in vitro* diagnostic medical devices and its various updates [50]. This directive is continuously under revision for updating

Table 1.8 Multianalyte profiling for diagnostics in cancer patients.

Product	Company	Profile	Clinical use	Status
MammaPrint®	Agendia	Microarray on 70 genes in a tumor sample	Prognosis in stage 1 and 2 LN^- breast cancer	Approved in 2/2007 by FDA
Oncotype DX® Breast Cancer Assay	Genomic Health	21 gene expression test by qRT-PCR in tumor samples	Prediction of chemotherapy response in early stage LN-ER^+ breast cancer	Oncotype DX® assays for breast cancer resp. colon cancer are for laboratory-developed assay service conducted in the CLIA-licensed Genomic Health clinical laboratory
Oncotype DX® Colon Cancer Assay	Genomic Health	12 gene assay	Prediction of colon cancer recurrence in stage 2 patients by a recurrence score	
OVA1®	Vermillion and Quest Diagnostics	5 protein assay on serum samples	Prediction of malignancy of ovarian cancer	Approved in 9/2009 by FDA

qRT-PCR – quantitative real time polymerase chain reaction.

reasons and for risk-appropriateness of its regulations on medical devices. The EC directive regulates the approval of diagnostics for high-risk applications in a special appendix. Those diagnostics outside of that clearance provision for high-risk applications are subject to analytical quality control within the responsibility of the test-kit providing organization and are CE-certified under the auspices of a special external authorized body. In addition, the EMEA guideline on clinical evaluation of diagnostic agents is to be considered [51]. Regarding companion diagnostics, FDA, Health Canada, and the EMA reportedly intend to jointly clarify the regulatory pathway on which companion diagnostics shall enter the market [33].

1.7 Summary and Outlook

New high-throughput technologies for genome sequencing/whole genome analysis or proteomics, advances in cellular and tissue imaging, and new technologies for *in vivo* imaging will continuously drive the acquisition of pathophysiologically relevant data. This will result in a variety of unprecedented biomarkers as novel candidates for use in pharmacological research and clinical diagnosis. Those accumulating novel data sets are highly complex and interrelated. Their interpretation

needs biomedical professionality and advanced analysis through the expertise of bioinformaticians [51]. Many exploratory biomarker data have still to reach a statistically validated significance. Moreover, novel candidate classes are worked on in clinical research, such as circulating DNA, various microRNAs, and circulating tumor cells. All of them will have to be submitted to a similar scrutiny for analytical and clinical validation.

Some significant advances have been achieved in the combination of selected biomarkers and biomarker signature with therapy selection for stratification in a patient population, especially in clinical areas of high medical need. Evidence for clinical utility in using novel biomarkers and companion diagnostics has been achieved in various indications [33, 52]. However, the highly dynamic progress in research on pathogenic molecules and regulatory mechanisms as disease drivers has outpaced the ability of the medical community and of regulatory authorities to understand and to implement appropriate reactions. The regulations for evaluation and approval by FDA or by the EU directive for *in vitro* diagnostic devices primarily target analytical accuracy and safety aspects of medical devices for biomarker use. Approval regulations for multivariate index tests on biomarker panels and understanding of their inherent algorithms for score/index calculation are still in an early stage. Translation of novel biomarkers to clinical practice needs more dedicated work to provide evidence of their clinical utility. Reimbursements for biomarker assays will follow sociomedical priorities and need thorough proof of an attractive benefit/cost ratio in healthcare.

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2
Quantitative Proteomics Techniques in Biomarker Discovery

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2.1
Introduction

In the last 10 years, proteome analytical techniques have made a quantum leap forward and have became powerful tools in the discovery of protein biomarkers. While in its early days proteomics mainly aimed at indexing the highest possible number of proteins present in a sample of interest, quantitative proteomics evolved into an analytical platform that has been used to address a broad variety of clinical problems. In fact, the expectation to discover protein biomarkers that would be helpful in the diagnosis of patients and disease management has always been a great motivation for the development of proteomics technologies. Several quantitative proteome analytical techniques have been established and countless biomarker candidates have been reported accompanying the technological advances in the field of proteomics.

Proteome is defined as the collectivity of all proteins that are present in a given entity (e.g., an organism, tissue, cell, or cell compartment) at a given time point under certain conditions. In the field of biomarker discovery, these entities could be tissues of diseased and non-diseased state (e.g., tumorous tissue and adjacent healthy tissue); cell culture and animal models have also been shown to be useful sample sources in biomarker discovery. Depending on the kind of entity under investigation the proteome can be highly dynamic. This is even more evident if post-translational modifications (PTMs) of proteins are also taken into account. The proteome precisely represents the status of the sample under examination at a given point of time, a key prerequisite for biomarker discovery. All quantitative proteomics approaches aim for a comparison of protein expression levels between different entities/samples. Two main kinds of approaches need to be distinguished: in top-down strategies the protein quantification is performed at the level of intact proteins. In bottom-up strategies proteins are digested and the quantification is performed at the peptide level. The quantitative results of the peptides that belong to one protein are then combined for protein quantification. Apparently, bottom-up strategies bear some methodological problems, as not all

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peptides are unique for only one protein but possibly may belong to several different proteins in a given proteome. In the simplest case such peptides can be excluded for quantification; however, much more sophisticated models to address this problem have been described [1].

As a result of differential comparisons researchers usually obtain a list of differentially expressed proteins, which is then filtered according to statistical metrics and the changes in expression levels, mostly expressed as relative changes. These differentially expressed proteins are expected to serve as biomarkers for the disease state under investigation. As a matter of fact, much more effort is necessary to finally find true biomarker candidates among the list of differentially expressed proteins. Often, expression levels of proteins are differentially associated to stress response or inflammatory processes, but are not specific for a certain disease [2]. In general, this does not necessarily mean that such proteins are useless as biomarkers, for example, if one thinks of a general marker for cancer. Principally, a biomarker is always defined by its individual usage – the clinical setting in which it is meant to be used (e.g., diagnosis, prognosis prediction, monitoring of treatment response). In addition, the assays in which the biomarker is supposed to be measured need to be specified. A biomarker can be specific for a disease and serve as a good marker for tissue-based diagnosis using immunohistochemistry (IHC), but might not be detectable in a patient's blood. The purpose of the biomarker has to be clearly defined and the biomarker discovery study has to be designed in an appropriate way. In fact, many published biomarker candidates are not being followed up because the clinical application of the marker is not well defined.

The initial proteome analytical quantification strategies were gel-based approaches and led to the development of the proteomics field [3]. Recently, mass spectrometry (MS)-based methods emerged to be high-throughput technologies frequently used in proteomics (Section 2.3). MS-based approaches utilize label-free as well as label-based quantification strategies, every strategy with its own characteristic advantages and disadvantages (Figure 2.1). A technique that very recently complemented the collection of proteomics methods is mass spectrometry imaging (MSI). Matrix-assisted laser desorption ionization (MALDI) MSI enhances quantitative proteomics by an additional dimension as it considers the spatial distribution of proteins in the tissue and the correlation to histological information [4].

2.1.1 General Considerations

There are several important things to consider when setting up a proteomics biomarker discovery study. First of all, the methodological approach has to be chosen. Here, it is of importance to consider the basic difference between bottom-up and top-down approaches. While bottom-up approaches are mainly used for biomarker discovery it still makes sense to consider top-down strategies as well. They may be used to identify disease-related changes in the expression of protein isoforms or aberrant PTMs, for example, glycosylation motifs.

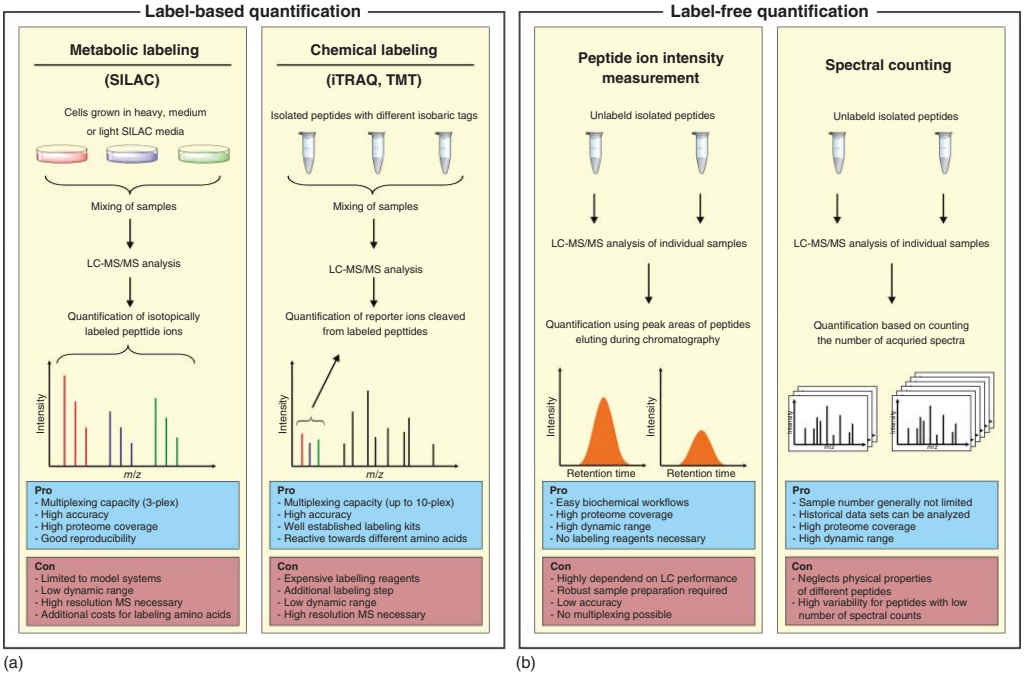

Figure 2.1 *Schematic representation of bottom-up proteomics techniques.* A typical minimal-labeling workflow is shown as a representative for a 2D-DIGE (2D-difference gel electrophoresis) experiment. Two samples are analyzed together with an internal standard in a multiplex experiment. Pros and cons of the technique are summarized in blue and red boxes, respectively.

Since bottom-up strategies are usually advantageous with respect to sample throughput, resolution, and proteome coverage, top-down strategies should be applied considering especially their beneficial aspects by analyzing intact proteins. Obviously, MALDI MSI should be used when information about histological distribution of proteins is relevant. Concerning proteome coverage and quantification accuracy, well-established methods have advantages over MALDI MSI.

Another important aspect of biomarker discovery studies is the choice of the appropriate sample; manifold kinds of samples have been used in proteomics studies. Here, one has to consider the given biological variance in the human population. The analysis of cell lines hardly represents the heterogeneity of diseases even if a high number of different cell lines are used. Mouse models usually have a uniform genetic background, thereby reducing biological variance. It has also been shown that not all findings from mouse models can subsequently be transferred to humans. On the contrary, cell culture and animal models provide samples that are usually easy to analyze because they fit the requirements of standard sample processing procedures. The samples can easily be reproduced and high numbers of biological replicates can be produced and analyzed. Sample material is generally not limited, but involves a question of investment in terms of money, time, and man power.

In contrast, the availability of clinical samples is generally limited by the prevalence of a disease, and samples are usually unique and not reproducible. Nevertheless, the usage of clinical samples is most advisable when performing biomarker discovery approaches. Clinical samples represent the disease in its whole complexity, and biological variance is considered. However, there are several aspects to consider when using clinical samples. A close collaboration with clinical partners is necessary, since samples must be examined regarding their quality. Tumor samples need to be annotated in order to mark areas of interest, as they might contain necrotic or highly inflammatory areas. Often diseases are staged into different degrees of severity, which need to be diagnosed by an expert. Clinical samples may also require alternative strategies concerning sample preparation. Depending on the type of sample they can contain big amounts of extracellular matrix macromolecules that cannot be solubilized, thus complicating the extraction of the proteins of interest.

It is important to note that the above-mentioned considerations focus on clinical tissue samples. Body fluids such as serum and plasma samples or urine are not taken into account because they are less suitable for biomarker discovery since they bear analytical pitfalls that complicate their analysis. Serum and plasma contain high amounts of only a few highly abundant proteins. In fact, the 20 most abundant proteins in plasma account for 95% of the protein content and the protein concentrations in plasma range over 12 orders of magnitude [5]. Albumin, for example, has a concentration of 35 – 40 mg ml^{-1} while interleukin 6 is found from 0 to 5 pg ml^{-1}. This makes the analysis of low-abundance proteins in plasma samples extremely complicated, and blood is not the sample of choice when performing biomarker discovery studies despite being easily accessible. Urine is also easily

available and seems to be obvious to use when addressing clinical problems related to the genitourinary system. However, protein concentrations in urine are highly variable due to dilution effects, and normalization of protein concentrations to creatinine should be considered. As blood and urine samples are relatively easy to obtain in high numbers and can be collected noninvasively they should be used for the verification/validation of biomarker candidates. Here, immunological methods such as enzyme-linked immunosorbent assay (ELISA) are often being used but the targeted proteomics approach, multiple reaction monitoring/selected reaction monitoring (MRM/SRM), is also recently evolving as an MS-based tool for biomarker verification [6].

2.2
2D-Difference Gel Electrophoresis

Although developed almost 40 years ago, two-dimensional electrophoresis (2-DE) is still one of the methods of choice when analyzing complex protein mixtures. In the first dimension, proteins are separated according to their isoelectric point while in the second dimension separation via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the molecular weight is performed. By this two-dimensional (2D) separation method thousands of protein spots can be separated, visualized, and quantified in a 2D gel. Separation of a maximum of 10 000 intact proteins by 2-DE has been reported [7], although it has to be mentioned that this resolution is difficult to realize on a routine base and also depends on the kind of sample. Apparently, 2-DE has given access to high-resolution proteome analysis and continuous development has consolidated 2-DE applications in proteomics. The greatest impact of these improvements is the introduction of difference gel electrophoresis (DIGE) [3]. DIGE circumvents some basic problems of 2-DE such as gel-to-gel variations and limited accuracy using different fluorophores for a multiplex analysis. DIGE is based on protein labeling with fluorescence dyes prior to 2-DE. This kind of protein labeling and separation allows multiple samples to be co-separated and visualized in one single 2-D gel (Figure 2.2). The most used labeling chemistry is the minimal labeling method, which allows separation of three samples in one gel. CyDye DIGE fluors have an *N*-hydroxysuccinimide (NHS) ester reactive group that covalently attaches to the epsilon amino group of lysine residues. Usually one dye is used for labeling of an internal standard that is separated together with two samples and improves the accuracy of quantification. One of the major drawbacks of this method is that relatively large amounts of samples are needed for labeling. About 50 µg of protein per sample are used for minimal labeling in standard protocols.

This disadvantage has been overcome by the development of a second labeling approach that is designed for use in situations where the quantity of samples is limited. DIGE saturation labeling is based on the covalent attachment of all protein cysteine residues prior to 2-DE. In contrast to DIGE minimal labeling where only

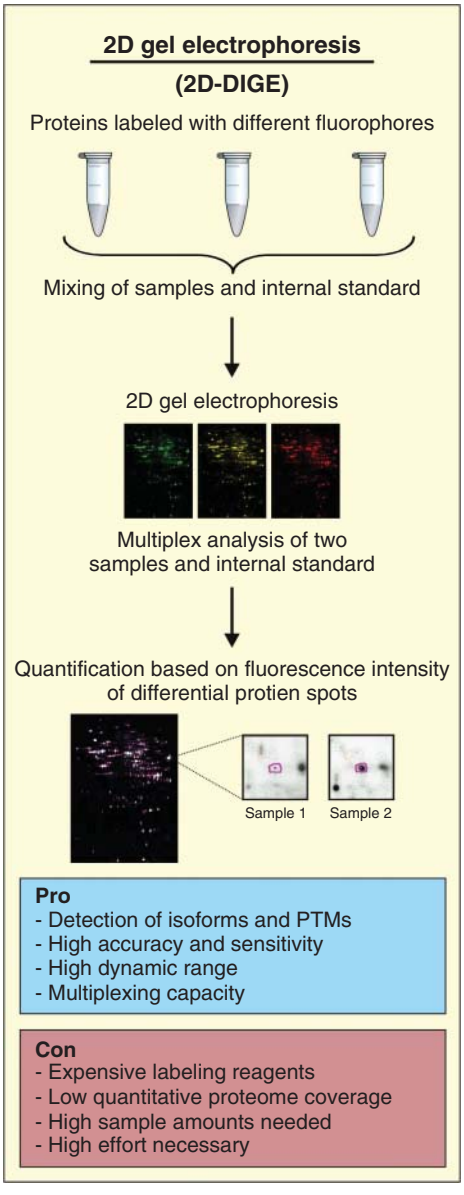

Figure 2.2 *Schematic representation of bottom-up proteomics techniques.* (a) Simplified workflows of label-based proteomics approaches are shown, including metabolic and chemical labeling. (b) Label-free proteomics quantification methods are shown, including peptide ion intensity-based quantification and spectral counting. Pros and cons of the techniques are summarized in blue and red boxes, respectively. Abbreviations used in this figure are SILAC: stable isotope labeling of amino acids in cell culture; TMT: tandem mass tag; iTRAQ: isobaric tags for relative and absolute quantification; LC: liquid chromatography; MS: mass spectrometer; and MS/MS: tandem mass spectrometry.

one lysine residue of approximately 3% of a protein species is labeled by a fluorescence dye, DIGE saturation dyes lead to complete labeling of all proteins (as long as they contain cysteins). Saturation labeling allows the highly resolved detection of as little as 1–3 µg of protein in a 2-D gel by separation of a maximum of two samples in one gel. As it is recommended to use an internal standard, this results in the quantification of one sample per gel. In combination with microdissection of specific tissue areas, saturation labeling has also been successfully applied in biomarker discovery [8].

In both approaches, differentially expressed proteins can be detected using image analysis software. The identification of proteins of interest can be carried out by isolation of the protein spots from the gel, tryptic digestion of the proteins, and identification using a mass spectrometer. The major feature of 2D-DIGE is that it represents a quantitative top-down proteomics approach meaning that intact proteins are separated and quantified. Owing to this characteristic DIGE can be applied in the detection of expression changes between different protein isoforms or PTMs, respectively. Since DIGE requires much effort in terms of realizing differential studies with reasonably high numbers of investigated samples, it should be used especially taking advantage of this aspect. DIGE offers a high accuracy of quantitative results and a high dynamic range. A drawback is that hydrophobic proteins are usually deficiently represented in gel-based approaches. For the DIGE method, investment costs are moderate compared to MS-based platforms, while running costs for dyes can be quite high.

2.3 Mass Spectrometry-Based Proteomics

2.3.1 Principles and Instrumentation

The tremendous technological advances in the field of mass spectrometry allowed MS to become an attractive technological platform for biomarker discovery. MS-based quantification methods can be applied to measure changes in expression levels of thousands of proteins in complex samples. In principle, a mass spectrometer always consists of three main parts: an ion source that creates ionized analytes such as peptides, a mass analyzer that measures the ionized peptides as mass-to-charge ratio (m/z), and a detector that records the number of ions at each m/z value [9].

2.3.1.1 Ionization Methods

All mass spectrometric analyses are carried out in the gas phase and peptides have to be ionized before being measured by the mass spectrometer. The most commonly used techniques to ionize and volatize peptides for MS are electrospray ionization (ESI) and MALDI. Unlike MALDI, ESI has the ability to ionize peptides directly out of a solution, which makes it compatible with liquid phase separation

techniques such as liquid chromatography (LC) or capillary electrophoresis (CE). Such techniques allow decreasing the complexity of a sample by separating analytes according to a certain characteristic (e.g., hydrophobicity, charge, size). This leads to a lower number of analytes measured in a single MS duty cycle and therefore allows the analysis of much more analytes, since the number of possible analytes for one duty cycle is limited. The ability to combine LC with ESI-MS allowed LC-MS/MS (liquid chromatography tandem mass spectrometry) to evolve into the preferred technology for the analysis of complex samples.

2.3.1.2 Mass Analyzers

In the context of proteomics, it can be said that the mass analyzer is the "heart" of the mass spectrometer. The key factors of mass analyzers are resolution, mass accuracy, sensitivity, and the time that is necessary for one measurement (duty cycle). There are several types of mass analyzers being used in proteomics: time of flight mass analyzers (TOF), quadrupoles, Fourier transform ion cyclotron (FT-MS), and ion trap/orbitrap analyzers. All mass analyzers have different designs and performances and each has advantages and limitations. For this reason, several elements are often combined in state-of-the-art mass spectrometers. Quadrupoles are frequently used as mass filters and combined with other mass analyzers (e.g., quadrupole-TOF, quadrupole-orbitrap).

2.3.2 Label-Free Protein Quantification

The major aim of proteomics in biomarker discovery is to examine changes in protein expression levels in biological systems associated with a particular disease. Therefore, usually a diseased type of sample is compared to some reference type of sample, or samples representing different states of a disease are compared. To enable the quantification of the protein expression levels in complex biological samples using LC-MS/MS, several different methods have been established. In general, these methods are characterized by either using an isotopic labeling strategy or being label free (Figure 2.1). Among the label-free methods two quantification strategies have been established and are widely used for biomarker discovery [10].

2.3.2.1 Area Under Curve (AUC) or Signal Intensity Measurement

This label-free quantification approach is based on measuring the peak areas (area under curve, AUC) for peptide precursor ions, also called peptide ion intensities. The AUC of precursor ion peaks has been shown to have a linear correlation with peptide abundances over a broad dynamic range [11]. All investigated samples are measured individually and subsequently the peptide ion peaks are matched according to their retention time and their m/z value. Depending on the physicochemical properties of peptides and the chromatography method, peptides elute from the LC column at specific retention times and subsequently ion abundances and m/z values are measured by the mass spectrometer. The intensity of a peak as a function of the retention time can be visualized as an extracted ion chromatogram

(XIC) and the AUC can be determined. The matching of precursor ions according to their retention time implies that this quantification approach requires a robust performance of the LC system. The LC performance limits the number of samples that can possibly be analyzed in one differential study, since retention time shifts occur within the time and complicate the matching of peptide peaks. In addition, this method needs a robust sample preparation workflow to allow the analysis of comparable sample amounts. For the realization of a label-free analysis the user relies on computational methods. The matching of peaks, peak picking, noise reduction, and also normalization of peak intensities is performed using software solutions [12].

2.3.2.2 Spectral Counting

The spectral counting approach is based on counting the fragment-ion spectra (MS/MS) acquired for peptides and the comparison of their quantity between different samples. This method relies on the observation that more abundant peptides will produce higher numbers of MS/MS spectra in data-dependent acquisition. Therefore, the number of spectra can be correlated to the abundance of the corresponding protein [13]. As the quantification strategy of this method depends on counting of acquired spectra rather than on physical measurement data, this approach is controversial. One of the limitations of spectral counting is that different peptides have different physicochemical properties, which might cause bias and variability in the MS measurements. In addition, spectral counting should consider the length of proteins as digestion of long proteins is likely to produce more peptides and consequently more MS/MS events. However, spectral counting was applied successfully in a multitude of settings, and many different strategies to improve the approach have been emerged. Absolute protein expression (APEX) was developed to consider both the number of peptide spectra for proteins and the probability of the peptides being detected by the mass spectrometer. In addition, normalized spectral abundance factor (NSAF) was introduced to improve spectral counting by considering the length of proteins [12]. One of the main advantages of spectral counting is that the size of data sets is generally not limited.

2.3.3 Label-Based Proteome Analysis

In addition to the aforementioned label-free approaches, label-based strategies are also widely used in the field of MS-based quantitative proteomics. In principle, such approaches utilize the labeling of proteins and peptides derived from samples of different experimental conditions with stable isotopes (2H, 13C, 15N, 18O) and a subsequent multiplex analysis of combined samples in a mass spectrometer. Depending on the applied approach particular mass shifts of differentially labeled samples are observable either at the MS1 or the MS*n* level and the quantitative information can be extracted from the ratios of isotopically labeled peptides (MS1 level) as well as of fragment ions or cleaved reporter ions (MS*n* level). Apart from this classification according to the level

of quantification, labeling strategies can further be categorized according to the applied labeling methodology, namely, enzymatic labeling, metabolic labeling, or chemical labeling strategies. While enzymatic labeling strategies play only a minor role in routine proteomics analyses, chemical and metabolic labeling approaches are widely used. Hence, well-established and robust protocols as well as several commercially available kits for labeling and software packages for data analysis are available for chemical and metabolic labeling.

The most prominent metabolic labeling strategy referred to as SILAC (stable isotope labeling by amino acids in cell culture) utilizes a quantification at MS1 level by comparing the signal intensities of peptide ions containing isotopically labeled variants of the amino acids lysine and arginine (one of these amino acids should generally be present in a tryptic peptide) [14]. Depending on the number of heavy isotopes in the labeled amino acids a triplex experiment can be generated. The fact that every protein containing arginine or lysine is metabolically labeled and therefore can be quantified leads to high proteome coverage that is accompanied by high accuracy and good reproducibility of quantification. However, disadvantages of SILAC are its low dynamic range and its limitation to cell culture models. To overcome this limitation SILAMs (stable isotope labeling by amino acids in mammals) and Super-SILAC approaches have been developed. While SILAM is still limited to studies with animal models, the Super-SILAC approach can be applied even to clinical samples [15].

A chemical labeling approach relying on a quantification methodology comparable to SILAC is dimethyl labeling. Here, quantitative information is again obtained by measuring the signal intensities of labeled peptides showing a particular mass shift at MS1 level. However, in this case, the isotopic label is not introduced by amino acids containing different isotopes. Instead, dimethylation of peptide amino groups (side chain of lysine, N-terminus) with formaldehyde and sodium cyanoborohydride is utilized to generate an isotopic coding suitable for triplex analysis [16]. Within the field of chemical labeling strategies several more approaches are available and widely used. These include ICATs (isotope-coded affinity tags), ICPL (isotope-coded protein labeling), iTRAQ (isobaric tags for relative and absolute quantification) [17], and TMTs (tandem mass tags) [18]. In particular, TMT and iTRAQ imply the labeling of proteins or peptides with isobaric tags consisting of a peptide-reactive group, a mass normalizer, and a reporter region. Usually, the tags are incorporated via active NHS esters at the amino group of lysine and the N-terminus of a protein or peptide, but new variants of TMT tags also allow for the selective labeling of sulfhydryl or carbonyl groups. As peptides labeled with differently coded isobaric tags are isotopomers they behave exactly the same within the LC-MS analysis (same chromatographic behavior and precursor mass). However, during fragmentation reporter ions of different masses are cleaved, whose abundances allow for a relative quantification between the differently labeled experimental groups. With the possibility to analyze 10 samples at the same time the newest generation of TMT tags enables the highest multiplexing capacity available.

Chemical labeling-based quantification strategies are characterized by a higher quantification accuracy compared to label-free approaches [19]. However, label-free quantification shows a wider dynamic range, which is beneficial when large differences in protein abundance are quantified. A main advantage of chemical labeling-based approaches is the possibility to set up multiplex experiments that allow reducing the number of necessary LC-MS/MS measurements. Labeling reagents are available in well-defined kits that are easy to use, and are therefore also associated with quite high costs and additional steps in sample processing.

Label-free approaches and isotope-labeling methods can provide quantification data for hundreds or even thousands of proteins. Owing to technical variability in the label-based methods and the relatively low precision of label-free techniques the expression levels of proteins of interest should be verified using alternative methods. Unfortunately, there is no assay that covers the majority of the human proteome and a great number of commercially available antibodies have a very bad performance. Thus, for verification and quantification, targeted MS-based approaches such as SRM/MRM are emerging in the field of proteomics as a gold standard for precise quantification [6, 20]. Very high accuracy can be achieved when stable isotope-labeled peptides, having the same properties as the natural endogenous peptides, are spiked into the samples as an internal standard. Targeted proteomics techniques can be used for quantification of specific and predetermined proteins and are therefore ideal tools to verify biomarker candidates in large sample cohorts. As a matter of fact, targeted proteomics begins to fill an analytical gap between biomarker discovery and high throughput assays such as ELISA that afford a long development time.

2.4 MALDI Mass Spectrometry Imaging

MSI is a fairly new and therefore constantly evolving technique in the field of proteomics and biomarker research. MALDI MSI is based on a combination of histological information of a tissue and its corresponding mass spectrometric information. The acquisition of spectra directly from tissue sections is possible, by moving the instrument stage in two dimensions within the mass spectrometer. Mass spectra are acquired for each x, y coordinate. The measurement provides spatially resolved information of analytes, such as proteins or peptides and their abundances in different areas. Hence, MALDI MSI enables the correlation of mass spectrometric information with histological information derived from classical histological techniques. For example, healthy and diseased tissue can be compared and proteins characteristic of a certain tissue can be identified. Usually, the instrument used for MALDI MSI is a MALDI TOF mass spectrometer. More sensitive analysis techniques with orbitrap mass analyzers or an ion-mobility spectrometry option enhance the accuracy of the results and can differentiate analytes within the millidalton range. Ion mobility can separate analytes with the same mass but different ion mobilities.

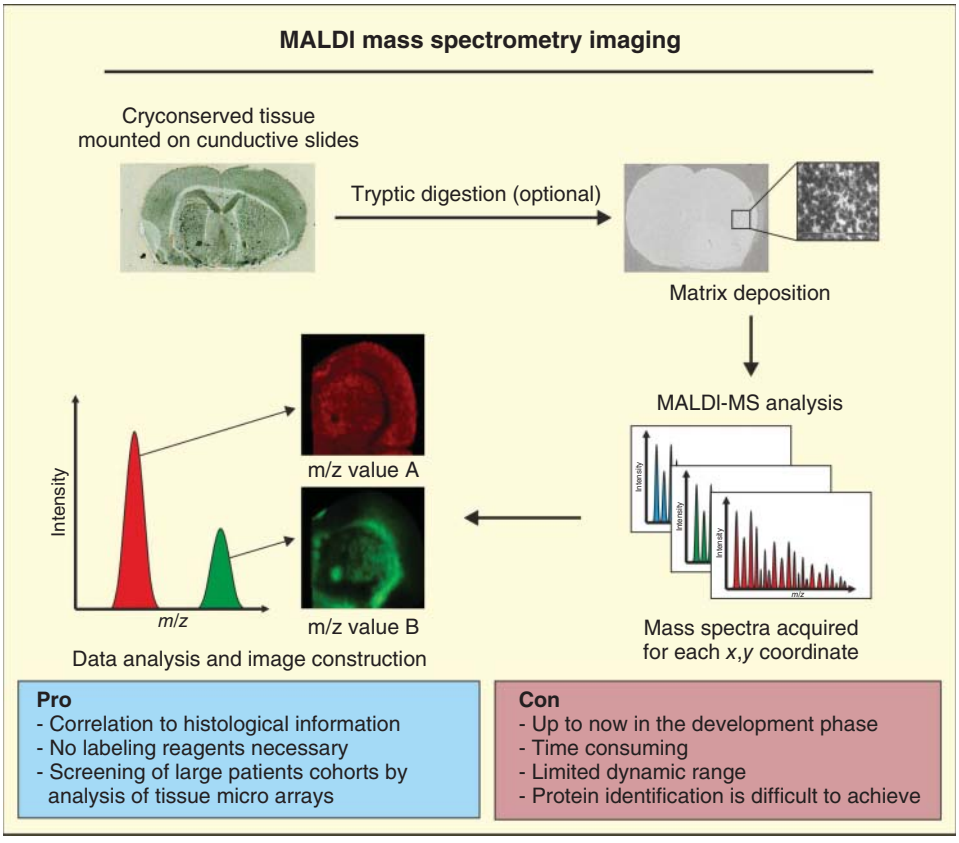

Figure 2.3 *Schematic representation of MALDI mass spectrometry imaging.* A representative workflow of a MALDI (matrix assisted laser desorption ionization) mass spectrometry (MS) imaging experiment is shown. Pros and cons of the technique are summarized in blue and red boxes, respectively.

A standard MALDI MSI workflow is shown in Figure 2.3. First, a cryoconserved tissue section is prepared and mounted on a conductive glass slide, since conductivity of the slide is a prerequisite for the MALDI process. The pretreatment of the samples concerning tissue storage, sectioning, and washing procedures are considered to be among the most critical steps in MSI experiments. Incorrect storage conditions can lead to protein degradation within the tissue and consequently change the protein composition of the sample. Different washing procedures were described to optimize the sample preparation and are important to remove contaminants such as salts or lipids [21].

The spatial resolution of MALDI MSI is defined by the raster width (i.e., "pixel size," geometric distance between spectra), which ranges from 10 to 200 µm. The spatial resolution is limited by the matrix deposition method. The matrix can be

applied by a spotting system, which achieves good extraction efficiency of analytes, whereas the spot to spot distance is mostly about 200 µm. The other widely used technique is the deposition of matrix with spraying devices, where the size of droplets and consequently matrix crystals that cover the tissue achieves a better resolution up to 20 µm [22]. In MALDI MSI experiments there is always a compromise between spatial resolution and analyte extraction efficiency. The better the quality of the matrix the better the analyte extraction efficiency, but the spatial resolution decreases due to bigger droplets on the tissue. The best spatial resolution can be achieved by sublimation of the matrix. Therefore the matrix is directly transferred from the solid phase into the gas phase and used for solvent-free coating of the samples. This procedure prevents spreading of analytes in the deposited matrix droplets. For proteins and lipids positive results with a 10 µm spatial resolution have been demonstrated [23].

In routine pathological diagnosis IHC is used for protein visualization and tumor detection and classification. By now, MALDI MSI cannot reach the spatial resolution of IHC down to cellular structures visualized by antibodies. A disadvantage of IHC is that multiplexing of different antibodies can be complicated and the number of antibodies is generally limited. Antibodies might be unspecific or not capable of detecting important PTMs. The limitations in standard diagnostic techniques have led pathologists to be open minded about new techniques to close these gaps in diagnosis. MALDI MSI is able to detect multiple markers simultaneously, and additionally protein modifications can be examined. Recently, the remarkable gain of knowledge about MALDI MSI has promoted interest in different research areas. Especially in medicine, multiple newly available tools have been used to address important clinical questions. Here, MALDI MSI might help answer so far unresolved clinical questions. For example, protein MALDI MSI is able to automatically differentiate between different tumor grades of papillary bladder cancer tumors. The World Health Organization changed the system of bladder cancer grading 10 years ago from a three-grade system (Grade1 = G1, Grade2 = G2, and Grade3 = G3) to a two-grade system (Low Grade = LG and High Grade = HG). Still, the former G2 papillary tumor is hard to classify into LG or HG and therefore an unbiased objective tool for classification of G2 is needed. MALDI MSI spectra from tissue sections of the well-defined and easy to distinguish G1 and G3 were compared and the differences were used to define an algorithm that allowed the automatic classification of the uncertain G2 grade by MALDI MSI in an unbiased and objective way [24]. This illustrates how the routine work of clinicians can benefit from MALDI MSI techniques. Not only single marker proteins are stained and evaluated manually by pathologists but multiple spectra representing peptides or proteins are also used for differentiation of tissue areas. MALDI MSI can be implemented into fully automated workflows as data analysis is based on computational methods. Possibly well-defined samples can be used to generate tissue fingerprints, which allow automatic identification of tissue types in a clinical specimen.

2.5 Conclusion

Since the development of quantitative proteomics the aim to discover protein biomarkers for diagnosis and management of diseases has been a great motivation for technological progress in the field. However, although countless biomarker candidates have been published only very few were translated into clinical routine [25]. Often, this is interpreted to indicate that proteomics technologies are not suitable to discover biomarkers. This is not true and in most cases other factors such as an inappropriate study design, analytical faults (e.g., sample pooling), or a deficient definition of the biomarker's purpose account for the missing follow-up and consequently missing implementation into clinical routine. In fact, proteomics technologies have never been as powerful as they are today. Enormous technological progress has been made and the development of new technologies is still ongoing. As MSI illustrates completely new techniques emerge and constantly initiate new approaches to resolve clinical problems. All presented proteomics technologies are efficient tools for biomarker discovery, each with the addressed pros and cons. For a biomarker discovery study the applied proteomics technique should be chosen with care according to the respective requirements. Experimental parameters should be considered and most importantly the application of the biomarker must be taken into account when setting up a differential study. As a result, the experiences gained in the field of biomarker discovery can help improve future efforts.

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3
Biomarker Qualification: A Company Point of View

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3.1
Introduction

Biomarkers are defined by the US National Health Institute (NIH) as characteristics that are objectively measured and evaluated as indicators of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. As such, they are useful in both daily clinical practice and during drug development.

Over the last decade, in conjunction with the advent of the "omics" era, an ever-increasing number of biomarkers were reported to be associated to various pathological and physiological conditions. Pharmaceutical companies are investigating companion diagnostics in order to implement personalized drug therapies, and medical diagnostic manufacturers are developing new diagnostic tools in order to answer specific medical needs. These initiatives were positively perceived and encouraged by the Health Authorities, which have been pronouncing the importance of biomarkers in translational medicine and for a more personalized healthcare.

It became rapidly a consensus by all the stakeholders in the industry that biomarker technologies will only deliver their promises if the technology achieves a high level of quality and standardization. Diagnostic companies, specialized contract research organizations (CROs), and biotechs are serving these increasing market needs of biomarker discovery and development.

For several years, academic and industrial actors have been using various technologies to establish correlations between biomarker data and clinical endpoints. These efforts were designed in order to make the assessment of an often complex or long-term outcome accessible through a relatively quick and inexpensive measurement such as a laboratory test.

Although a number of candidate biomarkers have been identified correlating with diverse clinical endpoints, only a very limited number have made their way to actual use by medical practitioners and industries of the healthcare sector.

One of the reasons identified for the lack of clinical adoption of new biomarker candidates is the lack of reproducibility often associated with the biomarker

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measures. This poor reproducibility of new tests has raised skepticism in front of new biomarkers. In particular, the industrial sector has urged regulators to establish industry guidelines for the formal qualification of biomarkers in drug development [1]. In parallel, intense discussions within several public–private initiatives recently created both in United States (critical path launched in 2004) and Europe (IMI-JU launched in 2008) allowed the health authorities to issue recently a formal regulatory process for pre- and clinical biomarker qualification for use in drug development processes.

This evolution transformed the biomarker development domain as a new industrial discipline and allowed the development of various aspects related to the formal qualification of biomarkers. This chapter discusses various key features related to this field.

3.2 Biomarker Uses

Biomarkers in clinical chemistry have been in use for many decades and are often well established for diverse clinical applications, for example, creatine kinase-MB (CK-MB) in the diagnosis of acute myocardial infarction. Cardiac troponin (cTn) complex, a protein involved in heart muscle contraction has also been in use since about 25 years, first as a marker of cardiac necrosis [2], and more recently, as a marker for the short-term prognosis after acute coronary syndrome [3].

Interestingly, C-reactive protein (CRP) has been discovered more than 70 years ago and is used as a marker of inflammation [4]. However, its clinical indication expanded recently when a more sensitive version of the test was made available, allowing its usage also as a marker of cardiovascular risks [5].

Such markers, whose levels indicate an inflammation or a different type of infection, provide a diagnostic aid to the complete anamnesis of a patient. They have to be interpreted together with other data, for example, serological markers in order to lead to a clinical endpoint.

The pharmaceutical industry is investing significantly in biomarkers to reduce resources spent on the drug development process, to assess the efficacy of new compounds, and to ensure their clinical safety. The common needs of the industrial partners for such markers and in particular for safety-related markers, incited partners from public and private institutions to join their efforts in this area, called the "pre-competitive field."

Many public–private joint efforts recently focused to identify and develop new biomarkers for kidney, liver, and cardiovascular toxicities induced by drug candidates. These three organ toxicities represent a high percentage of drug-induced organ injuries observed during the drug development process.

The liver functionality is currently assessed using standardized liver tests, which consist of a number of functional and leakage markers. These are usually aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), bilirubin, and albumin. ALT can also be increased in some individuals

without disease, displays a pronounced diurnal behavior, and can also be increased after strenuous exercise [6]. Markers such as transaminases taken on their own hence provide only limited insight into the state of health of a patient. Innovation in the field of biomarkers has been driven by a need for tools that display a greater clinical specificity than the conventional markers available.

Presently, the most common markers of kidney toxicity are creatinine and total urinary protein. However, the relationship between creatinine and the glomerular filtration rate is not linear: an increase in creatinine levels in urine occurs when irreversible kidney damage has already started significantly [7]. It is hence clear that early markers of kidney toxicity and kidney damage are greatly needed. Localization is also important: a marker released and detected in urine saves the patient and the practitioner complex procedures such as kidney biopsies. Cystatin C and neutrophil-gelatinase-associated lipocalin (NGAL) are examples of second-generation kidney biomarkers that have established their place in clinical practice [8, 9].

An important application of biomarkers is their usage as a parameter of decision making during drug development. From a regulatory point of view, biomarkers are considered as drug development tools and are subject to a formal regulatory qualification process. Theoretically, this can serve from patient population selection to surrogate safety of efficacy endpoint. When using biomarker-based decisions in a drug registration study, the sponsor is required to provide the health agency with sufficient performance data to support the use of the biomarker. Noteworthy, a simple correlation between the biomarker and the desired outcome can often give misleading results. Endpoints should be sensitive enough to measure the desired endpoint but other parameters are also carefully considered in the validation package. For instance, the feasibility of measurement in clinical setting, the analytical validation of the measurement method, and the interpretation process of the biomarker should also be addressed in the qualification package. In addition, specificity of the biomarker and the effect of common covariates such as age or comorbidities should be considered specifically in the frame of the intended use of the biomarker. Although there are no specific requirements from the regulators to establish a causal link between the biomarker and the parameter measured, a well-documented mechanistic relationship greatly increases the credibility of the biomarker package [10].

3.3 Biomarker Types

Biomarkers are commonly classified on the basis of the molecular type of the analyte measured.

According to their origins,

- *genes,* genes products (transcripts, miRNAs, nc RNAs, etc.)
- *proteins,* enzymes, posttranslational modifications
- *metabolites* (peptides, proteins, lipids, etc.).

According to an ICH guideline released on the topic (EMEA/CHMP/ICH/437986/2006), genomic (DNA) biomarkers encompass the detection of single nucleotide polymorphisms (SNPs), variability of short sequence repeats, haplotypes, DNA modifications, deletions, or insertions of single nucleotides, copy number variations, and cytogenetic rearrangements. At an RNA level the sequence, expression level processing, and the amount of microRNAs fall into this category (EMEA/CHMP/ICH/437986/2006).

Many genotypes described in the literature are associated with prediction of disease, disease outcome severity, and response to therapy. The prediction of disease is of course easier when the genetic link is well described. Early-onset Alzheimer disease, for example, displays Mendelian genetics and its onset is strongly linked with three genetic mutations in the *APP, PSEN1,* and *PSEN2* genes. Mutation of these genes change the production of Aβ peptide, the principal causative agent of the senile plaques that are characteristic of Alzheimer disease [11].

Proteins are another major class of molecular biomarkers. The practical advantage of protein biomarkers is that they can often be tested from a matrix that is easily accessible such as peripheral blood or urine, making patient biopsy or a more invasive sampling redundant. Protein biomarkers are often measured using immunoassay platforms or enzyme activity assays. Clot-based assays, used when a bleeding anomaly is suspected or to monitor anticoagulant therapy, are prime examples of enzyme activity assays. The first radioimmunoassay has been described in 1960 and automatized enzyme-linked immunosorbent assay (ELISA)s for clinical chemistry exist since the 1980s [12, 13].

Immunoassays, in particular ELISAs, have been classically used as one of the reference technologies to develop numerous biomarker tests. Even though universally available, the use of ELISAs in measuring protein biomarkers has its limitations, especially in terms of sensitivity, dynamic range, and sample volume. A number of novel platforms have been developed in recent years to address the shortcomings of the classical ELISA or to expand its use. These are often fluorescence or chemiluminescence based.

The advantages of emerging platforms can be the following:

- *Multiplexing*: several biomarkers can be measured in a single sample, saving sample volume and resources.
- *Improved assay range*: While the assay range of a classical ELISA covers typically 1 log of concentration, novel platforms have assay ranges of up to 5 logs. A higher probability of a measurement being in the assay range increases total precision and obviates post-measurement dilutions.
- *Improved sensitivity*: Biomarker measurements have been described even in the femtomolar range, increasing the utility of known biomarkers and opening up the potential for proteins to be biomarkers that are found in the matrix of interest at very low concentrations. Improved sensitivity also means more precise measurements and higher dilutions that save sample volume.
- *Increased assay speed*: While a classical sandwich ELISA typically takes 3 – 5 h to prepare, immunoassay-based platforms have been developed that can measure

biomarkers in minutes, which is especially important in an estrogen receptor (ER) setting.
- *Improved throughput*: Immunoassay automatization finally allows clinical chemistry laboratories to run tests at a high throughput and decreased operator variability.

Small-molecule metabolites are another type of biomarker. Blood glucose and cholesterol measurements may be the most prominent example in clinical practice. In terms of lipids, lipoprotein-associated and soluble phospholipase A2 measurements were proposed as biomarkers of cardiovascular risk. Regarding novel markers, glycine, lysophosphatidylcholine (LPC), and acetylcarnitine have been identified as potential biomarkers for the development of Type II diabetes, for example [14]. Some metabolites have been identified as potential drug development tools (DDTs). An example would a recent finding that lactate, phosphocholine, and glycerophosphocholine levels could be used to monitor treatment response to phosphatidylinositol-3-kinase (PI3K) pathway inhibitors [15].

3.4 Validation vs. Qualification

The need for improved diagnosis, better treatment, and accurate assessment of endpoints in clinical studies drives biomarker innovation. Increasing numbers of new potential biomarkers are published in the literature each year. The path from biomarker candidate to biomarker leads over validation and qualification. Validation is related with the analytical performance of the candidate – well-documented test characteristics ensuring that the biomarker can be measured to a degree of precision and accuracy that is sufficient for its final intended use. Qualification corresponds to the mechanistic link between the biomarker itself and its final intended use in pre- and clinical settings. Validation, that is, proving a satisfactory measurement, has to hence happen before a biomarker can be qualified. Biomarkers are said to be qualified when they have been shown to be fit to detect a given preclinical or clinical endpoint (*www.fda.gov*; *www.ema.europa.eu*).

3.5 Strategic Choices in Business Models

An increasing number of biomarker discoveries have been realized during the last two decades thanks to the availability of especially various X-omics approaches. Mass-spectrometry-based proteomics approaches have also yielded a multitude of potential protein biomarkers. However, the translation of potential biomarkers to diagnostic tools has only recently been increasing. In fact, the list of FDA-approved *in vitro* diagnostic tests and companion diagnostics that are based on proteomics is still relatively small (*www.fda.org*).

This low implementation rate from bench to bedside has been found to be due to technical limitations. For example, the human plasma proteome contains proteins at concentrations that spread 5 logs from albumin, the most abundant, to rare proteins [16]. As biomarkers are more likely to be found at very low concentrations, serum depletion of abundant proteins, enrichment, and shot-gun fragmentation approaches are used to expand the detection range. However, development has to keep pace with discovery. As it becomes feasible to measure subnanomolar concentrations on discovery platforms, classical immunoassays (ELISAs) do not reach this level of sensitivity [16]. In recent years, the work of technology-based companies and academia is gradually allowing the translation from discovery to the development of validated assay platforms. As mentioned previously, the translation of the classical ELISA to technologically novel immunoassay-based platforms is an example for this translation. Combining several detection technologies such as fluorescence and electrochemiluminescence is a strategy to improve sensitivity. Platforms that incorporate single-photon counting drive assay technology from an analog to a digital era. Through increase in sensitivity, they open up interesting perspectives to transfer biomarker discovery to development and clinical use.

As it becomes technologically more feasible to validate biomarkers, specialized biology laboratories, CROs, and diagnostic companies fulfill this role. Service in the development of biomarkers, which entails the development of an assay and biomarker validation, is of particular interest for pharmaceutical companies developing therapeutics. Biomarkers are developed here either for drug efficacy studies or incorporated into companion diagnostics. Developing biomarkers for use as companion diagnostic tools can be a challenging task, though. There is, for example, the increase in development costs for a companion diagnostic when compared to the therapeutic alone. As the increase in market potential is often not proportional, this has been one of the primary inhibitory hurdles in the past [17]. Other impediments in developing a companion diagnostic can be the technical difficulty of validating and qualifying a test performing the role of a surrogate end point and the laboratory proficiency of the developing diagnostic partner.

Once an assay for the biomarker is set up and validated, it needs to be qualified in a "fit-for-purpose" approach. CROs and other laboratories specialized in clinical sample testing are involved in a range of services that include biomarker qualification and biomarker testing in the frame of drug development studies.

Some diagnostic companies are medical device driven. They provide the automatization that is necessary for other stakeholders to reliably measure clinical samples.

3.6 Validation of Analytical Methods

Analytical validation of the measurement tool needs to take into account a number of steps: The sensitivity needs to be proved to be sufficient for the final intended purpose. Some proteomic markers, for example, have an endocrine or

signaling function in the biological matrix tested. They will be naturally occurring in physiologically healthy individuals and the difference between healthy and diseased individuals will be very low. Other proteomic markers are more unlikely to be found in healthy individuals as they are released upon necrosis or following tissue injury. The assay should hence be tuned so that the medical decision level of the marker lies ideally in the linear part of the assay range.

Detection limit is often defined as the point from which the signal can be discerned from the background to a certain probability. Precision usually decreases the closer a signal gets to the lower end of the measurement range of the assay. The lower limit of quantification is hence a point of prespecified minimally acceptable precision. The same is true for the upper limit of quantification, which, together with the lower limit of quantification spans the measurement range of the assay. Total imprecision or total error of a biomarker assay is the sum of all its systemic and random errors. Of particular importance in terms of random errors is the precision measurement of technical replicates within one assay (the intra-assay precision) and the reproducibility, that is, the precision of completely independent repeat measurements. Total error can be evaluated by comparing the method to a reference method should that be available. Another part that needs to be evaluated is interference of the measurement. Different factors that can cause interference, either because they have been shown to interfere previously for other assays (lipemia, rheumatic factors, heterophilic antibodies) or because of their homology with the protein biomarker, need to be assessed. Stability is also an important aspect in terms of robustness of the assay. The freeze–thaw, short-term, and long-term stabilities of the assay are parameters that are often tested.

3.6.1 Currently Applicable Guidelines for the Validation of Analytical Methods

The FDA has developed guidance for industry standards for the method development of assays that follow pharmacokinetic (PK) studies in small-molecule drug development [18]. Routine clinical chemistry testing laboratories follow the Clinical Laboratory Improvement Amendments (CLIA) guidelines to validate assays in the United States [18] and relevant ISO norms in Europe. In addition to these, GLP (good laboratory practice) rules exist for bioanalytical assays used in pharmacokinetic studies. However, even though the FDA has made biomarkers a focus and included them as a key item in its Critical Path Initiative, no set of definite recommendations has been issued for biomarker assay development and analytical activities. During the 2003 AAPS Biomarker Workshop, a reiterative "fit-for-purpose" validation process has been proposed. This "fit-for-purpose" approach describes a validation process that is customized to its intended use and that follows different phases of increasingly more stringent validation [19]. Differences between this path of validation with the GLP rules of pharmacokinetic validation and routine laboratory validation are partly based on the fact that reference material most often exists for the bioanalytical methods, while it does not for novel biomarkers. Furthermore, in pharmacokinetic methods the analytes (small molecules) are

exogenous, while in biomarker assays the analyte is mostly an endogenous protein or nucleic acid. The effect of the matrix is also more difficult to take into account in a biomarker assay than in a bioanalytical assay [18].

Guidelines and standards have also been published by CLSI, a volunteer-driven standards organization. Relevant guidelines are, for example, EP05-A2, EP06-A, EP07-A2, EP09-A2, and others that provide manufacturers of biomarker assays with recommendations for analytical validation (*clsi.org*).

The qualification of a biomarker as DDT is going through the CDER office of the FDA. CDER reviews novel DDTs and provides guidance. As such, it has published recommendations for manufacturers on how to qualify a DDT [20]. The European Medical Agency (EMA) has equally recognized the importance of biomarkers in drug development and also provides guidance and public opinions on biomarker qualification.

In terms of diagnostic tools, biomarkers require CE marking if they are *in vitro* diagnostic medical device (IVD) devices in Europe. In the United States, the analytical parameters of the IVD are checked by a 501(k) submission before the product is placed on the market. CE marking of a biomarker assay follows the European directive 98/79/EC, which is an approach that focuses on risk minimization and on the reliability of the manufacturer's own claims. In the United States, the CDRH office of the FDA regulates the validation process.

3.6.2 Laboratory Proficiency

Clinical samples have to be measured to qualify a biomarker as a DDT or to use the biomarker as a DDT in the actual drug development. Testing and clinical laboratories have to demonstrate an appropriate level of proficiency for that. ISO 17025 is thereby the main standard used for testing and calibration laboratories. The standard has a technical and a managerial part, and emphasizes continuous improvement, technical competence, and staying abreast with the technological state-of-the art. Biomarker DDTs as well as diagnostic devices are validated by the laboratory to fall into the scope of the accreditation. The standard hence includes elements of analytical validation.

GLP on the other hand describes a quality management system that is suitable for nonclinical safety tests and obligatory in most OECD countries. GLP regulation is described in the United States, for example, by 21CFR58 and in the EU Directive 2004/9/EC.

3.6.3 Establishment of Reference Ranges for Candidate Biomarkers

Knowing the physiological concentration of a protein is important for it to be a biomarker. Especially for novel markers for which new assays are being developed and validated, a reference range is often not available. The establishment of a reference range is linked to the use of the biomarker. During biomarker qualification,

a control population is tested from which a reference range can be deducted. A cohort of healthy subjects is chosen that is as similar as possible to the test cohort. This includes the subject's age, sex, ethnicity, and various routinely tested physiological parameters. In a biomarker assay intended to follow a therapeutic intervention, subjects treated with the standard therapy or, if ethical, untreated patients are included as controls. A fine definition and description of a reference range is hence always necessary when assessing biomarkers. Chronobiological and environmental conditions also need to be included. The time of the sample drawing, nutritional habits, exercise, and confounding factors such as ongoing therapeutic treatment, tobacco and alcohol consumption, and drug abuse should be known. In most protein biomarker assays the standard curve calibrator will not be exactly the same as the measurement for which a reference range is to be established. Circulating biomarkers have a particular pattern of posttranslational modifications and alterations. They can be partially degraded, alternatively spliced, contain cell- and species-specific posttranslational modifications such as glycosylations and others, or they can be bound to various binding partners. The chance that a standard curve calibrator will be identical to the endogenous molecule is hence very small. This leads to errors in the reporting of reference ranges. It highlights that not only the population specifics affect a reference range but also the biomarker measuring method and its calibrator need to be taken into account.

3.7 Clinical Qualification of Candidate Biomarkers

3.7.1 Methodological Approaches

Methodological considerations during the development of biomarkers are frequently overlooked. This often leads to the reporting of misleading biomarker performance data and lack of reproducibility of biomarker performance. This is exacerbated by the use of data mining approaches and predictive modeling tools that are prone to overfit a biomarker model to a particular study. It is then essential to ensure that the methodological robustness of a biomarker validation project is tested and maintained. A recommended approach is a multistep "funnel-like" approach including first the selection of a panel of biomarkers and then the characterization of the performance of the biomarker or the biomarker panel.

There is no consensus for the methodological steps associated with the qualification of biomarkers but one recommended approach is a multistep validation process. The SAFE-T consortium, an industrywide initiative intended to qualify biomarkers of drug toxicity has published a general qualification process for biomarkers [21]. This process is based on the conduct of three steps defined as "stage-gate," exploratory, and confirmatory studies. Essentially, the "stage-gate" cohort aims at demonstrating the feasibility of biomarker measurement in the appropriate context, the exploratory study aims at building a biomarker model

fit for the intended use, particularly in terms of sensitivity, and the confirmatory step aims at validating the biomarker performance and establishing the specificity of the marker, particularly with respect to possible covariates that may affect its later use.

Another common issue in the qualification process is the selection of a reference standard or gold standard to establish the performance of the biomarker. The "gold standard" is the best available method to measure a given endpoint under reasonable conditions [22]. It is to be noted that the gold standard for the definition of a disease may not be impractical or even virtually not usable in practice; for instance, histologic examination of the brain provide a definite diagnostic criteria for Alzheimer's disease but cannot be considered as a diagnostic tool as it is not possible to obtain a suitable sample in living patients. In this particular context, the best standard may be any criteria used clinically to diagnose patients. In such a complex case, the reference to be selected for the benchmarking of biomarker performance needs to be subjected to discussion and consensus with the regulators prior to the conduct of clinical studies. A similar discussion should address other complex cases such as diseases with no currently established diagnostic criteria. Possible solutions are the use of statistical methods that do not require any reference criteria to establish the performance of a marker, or the use of an independent adjudication committee that will make a classification decision based on the clinical data.

Another key regulatory requirement for the qualification of a biomarker as a drug development tool is the definition of "Context of Use" (CoU). The CoU can be considered as the equivalent of a drug indication in the field of biomarkers. The CoU clearly defines the scope of the biomarker use with precise details on the situation in which the biomarker can be used, the population concerned, the nature of the biomarker signal (measurement method, threshold), the clinical interpretation, and the decision to be made on the basis of a positive or negative biomarker signal (decision tree).

As of today, unlike for the qualification of drug development tools or for the qualification of a companion diagnostic biomarker, there is no formal qualification process on the type and level of scientific/clinical evidence to be provided for the qualification of a candidate biomarker as a standalone diagnostic device. Manufacturers have the legal obligation to document development and manufacturing of the device, maintain traceability, and show the clinical performance of the device. However, this demonstration of performance does not constitute proof of the utility of the biomarker in the clinical care of patients. Once a manufacturer releases a diagnostic product, the adoption of the device by the clinicians/users will then depend on the weight of evidence available, the market access to the device, and of course on the price and insurance reimbursement status of the device.

3.7.2 Study Size for Biomarker Performance Characterization

To ensure that data from the study will lead to an acceptable estimate of biomarker performance (mainly sensitivity and specificity values), sample size calculation for

a study cohort can be performed according to the method reported by Buderer [23]. This method permits estimating the minimum sample size required to compute sensitivity and specificity with a given 90% confidence interval with α error. For instance, with an $\alpha = 0.1$, we will be able to establish the minimum sample size to calculate performance of the assay with a 90% interval of confidence.

Therefore, with an expected sensitivity and specificity of 80% we would be able to calculate the following:

$$N_d = Z^2_{\alpha/2} \frac{\mathrm{SN}(1-\mathrm{SN})}{W^2 \times P} = 1.645^2 \times \frac{0.8(1-0.8)}{0.1^2 \times 0.5} \approx 87$$

where N is the number of patients with the disease, SN is the sensitivity, W is the width of the 90% confidence interval, P is the prevalence of disease in percentage (50%: cases and 50% controls in case control studies), and Z is the Z value from the normal law table with 90% confidence interval width of 10% $\alpha = 0.1$.

The corresponding formula for the specificity leads, in our case with $P = 0.5$, to the same minimum number of patients needed:

$$N_d = Z^2_{\alpha/2} \frac{\mathrm{SP}(1-\mathrm{SP})}{W^2 \times (1-P)} = 1.645^2 \times \frac{0.8(1-0.8)}{0.1^2 \times 0.5} \approx 87$$

where N is the number of patients with the disease, SP is the sensitivity, W is the width of the 90% confidence interval, P is the prevalence of disease in percentage (50%: cases and 50% controls in case control studies), and Z is the Z value from the normal law table with 90% confidence interval width of 10% $\alpha = 0.1$.

So, a total population of approximately 87 patients will be required.

However, the sample size required varies considerably with the sensitivity, and a similar reasoning can be applied for specificity.

As shown in Figure 3.1, the level of precision targeted is achieved in the worst case scenario with 135 patients and 135 matched controls. This situation is very unlikely to occur given that this would be the case of methods with no discriminating power at all. Numerous literature reports, and internal data, show that multivariate analysis of the markers allows relative stringent discrimination across the different populations.

Therefore, the project sample will be at least sufficient to calculate the performance of the marker, with the required precision.

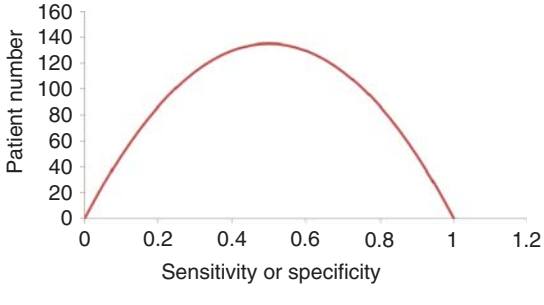

Figure 3.1 Number of patients to be included to determine performance parameters with a 90% confidence interval as a function of sensitivity or specificity of the biomarker.

3.7.3 Sample Quality and Biobanking

A point of growing interest is the sample quality required to conduct biomarker studies. Quality of sample will essentially depend on the methodologies used to harvest the samples and on the modalities of its subsequent storage. For a long time, these operations were considered relatively trivial and were not the object of particular attention. However, the advent of the use of large cohorts for the study of biomarkers unveiled the importance and complexity of sample management in biomarker studies and has greatly increased the emphasis on these topics. The management of samples is now considered a discipline on its own and also covers aspects related to the stability of samples and advanced sample data management. The importance of biobanking is exacerbated by the increased use of retrospective samples, and in particular samples collected in the frame of large international cohorts such as the Anglo-Scandinavian Cardiac Outcome Study or the Framingham Heart Study.

3.7.3.1 Sample Collection

Collection of samples should be performed using standardized protocols, particularly in the case of biomarker studies involving several centers. Particular attention needs to be paid to selecting the sample matrices. Table 3.1 lists some common parameters to be considered for the collection of the most usual biofluids.

The importance of these parameters should not be underestimated given that it was reported that the smallest changes can lead to significantly different biomarker values. Of course serum and plasma commonly show marked differences, but more subtle changes can also have a significant impact. Notably, the platelet content of the sample has a significant effect on the proteome and warrants standardization of plasma centrifugation processing. The use of a mechanical separator can also be considered in order to lower the platelet content of a sample but may affect the measurement of circulating microvesicles, an increasingly studied family of biomarker. The choice of the anticoagulant may also be critical. The choice of an anticoagulant needs to be driven by several factors including the stability of the studied biomarker in the presence of different anticoagulants and the effect of the anticoagulant on the analytical methods [24]. It is also important for the investigator to remember that the impact of the

Table 3.1 Most common sample collection modalities for serum, plasma, and urine.

Biofluids	Collection modalities
Serum	Use of coagulation activator, clotting time, addition of protease inhibitors, centrifugation speed
Plasma	Anticoagulant, addition of protease inhibitors, centrifugation speed, use of a mechanical separator
Urine	pH buffer, addition of protease inhibitor

different parameters may affect different methods in distinct ways; for example, immunoassays may not be affected in a situation where mass spectrometry-based techniques can be affected drastically.

More recently, the addition of preservatives such as anti-proteases to the sample has been increasingly used or recommended. While it may turn out to be very useful for some biomarkers, it adds costs and complexity to the collection of samples. Furthermore, the nature of the preservatives should also be considered given that their nature will depend on the matrices and the markers. For instance, while urine samples may benefit from pH stabilization, blood-derived samples may benefit from the addition of protease inhibitors. The nature of the protease inhibitors may vary as well depending on the target inhibitor: while large spectrum protease inhibitors may be useful for many markers, some proteins will require a specific cocktail. For instance, metabolic factors such as ghrelin, glucagon, or glucagon-like peptide 1 will require specific inhibition of the dipeptidyl peptidase IV. Finally, care should be taken that the inhibitors used do not affect the analytical method. Indeed, while commercial preservative cocktails were developed, their impact was specifically documented for mass-spectrometry-based methods. Their impact on ligand-based assays needs to be established.

From a practical point of view, it is obvious that at the start of a biomarker project, the investigator will seldom have the time and resources to address all these points. A common approach will be to select the variables that are expected to have the most important impact and consider collection of samples in various conditions. While it may seem that the work related to sample collection may exceed the capacities of a clinical center, the advent of many commercial solutions for samples collection have greatly eased this work. Several types of commercialized sample collection tubes are available for various matrices and are pre-filled with anticoagulants and various types of protease inhibitors.

3.7.3.2 **Storage of Sample**

The storage of biomarker samples is a critical issue as stability, in particular of protein biomarkers, can be greatly affected by storage conditions. Here again, it is not possible to establish standard criteria applicable for all biomarker projects. However, the investigator can establish a process based on good practices in order to minimize sample storage issues. In particular, the following points may serve as a guideline:

- The process should always minimize the number of freeze and thaw cycles the samples are exposed to. Some markers are extremely sensitive to freeze and thaw cycles and can be affected drastically. To do so, aliquoting of samples in low volume (however sufficient to perform the different measurement) should be performed at the clinical site whenever possible. If onsite aliquoting cannot be performed, then samples will be frozen and aliquoted the day of the first analysis.
- The tubes used for the storage of samples should carry sufficient labeling to identify samples unambiguously. Ideally, tube labels should be printed either

directly on the tube or on moisture- and frost-resistant label. Whenever possible, handwriting should be avoided. The use of a barcoding system is greatly recommended. These tubes bear a globally unique barcode on the bottom, allowing storage in 96 well-plate format and reading of a plate using a scanner reader. Such a solution can be associated with higher costs than standard tube hand labeling but are of added value because they decrease the processing time as well as the risk of error. One other advantage is the freezer space gain obtained through the use of this compact format.

- The storage temperature of samples should be at least −20 °C. Ideally, samples will be first frozen at −20 °C for a few hours and then transferred to −80 °C. This pre-freezing step is believed to decrease thermic stress for the sample but there is no evidence of its added value for biomarker stability. However, pre-freezing at −20 °C will greatly reduce the temperature variation in the final storage device. Liquid nitrogen storage or −150 deep-freezers are mainly used to store living cell samples for cellular or functional biomarkers.
- Temperature in the storage device will have to be monitored as a minimal quality requirement. The temperature monitored is only valid if performed using a temperature probe calibrated versus an international standard. Manual temperature recording at regular intervals is possible but inferior to continuous monitoring using an informatics system. Most biobanks use a wireless temperature transmitter and a centralized real-time temperature log.
- Real time temperature monitoring can also be supported by an alarm system. Alarm messages can be transmitted by phone or other means to an officer in charge of the management of the biobank.
- Biobanks need to ensure the continuity of sample freezing, so that in case of default of a storage device, the necessary back-up solution is available. Such solutions could include back-up devices in case of failure of equipment, or an electric generator in case of failure of the power grid.
- Another key requirement for a biobank is the maintenance of a data management system to record the life cycle history of the sample and sample information such as the matrices, volume, and other sample identification data. Such system is ideally based on a database software and includes good IT practice such as controlled access in order to guarantee data integrity.

3.7.3.3 **Clinical Data (Sample Annotation)**

Establishment of biomarker performance requires the biomarker measurement data to be analyzed and interpreted in the light of patient clinical data.

Clinical data may include patient medical history, demographic data, laboratory test results, imaging data, or any other data resulting from clinical observation. These data are sometimes referred to as sample annotation data.

Clinical data can be collected in a number of ways. The historical method was based on the records of the data in paper form, the so-called Case Report Form (CRF). The CRFs are datasheets constructed according to the study protocol and are used to record the data of each patient at the various visits. More recently, electronic data capture systems have been developed. These software systems

make use of a computer or a mobile device to enter patient data. Data are then subjected to cleaning, formatting, and storage in a clinical database. Nowadays, several suppliers provide electronic data capture systems with various business models: closed proprietary or open-source softwares are available. Data collection and data management system should ensure compliance with regulatory requirements, in particular, related to proper tracking of users, access control, and data integrity. In the United States, requirements for electronic systems are described in the Code of Federal Regulation Title 21 part 11 (21 CFR part 11). A longer option is the design of a "home-brew" system provided that the necessary expertise is available for the development of such software in compliance with existing standards.

3.7.3.4 Ethical Considerations

The collection, storage, and use of clinical sample are tightly controlled by legislations and regulatory standards. Current standards are based on the Declaration of Helsinki on human research but may vary from country to country and always require that the patients are clearly informed of the purposes and risks associated with the study. Patient information should pay particular attention to record of genetic data. Depending on the study, it may be pre-approved by an institutional board or an ethical committee prior to its initiation. Patient information and acceptance to participate in the study are to be recorded by using a signed informed consent, and biobanks are required to maintain traceability of the patient acceptance of each sample.

3.8 Biomarker Qualification in the 'omics Era

The advent of omic technology has allowed the measurement of a large number of biomarkers from a single sample. Current multiplex technologies can measure the presence of millions of SNPs, the relative expression of thousands of gene transcripts or hundred of proteins using minute amounts of sample.

The data deluge created by these techniques has called for advanced analysis techniques and has highlighted the power of biomarker combinations in order to measure a particular endpoint. Indeed, application of advanced statistical methods such as machine learning technologies could make use of large datasets in order to identify hidden patterns and biomarker combinations. On the basis of various mathematical algorithms, these methods will identify a biomarker model based on a subset of biomarker data that reflects best the endpoint of interest.

However, these techniques also have limitations that require specific considerations.

One key consideration is the problem caused by multiplicity. Multiplicity of measurements will result in the identification of a biomarker and the biomarker panel that are falsely correlated with the endpoint. The term "overfitting" refers to the fact that a model designed to achieve a high specificity and sensitivity on a first

(learning) dataset may only perform poorly in another dataset. The model overfit is often due to the coincidental association of the model with the endpoint. The risk of an overfitting phenomenon increases greatly with the number of analytes measured and is a common pitfall if the learning dataset is of reduced size.

The risk of overfitting needs to be addressed by taking the following measures:

- Sufficient sizing of a biomarker study and its biomarker dataset in the learning dataset.
- Use of cross-validation statistical methods that will attempt to compensate the risk of multiplicity and overfitting in the learning dataset.
- Replication of the data in the confirmatory study. In this study the investigator should have committed on a hypotheses *a priori* and test this hypothesis in the subsequent study.

3.9 An Example of a Biomarker Provider

The area of biomarker development has reached a level of technicity that requires specific expertise and sufficient skills and equipment specialization. These particular needs have opened the door to specialized CROs or biomarker product manufacturers.

A number of companies operate on business models built around biomarkers. These companies address different market in the field of biomarkers. For instance, many companies offer discovery services based on various technologies. For instance, the Canadian company, Capricorn, or Belgium-based Pronota have developed particular expertise around mass-spectrometry-based biomarker discovery.

Other companies have developed expertise centered on the measurement of biomarkers. Myriad-RBM is nowadays one of the world leaders in the domain by proposing multiplexed bead-based immunoassay measurements. Alternative technologies have also recently made their way to the market; for instance, Somalogic Inc., propose a multiplexed aptamer-like binding assay for serum biomarkers.

These CRO organizations have built core expertise around biomarker science and technologies.

Other companies have developed expertise based on the translation of biomarkers toward applications in various disease areas. This is the strategy adopted by Firalis SAS. The company has established several laboratories that cover the needs of biomarker development. Typical activities cover the synthesis and production of analytical reagent, the development, and validation of analytical methods using various technologies and a structure that supports biobanking and testing of clinical samples. During the first years of its inception, the company has made a tremendous effort to establish compliance with relevant industry standards and obtain the corresponding certification.

Relevant standards include ISO 13485 certification (medical device manufacturing), ISO 17025 accreditation (testing laboratory proficiency), NF S 96 900 (Requirements for biobanking), and Good Clinical Laboratory Practice (GCLP).

The company offers its expertise on a service-for-fee basis to industrial partners and through collaborative R&D projects to academic researchers. This model has allowed access of cutting edge technology to academic researchers and has permitted the company to develop close relationships with key opinion leaders in the field of cardiac medicine. Based on this research network and in parallel to its service offer, the company has launched a series of internal validation programs for biomarkers in different cardiologic applications.

This mixed business model is designed to allow the company to balance its financial needs while conducting the tremendous undertaking that constitutes a biomarker qualification program.

3.10 Conclusion

The development of biomarkers has emerged as a new discipline and requires a specific and highly specialized expertise. What was once seen as mere correlation studies has turned into a complex regulatory maze and tortuous path through various technical and scientific pitfalls.

The development of specialized companies and the growing acceptance of biomarkers in clinical practice are factors that will promote the continuous growth of the field in forthcoming years.

The use of increasingly complex technology such as sequencing will add a further layer of complexity and, the experience and know-how accumulated over the years, together with further elaboration of the regulatory pathways, will streamline and normalize the processes.

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4
Biomarker Discovery and Medical Diagnostic Imaging

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4.1
Introduction

The dawn of medical imaging was in the year 1895, when Wilhelm Röntgen discovered the X-ray [1]. For this discovery, he was awarded the Nobel prize in 1901 [2]. Since that seminal discovery, huge steps forward in science and engineering have given us the tools to look inside the body with ever-increasing detail and sensitivity. It is these discoveries that have led to the wide range of molecular diagnostic methods now available.

These methods can be *in vitro*, that is, biopsy or blood based, or *in vivo,* that is, imaging. In this chapter, the processes involved and the factors for consideration in the development of *in vivo* imaging techniques, harnessing the strengths of *in vitro* methods in development are discussed. Prior to that, we give an overview of the range of imaging modalities available to the clinician.

4.1.1
Imaging Modalities

4.1.1.1 Positron Emission Tomography (PET)

Positron emission tomography was first developed in the 1950s [3] and is based on the detection of positrons (positively charged electrons) due to isotope decay. An increasing range of PET (positron emission tomography) agents are being developed across the major disease modalities [4–6]. However, the most common agent used in the clinic is fluorodeoxyglucose (FDG); this is a glucose analog bound to fluorine^{18} [7]. FDG is a powerful tool for use as a general marker of tumor measures such as location and size. The glucose is taken up by the tumor cells where it is metabolized, so fundamentally FDG is a marker of cell metabolism [8]. The success of FDG centers around the fact that tumor cells and tissue will have higher metabolic activity than normal tissue and will preferentially take up FDG [6, 9]. In general terms, the strengths of PET are its spatial resolution and its depth of penetration allowing deep tissue or organ assessment and that it is fully quantitative.

Biomarker Validation: Technological, Clinical and Commercial Aspects, First Edition.
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Also of note is the fact that PET labels such as ^{18}F or carbon (^{11}C) have relatively short half-lives and reduce patients' exposure to radioactivity [10].

4.1.1.2 Single Photon Emission Computed Tomography (SPECT)

Unlike PET agents, SPECT (single photon emission computed tomography) isotopes such as technetium (^{99m}Tc) or iodine (^{123}I) are relativity long lived, with half-lives of hours or days [10]. SPECT agents release single gamma rays (compared to PET where two photons are released at 180° from one another). These gamma rays are detected using two or more rotating gamma detection units [11, 12]. Similarly to PET, the detected rays are then reconstructed to give a 3D image. SPECT imaging has been available for longer than PET; however, SPECT currently offers a lower resolution. To address this issue, dedicated breast, cardio, and neurology SPECT systems have been developed to maximize resolution. The longer lived radioisotopes allow longer scan times while the nature of the isotopes makes in-house production of agents from commercial precursors easier.

4.1.1.3 Computed Tomography (CT)

While PET and SPECT involve the injection of radiolabeled agents, computed tomography (CT) uses external X-rays to probe the body. In CT, an X-ray tube rotates around the patient with a series of detectors being used to measure X-rays that are not absorbed or that have been reflected or deflected [13]. These X-rays are used to generate a series of 2D images with computational analysis allowing an artificial 3D structure to be generated [14]. CT was initially used in the 1970s to generate low-resolution images, but recent developments with multiple detectors now allow far more detailed images to be generated [15].

CT scans can be performed with or without a contrast agent, which if used will generate a more detailed image. These agents cause a change in X-ray passage as they have a different density to the surrounding tissue [16]. Combined PET/CT and SPECT/CT systems have also been developed; these are powerful tools combining the spatial resolution of CT with the target specificity of PET and SPECT [17].

4.1.1.4 Magnetic Resonance Imaging (MRI)

Unlike the modalities covered so far, MRI (magnetic resonance imaging) does not require the use of a radiation source, be it a radioisotope or X-rays. The MRI scanner essentially consists of a large, highly uniform superconducting magnet into which a patient is placed. To image, a series of electromagnets are transiently activated around the patient to generate a series of magnetic fields [18]. The magnetic fields cause changes in the alignment of the protons of hydrogen nuclei. As these nuclei return to their original position they emit a radio signal, which is detected by an radiofrequency (RF) coil around the patient. These emissions differ between tissues, allowing different tissues and organs to be defined. As in CT, a tracer such as gadolinium can also be used to improve contrast [19].

4.1.1.5 Ultrasound (US)

In ultrasound (US), sound waves are used to image the tissues under investigation. US imaging is based on the generation and differential reflection or echo of sound waves when they hit the tissues under assessment [20]. The technology has progressed significantly since its introduction in the 1970s [21], with US imaging possible at relatively low cost and capable of being performed in a range of settings outside the hospital, although a high degree of skill is required for image interpretation. US is particularly effective in imaging the soft tissues of the body such as breast, muscle, and thyroid [21]. The technology is less capable of imaging dense tissues or tissues behind bone such as the lung. Therefore, a balance needs to be found between the strength of the waves used, the penetration depth, and the quality of the image.

While US can be used as a standalone technology, the use of microbubbles enhances the image [22]. Microbubbles are intravascular contrast agents [23], which when bombarded with ultrasonic waves contract and expand rapidly. This pulsation generates a much stronger reflected signal than the surrounding tissue. Microbubbles have been particularly effective in liver imaging and mapping tumor circulation as the bubbles are sufficiently small to enter small and chaotic vessels [24]. Examples of agents include Optison™, which is used to enhance poor echocardiograms, and Sonazoid™, which is used to image liver lesions.

4.2 Factors to Consider in Biomarker Selection for Imaging

The development of *in vitro* and *in vivo* diagnostics requires a clear understanding of the biology of the disease of interest. Common considerations include the range of cell-based biomarkers available, the expression level of these markers, and their specificity to the disease. The biologist has a wide range of tools to address this and can employ both traditional laboratory and *in silico* methods to find the ideal marker.

Often, the range of molecular targets available initially appears huge as both intracellular and extracellular markers can be considered. The number of markers decreases when expression level or marker specificity is considered [25]. The marker or markers of interest must be expressed to a sufficiently high level to be detected with some margin of error to account for technical variation; importantly, the marker must also be sufficiently different between health and disease. A word of caution is needed regarding new markers reported in the literature: these are often discovered in small sample sets using research level tools potentially using a range of enrichment or amplification methods. While such markers can be measured in such a setting, in a clinical laboratory processing hundreds of samples per day with high stringency such markers may prove unreliable. This in part explains the relatively small number of markers that have progressed from research to the clinic, for example, HER2 [26]. Another consideration when using

in vitro methods is the lack of location information they offer; that is, a blood-based expression assay may show cancer markers but will not show tumor location. It is at this stage that a molecular imaging agent is preferred.

When identifying an imaging agent marker, expression and specificity are again considered. In addition, several other factors must also be addressed, including the location of the marker in or on the cells (ideally on the membrane). Being located on the membrane allows easier binding of the imaging agent as the chemistries used may be incompatible with cell entry. Clearance speed of the agent via the kidneys, and so on, is also a consideration as the agent must be retained for a sufficient period for detection, but will ideally be excreted soon after. These questions are addressed later in the chapter.

4.3 Defining the Insertion Point of the Assay and Its Business Case

Before initial biomarker selection using literature and *in silico* methods, a number of other factors will have been considered. First of these would be to identify the unmet clinical need. This requires an understanding of the current clinical pathway for the disease from initial presentation through diagnosis and staging, progressing through therapy, and finally post treatment monitoring [27]. In some cases, the current clinical pathway is well developed with rapid and sensitive methods already available or guidelines from clinical bodies are clear on what they feel is the ideal pathway. It is advisable for the researcher to seek input from a range of clinicians at this very first stage to ensure that what appears an unmet need on paper is in fact something that the clinicians desire.

The marker chosen for an assay or agent could differ between these stages; this means that an early decision on insertion point is needed. While insertion points have traditionally been in detection or staging, increasingly diagnostics are being developed for therapy selection and efficacy [28]. This is due to our increasing knowledge of disease subtypes and the technology for that form of analysis. The assays can also be used to identify successful surgery as a marker in the blood may drop after surgery, with residual disease detected by a lack of decrease or an increase in the marker post treatment. Finally, after therapy has been successful assays can still be used for routine post screening for years after therapy to ensure the disease has not returned.

The issue of disease heterogeneity means that a single marker is rarely suitable for use at all stages of the clinical pathway. Specifically in cancer, the rounds of therapy used often mean that the cancer cells present after the second round of therapy can be noticeably different from those at initial presentation [29]. This means that either a very robust common marker must be used (which is likely already on the market) or a screen of several markers must be used to ensure that the whole disease is being assessed and not just a subpopulation of cells within it.

In industry, in addition to the needs of the clinician, there must also be an assessment of the potential size of the market for the agent or test as there must be

sufficient return on the initial investment to make the assay or agent viable. Tied to this is the need for an assessment of the intellectual property (IP) space to ensure the test being developed does not encroach on a preexisting patent. This assessment will involve patent agents working with scientists to assess the challenges presented by the current IP.

Also for consideration is the choice of imaging modality. As discussed earlier, there are a range of methods available. However, it is unlikely that the majority of hospitals would have access to all of these platforms. While many will have access to CT or MRI, the number who can access PET or SPECT systems will be lower. This will likely change in time, but for now the platform of choice must also be considered at the initial stages of a project if the finished product is to have a strong platform base in the clinic. As already discussed, the choice of modality will also have an impact on the choice of marker due to expression level, and so on.

4.4 Practical *In Vitro* Methods Used to Identify Biomarkers

Following an assessment of unmet need, business case, and IP landscape the search for the actual markers can begin. By this point, the researcher will have a clear understanding of the disease area and a shortlist of potential biomarkers for initial investigation. If this is not the case, then a literature review or *in silico* methods can be used to generate a list of markers for analysis. Once generated, tissues of interest must be collected. This may be via a clinical collaborator or a tissue bank. This stage also presents challenges, often due to the variability in collection or storage methods used between clinical centers [30]. At this initial stage of development, relatively small numbers of tissues are required so this may not present such a challenge if the tissues can all be sourced from one supplier. A selection of control tissues must also be obtained.

Following tissue collection, initial analysis of markers can begin. The choice of analytical method will be dictated by those methods available in the laboratory, but can commonly include western blotting or histology for protein detection, followed by enzyme-linked immunosorbent assay (ELISA) for quantification. Genetic markers, for example, single nucleotide polymorphisms (SNPs) may be assessed by quantitative polymerase chain reaction (QPCR), microarray, or sequencing methods. After development of the imaging agent, testing of the binding efficiency of the potential agent to the target can be performed using methods such as radioligand binding or autoradiography.

Once initial test tissues and controls have been located, markers defined and detected in these tissues, and the imaging agent has shown binding *in vitro*, the development stage can progress to *in vivo* methods, which are now discussed.

4.5 Preclinical Models

In vivo biomarker analysis may yield different results from *in vitro* or *ex vivo* experiments due to the enormous genetic, biochemical, and physiological complexity of a complete organism; thus, *in vivo* experiments in animal models become important stepping stones in the development of biomarker targeting tracers or therapeutics in man.

4.5.1 Model Species

A wide number of organisms have been used in biological research for various purposes: yeast may be used for cell process analysis, fruit flies for gene mapping, and zebrafish for developmental studies. For the study of human disease, mice have been used extensively as a model organism. The advantages to using mice are numerous: they share similar genetic, biochemical, and physiological traits with man; they are in unlimited supply; the genetic background can be selected and controlled and mice can be manipulated genetically using standardized tools and protocols. This allows scientists to test hypotheses relatively quickly and cost-effectively in sufficiently large numbers of animals in order to attain statistically significant results.

Although many other species are also used for preclinical work, we focus on the mouse as the key example due to the ubiquitous use and versatility of this small rodent. It is important to note that many of the key principles and considerations when using preclinical models, as described herein, are applicable to all species.

4.5.2 Inducing Human Disease and Relevant Biomarker Expression

Being mammals, mice are subject to various diseases that afflict humans, such as cancer [31] and diabetes [32]. These are known as "spontaneous" disease models. Other diseases, such as cystic fibrosis and Alzheimer's, do not naturally occur in mice but can be induced in these animals through genetic manipulation such as gene knock-in/-out or transgenic procedures. Other ways of inducing human diseases in mice include the use of immunodeficient mice to host human tissue, such as a cancerous tumor [33] or pharmacological interventions such as inducing Parkinson's disease using the neurotoxic compound 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [34]. These are known as "experimental" models.

In addition to spontaneous and experimental models, there are "negative" models comprising essentially healthy control animals and "orphan" models where the disease is specific to the animal model with no human analog.

There are many considerations when selecting an appropriate animal model for a disease. Ideally, the chosen model would show similar biomarker expression and activity characteristics to the human disease. In some cases, the same biomarker

may change expression in a number of different diseases, an example being the biomarker myeloperoxidase (MPO), which is upregulated in diseases as diverse as stroke, models of bacterial infection, and specific types of cancer [35–37]. In such cases where a biomarker is shared among different diseases, a model may be selected on the basis of ease of use, reproducibility, cost, or other practical considerations.

4.5.3 Genetic Manipulation

Initially, genetic manipulation on mice was relatively crude: selective breeding, spontaneous mutations, or non-directed mutagenesis (such as radiation or chemical exposure) would cause a mutation of phenotypic interest, which could be selectively bred to produce a new source of mice with a desirable trait. Over time, more powerful, directed gene manipulation approaches were developed to create custom-made mouse models for a wide range of specific diseases.

Transgenic mice are created by the insertion of a new gene into the animal's germline (without targeting to a particular locus). In most cases the addition of foreign DNA to the genome results in a gain of function, such as the production of a new protein or the expression of an existing protein at higher levels or in different cells. For example, this approach can be used to model human diseases caused by dominantly acting mutant proteins such as the expression of mutant tau and/or amyloid precursor protein to produce Alzheimer's disease mouse models [38, 39]. Both of these biomarkers are key diagnostic and therapeutic targets in this disease and other dementias.

Approaches utilizing homologous recombination can be used to target genes at a specific locus; this can create knockout mutations where a gene is disrupted by its removal or replacement at its original locus or knock-in mutations where a gene is added at a particular locus. Examples include "p53" mice in which the p53 tumor suppressor has been knocked-out, leading to increased rates of cancer [40], and various mouse Huntingdon's disease models where cytosine-adenine-guanine (CAG) repeats have been knocked into the coding region of the gene encoding the Huntingtin protein in order to mimic the human disease [41].

4.5.4 Pharmacological/Chemical Induction

Parkinson's disease is a human condition caused by the loss of dopaminergic neurons in the substantia nigra region of the brain. Mice do not suffer from Parkinson's disease but it has been found that the chemical MPTP is toxic to mouse dopaminergic neurons and that treatment of the animals with this compound can induce the disease in them. Loss of dopaminergic neurons results in the loss of the dopamine reuptake transporter (DAT), a specific cell surface biomarker for that cell type. Imaging DAT in man or MPTP preclinical models is an indication of the status of the dopaminergic system in substantia nigra [42].

In a similar manner, the injection of bacterial lipopolysaccharide (LPS) into a tissue can induce a potent inflammatory response akin to that raised in response to a bacterial infection and provide a model by which to examine biomarkers associated with the immune system and its activation [43].

4.5.5 Xenografts: Grafting Foreign Cells or Tissues

Tumors are often characterized by the overexpression of particular biomarkers. Often, these are growth factor receptors on the cell surface, such as HER2 or c-Met [44]. These cell surface biomarkers can be utilized as both therapeutic and imaging targets. The initial identification and characterization of a human cancer will be done using established *in vitro* techniques in a pathology laboratory on cancerous tissue surgically resected from a human patient. The cells of a cancer of interest can be cultured *in vitro* and stored in international cell banks where they are available commercially to researchers. Thus, scientists have access to a broad range of cancer cell types, expressing a variety of biomarkers of interest, which can be purchased, cultured, and used in their own laboratories.

These cells can be grafted into immunocompromised mice (e.g., the BALB/c strain) to form tumors of human cells in the animal; these are known as xenografts [45]. Tumor cells are grown *in vitro*, are highly concentrated by centrifugation, and are injected into the mouse. Alternatively, tissue fragments of existing *in vivo* tumors may be used to seed into naïve animals, or tumor cells may be injected via tail vein in order to mimic metastasis. Immunocompromised animal strains must be used so that they do not raise an immune response to the grafted human tumor cells. Once the tumors have developed to a sufficient size, they may be used for imaging or therapeutic studies. An imaging study may assess if a radiotracer targeted to the tumor biomarker can be used to visualize the tumor mass *in vivo;* a therapy study may monitor the size of the tumor in response to drugs designed to target the biomarker of interest.

4.5.6 Physical Induction

Diseases can also be modeled by physical manipulation of the animal or its tissues. An example is stroke, which can be induced by occlusion of the middle cerebral artery, stopping cerebral blood flow, and causing ischemia [46]. Similar manipulation of the cardiac blood vessels can cause myocardial infarction [47]. In addition, other physical interventions can also be used to induce stroke such as photothrombosis: following injection of a photosensitive dye (such as rose bengal), the brain is irradiated through the intact skull leading to photochemical occlusion of the irradiated vessels and resultant ischemia [48]. In these disease models, physical intervention and induction of stroke allows the study of biomarker changes as a result of the ischemia and other consequent downstream biochemical events.

4.6 Preclinical Analysis Techniques

A number of established *in vitro* techniques may be used to analyze biomarker expression in tissues resected from animal models. *Ex vivo* tissue analysis may include biomarker quantitation via ELISA or western blot, regional biomarker expression via immunohistochemistry, and biodistribution to track where prospective biomarker radiotracers are traveling and accumulating in the body.

During the development of a radiotracer, imaging studies utilizing the appropriate modalities are undertaken to observe the biomarker–radiotracer interactions in an animal model. Imaging modalities are essentially the same as used for human imaging (PET, SPECT, MRI, etc.) albeit with hardware scaled down for imaging small animals, such as mice. These smaller imaging systems are often referred to as "micro" systems, for example, micro-PET. Combined modalities can be used to provide a deeper understanding of disease processes, such as PET-CT, where PET provides functional imaging (pertaining to specific biomarkers or metabolic state) and CT provides anatomical and morphological data to give researchers a more complete picture of what is happening *in vivo.* As with human imaging, preclinical imaging studies can offer useful qualitative, quantitative, and longitudinal data, showing, for example, how a biomarker changes with disease progression or in response to therapy or other stimuli.

4.7 Translational Considerations and Restrictions

Despite the many similarities between mice, other mammal models, and humans, there are also sufficient differences that may confound the translation from the chosen animal model to man.

The biomarker under examination must have a highly conserved structure between human and the animal model species. This includes protein sequence (affected by gene sequence and splice variants) and post-translational modification (such as cleavage, lipidation, and glycosylation). The precise degree of homology required depends very much on the binding characteristics of a specific tracer to the biomarker. For example, one tracer may bind to a highly conserved region of a protein biomarker and therefore bind to both the human and mouse biomarkers. In contrast, another tracer targeting the same biomarker may bind to a more variable region of that protein and thus bind only to the biomarker in one or the other species.

The rapid expansion and open availability of sequence databases in recent years means that it has become straightforward to compare the gene and protein sequences of biomarkers across various species. An example is the National Center for Biotechnology Information (NCBI) "HomoloGene" online database [49]. This database contains automatically generated sets of homologous genes

and their corresponding mRNA, genomic, and protein sequence data from selected eukaryotic organisms.

As part of any *in silico* homology analysis, the existence of proteins of similar structure to the biomarker of interest can be probed. For example, the biomarker of interest may be part of a highly related protein family with conserved structural features. There is a risk that other proteins within that family may present nonspecific targets for a tracer instead of the intended biomarker. This risk may manifest itself differently across species. A pair of related biomarkers in man may not be sufficiently homologous to bind both to a particular tracer, but the equivalent biomarker pair may be more similar in the mouse and would therefore bind both with the same tracer. Identification of close homologies *in silico* can help drive decisions into the most appropriate preclinical model to use.

Aside from the biomarkers themselves, there will be additional genetic, physiological, and biochemical differences that will affect translation from mouse into man. Examples of such factors include different metabolic rates or the type of immune response raised to a particular stimulus. Where known, these factors should be taken into account when choosing the most appropriate model species for the assessment of tracer binding to a disease [50].

4.8 Other Uses of Preclinical Models

In vivo models are critical for determination of the characteristics of a particular tracer, including pharmacokinetic parameters such as rates of absorption, distribution, biotransformation, and excretion – that is, how the tracer behaves inside the body. Pharmacodynamics is the study of the biochemical and physiological effects of drugs on the body and the mechanism of action. Pharmacokinetics can be considered to be the study of what the body does to the drug and inversely, pharmacodynamics is the study of what the drug does to the body.

There may be several tracers that bind similarly well to a biomarker of interest during initial *in vitro* examinations. Their behavior *in vivo* will then be examined to give a much stronger indication of which tracer may be of most clinical utility. Because radiotracers will ultimately be used *in vivo* for imaging, it is more than just the binding to the biomarker that will determine whether it is useful or not.

Some radiotracers may be very quickly excreted or metabolized, making the imaging window impractically short. Other tracers may be found to accumulate in non-target organs, providing a nonspecific signal while at the same time reducing availability of the tracer for binding to the biomarker of interest. An ideal radiotracer would bind specifically to its target biomarker, would not be metabolized or accumulate in any organs, and be excreted in a timely manner yet allow sufficient time for imaging to take place.

Safety and toxicity studies are also undertaken in animals. This is so that adverse events and side effects caused by a radiotracer are identified and mitigated, for

example, by optimizing the dosage, so that such risks are minimized sufficiently to allow safe testing in man.

The knowledge gained of biomarker expression and the pharmacokinetics, pharmacodynamics, safety, and toxicity of the corresponding radiotracer in a preclinical model is an important step in the progression to clinical studies in man. It must also be understood that animal models have their inherent limitations in that they cannot behave identically to a human body, but give an important initial understanding nonetheless.

4.9 Nuclear Imaging Infrastructure

Nuclear imaging has specific infrastructure requirements not normally required by *in vitro* diagnostic methods for which a standard laboratory will suffice. Aside from the specific imaging hardware and related expertise, the short radioactive half-lives and scarcity of some of the radioactive elements are hurdles that must be overcome before the widespread use of such radiotracers in the clinic.

The short half-life of many PET radiotracers (e.g., 110 min for ^{18}F, 20 min for ^{11}C) means that a clinic wishing to use such isotopes must be sufficiently close to a cyclotron (the facility by which these radioisotopes are produced) so that the radioisotope does not completely decay during transportation between the cyclotron and the clinic. In developed countries, large hospitals may have access to their own cyclotron facilities.

In addition to the production of radionuclides themselves, their incorporation into the relevant tracer molecule – a process called radiosynthesis – must also be done to make a finished product. PET radiotracers are synthesized on automated platforms such as General Electric's TRACERLab™ or FASTLab™ systems. Such platforms will accept ^{18}F/^{11}C from a cyclotron and, by means of a pre-programmed synthesis methodology, radiolabel a non-radioactive precursor compound to produce the desired radiotracer. This radiotracer is then purified and sterility checked using established methods before injection into the patient.

The availability of SPECT radionuclides can also be problematic, with recent shortages of molybdenum (^{99}Mo) causing a knock on effect on availability of its decay product ^{99m}Tc, a SPECT radionuclide used in around 80% of diagnostic and therapeutic procedures. The recent ^{99}Mo shortage was caused by a temporary shutdown of both source reactors in Canada and the Netherlands, which are both nearing the end of their lifecycles. Alternatives being investigated include the identification of new reactors for ^{99}Mo production, reducing ^{99m}Tc dose, and therefore demand and use of alternative radioisotopes such as thallium (^{201}Tl) [51].

Other considerations when choosing to image biomarkers using nuclear imaging include safety regulations around production, storage, and disposal of radioactive materials, staff dosimetry to ensure they are not exposed to dangerous levels of radiation, and similarly, patient safety-especially with regard to the young and

pregnant or breastfeeding mothers. Longitudinal scans subjecting a patient to repeated doses of radioactive material must also have appropriate clinical justification [52].

However, it is an inescapable fact that the ability to consistently supply radiotracers, especially those of limited radioactive half-life, is geographically limited and is therefore an important consideration in the process of choosing the appropriate technology to detect a particular disease biomarker. Nuclear imaging is a valuable diagnostic technique currently limited to the developed world and therefore only of utility in imaging of "first world" diseases and their associated biomarkers.

4.10 Image Processing

Once the images from a nuclear imaging scan have been acquired, they must be processed and interpreted. This process of analyzing image data involves a subjectivity not normally associated with *in vitro*-based biomarker analysis where the output of a biomarker assay may be an unambiguous number.

At its most basic, processing may involve simple normalization of the signal against a known high or low signal area in the image, corresponding to a region with known high or low biomarker availability respectively. This is to produce images of the appropriate final signal intensity and correct for patient and dosage differences to ensure consistency across multiple patients, hospitals, and clinicians. Incorrect processing could lead to misleading images and inappropriate diagnosis.

Frequently, processing may also involve combining more than one imaging modality and the selection of "regions of interest" (ROI) to allow region-specific biomarker quantitation to take place. PET and SPECT images may be co-registered, or overlaid, against a CT image providing accurate anatomical data that PET and SPECT do not alone provide. The anatomical data allows the clinician or researcher to delineate the appropriate ROIs (a specific region of the brain, for example), and then analyze the PET/SPECT radiotracer signal that arises from the biomarker availability in that region. The combination of multiple techniques in this manner, the so-called multimodal imaging, can provide clinicians and researchers with greater diagnostic capability as they can accurately pinpoint biomarker changes associated with the disease state or response to a therapy [53].

4.11 Concluding Remarks

Taken together, the identification, validation, and application of novel biomarkers for use in molecular diagnostics both *in vitro* and *in vivo* is a multistage process. In

this process, the underlining biology and chemistry must be considered alongside the clinical application, business case, and infrastructure requirements. When all of these factors have been addressed, the chosen biomarker and its test or tracer can become a powerful tool in the clinic to diagnose and fight disease.

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5
Breath: An Often Overlooked Medium in Biomarker Discovery

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5.1
Introduction

A biological marker, or *biomarker*, is a measurable and quantifiable characteristic of a process that occurs within the body, as has been described in detail in the introductory chapters of this book. Biomarkers can indicate diverse processes, ranging from the normal (healthy) physiology to perturbations or dysfunction thereof. They can relate to the actions of organisms hosted within the body, for example the microbiome, as well as to presence and effects of exogenous entities that have entered the body through the environment, the diet, or through medical or pharmaceutical intervention.

When confronted with the term "human biomarkers", most people will immediately think of blood – perhaps also urine – as a medium in which they are present and can be detected. Indeed, blood is at the focus of most current biomarkers research, as is evident from other chapters of this book, and it is undoubtedly the obvious choice: blood is pervasive and in continuous motion throughout the body, and it is thereby exposed to any perturbations that are confined to local areas of the body, for example relating to specific organs, the digestive system, and the muscles. This pervasiveness of blood within the body and its perfusion through the human tissue means that any biomarkers that are generated locally can pass into the bloodstream and can be carried throughout the body with the systemic circulation. Analyzing blood-borne biomarkers is therefore advantageous because the biomarkers found in blood can be indicative of processes occurring in less accessible regions of the body.

Exhaled breath offers an alternative medium for detecting certain types of biomarkers. Further, in some respects, analyzing breath has distinct advantages over blood analysis. Before contemplating breath gas analysis, however, let us first briefly consider the lungs.

The primary function of the lungs is twofold: it enriches the blood with molecular oxygen (O_2) that is supplied to the cells of the body and simultaneously removes the excess carbon dioxide (CO_2) produced by these cells from the bloodstream. This process of O_2 enrichment and CO_2 removal, which was first discovered by

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Lavosier and Laplace in 1784 [1], is a physical process that takes place in the alveoli of the lungs and is dictated by the partial pressures of these two molecules in the alveolar gas and mixed venous blood [2].

Atmospheric (ambient) air is mainly composed of molecular nitrogen (N_2) at 78.08%, O_2 at 20.95%, water vapor (H_2O) at 1–4% and CO_2 at 0.04%, as well as other constituents such as argon at 0.95% [3]. By comparison, exhaled breath is largely made up of N_2 at 74.87%, O_2 at 13.68%, H_2O at 6.18%, and CO_2 at 5.26% [2]. Since inspired air is rich in oxygen and typically low in carbon dioxide these two molecules experience different diffusion gradients during alveolar ventilation: the O_2-rich inspired gas transfers O_2 to the blood and, conversely, the CO_2-rich venous blood transfers CO_2 to the alveolar gas that is then exhaled before the next ventilation process repeats anew. This gas exchange is the underlying principle of the presence of biomarkers in exhaled breath.

In addition to the main gas constituents, both ambient air and exhaled breath typically contain many hundreds, if not thousands, of volatile organic compounds (VOCs). In ambient air these can originate from any number of sources, broadly categorized as being either of biogenic origin, that is, from vegetation, trees, and so on, or anthropogenic, that is, from human-related activities, for example, vehicle emissions, factories, and power plants. These VOCs are diverse in nature and include common aldehydes and alcohols, terpenoids and aromatics, amines and thiols, but also more exotic and synthetic species can be in high abundance, such as the polycyclic aromatic hydrocarbons (PAHs) and phthalates, to name but a few [4, 5]. In short, VOCs in the environment are ubiquitous, diverse, and are present in highly variable and wide-ranging abundances.

VOCs in exhaled breath can generally be placed into two categories: *exogenous* VOCs, which are compounds that have entered the body from the external environment, and *endogenous* VOCs, which are compounds that are generated within the body. Both types of VOCs are of relevance in breath biomarker discovery, as is discussed below.

Returning to the lungs, similarly to O_2 and CO_2, VOCs in inspired and expired air undergo gas exchange during alveolar ventilation. Again, the phenomenon of partial pressure plays a role in this physical process: when (exogenous) compounds are present in higher concentrations in inspired air than in the blood, these partition into the blood and enter the systemic circulation. Conversely, higher concentrations of (endogenous) compounds in the blood than in the alveolar gas create a positive gradient toward the latter and these compounds subsequently exit the body during exhalation. This is the basic premise of the presence of biomarkers in breath and the justification for breath gas analysis.

5.1.1 Breath Analysis: Past and Present

Breath, or more specifically breath odor, was utilized as a diagnostic medium as early as two millennia ago when Hippocrates reported on the olfactory detection of *fetor oris* and *fetor hepaticus* on breath to indicate disease [1]. Indeed, before

the modern era of sophisticated analytical tools it was routine for a physician to smell a patient's breath for indications of conditions ranging from diabetes (via the associated "sweet" odor of acetone on the breath) to trimethylaminuria (relating to fish malodor syndrome). In today's medicine, analysis of exhaled breath has been largely overlooked among the complex array of other diagnostic tools. Nevertheless, the modern era of breath research started with the basic analysis of volatiles in the breath performed in the 1950s and 1960s, which identified the most abundant and ubiquitous of the breath volatiles, namely acetone, ethanol, and methanol [6–8]. This laid down the foundation for the pioneering work of Pauling and coworkers in the 1970s, who demonstrated the richness and complexity of volatiles in the breath via the gas chromatographic detection of approximately 250 different volatiles [9, 10]. Later, comprehensive studies by Phillips and colleagues that focused on specific diseases and their recognition in breath was a principle driving factor for the steady and growing interest in breath gas analysis over the last two decades [11–14] and the field is now rife with different methods and instrumentation for the comprehensive and targeted analysis of breath [15–17].

5.2
Breath Analysis Studies: Targets, Techniques, and Approaches

The study of breath as a biologically probative medium has certain pragmatic factors that differentiate it from the more traditional urine and blood assessments:

- Breath is a diffuse (mostly gaseous) medium, which requires specialty collection equipment, rather than standard syringes, vials, test tubes, and so on, as for the liquid media.
- Breath is a dynamic medium; a single exhaled breath initially is comprised of upper airway dead space, then lower airway (bronchial) gas, followed by the deep alveolar (end-tidal) volume engaged in gas exchange with the blood.
- Breath is comprised of inorganic and organic gases, water vapor, particles, cells, aerosols, and other large molecules and fragments.
- Breath can be analyzed online or offline, depending on the analytical method employed. The transfer and storage of samples for offline analysis requires different techniques depending upon which fraction of the breath is of interest.
- The processing of a breath sample for subsequent analysis is dependent upon the sampling medium and the particular analytes sought.
- The analysis of a processed breath sample depends on the goal of the experiments – discovery of whatever is there, or targeted analysis for documenting specific compounds.

An obvious major advantage of breath analysis over blood sampling is that it is noninvasive and practically inexhaustible. Further, samples can be provided easily with minimal effort, making it ideal for cooperation by sectors of the population that might otherwise not be able to provide samples, such as children

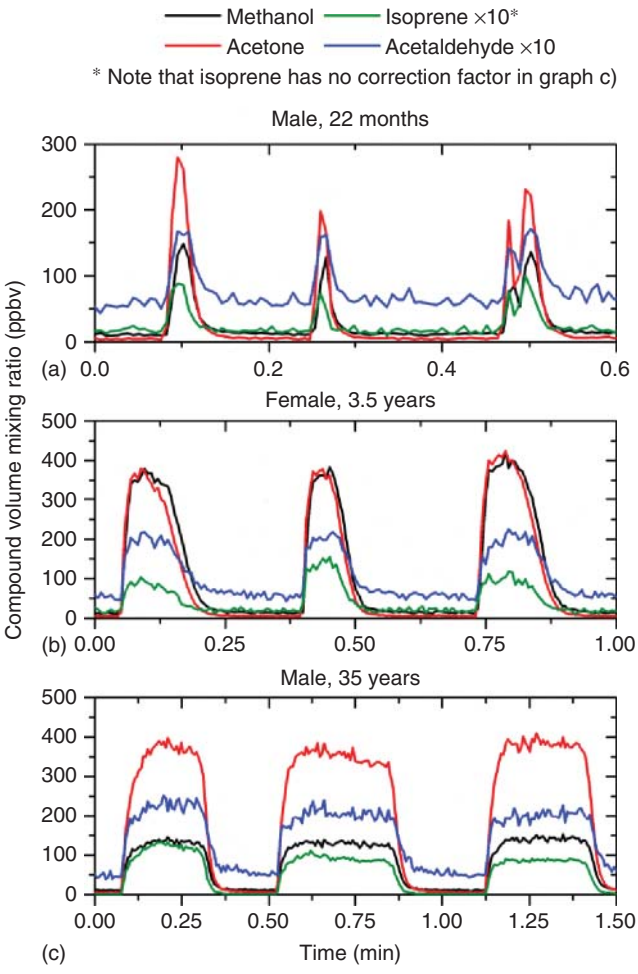

Figure 5.1 Breath profiles of three single exhalations sampled via a buffered end-tidal (BET) sampler coupled to a proton-transfer-reaction mass spectrometer (PTR-MS) for three subjects, namely, (a) a male toddler of 22 months, (b) a female child of 3.5 years, and (c) and male adult of 35 years. Profiles are shown for four selected endogenous compounds: methanol, acetone, acetaldehyde, and isoprene. The low concentrations between exhalations represent ambient air levels. Note the different axis scales.

or the elderly. As an example, Figure 5.1 displays exhaled breath profiles of selected compounds (methanol, acetaldehyde, acetone, and isoprene) taken from two young children (male, aged 22 months and female, aged 3.5 years) and one adult (male, aged 35 years). Breath profiles were sampled using a buffered end-tidal (BET) sampling unit [18] coupled to a proton-transfer-reaction mass spectrometer (PTR-MS) for chemical analysis [19, 20]. The breath profiles, which were recorded while the subjects provided single exhalations through a tube,

clearly display distinct signals for the individual compounds monitored and demonstrate good to high reproducibility for this analytical procedure, even for the breath profiles of the 22-month-old toddler.

In the following sections, we discuss the aforementioned pragmatic factors in more detail.

5.2.1 Exhaled Breath Gas, Condensate, and Aerosols

Traditionally, exhaled breath was considered useful only for the physical parameters or gas-phase constituents. For example, pulmonary function tests are only concerned with total volume expelled regardless of minor constituents. During exercise physiology tests, only respiration rate, oxygen absorption, and carbon dioxide elimination are of interest. More detailed breath tests are concerned with the levels of acetone, pentane, nitric oxide, carbon monoxide, malondialdehyde, and isoprene in breath as indicators of various health states including diabetes, lipid peroxidation, metabolic efficiency, and so on [21–28]. Measurements of isotopically labeled probe molecules, especially $^{13}CO_2$, have been used to determine certain metabolic pathways or disease states [29, 30]. Measurement of exogenous gas-phase compounds has been used to assess exposures to fuels or consumer products, and various trace level endogenous compounds have been used for pattern recognition for diagnosis of diseases [31–36].

More recently, breath analysis has been expanded to include the exhaled breath condensate (EBC) fraction [37]. The EBC is collected by having the subject breathe through an open-ended chilled tube; the water vapor, water-soluble organics, and other low volatility constituents condense on the inner surface of the tube and the bulk of the exhaled gases are expelled. Subsequently, the condensate is collected into a small vial for analysis [38]. Initially, the pH of EBC was used to assess asthma state and drug treatment efficacy [39]. More recently, EBC has been used to assess endogenous polar volatile organic compounds (PVOCs) that are indicative of certain human metabolism pathways for preclinical disease diagnosis but are difficult to measure in the gas phase [40].

The most recent applications of exhaled breath analysis involve the exhaled breath aerosols (EBAs) [41, 42]. Generally, the aerosol fraction is a subset of the EBC under the proper conditions; however, the EBA can be collected separately using the appropriate filter, thus avoiding the gas-phase and the liquid-phase constituents completely. The use of disposable filters, as in medical pulmonary tests, allows the efficient and inexpensive capture of exhaled proteins, cellular fragments, and bacteria for subsequent analysis. This approach is of particular value for population-based studies as it is completely noninvasive, fast, can be done anywhere, and can be self-administered with minimal training. In addition to measuring inflammatory markers, the EBA can be cultured to fingerprint the pulmonary microbiome [43].

5.2.2 Sampling Techniques and Analytical Tools

At the present, choices of distinct methods for breath sampling are under considerable discussion and even controversy [44, 45]. The basic techniques for sampling gas-phase breath all incur variability from the subject; individuals will tend to hyperventilate or breathe differently to a certain extent when being tested. Most standard breath collection devices have minimal control; subjects are just told to provide a single breath or multiple breaths into a canister, a polymer bag, or some other gas collection device. To better standardize collection, there are now instruments available that provide feedback to the subject to stabilize rate and depth of breathing, and that automatically select specific parts of the breath, for example alveolar (deep lung) breath, for collection. Similarly, EBC can also be collected in a number of different ways. A common approach is to ask a subject to breathe "normally" through a chilled tube for 10 min without much concern for total volume. Newer instruments now incorporate some form of cadence cues and volumetric measurement. The temperature of the chilled section is also modified for different analyte streams. For example, when the EBC is used for pH measurement, the thermal sink used is generally at freezer temperature (−10 °C, or so) and is sufficient to collect water vapor. However, for capturing PVOCs efficiently, a dry ice method at −80 °C is more effective [40]. Capturing EBA is still the simplest technique; it is performed at ambient temperature typically with a disposable filter and there is no particular technique used other than "blow through this." In the future, it is expected that some form of handheld pulmonary test instrument (measuring exhaled volume) will be used to standardize the samples.

Precise and efficient breath sampling is a prerequisite for accurate breath analysis, but the exact method of sampling will inherently depend on the analytical system used [45, 46]. Analytical tools for breath analysis run the gamut from simple point-of-care systems to extremely complex laboratory instruments. There are some basic categories:

- *Electrochemical sensors*: these are hand-held instruments for the gas phase that use arrays of chemical sensors that provide a pattern of responses, but do not identify specific compounds. The patterns may be "trained" using standards. The instruments are generally used for real-time (online) analysis.
- *Optical spectroscopy instruments* (tunable diodes, quantum cascade lasers, etc.): these are typically small bench-top instruments that provide very specific measurements of targeted gas-phase compounds. They are designed specifically for a few compounds, and may include multiple lasers to cover different compounds. These instruments are generally used for real-time (online) analysis.
- *Online mass spectrometry (MS)*: three types of MS instruments are dominantly used for direct breath analysis – ion mobility spectrometer (IMS), PTR-MS, and selected ion flow tube mass spectrometry (SIFT-MS). Each can monitor breath from a flowing sample for specific ions that are related to specific

compounds. There is no pre-separation (chromatography) step. Typical resolution is 1 amu, but newer PTR (proton-transfer-reaction) instruments are now employing time-of-flight mass spectrometry (ToF-MS) making them as specific as some gas chromatography-mass spectrometry (GC-MS) instruments.

- *Laboratory-based mass spectrometry (MS)*: the "gold standard" for analyzing gas-phase volatiles in breath is GC-MS. These instruments include standard large bench-top instruments using single quadrupole mass spectrometry (QMS) and ion trap MS at 1 amu resolution, GC-MS × MS, sometimes referred to as "triple-quad", wherein parent ions are further fragmented to achieve superior identification, and finally GC-ToF or GC-QToF wherein mass resolution is greatly enhanced for compound speciation.
- *Liquid chromatography mass spectrometry (LC-MS)*: this technique is only used for analyzing larger molecules from EBC and EBA that are not easily converted into gas phase, even at higher temperatures. This is a somewhat rare application for breath work.
- *Immunochemistry platforms*: these analyses are primarily for the EBC medium wherein specific proteins and other messenger molecules are sought. Typically, compounds are captured with some form of affinity well (sandwich enzyme-linked immunosorbent assay, or ELISA) and detected using photon emission. One major application is the detection of cytokines as pulmonary inflammation markers.

Of these tools, the most commonly used instrumentation for breath analysis is the laboratory-based "single quadrupole" GC-MS. This is a good work-horse instrument with sufficient sensitivity and specificity for organic compounds in breath. GC-MS can be used in selected ion mode for targeted analysis or scan mode for discovery analysis.

There are distinct sensitivity and specificity advantages for the large laboratory-based instruments but these require capturing, storing, and transporting samples to a central location. The more portable instruments such as PTR-MS, SIFT-MS [47], and IMS [48] can be brought to the patient, but sacrifice some performance, especially in terms of compound identification. Their main advantage, however, is that samples can be analyzed directly thus being able to quantify many reactive species that are difficult to store. The optical instruments have high specificity for a few compounds and can be brought directly to the subject, but the current technologies do not have the sensitivity of the GC-based instruments nor can they be easily modified to measure different compounds.

5.2.3 Discovery Versus Targeted Study Approaches

Analysis of biological materials, especially breath, blood, and urine, falls into one of two philosophical categories: *discovery* or *targeted* [43]. The principle differences between discovery and targeted approaches are represented graphically in Figure 5.2.

Figure 5.2 Depiction of targeted and discovery modes of research as they impact the public (center). The left side illustrates targeted research wherein known sources and their respective compounds are chosen specifically to assess exposures. The right side illustrates discovery research wherein samples of breath, blood, and urine are analyzed for as many compounds as possible to discern potential impacts from exposures and metabolism. (Graphic created by US EPA for [43].)

Discovery experiments are designed to measure and catalog all compounds in a sample without preconception as to what may be important. These types of experiments have three purposes; the first is to learn which compounds are actually present in a sample, the second is to determine what their patterns are with respect to each other, and the third is to develop a knowledge base of the levels and the variability in different populations. A major application for discovery experiments is to compare samples from case-control cohorts and discern differences that may ultimately be probative in early detection of disease or for environmental exposure reconstruction.

Targeted experiments are generally an implementation of discovery results. The purpose is to focus on getting a specific answer. For example, if discovery had determined that of all of the thousands of compounds in jet fuel, a pattern of four *n*-alkanes and naphthalene were a definitive pattern for exposure, then there is no need to look for anything else in breath to assess contact with jet fuel. Now, the analysis can be targeted and made very specific and sensitive by targeting the MS for only those five compounds [32].

Discovery and targeted experiments are not mutually exclusive from a pragmatic standpoint, but just describe the intent of the study. For example, suppose one wants to discover if inflammatory cytokine biomarkers are present in breath; in this case, a standard immunochemistry panel of 10 representative cytokines can be used, without preconception, to discover if there is value in this approach. Certainly, this experiment is stratified to only those 10 cytokines for which there is an available analytical panel, but the intent is to discover some underlying truth about cytokines in general. Suppose that these discovery experiments show that there are indeed different patterns in the 10 cytokines that can be linked to certain types of inflammatory response. Based on the discovery results, a targeted

approach can now be implemented for those 10 cytokines to classify unknown samples with respect to inflammation. In either case, the analytical scheme is exactly the same, but the intent and the interpretation of the results define the experiment as discovery or targeted.

Usually, the distinction between discovery and targeted experiments is much more pronounced than the cytokines example above, but one must always be cognizant that there is no pure discovery or pure targeted approach. This is especially true in breath analysis because of the complexity of breath composition. All breath studies are stratified to some extent in their sampling and analytical approach depending upon some preconception of analyte interest. For example, if one were interested in human metabolism, then the analytical scheme for discovery study would target the hundreds of polar VOCs such as alcohols, ketones, esters, aldehydes, and other oxygenated organics in breath; if one were interested in exogenous fuel exposures, the analytical scheme for the discovery study would target the hundreds of nonpolar hydrocarbons, and so on, known to be in various fuels. In both cases, the intent is discovery, but the approach has a targeted component because the analytical schemes are different to accommodate the polarity of the analytes.

Similarly, even a purely targeted study design can exhibit discovery characteristics. Suppose one is investigating a particular metabolic pathway using a targeted aldehydes method and discovers that a subgroup of samples has a distinctly different pattern. This could indicate the presence of an unanticipated pathway and perhaps precipitate a second round of discovery experiments that would then include many more analytes.

5.3 Biomarker Confounders

The current major limitation to progress in breath research – and as a consequence, its implementation in the clinical setting – is the ubiquity of biomarker confounders, both known and unknown. In this context, confounders are signals that are elicited in the analytical detection system but are not related to the subject matter being targeted. Confounders can arise from a number of sources, but are predominantly reflective of suboptimum breath sampling or extraneous factors affecting the breath composition [49, 46]. These are discussed in more detail in this section.

5.3.1 Sampling Impact

Breath can be sampled via two approaches: either directly (or online), with immediate analysis, or indirectly (or offline), with interim storage before analysis. Each approach has its pros and cons, but both are liable to introducing confounders into the analysis of breath. We treat each approach separately below, focusing on the analysis of gas-phase breath constituents.

5.3.1.1 Online Breath Sampling and Direct Analysis

The direct analysis of breath is dictated by the analytical features of the breath analyzer employed, which must have a rapid response time and sufficient throughput capabilities. In its simplest form, the test subject/patient exhales into a mouthpiece (or via a nosepiece) and the gas is transferred directly to the detection system of the analyzer. The potential for sampling-related confounders is thereby principally limited to gas-phase emanations from the materials – such as the sampling tubes – used in the gas transfer lines, interaction of breath constituents with these materials, and carry-over effects from previous samples during the sample transfer. Each of these possible sources of artifacts can be minimized by appropriate measures, such as via the use of suitable (inert) materials for the whetted parts of the transfer line, adequate heating, and sufficient cleaning between samples, as necessary. Because of the ability to overcome many of these potential interferences, online sampling is arguably the preferable of the two methods. In many cases, however, online analysis is not a viable option due to a lack of rapid or direct analytical capability of the analyzer or due to logistical complications of bringing the instrument to the patient bedside. As such, sampling for offline analysis is by far more widely employed than online sampling.

5.3.1.2 Breath Sampling for Offline Analysis

In offline analysis a breath sample is taken and stored in a suitable container prior to analysis. There are several aspects of this procedure that are critical to reducing the likelihood of generating confounders. Initially during sampling, the same criteria as for online analysis must be adhered to, that is, the use of inert materials and a heated sampling system. The next hurdle to achieving clean sampling is imposed by the sample storage receptacle, which must equally be of an inert material to limit the degradation or adsorptive/absorptive losses of compounds in the stored sample. Handling of the sample is also a critical issue. Currently, the most widely used sampling receptacles are polymer bags made of Tedlar or Teflon. These are reasonably durable, relatively cheap, and can be reused after undergoing appropriate cleaning and conditioning measures. However, their correct handling and storage is imperative if the analysis of their contents is to be trusted. Studies have shown that the concentrations of many VOCs stored in such bags diminish over time, even within 24 h, which has been attributed to diffusion through the bag walls and degradation due to oxidation [50, 51]. Some of these effects can be suppressed by maintaining the stored sample at an elevated temperature – particularly during analysis of the sample – and minimizing the storage time before sample analysis. Comparative analysis of a similar bag filled with pure air also helps identify confounding volatiles emanating from the bag material itself, although this will not reveal contamination of the sample bag itself [51].

Other storage systems for breath samples include absorbent traps (e.g., with organic polymers such as Tenax) that offer sample enrichment, or needle traps [16, 33]. Again here, memory effects can play a role in presenting confounders in the analysis. Additionally, as with bag samples, recovery of the entire sample for analysis can also be an issue, that is, by incomplete desorption of volatiles from the

solid-phase traps. Thus, again there are limitations presented by these methods that are yet to be overcome.

Breath sampling for storage and offline analysis will remain a primary method for breath analysis because of the typically greater logistical effort involved in taking the analytical instrument to the patient rather than the breath sample to the machine. Therefore, great care must be taken in the choice of sampling receptacle, its handling and storage, and the subsequent analysis of the sampled contents in order to avoid the numerous potential confounders presented by this method. Nevertheless, sample storage is currently the gold standard for breath analysis and has been implicit in many major discoveries.

5.3.2 Contributions from the Exposome

The "exposome" is a relatively recent concept – first introduced in 2005 – that attempts to establish a measure of a person's exposure history [52]. In this context, exposure relates not only to extraneous factors such as foreign bodies that have been inhaled, ingested, or taken up via the skin, but also exogenous compounds produced within the human body by foreign organisms, such as the microbiota. Human exposome has been defined as "*the cumulative measure of environmental influences and associated biological responses throughout the lifespan, including exposures from the environment, diet, behavior, and endogenous processes*" [53]. Given the continual dietary burden of foreign substances entering the body, as well as the persistent exposure to the ubiquitous compounds present in our everyday environment, the exposome is a major contributor to exhaled breath and must be taken into consideration in view of identifying confounders [54], although it is noteworthy that breath analysis also has potentially high utility in environmental health science in relation to characterizing elements of the exposome [55].

Clearly one problem with eliminating such confounders is the exercise of identifying them as such in the first place. There are currently no definitive methods to achieve this, but certainly work to establish and catalog the volatiles in "unremarkable" breath profiles, that is, of healthy individuals, is a step in the right direction [56, 43, 57]. By gaining knowledge on typical breath profiles, a baseline can be established that will allow differentiation of these with "remarkable" profiles, that is, those that are outside the norm. Further to this, test subjects should ideally be questioned extensively on recent dietary intake and in particular in relation to potential extensive exposure to certain environments. Such background information is a key to designating the origin of compounds in breath profiles that might initially appear to be remarkable, but that might actually only come from our last meal or from the vehicle emissions inhaled on the journey to the laboratory or clinic before providing a breath gas sample. Characterizing the exposome remains one of the greatest challenges to progress in breath gas, but already endeavors to tackle this issue are being undertaken.

5.4 Biomarkers in Breath

Volatile organic compounds are ubiquitous in the human body. A recent compendium of all VOCs that have been detected as emanating from the human body – collectively known as the volatilome – included a list of 1840 VOCs [58]. Strikingly, only 12 single compounds (equating to only 0.7% of the total) were common to all bodily media included in the compiled list, namely, in blood, breath, urine, saliva, feces, skin secretions, and human milk. Further, 872 different VOCs have been detected in breath compared to only 154 in blood. In comparison with blood, the number of accepted biomarkers in breath is limited. This can be attributed to a whole array of complex issues with breath analysis that have been discussed elsewhere in this chapter.

5.4.1 Inorganic Breath Biomarkers

Notably, breath analysis already has widespread and routine use in hospitals throughout the world. The practice of capnography is the measurement of CO_2 – more specifically the determination of its partial pressure – in breath during breathing. Capnography is a primary parameter that is monitored in ventilated patients under general anesthesia or moderate or deep sedation, during surgery or in intensive care units [59]. The elimination via the breath of CO_2 generated during cell metabolism provides an indicator of normal respiration; deviations from the expected capnogram can signify complications relating to the blood circulation or breathing, or arising from medical care, such as ventilation failure caused by malposition of intubation tubes. Exhaled CO_2 is by far the most widely monitored breath biomarker.

Nitric oxide (NO) is an inorganic compound that is produced endogenously and has been attributed to playing an important role in physiological regulation of airway functions such as blood flow in the pulmonary circulation [60]. NO was observed to be present in exhaled breath (of humans and animals) for the first time in 1991 [61], and this was soon followed by indications that its concentration in exhaled breath is higher in patients with airway inflammation and asthma [62]. Following these initial discoveries there was a hive of activity in characterizing NO in asthma, in the development of new and improved technologies for its detection, and in honing and standardizing the sampling procedures to improve interclinical reproducibility of results, which ultimately led to guidelines for standardized NO sampling being published in 1999, with a revised version released in 2005 [63, 64]. The procedure of measuring exhaled NO received approval from the US Food and Drug Administration (FDA) in 2003 and today it is a standard practice for identifying and monitoring airway inflammation in asthmatic patients.

FDA approval has also been granted for a breath measurement to test for *Helicobacter pylori* infection. The test involves measuring the ratio of $^{13}CO_2$ to $^{12}CO_2$ in breath after the patient has consumed a ^{13}C-labeled urea solution: the solution is hydrolyzed into ammonia and ^{13}C-labeled CO_2 by the enzyme urease that is

produced in large amounts by *H. pylori* and the exhaled $^{13}CO_2$ is then detected and compared to its value before consumption of the solution [29, 65]. This is an excellent example of a simple yet highly specific and effective breath test that is already in routine clinical use.

5.4.2 Organic Biomarkers in Breath

The intricacies and complications involved in unequivocally detecting and quantifying specific VOCs in exhaled breath in a reproducible manner have been discussed in this chapter. This impediment, which is compounded by the current lack of standardization, has meant that there is currently paucity in the number of organic biomarkers with sufficient specificity and sensitivity to be distinctly associated with health status or individual diseases. Nevertheless, several breath VOCs have found usage in indicating physiological conditions.

For most people, the term "breath test" conjures up images of a police officer flagging down motorists and armed with a breathalyzer. Indeed ethanol, as used in the breath alcohol test for inebriated drivers, is likely the most frequently measured breath volatile today. Ethanol was among the first volatiles detected in early breath research [6] and has since been widely investigated with respect to alcohol consumption; breath concentrations of ethanol show a remarkable linear correlation with their concentration in blood, which has been the main premise and justification for approved use of the breathalyzer test for legal purposes [66, 67].

Another exogenous volatile compound that is frequently observed in breath is acetonitrile, which can be almost exclusively attributed to smoking. Benzene is similarly a key biomarker for recent smoking activity, and breath tests for both compounds have shown high specificity and sensitivity to smoking habits, with excellent predictive receiver–operator characteristic (ROC) curves [68]. Of course, as with most breath biomarkers, even those with high specificity, there is still a potential for confounders, in this case from inhalation of other biomass-burning incidents, which must be borne in mind prior to unique attribution of a compound to any specific physiological condition [35, 54].

Detection of cancer has been at the forefront of modern breath research. In particular, lung cancer has received disproportionate attention. This is primarily due to its high prevalence and the widely held belief that if there are biomarkers to be found in breath, then a disease affecting the lungs will be the best target due to the proximal production of oxidation markers and their direct and uninhibited release into the airways, which circumvents the additional potential complications imposed by processes involved in blood-borne biomarkers. In reality, the detection and identification of exhaled compounds that are unique to lung cancer remains elusive. This can be attributed to a number of issues, including a lack of standardized sampling and analysis procedures, failure to consider confounding variables associated with extraneous factors such as exposure to the clinical environment or smoking habits, and inappropriate use of data-mining tools that can deliver biomarker results to non-cross-validated data [35, 69]. The absence

of clear biomarkers for lung cancer should not act to discourage breath research from further endeavors to detect this major affliction on the breath, but rather should encourage due diligence in conducting such breath studies to increase the likelihood of success.

Most breath studies that have successfully shown associations of breath biomarkers with specific diseases have been based on a suite of volatiles, rather than individual biomarkers, often unidentified, with positive correlations made via diverse chemometric methods. Such studies have been made for lung cancer [34, 70], breast cancer [71], tuberculosis [72], and liver disease [73], to name but a few. Although identification of single, specific biomarkers is desirable, this is an unlikely outcome of breath research due to the great complexity of disease-generated volatiles and their high degree of commonality in processes involved in their production, such as lipid peroxidation. Therefore, the use of several biomarkers in combination with data-mining procedures is evidently the most promising in terms of clear detection of disease in breath.

Another line of biomarker research is to investigate and compare characteristic compounds *in vitro* in the headspace of bacterial or viral cell cultures and to probe their presence in breath during host infection [74]. This has been recently demonstrated, for example, for *Staphylococcus aureus* and *Pseudomonas aeruginosa*, the latter playing a role in infections of the cystic fibrosis lung [75]. The study demonstrated clear differentiation of the two bacteria in infected mice, a discrimination of infected versus uninfected mice, as well as a separation of different strains of *P. aeruginosa*. Clearly, such results are of high utility and offer a new perspective in breath research by including the analysis of volatiles generated by pathogens within the body. In another study, the volatile *in vitro* fingerprints of human tracheobronchial cells cultured with human rhinovirus (HRV) were investigated. More specifically, a comparison was made between cells treated with heat-killed HRV and poly(I:C), which is a TLR3 agonist, and controls [76]. The analyses revealed a differential expression of aliphatic alcohols and branched alcohols, which were postulated as being direct biomarkers of HRV infection. No differences were observed between the heat-killed HRV, poly(I:C), and control samples. This study again highlights the potential of breath analysis for diagnosing viral infections.

The list of diseases for which breath analyses have been performed is seemingly endless and certainly it will continue to expand as new technologies and methods emerge. Already there are several breath tests that are in regular clinical use and it is only a matter of time before this repertoire will expand. For a comprehensive treatise of the most common and successful breath tests, the interested reader is referred to review articles available in the literature [23, 15, 77].

5.5 Outlook for Breath Analysis

Ironically, breath analysis is simultaneously the oldest of the diagnostic medical techniques and also the least used in contemporary medicine. Analytical

applications for blood and urine have matured and now the biological medium of exhaled breath has become the focus for innovation. This is particularly apparent in the development over the past decade of the real-time instruments invoking lasers and chemical ionization MS (PTR-MS and SIFT-MS) that have been adapted to specifically address the issues of breath analysis such as temporal profiling and measurement of reactive species.

The expectation is that breath instrumentation for sampling and analysis will continue to experience technological advances primarily because breath is no longer relegated to the measurement of a few gas-phase environmental contaminants and biological (endogenous) compounds. In addition to the contemporary gas-phase methods for reconstructing environmental exposures and documenting the human exposome, the arena for breath analysis is embracing many new applications, including the following:

- testing metabolic pathways using isotopically labeled probe molecules
- genotyping the colonized pulmonary microbiome
- diagnosing opportunistic pulmonary infections
- recognizing VOCs and PVOCs patterns of preclinical disease state
- direct detection of inflammatory markers
- biomonitoring of disease progression and pharmaceutical efficacy
- linking exogenous (environmental) stressors to health effect using pharmacokinetic models
- monitoring organ function using specific enzyme pathways and metabolite production.

In all of the existing and new endeavors, there is an underlying quest for continuous discovery by collecting and cataloging new measurement data to gain as much knowledge as to "what is normal". Only when we understand the totality and variability of the breath exposome in groups of "unremarkable" or "nominally healthy" subjects can we begin to put individual measurements into the context of "remarkable" for diagnostic purposes.

A second major change in the outlook for breath analysis is the expansion of the definition of "breath" as coming from an intact animal or human, to the concept of cellular respiration. Under this framework, "breath" can include *in vitro* gas-phase measurements of cell lines and bacteria, as well as emanations from skin, urine, blood, feces, and other biological media. Such inclusion will allow researchers to focus on specific microscale systems for discovery, and then apply the results to targeted *in vivo* studies.

The overall outlook for breath analysis is positive. It is a biological medium that is only now becoming better understood and possesses some major advantages of which the prominent ones are noninvasiveness and inexhaustibility. In contrast to other biological media, breath is also the direct link into pulmonary function and is likely to be prominent in the future for finding preclinical signs for elusive diseases such lung cancer, asthma, COPD (chronic obstructive pulmonary disease), and emphysema before they exhibit serious, or even irreversible, stages.

Certainly breath analysis is coming of age and will see more widespread clinical implementation within the next decade.

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6
HTA in Personalized Medicine Technologies

Franz Hessel

6.1
Introduction

There is a widespread core belief that the concept of genetically stratifying therapy improves the efficiency of healthcare, by treating the right patient with the right drug at the right time, combined with substantial savings in healthcare costs by avoiding ineffective treatments and unnecessary side effects. Personalized medicine seeks to decrease the burden of disease by targeting prevention and treatment more effectively. By improving the ability to predict and account for individual differences in diagnosis and therapy response, personalized medicine promises to reduce the duration and severity of illness. At the same time, it may reduce healthcare costs by improving our ability to quickly and reliably select the effective therapy for a given patient while minimizing the costs associated with ineffective treatment and avoidable adverse events [1].

In times of worldwide economic pressures, healthcare systems are increasing the hurdles for pharmaceutical companies to achieve reimbursement for new drugs at premium prices for large patient populations. Personalized medicine is sometimes regarded as a potential solution to all fundamental problems of healthcare such as the patent cliff and the scarcity of healthcare resources. Even the US regulatory body Food and Drug Administration (FDA) aims to "pave the road for personalized medicine" [1]. However, the picture is not completely clear. Other stakeholders such as some European payers and reimbursement decision-making bodies show a greater amount of skepticism. They are afraid that the era of blockbusters is replaced by a new era of genetically stratifying niche busters with lots of additional high-priced drugs for an increasing number of smaller subpopulations of patients combined with additional high-priced biomarker tests. The overall consequences might be only slightly improved patient outcomes and a massive budget impact. A scenario of dozens of genetically different subgroups of patients who all, a few years ago, had the same diagnosis is already a reality, for example, in colorectal or non-small-cell lung cancer.

Currently, however, we are far away from such a scenario. So far, any specific therapy is available only for a few of the many subgroups and it might be a myth

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that the pharmaceutical industry will be able to develop new and efficient drug therapies for every individual patient or genetically differentiated patient subgroup. Looking at the number of new drugs approved by the FDA and the EMA (European Medicines Agency), there is an increasing percentage of personalized medicine technologies but so far the landscape has not changed dramatically. There is a number of success stories of impressive gains of patient outcomes but the development of genetically stratifying patients' therapy also bears some risks and uncertainties for pharmaceutical and diagnostic manufacturers. Neither the cost-effectiveness of personalized medicine technologies nor the efficiency of the drug development process is in every case enhanced, but in some cases there are promising chances that both objectives are achieved. It can also be stated that for the evaluation of personalized medicine, "one size fits all" does not apply; the assessment should rather be performed on a "case by case" basis [2].

There is a necessity for manufacturers to predict the success of new products during the development process and there is a necessity for healthcare decision-making bodies to identify promising technologies. Innovations that demonstrate to be useful for the overall health care system should be rewarded with a premium price (value-based pricing). Both clinical and economic advantages in comparison with existing therapies should be considered as relevant in the evaluation. Medical benefit can consist of a lower rate of non-responders, fewer side effects, and new therapeutic options for specific patient populations, each manifested in additional patient-relevant benefits such as improvements in morbidity, mortality, and quality of life. Economic benefits from the perspective of insurers can be lower treatment costs, for example, caused by avoided treatment of non-responders. The specific criteria that will be applied depend on the national assessment procedures and regulations.

The aim of this chapter is to describe the challenges in evaluating genetic biomarker tests and personalized medicine technologies for healthcare reimbursement and pricing decisions. This includes considerations of health technology assessment (HTA) and health economic evaluation. First, a brief overview of the core principles and the implementation of HTA is given before the specific aspects of HTA and health economic evaluation of genetic biomarker tests and personalized medicine are discussed.

6.2 Health Technology Assessment (HTA)

The term technology assessment was created in the United States in the mid-1960s from a discussion about the critical role of technology in modern society and its potential for unintended, and sometimes harmful, consequences. Health technologies have been evaluated for safety, efficacy, cost, and other consequences of their use long before. The implementation of technology assessment as a systematic process coincided with the introduction of specific health technologies widely discussed in public such as medical devices (e.g., CT scans), techniques

for *in vitro* fertilization, and drugs to modify human behavior. Those technologies were among the topics of the first technology assessments.

From the very beginning, the terms technology and assessment were used in a broader sense as any medical intervention and "any process of examining and reporting properties of a medical technology used in health care, such as safety, efficacy, feasibility, and indications for use, cost, and cost-effectiveness, as well as social, economic, and ethical consequences, whether intended or unintended" [3]. However, beyond the pure proof of efficacy and safety for approval purposes HTA is a form of policy research that examines short- and long-term social consequences (such as societal, economic, ethical, legal aspects) of the application of a specific technology. The goal of technology assessment is to provide policy-makers with information on policy alternatives [4, 5]. These core principles of HTA clearly differentiate it also from comparative effectiveness research (CER) and clinical guidelines [6].

A further core characteristic of HTA is the strict orientation toward the principles of evidence-based medicine (EbM). The term EbM refers to the use of the currently best, publicly available – for some institutions, the best possible – evidence from scientific and medical research. EbM combines evidence from the scientific literature and to a smaller extent also from other sources, with clinical experience and observations as well as patient preferences [7]. This approach for bedside decision-making about the care of individual patients is transferred to clinical guidelines for clinical decisions and to HTA for the purpose of health policy and coverage decision-making.

Over the last few decades, HTA was more and more linked to the decisions about reimbursement and pricing of pharmaceuticals and medical devices. All industrial countries and an increasing percentage of the emerging countries have implemented official HTA institutions that make recommendations or compulsory decisions. The algorithms and the structures of the evaluation processes are based on transparent method guidelines. Although there is quite a long tradition of HTA in Scandinavian countries and the Netherlands, the British NICE (National Institute for Health and Care Excellence), the French HAS (Haute Autorité de santé), and the German IQWiG (Institute for Quality and Efficiency in Healthcare) are regarded as the most important and influential HTA bodies in Europe.

As HTA is intended to support health policy decision-makers it should be conducted at a time when the diffusion and use of the technology can still be influenced. On the other side, sufficient evidence must be available yet. This challenge to balance between the fact that a decision has to be made and the knowledge of all consequences of the use of the technology is not fully based on high-evidence studies yet let some HTA bodies (e.g., the British NICE) include decision analytic modeling techniques into the evaluation process.

HTA is not a one-time evaluation. Sometimes it might be necessary to revise the HTA recommendations when new information becomes available and many HTA institutions have regular schedules to update their reports. Others differentiate the time of assessment through different approaches such as horizon scanning, early

HTA, and HTA of existing well-established therapies. Such stepwise approaches are especially reasonable for test-drug combinations in which the biomarker test or test combination is optimized after the launch of the drug and over a longer time period.

Besides the evaluation of the external validity of the scientific evidence – in other words, whether the study results are valid for the specific research question, the specific patient population, and the specific healthcare setting – the internal validity of the detected studies is an essential part of HTA recommendation. The most intense discussion about the IQWiG evaluations in Germany is focusing on methodological aspects and study designs. Questions of the appropriateness of the comparator technology (e.g., standard of care, doing nothing, placebo), the length of follow-up time, the percentage of missing values, the quality of blinding and randomization, the sample size, the patient characteristics, and many more are typical aspects of the evaluation of the methodological quality. In HTA, these aspects usually are documented using standardized checklists with some knock-out criteria and some aspects leading only to minor limitations. Only if all relevant quality criteria are fulfilled are the study results considered for the final recommendations. If the quality limitations of the available, potentially favorable studies are regarded to be so relevant that it cannot be ruled out that the results are heavily influenced by study biases, the result of the HTA process might well be, "There is no evidence of an additional benefit." In consequence, the technology might not be reimbursed or reimbursed only at the price level of the existing standard therapy.

Besides these methodological aspects, the most important criterion to classify the level of evidence (LoE) of a HTA recommendation is the type of study. According to EbM, prospective randomized controlled trials (RCTs) and systematic reviews of RCTs are assigned to the highest LoE I, followed by non-randomized comparative trials up to case studies and expert statements (LoE IV–V) [8]. For the evaluation of the medical benefit of pharmaceuticals, international HTA bodies usually accept only studies with LoE I.

There is an ongoing discussion as to which outcome measures should be considered as essential in the decision-making process in HTA. There could be a significant difference in a certain laboratory parameter such as blood glucose level or virus load, but no difference in survival rate. Consequently, some HTA bodies might not consider these differences in laboratory test results as clinically relevant.

It is common consensus that the measures should be relevant for the health of the patients, but the acceptance of surrogate parameters and appropriate measures for morbidity differs between countries and HTA institutions. For example, the German social law sets a framework by referring to mortality, morbidity, and quality-of-life, but so far biomarker test results or progression-free survival are often not considered as patient-relevant outcome measures.

Epidemiological data and costs are commonly excluded from EbM reviews but their inclusion is frequently recommended in HTAs [6]. Often the question addressed is, "Is the technology worth the money?" In some countries this question is approximated by estimations of the budget impact or

cost-effectiveness – for example, in the United Kingdom by the results of cost-per-QALY (quality-adjusted life year) models – while other countries such as Germany do not explicitly address health economic measures. Although the international method guidelines for HTA recommend including health economic consequences of the use of specific technologies, how and to what extent health economic studies are regularly included in the HTA process varies widely.

According to the international principles of HTA [6], the link between the identification and description of the scientific evidence and the healthcare decision-making process itself needs to be transparent and clearly defined. A clear distinction should be made between the assessment and the resulting decisions, the appraisal. This link will be different in various settings and healthcare systems, but in all cases the underlying structure and processes should be transparent.

Many HTA bodies accompany the summaries of their results with an estimation of the strength of the underlying data by categorizing it, for example, into "in hint for an effect," "small," or "strong effects." Usually, recommendations for further research are made.

6.3 Validation and Evaluation of Biomarker Tests

In general, *in vitro* diagnostic tests have been influencing therapy decisions for a long time and traditionally they are often seen either as a commodity or in some cases as a scientific playground for specialized laboratory physicians without practical relevance to routine care. This changed to greater attentiveness toward *in vitro* diagnostic tests since the identification and broad use of genetic markers. In case of *in vitro* diagnostics used in personalized medicine the tests generally are intended to identify the presence, absence, or amount of a biomarker. If the diagnostic test is inaccurate, the treatment decision based on the test may not be optimal. Therefore, to a great extent the success, particularly of the clinical validity of genetically stratifying therapy approaches, depends on the identification of the appropriate predictive biomarker and the quality of the technology used.

Probably due to their use in forensic science, genetic test results seem to convey the image that they represent the "absolute truth." In the reality of clinical routine care they rarely offer a clear yes – no result concerning long-term clinical outcomes. They often just classify the risk and in many cases they are just one piece of the diagnostic puzzle.

From the perspective of evaluation of healthcare programs it is important to differentiate between the attributes of the test itself and the factors due to the companion therapeutic intervention or the further circumstances of treatment such as compliance.

In the evaluation of biomarker tests there are three basic aspects to be considered: The analytical validity of the marker, the clinical validity, and the clinical utility. Analytical validity can be summarized with the questions "Can I trust the results of the test?" or "Does the test really measure what it is supposed to

measure?" Clinical validity means the degree to which a test result can be used to correctly identify patients with the target condition. Finally, clinical utility describes the actual consequences of the whole treatment strategy on patients' clinical outcomes.

Fryback and Thornbury [9] presented a hierarchical model of the efficacy of diagnostic tests in their classic 1991 article and their classification is still referred to by international HTA bodies. They classify the technical efficacy of a test as level 1 and the diagnostic accuracy and the analytic performance of the test (e.g., PPV (positive predicative values), NPV (negative predicative values), AUC (area under curve), or ROC (receiver operating characteristic)) as level 2. Level 3 is defined as the extent to which the test helps make the diagnosis (diagnostic thinking efficacy), whereas level 4 represents the therapeutic efficacy, in particular, whether the test result is helpful, or may be required, for further management of the patients. The highest levels 5 and 6 represent patient outcome efficacy and societal efficacy. Usually, HTA of diagnostic technologies require convincing evidence of levels 5 and 6 for a recommendation of the test to be used in routine healthcare.

There are established methodological standards for studies on the analytic validity of biomarker tests such as the STARD (Standards for Reporting of Diagnostic Accuracy) initiative on accurate and transparent reporting of study results [10] and the QUADAS (Quality Assessment of Diagnostic Accuracy Studies) checklist for systematic reviews [11]. The main components of the validation of genetic tests have been summarized also by the US Center for Disease Control (CDC) in the so-called ACCE wheel. The name ACCE is derived from *A*nalytic validity [2], *C*linical validity [3], *C*linical utility, and [4] *E*thical, legal, and social implications [12]. Figure 6.1 shows the criteria for evaluation in more detail. It becomes obvious that the process is multidimensional and besides the analytic performance of a test a considerable number of further measures are taken into consideration.

The first evaluation approach from Fryback and Thornbury has been developed generally for diagnostics, whereas the ACCE specifically focuses on genetic tests. Similarly to any other diagnostic test, modern genetic biomarker tests should show a high analytic performance, but with regard to patient clinical outcomes, the clinical validity of the test – again whether it is a biomarker or a conventional laboratory test – is often not satisfying. This can be due to many reasons such as sensitivity and specificity of the test or other quality aspects of the test itself, the platform or the sample, and also because of insufficient correlation between test result and therapy effectiveness.

6.4 Health Technology Assessment of Personalized Medicine Technologies

First of all, from a scientific point of view there is no reason why a specific medical technology should be investigated with different methodological approaches or a different standard of the methodological quality. There can be no "discount" on the quality of the studies or the quality criteria of HTA institutions concerning the LoE

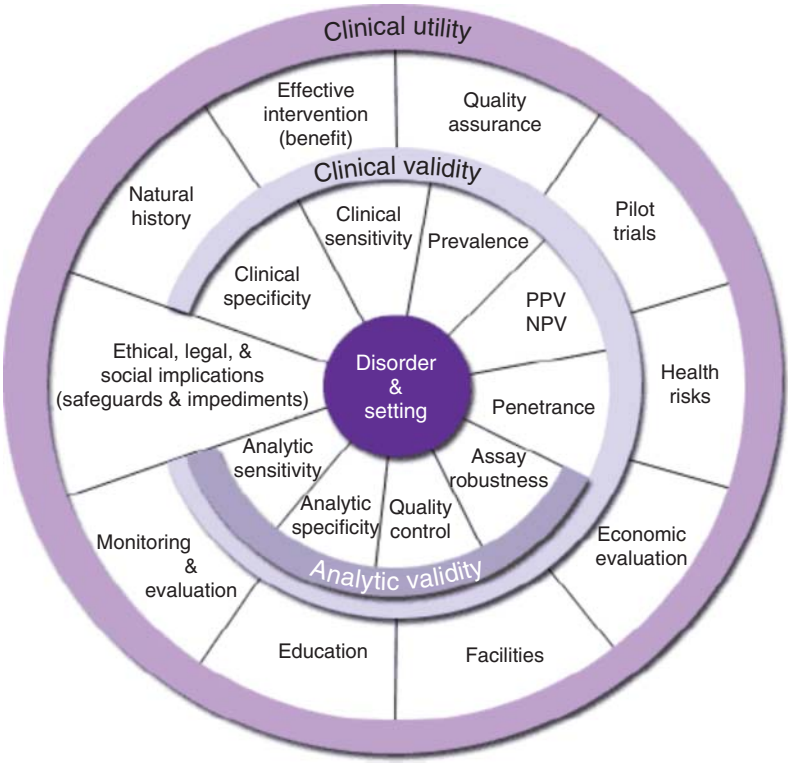

Figure 6.1 ACCE wheel [13].

rating just because the topic is more complex or because it is more difficult to conduct the appropriate scientific studies. The question is whether all fundamental principles of HTA can be applied also to personalized medicine technologies and whether there are any special issues to consider in HTA of personalized medicine.

An obvious issue is the fact that – compared to pharmaceuticals – we are dealing with a more complex intervention consisting of at least two more or less permanently connected parts, the test and the therapeutic consequence of the test result. Both components are mutually dependent. A perfect test could be without any benefit if the therapeutic consequences are not beneficial, and a therapy, highly effective in a subgroup of patients, could do more harm than good without an appropriate selection of the responders if the total populations suffers from adverse events, and only a – potentially very small – subgroup responds to the therapy. Reimbursement bodies who intend to select the subgroup of patients that benefits most face the challenge of separating the consequences of the single components of the intervention. The whole intervention should therefore be evaluated using an adequate study design to differentiate the effects of both parts, the test and the pharmaceutical therapy.

Several expert groups tackled the question whether the general HTA principles are also applicable to personalized medicine or should HTA procedures and methods differ in the evaluation of personalized medicine technologies. [2, 14–16]. There seem to be a broad consensus that in general, all principles, recommendations, and methods of HTA are applicable and valid also in the field of personalized medicine. The fundamental EbM-based evidence requirements remain. Causality should and could only be demonstrated with RCTs although it has to be taken into consideration that non-randomized controlled intervention trials, observational studies, and decision-analytic modeling might contribute important additional pieces of knowledge, especially to investigate the real-world use of technologies. These studies of lower levels of evidence should also be included in the HTA process.

There is an ongoing discussion in the international scientific community about the role of biomarker studies with a so-called prospective-retrospective design. Following the concept of Simon and Hayes [17, 18] prospective-retrospective studies are prospectively planned analyses of archived material of completed RCTs. The new analysis is designed to answer new research questions – usually the clinical validity of a new biomarker testing strategy accompanied by a drug therapy – using only the archived samples of patients with the specific test-therapy combinations to be compared. According to Simon, under certain circumstances the results of prospective-retrospective studies in HTA should be classified as level I evidence, comparable to the results of RCTs. So far, HTA institutions did not specifically comment on this issue but with the increasing number of genetically stratifying therapies entering routine healthcare over the next years and the growing number of prospective-retrospective analyses of archived samples it can be assumed that they will specify their method guidelines. In some countries such as the United Kingdom, Canada, and Spain the HTA institutions indirectly followed the arguments of Simon *et al.* [18] by giving positive recommendations concerning the use of genetic marker tests (e.g., Oncotype Dx) whose scientific evidence was derived from prospective-retrospective trials.

With regard to patient outcomes there is no fundamental difference. In general, the evaluation always should include patient-relevant, long-term morbidity and mortality as well as utilities, particularly quality of life. However, there might be some additional outcome aspects for the evaluation of personalized medicine. There is a certain discussion in the literature about the value of knowing whether one has a disease, and in particular knowing whether one is responding to a specific treatment. Another special aspect of HTA of biomarker tests might be the fact that the family of the patient might also be affected by the test result. Therefore, the full scope of short- and long-term consequences – potentially beyond the lifetime of the affected patient – should be included in the assessment.

The possibility of using different testing strategies, for example, testing for one marker or marker combinations, potentially in sequence, as well as non-personalized approaches, bares the problem of comparing multiple strategies. The role of indirect comparisons might increase and more frequent updates of the assessment might become necessary.

In general, personalized medicine by definition reduces the number of patients available for studies. Smaller sample sizes might be inevitable. In high-prevalence diseases no such recruiting problems should occur but there is an increasing number of personalized medicine examples in which the small number of existing patients for the specific indication makes the conduction of large clinical trials very time consuming, if not impossible. HTA institutions should take this inevitable problem into consideration by accepting smaller sample sized and specific statistical methods. Potentially, the existing orphan-drug regulations also have to be reconsidered.

A further point to consider is the role and the methods of decision-analytic modeling, which is an integral part of the HTA process in several countries [19]. On first sight, it seems to make no difference whether a health economic model is designed and calculated for a personalized medicine strategy or for a conventional one-step treatment approach. Appropriate model designs that include all relevant alternatives, especially also the test-negatives receiving treatment, have been developed and demonstrated to be feasible. A recent paper of Annemans and colleagues summarizes a number of further, more or less specific points to consider in health economic assessments of personalized medicine technologies, which are also valid for the evaluation of genetic biomarkers alone [20]. An important point is being clear about the technology to be evaluated. In studies on pharmaceuticals this aspect seems to be obvious but in studies on test-drug combinations the technical details concerning the diagnostic are often missing. As long as the study is designed to evaluate the pharmaceutical the test result is often assumed just to be "given." Questions of test or sample quality, laboratory size, or the platform used, often also concerning the specific technology such as IHC (immunohistochemistry) or FISH (fluorescence *in situ* hybridization), are not taken into consideration. The scope of the economic evaluation should also include these aspects.

Furthermore, it is recommended to include at least the current practice as an alternative strategy. Thus, a "do-nothing," particularly a "no-test" scenario, should regularly be considered, at least if the test strategy is not yet completely implemented in routine care. Further alternative scenarios could be parallel tests or test sequences. It is technically feasible to conduct health economic studies on genetic biomarkers according to the established quality standards. The increased uncertainty of the model structure, the input data, and consequently the model results must be faced with adequate instruments and extensive sensitivity analyses.

Summarizing this chapter, it clearly can be stated that the fundamental principles of HTA and health economic evaluation are also valid and feasible for the evaluation of personalized medicine and genetic biomarker tests. Nevertheless, a number of points have to be addressed when performing the assessments, in the form of careful revisions of the existing HTA and health economic guidelines. This process as well as conducting HTAs definitely requires an interdisciplinary team, including academia, industry, and payers, and will last definitely for the next 5 years.

6.5 Concluding Remarks

Following the definition of the European HTA Collaboration EUnetHTA HTA is a multidisciplinary process that summarizes information about the medical, social, economic, and ethical issues related to the use of a health technology in a systematic, transparent, unbiased, robust manner. The assessment of personalized medicine or genetic biomarker tests is principally feasible according to the general principles of HTA, although some modifications seem to be necessary. The main challenges of HTA of personalized medicine are the smaller sample sizes and the necessity to evaluate a multistep intervention with two or more co-dependent technologies – usually test and drug – of which the different components might be funded in different programs, particularly according to different schemes. In 2011, Australia became the first country to introduce a HTA structure that is explicitly designed HTA to smoothen the process of personalized medicine. Most of the other countries are still in the adaptation process.

In general, the HTA and reimbursement processes should be aligned as far as possible. If a healthcare system decides to reimburse a drug the companion diagnostic test should also be reimbursed at the same time. If the scientific evidence for the use of a specific marker test is not sufficient at the time of drug launch a temporary or conditional reimbursement should be taken into consideration. The HTA process of the personalized medicine technology should include both the diagnostic test and the drug in a coordinated, integrated way. Especially, the quality of the test including analytic and clinical validity should be included in the evaluation process.

Personalized medicine approaches can only show optimal benefit for healthcare systems if the price and the value of the technology are aligned. Only technologies with a high additional benefit according to HTA should be funded at a premium price adequate for the specific country. In consequence, prices could vary between countries and between indications.

Most reimbursement bodies primarily look at medical benefit, particularly the evidence level of the results of studies on clinical efficacy. The relative low number of genetic biomarker tests successfully adopted for routine care might be due to a combination of the high expectations of reimbursement bodies concerning the LoE and the business model of diagnostic manufacturers. As long as value-based pricing approaches are not used for IVDs (*in vitro* diagnostics) equally to pharmaceuticals, the companies' financial resources for clinical and health economic studies demonstrating the value for money remain limited. The resulting lack of high-level evidence study results again prevents reimbursement of genetic biomarkers at a premium price [21]. From a point of view of a HTA body, this is correct. However, it is unsatisfying for the manufacturing industry and the patients if likely beneficial technologies are not given a chance for successful implementation.

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7
Bone Remodeling Biomarkers: New Actors on the Old Cardiovascular Stage

Cristina Vassalle, Silvia Maffei, and Giorgio Iervasi

7.1
Introduction

Both osteoporosis and cardiovascular disease (CVD) are major public health problems leading to an increased morbidity and mortality rate. Growing data suggest the relationship between osteoporosis and CVD, through mechanisms not fully elucidated but likely related to common risk factors, common pathophysiological mechanisms, or both. In addition to menopause and advanced age, other risk factors for CVD, such as dyslipidemia, hypertension, and diabetes, have also been associated with increased risk of low bone mineral density (BMD). There are common underlying molecular pathways in the physiopathology of these two diseases. Oxidative stress (OxS) and inflammation are key factors in both atherosclerosis and osteoporosis. Elevated homocysteine (Hcy) concentration is associated with both CVD and osteoporosis. Nitric oxide (NO), in addition to its known atheroprotective effect, appears also to play a role both in osteoblast function and bone turnover. Impaired mineral metabolism appears to be associated with bone disease and also with vascular calcification. Many traditional biochemical markers of bone remodeling retain significance for atherosclerosis, CV risk, and vascular calcification. In addition, recent data suggest how bone metabolism affects energy balance, with important effects on the onset and development of CVD and its associated comorbidities. Gene deletions and variants have a pathogenic role in CVD and vascular calcification as well as in osteoporosis.

This chapter highlights the available literature on pathophysiological similarities between osteoporosis and CVD and presents an overview of the available evidence that supports the interaction between these conditions. Prevention and treatment strategies for one of the two diseases may be beneficial for the other one. A better understanding of these mechanisms may help in the advancement of common preventive and therapeutic interventions targeted at both conditions.

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7.2 Cardiovascular Disease and Osteoporosis: Common Risk Factors and Common Pathophysiological Mechanisms

One line of evidence that confirms the relationship between CVD and osteoporosis is the observation that both diseases share many risk factors and comorbidities such as diabetes, chronic kidney disease (CKD), hypertension, smoking, or low level of physical activity (Table 7.1) [1]. Nonetheless, it is also important to remind that there is discordance for some risk factors, as there are fewer fractures in men and in obese subjects, which are recognized risk factors for CVD. Indeed, there are interesting issues in bone loss and CVD in relation to gender, as estrogens are protective for both conditions in premenopausal women. With regard to CVD, the lower risk for women has been reconsidered [2]. In fact, after menopause changes occur, and the risk of CV events exponentially rises, and the advantage gap for women decreases, and becomes progressively smaller, until it overcomes the risk for men in advanced age [2]. Sex-related differences are also evident for bone, as BMD well correlates with estrogen, and early postmenopausal bone loss is due to direct consequences of estrogen fall [3]. Moreover, bone loss is likely the main reason of BMD and risk of fracture in female subjects after menopause, and increases at a higher rate in elderly women with respect to men. Conversely, men have higher bone density, and in older men BMD is more dependent on BMD peak [3]. Thus, more recent research lines are more focused on studying the effect of estrogen presence in women and the consequences of estrogen lack in postmenopausal women evidencing different gender-related aspects in the onset and progression of both diseases in elderly subjects, rather than consider a real lower risk for one sex with respect to the other [3].

Table 7.1 Main common risk factors for bone and cardiovascular disease.

Pathogenetic factors	CV effects	Bone effects
Aging	−	−
Male gender	− (−/+)	+ (+/−)
Obesity	− (−/+)	+ (+/−)
Hypertension	−	−
Diabetes	−	−
Dyslipidemia	−	−/+ (−)
Chronic kidney disease	−	−
Smoking	−	−
Physical activity	+	+
Inflammation	−	−
Oxidative stress	−	−
Nitric oxide	+	+
Homocysteine	−	−
OxLDL	−	−
Estradiol	+	+

The relationship between obesity and the bone or the CV system is also extremely complex, as adipose tissue is no longer considered an inert tissue storing fat, but an active endocrine tissue that affects a variety of pathophysiologic processes, including immunity and inflammation. Obesity is without doubt correlated with hyperinsulinemia due to insulin resistance and to a low-grade chronic inflammatory state. Conversely, other molecules, such as adiponectin, actively secreted by the adipose tissue, have anti-inflammatory properties, and exert multiple protective effects on the (CV) system [4]. In particular, adiponectin also promotes bone remodeling through positive effects on osteoblast proliferation and differentiation [4]. Thus, the balance of the adipose tissue numerous biological effects likely determines if consequences will be positive or negative. Given the association of obesity with CVD, obese subjects are expected to develop adverse outcomes after a cardiovascular event compared to individuals with normal body mass index (BMI). However, many results evidenced a U-shaped relationship between obesity and mortality from various diseases, including myocardial infarction and heart failure (HF) [5]. These findings indicate that patients with higher BMI have better short- and long-term mortality rates, the so-called "obesity paradox" in CVD [5]. On the other hand, a higher BMI exerts greater mechanical load on the bone, which increases its BMD to compensate this effect. Conversely, as low BMI is a recognized risk factor for future fracture, a meta-analysis from 12 prospective population-based cohorts showed an inverse relationship between BMI and fracture risk [6]. Nonetheless, circulating levels of protective molecules, for instance, adiponectin, are inversely correlated to adiposity in obese subjects [7, 8]. In this context, very recent data showed a significant number of fractures in obese subjects [9]. Specifically, obesity appears associated with certain types of fractures, with a higher risk of upper arm and ankle fracture, but a lower risk of developing wrist fractures in these patients [9]. Thus, as for the CV system, these findings underline the need for more information on balance of underlying molecular mechanisms to better understand how adipose tissue might affect bone health.

Many common pathogenic factors have been proposed to promote atherogenesis and osteoporosis by acting on both vascular and bone cells. Pro-inflammatory cytokines such as IL-6 and TNF-α have a recognized proatherogenic effect, and also represent major factors for activation of osteoclasts, showing stimulation of bone resorption and inhibition of bone formation [10–14]. Moreover, recent data from the Health Aging and Body Composition Study and the Women's Health Initiative (WHI) Observational Study have shown that elevated levels of these cytokines or their soluble receptors were related to an increased risk of hip fractures in elderly subjects [15, 16]. In addition, CRP levels have been found to be negatively related to BMD and positively to fracture risk [17–20]. However, data from the Framingham Osteoporosis Study showed that IL-6, TNF-α, and CRP did not show any association with BMD in men [21]. Among premenopausal women, there were significant inverse associations between IL-6 and trochanter BMD, and between CRP and femoral neck and trochanter BMD, while TNF-α was positively associated with spine BMD [21]. In postmenopausal hormone therapy (HT)

users, CRP was positively associated with femoral neck BMD, in contrast to postmenopausal women not using HT [21]. Notably, inflammatory parameters may be more significant considering the bone loss rate rather than the absolute BMD value at a single time point. A significant relationship between IL-6, CRP, and TNF-α levels and 3-year BMD change has been evidenced in a cohort including men and women (age: mean 63 years, range 52–78), and also showed that variation in the low levels of inflammatory markers, especially IL-6, predicts bone loss and resorption [22]. Interestingly, recent data from the European Prospective Investigation into Cancer in Norfolk study (EPIC-Norfolk) showed a U-shaped association between CRP and fractures (all types and hip fractures) with the lowest risk of fracture of all types observed for participants with intermediate CRP levels in the range of $1-2\ \mathrm{mg\,l^{-1}}$ [23].

Another important variable related to inflammation and determinant of both bone loss and CVD is OxS. Many oxidative stress biomarkers are recognized predictors for CV risk and morbidity due to CV events [24, 25]. Moreover, OxS is associated with a faster bone turnover driven by higher bone resorption and lower rate of formation. In particular, the differentiation of monocytes to osteoclast precursors appears mediated by the monocyte chemotactic protein-1, which involves production of reactive oxygen species [26]. Conversely, an elevated OxS decreased osteoblast differentiation and induced osteoblast cytotoxicity [27]. Accordingly, hydroperoxides are independently associated with decreased BMD in the total body, lumbar spine, and the total hip [28]. Moreover, a significant positive association between serum levels of hydroperoxides and C-terminal crosslinking telopeptides of type I collagen (CTx-1, a marker of bone resorption) was also observed in a cohort of 167 postmenopausal women, confirming a role for OxS by enhancing bone resorption rate [28].

The endothelial NO synthase signaling pathway, a key factor in the modulation of atherosclerosis, has also a role in bone remodeling by decreasing osteoclast formation through repression of the receptor activator of nuclear factor kappa-B ligand (RANKL) as well as through reduction of the resorptive activity [29, 30]. Moreover, interesting data suggest that bone marrow (BM) is an important factor in the regulation of myocardial remodeling, with eNOS of the BM identified as a regulator of myocardial angiogenesis and fibrosis in pressure-induced cardiac hypertrophy [31].

High level of homocysteine (HHcy) is a recognized risk factor for CVD, which also increases osteoclast activity and decreases osteoblast activity with direct effects on bone matrix [32–34]. The principal mechanisms by which HHcy may exert its actions included endothelial dysfunction, reduced NO bioavailability, smooth muscle cell (SMC) proliferation, mitochondrial abnormalities and OxS, and increased platelet aggregation [34, 35]. Hcy binds directly to the extracellular matrix (ECM) and reduces strength at the bone level [34]. However, although majority of clinical studies confirm that HHcy affects bone density, others have not found significant associations [36–41]. Recent experimental data on mice fed with a homocystine-supplemented diet, which leads to severe HHcy, showed decreased bone quality and impaired fracture repair [42]. There is also clinical

evidence that postmenopausal women with heterozygous mutation in MTHFR (methylenetetrahydrofolate reductase) and hyperhomocysteinemia demonstrate a decrease in BMD [43].

There are many parallels between the mineralization of bone and vascular calcification [44]. As in bone, the minerals formed are crystals of calcium hydroxyapatite. In a calcified artery, there are cells that have the appearance of osteoblasts, osteoclasts, chondrocytes, and even hematopoietic BM, with the potential to form mineralized nodules that contain bone matrix proteins, such as osteocalcin (OC), osteopontin (OPN), and alkaline phosphatase (ALP) [45–47]. Oxidized low density lipoprotein (OxLDL) may have opposing effects on vascular and bone cells *in vitro*. In particular, accumulation of oxidized lipids in the subendothelial space of arteries leads to arterial calcification by induction of alkaline phosphatase, a marker of osteoblastic differentiation [48]. OxLDL also plays a major role in the onset and development of atherosclerotic plaques and the ratio RANKL/OPG (osteoprotegerin) is increased in calcified arteries [49]. Conversely, its accumulation inhibits osteoblast differentiation at bone level by depressing the induction of ALP activity and reducing mineralization in preosteoblastic bone cells, as evidenced by the inhibitory effect on the induction of ALP and calcium uptake [48]. More recent data confirm that OxLDL may inhibit differentiation and mineralization of osteoblasts and upregulate osteoclast differentiation by enhancing RANKL expression in human osteoblast-like cells, with a negative role in bone remodeling [50]. Other *in vitro* results indicate that OxLDL induced OxS and reduction in the Wnt signaling required for the differentiation and survival of osteoblasts [51]. Besides bone cells, different cell types in the arterial wall, including fibroblasts and endothelial and vascular SMCs, express all members of the RANK/RANKL/OPG triad, with the ratio RANKL/OPG increased in calcified arteries [52]. OxLDL increases RANKL level in human vascular cells by the generation of reactive oxygen species [52]. In rats, RANKL was found to directly enhance vascular SMC calcification both *in vitro* and *in vivo* by binding to RANK and increase levels of the bone morphogenetic protein 4, a protein that belongs to the transforming growth factor β superfamily and involved in the endochondral bone formation and vascular calcification [53]. Experimental data indicate that an atherogenic high-fat diet reduces bone formation by blocking the differentiation of osteoblast progenitor cells [54]. Moreover, very recent experimental data demonstrated that hypercholesterolemia promotes the development of an osteoporotic bone phenotype in mice, including an increase in osteoclasts, loss of trabeculae, thinning of trabeculae and cortex, and reductions in failure load and energy to failure [55]. Nonetheless, in contrast to the experimental evidence, majority of clinical studies reported that lower BMD is associated with an atherogenic lipid profile, although others found no association, or even a positive relationship [56–61].

Both atherosclerosis and osteoporosis may result from estrogen deficiency after menopause [62, 63]. It is well documented that estrogen deprivation after menopause induced an increased high bone turnover and accelerated bone loss, with upregulation of osteoclast formation and differentiation [64]. On the other hand, postmenopausal hormone replacement therapy is beneficial on bone

metabolism and is considered a therapy for postmenopausal osteoporosis [65]. Moreover, estrogen deficiency related to aging has been identified as one major determinant of bone loss also in men [66]. Conversely, the beneficial effects of endogenous estrogens on the CV system are also well established [63, 67]. Bone and coronary arteries are target organs for estrogens, as shown by the expression of estrogen receptors on osteoblasts, osteoclasts, and coronary artery SMCs [66, 68]. Thus, the intensity of estrogen deficiency would in turn activate different estrogen targets involved in the regulation of postmenopausal osteoporosis, atherosclerosis, and possibly arterial calcification as well, including inflammatory parameters (cytokines and chemokines) and osteoclast regulators (OPG) [69]. In particular, reduced estrogens induce an increase in these molecules together with a decrease in OPG, a reduction in serum vitamin D (vitD), increased inflammatory and oxidative processes, and reduced NO bioavailability, key factors for the progression of bone loss and atherogenesis [35, 70]. Moreover, estrogens appear inversely related to levels of Hcy and lipids, especially OxLDL [71, 72]. A loss in estrogen levels is associated with an increase in parathyroid hormone (PTH), which accelerates the process of bone loss and facilitates soft tissue calcium deposition, also in vessels [73, 74]. Conversely, another mechanism by which estrogen may be beneficial against bone loss is by maintaining telomere length, a key emerging risk factor for both osteoporosis and CVD [75, 76].

Interestingly, in the clinical setting of the Rancho Bernardo study, the significant inverse association of BMD with coronary artery calcification in women taking HT and the lack of association between BMD and coronary artery calcification in non-HT users suggested that the association between coronary artery calcification and bone calcium might be largely affected by estrogen levels [77]. In this context, it may be conceivable that the rate of early postmenopausal bone loss could be used also as a predictive factor for other non-osteoporotic diseases. In the WHI Study, women treated with long-term estrogen therapy developed lower coronary artery calcification than those who received placebo [78]. Estrogen has been found to inhibit vascular calcification acting through RANKL counteraction [79]. Accordingly, experimental data confirm that RANKL contributes to vascular calcification, essentially acting on bone morphogenetic protein-2, an inducer of calcification, and matrix Gla protein (MGP), a calcification inhibitor [79]. Estrogen acts mainly through estrogen receptor α to counteract these effects [79].

Although androgens may affect bone and vascular health, their effects are likely gender-specific and their roles are more multifaceted than originally perceived [80–84].

7.3 Biomarkers of Bone Health in CVD

7.3.1 Cathepsin K

Cathepsin K (CatK) belongs to the cysteine protease family originally identified as lysosomal enzymes [85]. Specifically, CatK, initially considered to be selectively

expressed in bone, is the major protease of osteoclasts, responsible for bone resorption, with proteolytic activities against several ECM components such as collagen I and II, elastase, osteonectin, and OPN [86] (Figure 7.1). More recent data have revealed additional roles for CatK in other pathological conditions, including cancer, and autoimmune, infectious, and CVD [84]. In particular, the role of CatK in atherosclerosis has raised great interest [85]. In fact, ECM remodeling is an important process in atherosclerotic plaque turnover and stability [85]. CatK appears regulated by oscillatory shear stress, which may trigger a cascade of events leading to increased and progressive atherosclerotic plaque [87–89].

CatK expression in normal arteries is low. However, CatK has been found in plaques prone to rupture where it can mediate extensive matrix breakdown [90]. Early human atherosclerotic lesions showed CatK expression in the intima and medial SMCs, whereas in advanced atherosclerotic plaques CatK appears mainly localized in macrophages and SMCs of the fibrous cap [90]. Other data indicate that both CatK mRNA and protein levels are high in advanced stable human atherosclerotic plaques compared with early atherosclerotic lesions and lesions containing a thrombus. In these lesions, CatK appears localized in SMCs and macrophages, and also in endothelial cells (ECs) [91]. Moreover, recent data suggest that human macrophage foam cells may degrade the atherosclerotic plaques through CatK-mediated processes, including the release of CTx [92].

Knockout results indicated a reduction in atherosclerotic plaque area in CatK-deficient mice, and a significant reduction in the number of advanced lesions [91]. Although deficiency of CatK exerts a protective role, reducing plaque progression and increasing fibrosis also aggravates lipid uptake and macrophage foam cell formation, with adverse effects on plaque stability [91, 93, 94]. Other results suggest a role for cathepsins in restenosis and neointima formation, as increased mRNA and protein levels of CatK have been observed in the carotid artery after the balloon injury model of restenosis in rats [95]. CatK activity resulted in early arterial calcification, and its expression and activity are upregulated in stenotic aortic valves [96, 97].

CatK has also been proposed to be a novel marker of adipogenesis, and is involved in the pathogenesis of obesity by promoting adipocyte differentiation [98, 99]. CatK is overexpressed in adipose tissues of obese subjects [98]. Deficiency or selective inhibition of CatK reduces preadipocyte differentiation and adiposity and increases glucose metabolism in mice under high-fat diet treatment [98, 99]. Moreover, recent data showed that CatK contributes to the development of obesity-associated cardiac dysfunction, as CatK knockout partly reversed the impaired cardiomyocyte contractility and deregulated calcium handling associated with high-fat diet [100]. In addition, CatK knockout reduced glucose intolerance, improved insulin-stimulated Akt phosphorylation, and inhibited the expression of cardiac hypertrophic proteins and apoptotic markers increased by the high-fat feeding [100].

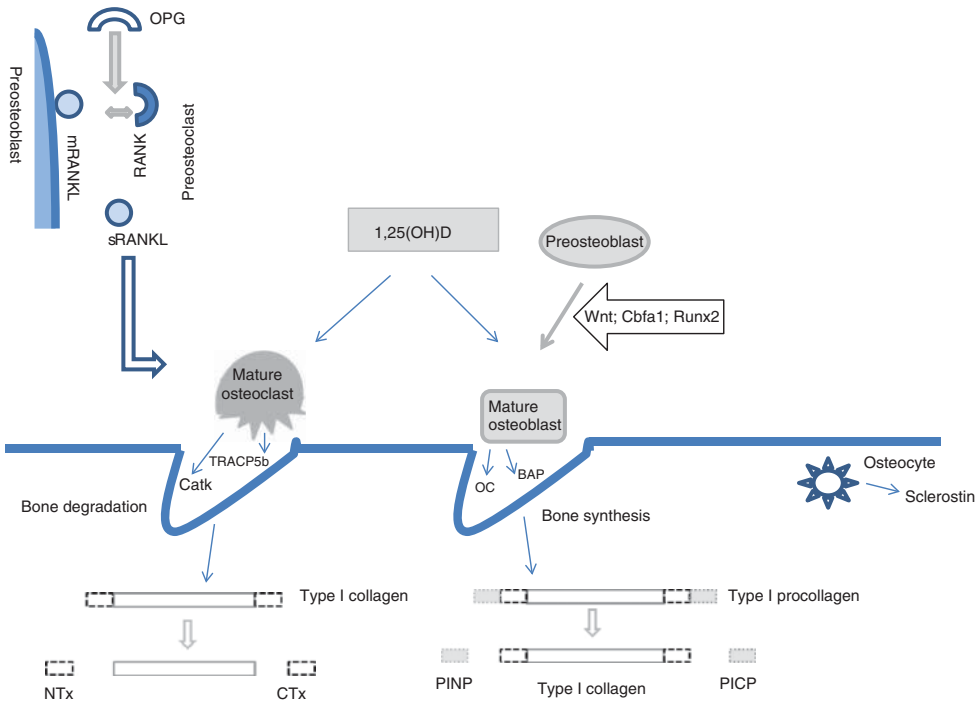

Figure 7.1 Schematic representation of bone turnover biomarkers released during the activity of a basic multicellular unit (anatomic structure including osteoblasts and osteoclasts). The RANK/RANKL interaction determines activation of nuclear transcription factors and the activity of genes essential for osteoclast activity. If the interaction is prevented by osteoprotegerin (OPG) the osteoclast is not activated. Common bone resorption biomarkers produced by osteoclasts: the osteoclast enzyme tartrate-resistant acid phosphatase 5b (TRACP5b), cathepsin k (CatK) an osteoclast protease, which produces the degradation products of type I collagen (CT_X, NT_X) during bone degradation. Markers of bone produced by osteoblasts: the precursor procollagen of type I collagen molecule is secreted by osteoblasts. The extension peptides at each end of the procollagen molecule, procollagen type I N-propeptide (PINP) and procollagen type I C-propeptide (PICP), are cleaved by enzymes during bone matrix formation and released into the circulation. Osteocalcin (OC) is one of the most common non-collagenous proteins in bone matrix produced by osteoblasts during bone formation. It is excreted by the kidneys and its fragments may also be measured in urine. During the phase of osteoid maturation followed by mineralization, alkaline phosphatase is secreted into the extracellular fluid by osteoblasts, and its bone derived isoform (BAP) can be measured in serum. 1,25(OH)D (vitamin D) promotes bone growth and remodeling acting on osteoclasts and osteoblasts. Wnt signaling is required for the differentiation and survival of osteoblasts, while Cbfa1 (core-binding factor a1) and Runx2 (Runt-related transcriptional factor-2) are two factors that induce differentiation of osteoblasts, and contribute to the mineralization process. Sclerostin is a glycoprotein produced by the osteocyte. OPG, osteoprotegerin; CatK, cathepsin k; TRACP5b, tartrate-resistant acid phosphatase 5b; OC, osteocalcin; BAP, bone alkaline phosphatase; CTx, collagen type 1 cross-linked C-telopeptide; NTx, collagen type 1 cross-linked N-telopeptide; PINP, N-terminal propeptide of human procollagen type I; PICP, C-terminal propeptide of human procollagen type I.

In humans, significant elevation in the levels of CatK was observed in patients with AMI (acute myocardial infarction) [101]. Moreover, CatK levels were found to be independent predictors of coronary artery disease (CAD), and correlated positively with percent plaque volumes and inversely with percent fibrous volumes by intravascular ultrasound [102].

There is no doubt that CatK is a valid and attractive target for therapies against osteoporosis, but is also useful for other extraskeletal diseases. In particular, given the association that CatK has with CVD and metabolic disorders, CatK-inhibitor drugs are particularly interesting in the context of these pathologies. Some inhibitors (odanacatib, ONO-5334) have recently passed preclinical studies and are presently in clinical trials at different stages of advancement; others are still under development [103].

7.3.2 Tartrate-Resistant Acid Phosphatase

Tartrate-resistant acid phosphatase 5b (TRACP5b) is a proteolytically processed isoform produced by osteoclasts, and represents a sensitive marker of bone resorption. This factor has been found in osteoclast-like cells of monocyte-macrophage lineage in the atherosclerotic plaque [104]. Moreover, it is independently associated with arterial stiffness together with the bone-specific alkaline phosphatase (BAP) and is a predictor of adverse CV outcomes in CKD patients [105, 106]. TRAP5b also represents an independent factor correlated with hsCRP (high-sensitivity C-reactive protein) and inflammation in hemodialized patients [107].

7.3.3 Sclerostin

Sclerostin is a glycoprotein produced by osteocytes. This molecule inhibits the Wnt signaling pathway, regulating osteoblast activity and leading to decreased bone formation [108, 109]. Higher serum sclerostin levels are associated with lower risk of fracture, higher BMD, and lower bone turnover rate in the MINOS study [110].

Sclerostin is upregulated in experimental models of vascular calcification [111]. Moreover, a strong association of sclerostin with calcifying aortic heart valve disease in hemodialysis patients has been found [112]. Recent data from CKD patients showed higher sclerostin levels in patients with aortic calcifications [113]. However, after adjustment in multivariate analysis, lower, and not higher, sclerostin levels were associated with aortic calcification [113]. In view of these contradictory results, additional clinical and experimental studies are still needed to clarify whether sclerostin is an adverse or a protective factor against progression of vascular calcification.

7.3.4 Fibroblast Growth Factor 23

Fibroblast growth factor 23 (FGF-23) is mainly produced by osteocytes and regulates phosphorus and vitD metabolism [114].

High FGF-23 levels promote left ventricular hypertrophy and are independently associated with greater risk of CV events and mortality in CKD e non-CKD cohorts [115–120]. High FGF-23 levels have been associated with vascular calcification and CV mortality, and with the extent and severity of CAD in patients with CKD [121, 122]. Recent findings demonstrated that FGF-23 directly induces left ventricular hypertrophy, suggesting that FGF-23 may not simply be a biomarker of CV risk but rather a key mediator of cardiac injury [123].

Recently, a negative correlation between serum levels of FGF-23 and HDL (high density lipoprotein) and a positive correlation between FGF-23 and triglycerides, BMI, waist/hip ratio and visceral fat, and also with the risk of metabolic syndrome have been shown in Caucasian populations [124].

7.3.5 Osteopontin

OPN is produced by osteocytes, osteoblasts, and osteoclasts and represents a key component of the mineralized extracellular matrices of bones [125]. This protein retains both proinflammatory and anticalcific effects. It is upregulated in a variety of acute and chronic inflammatory conditions, including atherosclerosis, where it is likely involved in the recruitment and retention of macrophages and T-cells toward the inflamed sites [125]. High levels of OPN mRNA and proteins were also reported in atherosclerotic plaques [126].

OPN is independently associated with the presence and severity of CAD [127–130]. OPN is also upregulated in experimental HF models, and provides significant prognostic information in patients with chronic HF [131, 132]. Moreover, it is upregulated after AMI and stroke, and is involved in restenosis processes [133–136]. OPN appears involved in left ventricle remodeling, and its production at sites of tissue injury during the inflammatory and remodeling phases may modulate neutrophil migration, macrophage recruitment and phagocytosis, regulation of inflammatory gene expression, cardiac fibroblast adhesion and proliferation, myofibroblast differentiation, ECM deposition, cardiac myocyte apoptosis and hypertrophy, and angiogenesis [137, 138].

Besides its proinflammatory effects, OPN is a potent inhibitor of mineralization, preventing ectopic calcium deposits and inhibiting vascular calcification [139]. OPN inhibits *in vitro* calcification of SMCs [140]. Conversely, increased circulating or valvular tissue levels of OPN in patients with aortic valve calcification and stenosis have been found, where these apparently controversial results with respect to experimental findings may depend on post-translational modifications of the molecule [141]. In fact, emerging data suggest that post-translational modifications of OPN greatly affect its biological functions, and proteolytic cleavage

by thrombin and matrix metalloproteases may enhance its adhesion capacities [139, 142, 143]. Accordingly, the dephosphorylation of OPN has been found to correlate with severe valvular calcification in patients with calcific aortic valve disease [144].

7.3.6 Osteocalcin

OC, a product of osteoblasts, has attracted much attention as a hormone affecting glucose metabolism and fat mass [145]. In fact, based on experiments conducted on OC-deficient mice, it has been proposed that the uncarboxylated form of OC is a pivotal factor by which bones influence insulin secretion and insulin action, by increasing insulin release and sensitivity and energy expenditure, decreasing visceral fat [146]. Moreover, mice that overexpressed Esp, a gene that encoded a receptor-like osteotesticular protein tyrosine phosphatase (OST-PTP), presented metabolic characteristics similar to OC-deficient mice, and developed insulin resistance, became fat, and presented reduced levels of undercarboxylated OC [146]. Conversely, Esp-deficient mice were hyperinsulemic and hypoglycemic, and showed increased insulin sensitivity [146].

In humans, OC levels are associated to the degree of insulin resistance and to insulin release, and also a beneficial effect of weight loss [147]. Accordingly, in healthy elderly individuals ($n = 380$), an inverse relationship was found between OC and fasting plasma glucose, fasting insulin, and homeostasis model assessment-estimated insulin resistance (HOMA-IR) [148].

In patients with type 2 diabetes (T2DM), serum OC levels were negatively related to glucose and fat mass and atherosclerosis surrogates (brachial-ankle pulse wave velocity and carotid intima-media thickness, IMT), and positively to total adiponectin levels [149]. OC levels were inversely associated to the presence and severity of CAD, and with fasting and post load (2 h) glucose and hemoglobin A(1c) values in a Chinese population [150]. Low OC levels represented an independent risk factor for carotid atherosclerosis in patients with T2DM, and inversely correlated with CRP [151]. Decreased levels of OC were associated with myocardial infarction in very young patients (≤40 years) [152]. Serum OC was also negatively associated with subclinical atherosclerosis in a large Chinese postmenopausal women population ($n = 1319$) [153]. However, other authors have reported different results, including a higher prevalence of carotid atherosclerosis in healthy postmenopausal women with elevated OC and low BMD [154].

Total OC values in humans appear inversely associated with measures of glucose metabolism, although it is important to remind that the evaluation of total OC includes both the carboxylated (cOC) and uncarboxylated forms (ucOC) [155]. The un-OC form appears mainly associated with enhanced β-cell function, while the carboxylated form is more involved in improved insulin sensitivity in male subjects [156]. Recently, one possible causal effect has been proposed, suggesting that ucOC stimulates insulin secretion and regulates energy metabolism in insulin

target tissues, an effect that is largely mediated by the OC-mediated Glucagon-like peptide-1 release from the gut [157]. Very recent data indicate that a value of ucOC/cOC index <0.3 is associated with markers of poor metabolic control in patients with T2D [158].

Interestingly, a higher percentage of circulating endothelial progenitor cells (EPCs) express OC in patients with coronary atherosclerosis compared with subjects with normal endothelial function [159]. Very recent data showed that early, highly active EPCs, carrying the osteoblastic marker OC, are strongly associated with unstable CAD. Therefore, this particular subset of EPCs could mediate vascular calcification and abnormal vascular repair and may identify patients with a more unstable phenotype of atherosclerosis [160].

7.3.7 Osteoprotegerin

OPG is a glycoprotein, which binds the RANKL, and can reduce the production of osteoclasts by inhibiting the differentiation of osteoclast precursors into mature osteoclasts. In epidemiologic studies, low OPG levels were related to higher prevalence of osteoporosis and vertebral fractures [161]. Based on experimental studies, OPG may be considered a protective factor for the vascular system that prevents vascular calcification [162, 163]. Conversely, OPG-deficient mice were found to develop early-onset osteoporosis with severe trabecular bone loss and a high incidence of fracture and also an increase in vascular calcifications of the aorta and renal arteries [164]. *In vitro* studies evidenced other anti-atherosclerotic OPG effects, such as regulation of B-cell maturation, and anti-apoptotic activity [165]. The protective vascular effects of OPG are also evident from a recent study suggesting that OPG represents a strong inhibitor of artery calcification induced by warfarin and vitD [166]. However, regardless of the evidence for anti-atherogenic properties in experimental models, mRNA and protein expression of OPG and RANKL have been detected in atherosclerotic plaques in humans, as well as strong expression of OPG/RANKL/RANK was found within thrombus material obtained at the site of plaque rupture during AMI, with a possible role in the destabilization of the atherosclerotic plaque [47, 167, 168]. Circulating levels of OPG correlate with CV risk factors, including hyperlipidemia, endothelial dysfunction, diabetes mellitus, and hypertension [169–171]. OPG has also been related to markers of subclinical atherosclerosis such as carotid IMT. In particular, in the Copenhagen City Heart Study ($N = 5863$), patients with clinical atherosclerosis had higher mean OPG, which remains independently correlated with atherosclerosis also after multivariate adjustment for traditional factors and CRP [172]. Moreover, in the control group without clinical atherosclerosis, OPG was independently associated with hypertension, diabetes, hypercholesterolemia, smoking, and subclinical peripheral atherosclerosis (ankle brachial index) [171]. In the Framingham Heart Study, OPG together with OPN levels is associated with arterial stiffness and the

presence and severity of CAD. Atherosclerotic risk factors, such as age, smoking, and new diabetes, were associated with increasing OPG concentrations [173]. In postmenopausal women without CVD, high OPG levels are positively related to markers of subclinical arteriosclerosis (markers of endothelial function and arterial stiffness) [174]. OPG also appears related to microvascular dysfunction, as OPG concentrations were higher in patients with impaired coronary flow reserve, decreased diastolic function, and increased ventricular arterial coupling in AMI patients [175].

In CAD, elevated OPG levels are associated to disease presence and severity [176, 177]. Accordingly, recent data suggest that circulating OPG and OPN levels are positively associated with arterial stiffness, and the extent and severity of CAD, independently of other known CV risk factors [127]. Moreover, a recent update of available data confirmed the association of OPG with stable CAD, acute coronary syndrome (ACS), and cerebrovascular disease [171].

OPG also correlates with the severity of peripheral artery disease, and appears associated with increased risk of all-cause mortality, CVD mortality, and myocardial infarction in the general population and in ischemic patients [178–183]. In particular, in The Fourth Copenhagen City Heart Study, OPG represents an independent predictor of the combined end-point of hospitalization for ischemic heart disease, stroke, and all-cause mortality, and its combination with hsCRP makes improved contribution to prognosis than the individual effect of the two biomarkers [184].

Interesting findings arise from a recent study, conducted in patients with ST elevation myocardial infarction (STEMI). Patients with higher OPG levels displayed higher neutrophil/lymphocyte ratio, admission troponin, admission glucose, and high-sensitivity C-reactive protein. Moreover, multiple logistic regression analysis revealed OPG as an independent predictor of major adverse cardiovascular events as well as eGFR (estimated glomerular filtration rate), number of obstructed vessels, and corrected TIMI (thrombolysis in myocardial infarction) frame count [185]. Increased plasma OPG levels were also independently associated with more severe stroke and poor functional outcome [186]. Furthermore, high OPG levels on admission retain prognostic significance in HF patients and are significantly associated with subsequent HF development in ACSs [187–190].

OPG has also been proposed as a marker of atherosclerosis in diabetic patients [191]. In fact, OPG levels are significantly increased in adult patients with type 1 diabetes (T1DM) or T2DM and in patients with previous gestational diabetes, and its role as a mortality predictor appears to be more important in diabetic patients than in the non-diabetic subjects [180, 192–195]. In children with T1DM, OPG was significantly increased in comparison with the non-diabetic subjects, and associated with the HbA1c levels [196]. These data were further confirmed by experimental results [197]. OPG levels were observed to increase concurrently with the severity of diabetic complications [198, 199]. As in the general population, serum OPG levels are related to the presence and severity of coronary calcification in patients with T2DM [200]. However, in the Framingham Heart Study, OPG was not predictive of the incidence of T2DM [201].

In view of the controversial experimental *vs* clinical results, elevated OPG levels may represent an ineffective compensatory and counter-regulatory mechanism in order to prevent further bone loss and vascular lesions, and as such a marker of disease for the CV system. It is also possible that in this case, OPG may not be effective in neutralizing RANK/RANKL interaction, particularly at high RANKL concentrations. Nonetheless, the observation that atherosclerosis risk factors appear associated with OPG, independent of measures of atherosclerosis burden, also suggests a pathogenic rather than a compensatory role for OPG in the vessel [202]. Accordingly, some data suggest that OPG released by ECs and vascular SMCs in response to TNF-α may upregulate EC adhesion molecule response by induction of angiopoietin-2, enhancing the adhesion of leucocytes to the EC line [203, 204].

7.3.8 Vitamin D

VitD is a secosteroid hormone, recognized as a factor promoting bone growth and remodeling [205]. In humans, the major source of vitD is from exposure of skin to sun, with a smaller contribution from diet [205]. Skin exposure to solar UV irradiation drives the conversion of 7-dehydrocholesterol to previtamin D3, which is then isomerized to vitamin D3 (cholecalciferol). Cholecalciferol, bounded to vitamin D-binding protein (DBP), enters into the blood and first hydroxylates in the liver to 25(OH)D, and then to 1,25(OH)2D, the active hormone, by 1a-hydroxylase in the kidney [205].

Apart from the known skeletal effects, many other vitD actions on different "extraskeletal" diseases have been shown, including inflammatory and autoimmune conditions, cancer, and CVD [205]. In fact, many data indicate how low vitD levels may affect the renin–angiotensin–aldosterone system, insulin resistance, vascular calcification, inflammatory processes, and endothelial function [206]. Accordingly, a recent meta-analysis confirmed the inverse correlation of vitD with CV risk factors, including diabetes, hypertension, and dyslipidemia [207, 208]. In clinical studies, low 25(OH)-vitamin D, 25(OH)D, was found to be associated with the risk of ACSs, stroke, HF, and peripheral arterial disease, and prognostic for adverse event and in-hospital mortality for ACS patients [209–216]. Recent trials including large populations and meta-analyses of available data confirmed the key role of low vitD for cardiovascular risk and cardiovascular and overall mortality [217, 218]. Specifically, in The Copenhagen City Heart Study ($N = 10\,170$ men and women, 29-year follow-up) lower levels of vitD were associated with higher risk of myocardial infarction, early death, and fatal CHD events. In the meta-analyses, a significant higher risk of CHD and death in the lowest quartile of 25(OH)D levels was evidenced [217]. Moreover, in the Whitehall study (5409 elderly men, 13-year follow-up) higher 25(OH)D values were inversely and linearly correlated with overall and cardiovascular mortality [218]. Lower overall and cardiovascular mortality in the top with respect to the bottom quartile of 25(OH)D was evidenced

in meta-analysis including 12 (4632 CV deaths) and 18 (11 734 overall deaths) prospective studies [218].

Nonetheless, the efficacy of vitD supplementation in reducing CV risk and outcome is still discussed [213]. For example, in the WHI study, vitD and calcium supplementation had no significant effect on CV events during 7 years of follow-up [219]. It is noteworthy that the dose used in the WHI was very low. In this context, a recent meta-analysis suggests that vitD supplementation at moderate-to-high doses may reduce cardiovascular risk [220].

In addition, although the strength of the associations of vitD and cardiovascular risk and events are well established, a causal role still remains to be proved [213, 221, 222]. Older subjects or those spending less time outdoors have a higher risk of developing CVD, and may have lower 25(OH)D levels coincidentally [212]. Also, obesity, a recognized risk factor for CVD, may be associated with a lower vitD status due to the lipophilic nature of vitD and its sequestration in the fat [213]. Low 25(OH)D levels are related to CV risk factors, and thus it may simply be a bystander due to the presence of these CV determinants, with possible limited significance as risk marker in CVD [223]. Nonetheless, vitD exerts multiple effects on various molecular pathways, which may justify its direct role in CVD [206]. Indeed, a large prospective trial ($n = 3296$ men and women) has identified a significant relationship between low vitD levels and increased activity of the renin–angiotensin system [224]. VitD also has a direct effect on calcium influx and affects myocyte contractility [225]. VitD has been also associated with vascular calcification, although some data support a protective rather than an adverse effect [221]. In addition, vitD affects NO levels, inflammatory parameters, angiogenesis, platelet aggregation, and insulin resistance and fasting glucose values [221, 226]. Recently, another mechanism by which vitD may exert its beneficial effects has been identified in the antioxidant properties of this molecule. In fact, vitD has been found to prevent EC death through modulation of the interplay between apoptosis and autophagy, by inhibiting superoxide anion production and inducing NO release, affecting mitochondria function, and cell viability, activating survival kinases [227].

7.3.9 Other Factors

A procollagen marker with higher prognostic potential is the N-terminal propeptide PIIINP, an index of type III collagen turnover rate, higher levels of which predict an adverse outcome after a myocardial infarction and in chronic HF [228]. However, type I collagen is an important component of bone matrix, and osteoblasts produce its precursor, the procollagen molecule, during bone formation. Procollagen type I *N*-propeptide (PINP) and procollagen type I *C*-propeptide (PICP) are the extension peptides at each end of the procollagen molecule, indices of type I collagen synthesis, and are cleaved by enzyme activity during bone matrix formation, and released into the circulation. The PICP has been found a predictor of adverse outcomes following myocardial infarction

and in chronic HF [228]. Conversely, the N-terminal crosslinking telopeptides of type I collagen (NTx) and CTx are considered bone resorption indices, and represent proteolytic fragments of bone collagen matrix. ICTP is a fragment always produced through action of matrix metalloproteinases at the C-terminal extremity of type I collagen, longer than CTx.

Bone turnover biomarkers are influenced by glycemic control status. OC and CTx-I were lower in TIDM (type 1 diabetes mellitus) children than in healthy controls [229]. Moreover, a negative correlation between CTx-I levels and HbA1c was observed in T2DM male subjects [230]. In addition, weight loss has been associated with significant variation in bone formation as well as resorption biomarkers [231–236].

Changes in the collagenous matrix may contribute to the development of valve stenosis. In a study where the concentrations of propeptides and telopeptide structure of type I (PINP, PICP, and ICTP) and those of type III collagens (PIIINP and IIINTP) were evaluated, aortic valve calcification was found associated with a marked increase of ICTP in type I collagen [237]. Recent data suggest that CAD patients had higher CatK and ICTP : I-PINP ratios than control subjects [102].

In a large cohort of men at high CV risk, a U-shaped association of CTx levels, together with OC values, with CV death and overall mortality was observed, suggesting that there may exist an optimum, intermediate range that identifies highest benefits for both biomarkers [238].

Cbfa1 (core-binding factor a1) and Runx2 (Runt-related transcriptional factor-2) are two factors that induce differentiation of osteoblasts and contribute to the mineralization process. These factors appear to have a role in the vascular SMCs calcification [239, 240]. In particular, Cbfa1-expressing cells were rarely observed but were found in the deep part of atherosclerotic plaques under the necrotic cores [241]. Molecular imaging techniques *in vivo* have detected the key cellular events in early aortic valve disease, including EC and macrophage activation, proteolytic activity, and osteogenesis. In particular, expression of osteopontin, OC, and Runx2/Cbfa1 was observed in inflamed valves, suggesting active processes of osteogenesis [242]. Recent data also suggest that the differentiation of vascular SMCs toward osteoblasts may be mediated by extracellular signal-regulated kinase (ERK) signaling pathway, which induced the expression of several bone markers, including ALP, OC, OPG, and Cbfα1/Runx2, as well as calcification [243]. Moreover, deletion of the Runx2 exon 8 in SMC inhibits vascular calcification *in vivo*, and blocks the upregulation of RANKL, macrophage infiltration, and the formation of osteoclast-like cells in the atherosclerotic lesions [244].

MGP is expressed at high levels in SMCs, with a role as a calcification inhibitor in cartilage and vasculature, and limits calcium phosphate deposition in the vessel wall. The MGP-deficient mice present with short stature, osteopenia, and fractures and prematurely die as a result of arterial calcification, which leads to blood-vessel rupture [245]. MGP overexpression reduced vascular bone morphogenetic proteins (BMP) activity, atherosclerotic lesion size, intimal and medial calcification, and inflammation [246]. Conversely, MGP deficiency increased BMP activity, which may explain the diffuse calcification of vascular

medial cells in MGP-deficient aorta and the increase in expression of activin-like kinase receptor 1 and vascular endothelial growth factor [246].

However, increased MGP expression has been observed in calcified media and atherosclerotic lesions, where MGP most likely is expressed as an inhibitory counteraction to the calcification process [247, 248]. In vessels from patients with diabetes, MGP levels are lower than in normal vessels, which suggests that reduced MGP in diabetes may predispose to calcification [249]. Nonetheless, other data indicate elevated MGP levels in T2DM and T1DM patients with ischemic heart disease and severe atherosclerosis, where it may reflect counteraction to the calcification processes in the vascular wall [250, 251].

As for OC, the desphospho-uncarboxylated matrix Gla protein (dpucMGP) and desphospho-carboxylated matrix Gla protein (dp-cMGP) forms can be found in the blood, where dp-ucMGP represents the fully inactive form. Levels of dp-ucMGP, associated with a poor vitamin K status, have been found to be a more promising marker for CV risk and severity and valvular calcification detection [252–254]. Very recent data evidenced that circulating dp-ucMGP is independently associated with the risk of all-cause and CV mortality in the general population, and in patients with ischemic disease and CKD [255, 253, 256]. So, this biomarker appears promising as an additional tool in the assessment of CV risk.

However, given the number and variety of new biomarkers proposed in the last few years, it is difficult to choose which ones may really improve the actual clinical evaluation beyond traditionally utilized biomarkers [257]. To this purpose, more complex and complete evaluation and statistical approaches could be applied to evaluate the real clinical relevance of each new proposed biomarker, and answers obtained to mandatory questions regarding at which stage of the disease its use may be more informative, and in which subsets of the population requiring further risk stratification it must be applied [257].

7.3.10 Genetic Factors

Different genes and their polymorphisms have been involved in both atherogenesis and bone loss. Wnts are glycoproteins crucial for the development of many tissues, including bone, which facilitate the differentiation of osteoblasts from mesenchymal progenitors, with consequent increase of BMD. The missense mutation of a receptor for Wnt proteins, LRP-6, leads to the early development of both atherosclerosis and osteoporosis [258]. Moreover, common intronic variants in the Wnt-responsive transcription factor TCF7L2 induce an altered insulin secretion and higher risk of T2DM [259].

The link between OPG and CVD was supported by the observation that mice lacking the OPG gene were found to develop early-onset osteoporosis and calcification of the aorta and renal arteries [164]. An OPG polymorphism (T950C) is associated with serum OPG levels, and appears related to vascular morphology

and function and CAD [260–262]. However, genotyping of four polymorphisms (A163G, G209A, T245G, and T950C) in the promoter region of the OPG gene was performed in 251 healthy women and in a cohort population including 100 patients who underwent coronary angiography, without observing any associations between OPG promoter genotypes and aortic calcification, serum OPG levels, or CAD [263]. Recently, three polymorphisms (T245G, T950C, and G1181C) in the OPG gene were associated with high serum OPG levels, and were found more frequently in patients with carotid plaques, where they might be potential markers for plaque instability [264]. The same polymorphisms appear to be associated with a history of ischemic stroke in diabetic patients [265]. Moreover, the C allele of the T950C polymorphism has been recently associated with increased risk of CVD in diabetic patients [266]. In another interesting study, the potential role of estrogen signaling on this association was assessed through putative target genes (OPG and interleukin-6), showing that carriers of the OPG-1181C/C genotype had a significantly increased risk of intracerebral hemorrhage ($P = 0.005$) [267].

Mice lacking the gene for MGP showed vascular calcification, osteopenia, and fractures [245]. MGP polymorphisms (−138 T > C, −7G > A, and Thr83Ala) could predict a higher risk of bone loss and progression of vascular calcification, especially in men [268]. Increased risk of myocardial infarction associated with MGP ThrAla83 genotype appears correlated to faster progression of subclinical coronary atherosclerosis [269]. Moreover, other data suggest that MGP genetic variants (rs4236, rs1800801, and rs1800802) showed association with calcification on arterial wall but not with calcification in atherosclerotic plaques as determined by computed tomographic angiography [270].

In the CARDIA study, the individual effects of the MGP and OPN polymorphisms (MGP T-138C and OPN T-443C) on coronary calcification are weak and not statistically significant. Moreover, BMD differences at both the hip and spine do not vary statistically by genotype for any of the polymorphisms studied [271]. Nonetheless, in 470 patients with ischemic stroke, the same OPN T-443C polymorphism was associated with increased IMT [272]. More recent data identify several haplotypes of OPN gene as risk factors as well as protective factors for large artery atherosclerosis (LLA). In particular, the haplotype TCA, at the loci C2140T, C5891T, and A7385G conferred the highest risk (2.09 for LAA, $P < 0.05$), while the CG at the loci C1013T and A7385G was the most protective haplotype (OR = 0.66, $P < 0.05$) [273]. Other results showed that the OPN locus is likely to associate with CVD-related phenotypes, as the allele D147D C is independently and significantly associated with lower apoB levels, that its allele frequency was significantly lower in patients with brain infarction compared to controls, and that C allele carriers had a significantly lower frequency of presence of carotid plaques [274].

Children with Kawasaki syndrome carrying the rs3832879 of the FGF-23 gene showed higher serum FGF23 levels and developed coronary artery dilatations or aneurysms more frequently, which suggests the potential contribution of this genetic factor to the development of CV complications [275].

A recent genome-wide study identified new pathways involving periostin (matricellular protein) and leptin (an adipose tissue-derived hormone) in bone

remodeling by osteoblasts [276]. These factors retain a recognized role in CV pathophysiology and might represent attractive biomarkers to study processes related to CVD onset and development [277, 278].

The first studies that evaluated the BsmI VitD receptor (VDR) polymorphism have reported the association of this VDR gene variant with the prevalence and severity of CAD and T2DM [279, 280]. However, in another study conducted in a large population ($n = 3441$), this variant did not associate with prevalence and severity of CAD [281]. In the San Antonio Family Osteoporosis Study, the same variant was independently associated with IMT [282]. Recent data also suggest that the B alleles of the BsmI polymorphism are correlated with vitD levels, and this alteration in BB genotype produces an increase in left ventricle mass in patients with end-stage renal disease [283]. A systematic genotyping for 15 haplotype tagging single-nucleotide polymorphisms (SNPs) of the VDR gene revealed an association of haplotypes in blocks 2, 3, and 4 of the VDR gene with the risk of clinical restenosis in patients after percutaneous coronary intervention [284]. In addition, in the DIABHYCAR cohort, the AAC haplotype, including the minor allele of BsmI, major allele of ApaI, and minor allele of TaqI of VDR, was associated with an increased risk of CAD in T2DM patients, independently of other known CV risk factors [285].

Several studies have also investigated the relationship between genetic variants of the DBP, the major plasma carrier for vitD and its metabolites, in pre- and clinical diabetes cohorts [286]. Data have been recently reviewed, and although some findings testify to this association, other studies did not find any differences between allele, genotype, and haplotype frequencies of DBP SNPs and T2DM [286]. Thus, at the moment, available findings fail to support a strong role for DBP in the physiopathology of diabetes.

7.4 Conclusion

CVD and osteoporosis are major causes of morbidity, mortality, and disability. Traditionally, these two diseases were considered unrelated and their coexistence was attributed to age-related processes. However, as we go further into bone and CV physiology, it becomes evident that a closer relationship exists between these conditions Table (7.2). In fact, common molecular, cellular, and biochemical processes appear involved in their pathogenesis. Moreover, gender, life-style habits as well the individual's genetic susceptibility may affect such diseases.

Presently, interpretation of available data is still affected by different methodological limitations. They include representativeness of the cohorts, quality of the registers and data obtained from questionnaires, severity of disease, number of events (which affect the statistical power of the analysis), their temporal closeness, and availability of data on potential confounders. Moreover, official indications or guidelines for clinical practice are still lacking. In any case, study of the common mechanisms involved in these diseases may contribute to a better understanding of common pathophysiological pathways underlying both conditions,

Table 7.2 Main traditional bone health biomarkers in CVD.

	Bone	Low bone mass	CVD	Putative involvment in CVD	References
Procollagen type I N propeptide (PINP)	Collagen synthesis products	↑	↑	CAD	[102]
Procollagen type I C propeptide (PICP)		↑		Prognostic role after MI and HF	[228]
Cross-linked carboxy-terminal telopeptide of type I collagen (ICTP)		↑		Valvular calcification	[237]
Osteocalcin	Matrix protein	↑	↓/↑	Arterial stiffness and atherosclerosis and CAD	[150–154]
				Glucose metabolism, insulin secretion and action	[146–148, 155–158]
C-terminal crosslinking telopeptides of type I collagen (CT_X)	Cross-linked telopeptdes of type I collagen	↑	↓	T1DM, T2DM	[229, 230]
Tartrate-resistant acid phosphatase	Osteoclast enzyme	↑	↑	Arterial stiffness and CV mortality and morbidity in CKD	[105]
Catepsin K	Osteoclast enzyme	↑	↑	Advanced plaque/CAD/AMI	[91, 101, 102]
				Restenosis and neointima formation, valvular calcification	[95–97]
				Adipocyte differentiantion	[98–100]
Fibroblast growth factor 23	Osteocyte protein	↑	↑	Cardiovascular events and mortality	[115, 120]
				Vascular calcificaton, CAD extent and severity of in CKD	[121, 122]
				Left ventricular hypertrophy	[123]
				Metabolic syndrome	[124]

Osteopontin	Extracellular structural protein linking hydroxyapatite	↑	↑	CAD presence and severity	[127–130]
				HF, MI, stroke, restenosis	[131–136]
				Valvular calcificaton	[139–144]
Osteoprotegerin	RANK/RANKL/OPG system Inhibition of osteoclast differentiation	↑	↑	Atherosclerosis	[167, 168, 174]
				CV risk factors	[169–172]
				CAD, MI, HF, CV, and total mortality	[178–183, 187–190]
				Vascular calcification	[166]
				T1DM, T2DM	[191–197]
Vitamin D	Homeostasis of calcium and phosphorus	↓	↓	CV risk and CV and overall mortality	[205–218]
				T2DM, CAD, ACS, HF, and cerebrovascular disease	[205–216]
				Vascular calcification	[221]
				Endothelial dysfunction	[206]
Sclerostin	Osteocyte enzyme	↓	↓/↑	Vascular calcification	[111–113]

and help develop new strategies for multiple-purpose preventive and therapeutic interventions targeted at reducing both bone loss and atherosclerosis progression. In this holistic medical approach, it is conceivable that patients with severe forms of osteoporosis would benefit from a detailed assessment of the CV status, whereas patients with severe CVD would benefit from the assessment of BMD and other bone-related parameters.

Interestingly, emerging data showed how common molecular and cellular mechanisms and pathways underlie aging as well as main chronic and degenerative diseases, including CV and bone conditions and also neurodegenerative disease and cancer. Thus, future research should be focused on the study of how biological and molecular determinants may drive the progression toward a certain disease and explain why a subject will develop a pathological condition rather than another one.

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8
Identification and Validation of Breast Cancer Biomarkers

Kori Jackson and Edward Sauter

8.1
Introduction

Breast cancer is the second most common cancer worldwide, and the most frequent cancer among women [1], comprising 25% of all cancers. Its incidence is rising in both the developed and the developing world. Breast cancer is the most frequent cause of cancer death in women in less developed regions and second most frequent in developed countries, after lung cancer [1].

Clinical examination and mammography, the currently accepted breast cancer screening methods, miss almost 50% of breast cancers in women younger than 40 years, approximately 25% of cancers in women aged 40–49 years, and 20% of cancers in women over 50 years old [2]. Early detection is crucial to improving the survival of individuals with breast cancer, as most deaths are due to disease progression and metastasis [3]. The currently known major breast cancer risk factors are age, genes, age at onset of menarche, body mass index (BMI), birth order, parity/nulliparity, diet, exercise, lactation, breast density, and use of hormone replacement therapy. Despite the accumulation of knowledge with respect to risk factors, currently known risk factors address only 15–55% of risk for breast cancer. Biomarkers hold promise to aid breast cancer diagnosis, prognosis, and prediction. Criteria that must be met to substantiate a highly predictive biomarker are stability, reproducibility, sensitivity, detectability, and specificity. Two breast cancer assays (i.e., multi-gene panel analysis) in clinical practice that assess response to treatment and prognosis are Oncotype DX and Mammaprint. Unfortunately, these assays require tissue samples and can only be used on early stage (0–2) cancers. It is imperative to identify new, noninvasive biomarkers. Body fluids provide the most promise in this respect, especially when evaluating healthy individuals. In this chapter, we briefly review current treatment modalities, and indicate biologic and other markers that are in clinical use. Further, we outline the challenges associated with the development of new clinically useful biomarkers, and review some of those under development.

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8.2 Current Detection and Treatment Modalities

8.2.1 Detection: In Clinical Use

8.2.1.1 Physical Examination

Physical examination by the individual is called breast self-examination (BSE); that by a health care provider, clinical breast examination (CBE). Most irregularities felt both by patients and their health care providers are not malignant, but can lead to anxiety. Neither BSE nor CBE has been convincingly demonstrated to provide significant benefit in early breast cancer detection. For this reason, the U.S. Preventive Services Task Force (USPSTF) recommends against teaching BSE, and concludes that current evidence is insufficient to determine if CBE adds additional benefit to screening mammography in women 40 years or older [4]. The American Cancer Society now advises the use of BSE as an optional screening tool.

8.2.1.2 Breast Imaging

- *Mammography*: Mammography uses a low dose of ionizing radiation to produce an image of internal breast morphology. If one or more lesions identified from the image obtained are deemed suspicious for cancer, a biopsy and pathologic assessment are performed to determine if they are malignant. The sensitivity of mammography is related to breast density; higher density decreases sensitivity. Specificity of mammography is decreased by prior surgery and breast irradiation. The USPSTF currently recommends biennial mammographic screening for women 50–74 years of age, and states that the decision to begin mammography screening earlier than age 50 is an individual choice. On the other hand, the American Cancer Society recommends annual mammography for average risk women starting at age 40 and continuing for as long as the woman is in good health.
- *Ultrasound*: Ultrasound uses sound waves to map the internal morphology of the tissues it is assessing. According to the American College of Radiology (ACR) Practice Guidelines (2011 revision), breast ultrasound is appropriate for (i) the evaluation and characterization of palpable masses and other breast-related signs and/or symptoms; (ii) evaluation of suspected or apparent abnormalities detected on other imaging studies, such as mammography or magnetic resonance imaging (MRI); (iii) initial imaging evaluation of palpable masses in women under 30 years of age who are not at high risk for the development of breast cancer, and in lactating and pregnant women; and (iv) as a supplement to mammography in certain high-risk groups of women such as those with dense breast tissue, whether the mammogram is normal or suspicious for malignancy.

- *MRI*: This modality scans breast tissue using magnetic energy and radio waves to produce a detailed image of the internal morphology of the breast. Current indications for breast MRI based on 2013 ACR recommendations include screening high-risk patients, screening the contralateral breast in women newly diagnosed with breast cancer, assessing extent of disease in women with *in situ* or invasive carcinoma both before and after surgery, in evaluating response to neoadjuvant chemotherapy, and in assessing breast cancer recurrence when clinical, mammographic, or ultrasound findings are inconclusive.

8.2.2 Detection: Being Evaluated

Early detection is a major factor contributing to the 2.3% annual decline in breast cancer death rates over the past 10 years [5]. Nonetheless, in 2014, 40 430 women in the United States will die from breast cancer [6]. Additionally, there are 2.5 million breast cancer survivors in the United States who remain at increased risk for recurrence for at least 20 years after diagnosis. While the best currently accepted early detection approaches are based on imaging, the quantification of biochemical markers in accessible tissues and/or fluids is an attractive alternative. Such approaches have the potential to be cheaper, faster, safer, and to be adopted more widely; further, if the right marker(s) can be found, they promise to offer greater sensitivity and specificity.

There has been considerable effort in the discovery arena targeted at identifying novel biomarkers that might ultimately offer clinical utility, but few candidates have been subjected to rigorous verification and/or validation. This has been the stumbling block in almost all studies. Validation requires the analysis of hundreds of samples to adequately survey the variability in biomarker expression that is present in patient samples. If assays are not optimized, even larger sample numbers are required to tease out the biological variability that is masked by analytical imprecision.

The evaluation of changes in both DNA and proteins in body fluids and tissue shows considerable promise in the diagnosis and management of breast cancer, but analysis of body fluids is preferred for diagnosis because sampling is minimally invasive and ongoing assessment is practical. Analysis of fluids also has promise, either alone or in combination with tissue analysis, for determining if breast cancer will recur.

Despite the potential already demonstrated, researchers have not delivered validated biochemical markers that can be used to optimally diagnose and manage breast cancer. Only three of the Food and Drug Administration (FDA)-approved markers available can be measured and assessed longitudinally; the other six are tissue-based and therefore invasive (Table 8.1). Notably, there are no FDA-approved biomarkers for breast cancer diagnosis or screening.

Table 8.1 FDA approved biomarkers for breast cancer[a] .

Marker	Type	Source	Clinical use
CA 15-3	Glycoprotein	Serum	Monitoring
CA 27.29	Glycoprotein	Serum	Monitoring
HER2/neu	Protein	Serum	Monitoring
Cytokeratins	Protein (IHC)	Tumor	Prognosis
ER and PR	Protein (IHC)	Tumor	Selection for hormonal therapy
HER2/neu	Protein (IHC)	Tumor	Prognosis and selection for therapy
HER2/neu	DNA (FISH)	Tumor	Prognosis and selection for therapy
Mammaprint	Multigene	Tumor	Prognosis
Oncotype DX	Multigene	Tumor	Prognosis and selection for therapy

a) Modified from Medscape Oncology, biomarkers in cancer staging, prognosis, and treatment selection: the biomarker paradox.

8.2.2.1 Bodily Fluid Analyses

The three markers approved for monitoring breast cancer (Table 8.1) have been evaluated for diagnosis, but have not been found useful. For example, cancer antigen (CA) 15-3 is elevated in <10% of newly diagnosed breast cancer patients [7].

Three breast-specific fluids that are being evaluated for predictive biomarkers are nipple aspirate fluid (NAF), breast milk, and ductal lavage (DL). NAF contains concentrated levels of proteins, carbohydrates, and lipids that are breast specific. DNA and RNA are also present, both free and intracellular, although the concentration of the nucleic acids is quite variable. Breast milk contains high concentrations of proteins, whose primary function is feeding an infant, but there are also proteins, carbohydrates, and lipids with potential cancer biomarker usefulness. DL is collected through the insertion of a microcatheter into the nipple, providing irrigating fluid, and analyzing the effluent. There are more cells than in NAF. The dilution factor through irrigation for analysis of proteins lipids and carbohydrates is somewhat uncertain, as often not all of the irrigant is collected.

8.2.3 Treatment: In Clinical Use

8.2.3.1 Surgery and Radiation

Surgical therapy, whether it is excisional biopsy or mastectomy, is an important part of the treatment of early stage breast cancer. Radiation is generally indicated for women who undergo excisional biopsy, and for some women (close or involved margin, positive lymph nodes) who undergo mastectomy. The primary marker of risk of local disease recurrence is pathologic assessment of the specimen margins. It is known that an involved margin significantly increases recurrence risk. For margins that are not involved, the size of the margin that is required to minimize disease risk has long been debated. A recent consensus statement from the Society of Surgical Oncology and the American Society for Radiation Oncology concludes

that so long as cancer is not present at the inked margin of surgical resection and adjuvant radiation is delivered afterward, that provides optimal treatment to minimize recurrence (i.e., larger margins provide no additional benefit) [8].

Molecular assessment of cancer specimens has been performed to determine its usefulness in predicting local recurrence. Brennan *et al*. evaluated tissue specimens from individuals who had undergone surgical resection for head and neck cancer with histopathologically negative margins. Of 25 patients deemed to have undergone tumor resection with negative margins based on histopathologic assessment, 13 contained a p53 mutation in at least one tumor margin. Of these 13 individuals, tumor has recurred locally in 5, compared to 0/12 individuals in whom the margins were negative for p53 mutations [9]. Notably, p53 mutations are present in approximately 50% of head and neck cancers (not 100%), and are less common in breast cancer. There are no molecular markers to predict local recurrence that are in routine clinical use.

8.2.3.2 Systemic Therapy

Chemotherapy frequently targets a cell division process, since cancer cells generally divide more rapidly than normal cells. However, normal cells divide, some rapidly, such as those in the intestinal tract and in the bone marrow. As a result, side effects from chemotherapy often occur in these body systems. Targeted therapy attempts to minimize toxicity by attacking a specific target, such as a protein or tyrosine kinase. While side effects may be mitigated, they are rarely, if ever, eliminated entirely. Treatment is based on pathologic features such as tumor size, grade, and stage, as well the biomarkers discussed below. There has been an explosion in biomarker development to determine a patient's risk of disease recurrence, and thereby, if systemic therapy (chemotherapy or biologic therapy) is warranted to decrease this risk.

Biomarkers in Tissue

1) *Hormone receptor status*: Estrogen receptor (ER) is involved in estrogen signaling. Cancers that express ER are estrogen dependent, whereas cancers that do not express ER are estrogen independent. Two thirds of invasive breast cancers express ER [10] and are classified ER+. Expression of ER is measured via immunohistochemical (IHC) assays. The progesterone receptor (PR) becomes activated when it interacts with the steroidal hormone progesterone. Approximately 65% of breast cancers that are ER positive are also PR positive. Expression of PR is measured via IHC. Tumors that are ER+ and/or PR+ generally respond to antihormonal therapy with tamoxifen or an aromatase inhibitor [11].
   Tamoxifen is FDA approved to treat *in situ* and invasive breast cancer. Its structure is similar to that of estrogen. It binds to ER as does estrogen. In some organs, its predominant effect is to inhibit estrogen signaling; in other organs, it stimulates signaling. It is therefore called a selective estrogen receptor modulator (SERM). It is effective in both pre- and postmenopausal women.

Aromatase inhibitors prevent estrogen production by disrupting the action of aromatase, which is a critical enzyme in the conversion of adrenal hormone precursors to estrogen. Three aromatase inhibitors (arimidex, letrozone, and exemestane) are FDA approved in post- but not premenopausal patients who have a tumor that is hormone receptor positive because the ovarian production of estrogen is not blocked by inhibiting aromatase.

2) *Heregulin (HER)2*: Also known as epidermal growth factor receptor (EGFR)II, HER2 is considered an "orphan-molecule" since it does not interact with a specific ligand, but rather dimerizes with an EGF-ligand-bound complex. Twenty percent of breast cancers overexpress HER2, most often due to HER2 gene amplification, and are thereby considered HER2 positive [10, 12]. Overexpression of HER2 causes the cell signaling pathway to be upregulated, leading to uncontrolled cell growth. HER2-positive breast cancers are generally aggressive and their prognosis is worse than patients whose breast cancers do not overexpress HER2 [13]. FDA-approved agents targeting HER2 include trastuzumab, ado-trastuzumab emtansine, lapatinib, and pertuzumab.
3) *Ki67*: This protein is a marker of cell proliferation. The fraction of cells that stain positive for this protein reflects cells that are proliferating (in G_1, S, G_2, or mitosis). Ki67 does not stain resting cells (those in G_0). The fraction of tumor cells dividing correlates with the rate of tumor growth and tumor aggressiveness [14].
4) *Oncotype DX*: Fixed tissue is used for this 21 gene expression biomarker panel, which is used to determine chemotherapy benefit and likelihood of recurrence in early-stage (ductal carcinoma *in situ*, stages I, and II node negative, ER positive) breast cancer.
5) *Mammaprint*: This 70-gene biomarker panel uses fresh or fixed tissue to determine the likelihood of recurrence of breast cancer within 10 years after diagnosis and response to treatment with chemotherapy. Mammaprint can be used to analyze both ER-negative and -positive early-stage (i.e., stage I or II) node-negative (US criteria; international criteria allow up to three positive nodes) invasive cancers.
6) *BRCA1* and *BRCA2*: The tumor suppressor genes *BRCA1* and *BRCA2* have a multitude of cell functions including regulation of division checkpoints (i.e., gate-keeping), genomic stability, DNA repair, and transcription. These genes are a model for autosomal dominant inheritance of breast cancer, and mutations within these genes correlate to the majority of inherited breast cancers. Inherited breast cancer in turn accounts for 2–5% of all breast cancers. Lifetime risk of developing breast cancer with a *BRCA1/2* mutation ranges between 20 and 80%, depending on the population under analysis. *BRCA1/2* mutation status is assessed when determining if a patient has a strong family history of breast and/or ovarian cancer, especially when the cancers present at or below the age of 50. *BRCA1/2* status is quantitated via genetic testing of a blood or saliva sample from the participant.

The major limitation with *BRCA1/2* testing is getting an ambiguous genetic result, a so-called "variant of unknown significance (vus)." This results when the mutation identified has not been previously associated with cancer risk. In a recent study [11], it was found that 10% of women who participated in genetic testing for *BRCA1/2* gene mutations returned a "vus" result.

Body Fluid Analyses CA 15-3 (MUC1) and CA 27.29 levels in serum are FDA approved to monitor patients with breast cancer. American Society of Clinical Oncology guidelines recommend using these tumor markers in conjunction with imaging and clinical examination to assess treatment response/failure. A confirmed increase of ≥25% has been suggested as clinically significant.

8.2.4 Treatment: Being Evaluated/Newly Available

8.2.4.1 Biomarkers in Tissue: Single Markers

1) *p53*: This tumor suppressor protein is involved in cell cycle gate keeping and the apoptosis pathway. Individuals who have germline mutations in *TP53* are said to have Li–Fraumeni syndrome. The mutations can be inherited, and can arise de novo during embryogenesis or in a parent's germ cells. This syndrome is rare, with approximately 400 known families. Patients with Li–Fraumeni syndrome are at higher risk for early-onset breast cancer than any other cancer type. The primary limitation of performing genetic screening for germline p53 mutations is their rarity, making it difficult to justify the cost of genetic testing.
2) *Ataxia Telangiectasia (AT)*: AT is an autosomal recessive disorder that affects one in 40 000–300 000 children [15]. The protein that is defective in AT is responsible for detecting DNA strand breaks, recruiting proteins to fix the break, and preventing the cell from making new DNA until the repair is finished. AT is a pleiotropic disease with multiple manifestations, one of which is an increased risk of cancers, especially lymphoma and leukemia, but also breast cancer. Further, women who are heterozygous or homozygous for AT have double the risk of developing breast cancer when compared to the general population [16]. However, the relative infrequency of the mutation in the general population limits its usefulness as a screening tool to identify individuals at increased risk.
3) *Phosphatase and Tensin homolog (PTEN)*: PTEN is a tumor suppressor gene which, when mutated, can contribute to the development of a variety of cancers, including breast cancer. It functions as a tumor suppressor by negatively regulating the Akt/protein kinase B (PKB) signaling pathway [16]. Approximately 50% of breast cancers have loss of PTEN protein expression, and loss of PTEN expression is associated with lymph node metastases and poor survival [17]. Individuals with Cowden's disease, which is associated with germ line mutations in PTEN, have a lifetime risk of 25–50% of developing breast cancer [18]. Polymorphisms in PTEN in individuals at increased

breast cancer risk based on family history but lacking PTEN mutations have not been consistently found to significantly contribute to breast cancer [19].

8.2.4.2 Biomarkers in Tissue: Gene Panels

1) *BROCA*: This 48 gene mutation panel focuses on ovarian and breast cancer. BROCA is most useful for analyzing patients with a suspected cancer predisposition. An advantage of the BROCA gene panel is that specific gene testing can be selected or the investigator can opt for the entire panel.
2) *BreastNext*: This 18-gene panel developed by Ambry Genetics is very similar to the BROCA panel in that it analyzes cancer risk and is best suited for patients with a suspected hereditary predisposition to breast or ovarian cancer. Like BROCA, this panel offers the option of specific gene testing or analysis of the entire panel. A further advantage to BreastNext is that it includes duplication and deletion gene analysis. Two genes analyzed in this panel that are not analyzed in the BROCA panel are NF1 and NBN.
3) *myRisk*: This 25 gene panel includes genes linked to eight cancers: breast, colorectal, ovarian, endometrial, gastric, pancreatic , melanoma and prostate. One of the genes in the panel, PALB2, has been linked to early onset breast cancer.

8.3 Current Biomarker Limitations

The assessment of biomarker expression is influenced by (i) tumor heterogeneity, (ii) treatment effect, and (iii) whether the tumor is new or recurrent. Expression of tumor markers can differ in patients with newly diagnosed, untreated breast cancer between core biopsy and surgical resection specimens from the same patient, most often due to inadequate tumor evaluation in the core specimen. Additionally, treatment can lead to changes in expression; resistant subclones that survive can become predominant after treatment, and tumor DNA, which is inherently unstable, can change over time, leading to changes in primary vs. recurrent/persistent tumors. Moreover, some breast tumors are biclonal, containing both basal and luminal clones with distinct genetic alterations [20]. When mutation driven tumor signaling by one clone is blocked, signaling by a different mutation within the tumor maintains tumor growth. The molecular phenotype of primary vs. recurrent tumors can differ due to treatment of the primary and the innate instability of tumor DNA.

8.3.1 Tumor Heterogeneity

When a diagnosis of breast cancer is made, IHC analysis of ER, PR, and HER2 is generally performed on the core biopsy specimen. Recent studies provide evidence

that there is significant intratumoral heterogeneity, 5–30% for HER2, and lower for ER [21]. A small core biopsy may not accurately represent the ER, PR, or HER2 status of the entire tumor. Moreover, expression may differ between a primary and a recurrent tumor as well as between the primary tumor and its metastasis(es) [21].

Heterogeneity between core biopsy and surgical resection specimens has also been found in the proliferation marker Ki67, in which there was no intervening neoadjuvant therapy. In one study, the average proliferation difference was 3.9% ($N = 200$ cells, $p = 0.046$), with biopsy specimens having a higher proliferation rate than surgical excision specimens. With the assessment of 800 additional (total 1000) cells, the difference was negated. The authors proposed that this was due to hot spot sampling in the core specimens, which was exhausted with a larger sampling pool, resulting in a lower proliferation count. Treatment can also contribute to acquired heterogeneity because it can alter the tumor phenotype and change the ER/PR and HER2 status of the original tumor. These findings suggest that overall under sampling of the primary tumor may be occurring.

8.3.2 Treatment Effect

It is known that both endocrine and chemotherapeutic cancer treatment can change tumor phenotype. A recent report of neoadjuvant endocrine therapy in postmenopausal women with ER+ breast cancer found that the selective ER down regulator fulvestrant decreased Ki67 and ER, but not PR, expression after 4 weeks and all three markers after 16 weeks of treatment in a dose-dependent manner [22]. In a study of 209 women who received neoadjuvant chemotherapy for breast cancer, changes in tumor phenotype were evaluated. Tumor grade changed in 35%, and ER, PR, and HER2 expression in 43, 55, and 27% of cases, respectively [23].

8.3.3 Primary Versus Recurrent Tumor

The molecular phenotype of primary versus recurrent tumors can differ due to treatment of the primary and the innate instability of tumor DNA. If the location of recurrent disease is different from the primary, a particular alteration in the recurrent tumor may have selected for disease spread to the new location. Macfarlane *et al.* retrospectively assessed, using a similar approach to assess expression in both specimens, the molecular phenotype of the original tumor and the tumor metastasis of 160 patients with biopsy-proven relapsed breast cancer [24]. There was 19% discordance in the ER/PR or HER2 status between the primary and relapsed lesions. Further, 5% of tumors had a receptor change from ER positive (+)/PR+ to ER negative (−)/PR−; 9% from ER−/PR− to ER+/PR+. For HER2, 4% of the tumors changed from positive to negative and 1% from negative to positive. A second study evaluating Swedish patients with biopsy proven relapsed/metastatic disease observed discordance for ER, PR, and HER2

of 32, 41, and 15%, respectively [25]. Notably, in the Swedish study there was an overall survival advantage in women with stable ER+ tumors vs. those whose tumors changed from ER+ to ER− at relapse.

8.4 Future Biomarker Discovery Targets

According to Tang and Gui [26], the success of present aims and future frontiers of breast cancer biomarker research hinges on four principle areas in biomarker development: first, by combining newly discovered biomarkers with established systems cancer diagnosis can be optimized; second, biomarkers should be implemented for establishing early determination of response to treatment, recurrence, and survivorship; third, biomarkers should be implemented to guide therapies in patient targeted medicine; lastly, biomarkers should be used to determine the highest quality drug candidates for the development of new therapies.

A major barrier to progress in the development of biomarkers for early breast cancer detection relates to the heterogeneity of the disease. Biomarker studies to date have generally been small, and evaluate one or a few markers in a single sample type. Further, a single sample collection is the basis for the conclusion of the study, which may not adequately account for random variation in the marker analyzed in a given individual. For individuals with new or recurrent breast cancer, more progress has been made since a patient's tumor can be phenotyped. Because of the inherent instability of tumor DNA, as well as treatment effect, changes in biomarkers are possible (as already discussed). Nonetheless, new biomarker development, for both the early detection and treatment of breast cancer, is an active area of investigation. Among the biomarkers under evaluation, we will review autoantibodies, inflammatory response molecules, proteins found in noninvasive fluids and other molecular components, DNA methylation (i.e., CpG Islands), benign breast disease, and pregnancy-associated breast cancer biomarkers.

8.4.1 Autoantibodies

These provide a promising prospective approach to biomarker discovery because they profile the host response to tumor antigens. This response allows the detection of signals from the tumor and allows researchers to improve cancer detectability via serum. CA5-3 and CA27.29 proteins are FDA approved to monitor patients with breast cancer for tumor recurrence or progression (Table 8.1).

8.4.2 Inflammatory Markers

It is clear that pathways involved in the inflammatory response are critical to cancer development. Assessment of inflammatory molecules critical to breast cancer

development and/or progression could contribute to response to treatment, diagnostics, and target treatments. Some of the inflammatory markers currently under evaluation include IGF-1, IL-6, and TNF-alpha.

8.4.3 DNA Methylation

Studies have shown via CpG Island analysis that excessive methylation in cancer cells serves essentially a purpose equivalent to a mutation because it silences tumor suppressor proteins. Therefore, DNA methylation is a promising target for treatment biomarkers.

8.4.4 Benign Breast Disease

Recent studies suggest that benign breast disease should be treated as a precursor to breast cancer. In addition, the following genes have not yet been studied in humans but provide promising molecular data for potential breast cancer biomarkers: CCND1, MYCC, VPACI, and BCL2.

8.4.5 Pregnancy-Associated Breast Cancer

Biomarkers associated with pregnancy-associated breast cancer are connected to the inflammatory pathways during breast involution. Matrix metalloproteinase 2, 6, and 9, as well as transforming growth factor (TGF) beta 1, 2, and 3, are overexpressed in the involuting breast, as well as in women with breast cancer [27]. These new gene targets in combination with known diagnostic gene targets (i.e., VPAC2, insulin-like growth factor 1 receptor (IGFIR), EGFR, and HER2) could optimize current diagnostic, prognostic, and predictive techniques overall.

8.4.6 Challenges with New Biomarker Development and Validation

In most of biomarker development studies, sample sizes are small and are not completely representative due to selection bias and availability. Biomarker assessment in healthy individuals without known cancer are generally limited to body fluids, both because there is no tumor to collect and because institutional review boards are reluctant to approve invasive approaches in healthy individuals. Participants may be noncompliant with the study protocol, making their sample assessment unreliable. Successful body fluid sample collection, especially breast-specific body fluid collection such as NAF or DL, requires learning the technique and practice. Inadequate body fluid or tissue collection can limit biomarker assessment. Antigen activity often decreases over time in fixed tissue.

Perhaps the biggest weakness in biomarker development is validation. It is likely that useful candidate markers have been reported, but the critically important verification and validation studies have not yet been performed. This is in part because the groups that are experts in biomarker discovery studies are ill-equipped to validate their own findings. Validation requires a transition to high throughput and precise analytical methods and access to large cohorts of well-defined clinical samples. Cost is a major hurdle, much of that involving patient recruitment. As such, we propose that a diverse range of molecular types (e.g., peptides, proteins, carbohydrates, DNA, RNA, metabolites) should be analyzed simultaneously from participant samples to optimize yield. Assessment of multiple candidates in multiple sample types in the same cohort, and at the same time, allows a valid and direct comparison of their performance characteristics.

8.4.7 Sample Type Selection for Validation Studies of Diagnostic Biomarkers

8.4.7.1 Why Ductal Lavage for DNA Analysis?

Both DL and NAF are collected from the breast ducts, the cells that give rise to breast cancer. The samples are not diluted by other non-breast epithelial cells or proteins, as is the case with the analysis of blood products such as serum. We have observed on some occasions that a biomarker will be predictive of breast cancer in DL or NAF samples but not in serum collected at the same time. The cellular content of DL is greater than that of NAF, allowing more efficient detection of DNA-based markers. On the other hand, the extracellular protein content varies based on the ratio of neat intraductal sample to irrigant, which is difficult to quantify.

8.4.7.2 Why Nipple Aspirate Fluid for Protein Analysis?

Nipple aspiration is a noninvasive, low-cost procedure that provides a sample containing a relatively small set of breast-specific proteins shed from the breast ductal epithelium – the cells that give rise to cancer. We are able to obtain NAF in over 80% of pre- and postmenopausal subjects [6]. Because NAF proteins are secreted, they represent the final processed protein form including post-translational modification, and further, although diluted significantly, these same proteins may serve as potential markers in the general circulation. Validation of the putative protein markers identified in NAF and tissue will be undertaken in NAF and/or serum because these samples are too minimally invasive to collect in a routine clinical setting.

8.4.7.3 Why Circulating Samples for Protein Analysis?

Serum is routinely used in clinical chemistry, is minimally invasive, and in some settings is the most practical sample to collect. Even though a marker may be diluted markedly in serum, much larger volumes (1 – 5 ml) can easily be obtained. In other words, a marker detected in NAF could be diluted by a factor of 1000 or more and still be detected in routinely collected volumes of serum.

8.4.7.4 DNA Candidates

There are several approaches to measure DNA alterations to predict cancer, including loss of heterozygosity, microsatellite instability, assessment of mitochondrial DNA, and methylation changes in DNA, but the last appears most promising for the assessment of breast cancer, and investigators on this proposal have considerable experience in the technique *I-C2*.

8.4.7.5 RNA Candidates

RNA is a powerful tool to assess marker expression, but degrades more readily than DNA or protein. While micro (mi)RNAs are more readily detected than larger stretches of RNA in body fluid samples, there is yet no agreement in the breast cancer community on the optimal miRNAs to analyze for cancer detection or prognosis.

8.4.7.6 Protein Candidates

Several proteomic approaches have identified candidate markers and/or profiles for breast cancer diagnosis. Interestingly, many of the proteins that have been identified are different from their associated genes as found by nucleic-acid-based studies. This underscores the importance of performing studies on multiple molecular types and highlights the fact that differences between normal and cancer samples may be best reflected in post-translational modifications such as glycosylation or truncation [28].

Although promising, proteomic analyses of breast tissues suffer for two reasons: (i) the sample is not homogeneous and (ii) an invasive procedure is required to obtain the sample. Sample heterogeneity means that large sample numbers are required to establish biologically relevant differences. Nevertheless, 49 protein markers in tissue have been reported that show promise in the diagnosis of hormone-sensitive breast cancer [18]. Because some of these proteins are actively secreted, they have the potential to serve as breast cancer markers when measured in the general circulation.

An attractive alternative to tissue is to seek biomarkers in biological fluids. Fluids, especially if they are produced locally, offer the advantage that they are more homogeneous, markers are concentrated, and the samples can be collected less invasively. We have identified three candidate diagnostic signatures in NAF (Table 8.2) that have not undergone extensive validation: Thomsen–Friedenreich (TF), urinary plasminogen activator (uPA), and uPA inhibitor (PAI-1). Carcinoembryonic antigen (CEA) has also been associated with breast cancer (Table 8.2).

There are multiple different sets of candidate protein biomarkers in studies of different tissues and fluids utilizing different analytical methods. Despite this wealth of information, there has been no concerted effort to objectively review, consolidate, and validate these findings. Table 8.2 is not meant to serve as a comprehensive list of published findings, but it does serve as an illustration of what has already been discovered but not validated. Available markers show potential to diagnose breast cancer; others appear useful in predicting response

Table 8.2 Putative protein biomarkers available for validation.

Marker	Source	References	Clinical use
TF	NAF	[21]	Diagnosis
uPA	NAF	[22]	Diagnosis
PAI-1	NAF	[22]	Diagnosis
CEA	NAF	[23]	Diagnosis
MIS	Ser	[19]	Diagnosis
Annexin A2	Tissue	[18]	Diagnosis
Cathepsin D	Tissue	[18]	Diagnosis
Cellular retinoic acid-binding protein 2	Tissue	[18]	Diagnosis
Keratin 7 (sarcolectin)	Tissue	[18]	Diagnosis
Peptidyl-prolyl *cis-trans* isomerase B (PPIase) (Rotamase) (Cyclophilin B)	Tissue	[18]	Diagnosis
Protein DJ-1 (Oncogene DJ1) (Parkinson disease protein 7)	Tissue	[18]	Diagnosis
Alpha-1-antichymotrypsin	Tissue	[18]	Diagnosis
Alpha-1-antitrypsin	Tissue	[18]	Diagnosis
Ferritin	Tissue	[18]	Diagnosis
Serotransferrin (transferrin)	Tissue	[18]	Diagnosis
CA 15-3	Serum	FDA approved	Prediction/prognosis
CA 27-29	Serum	FDA approved	Prediction/prognosis
HER2/neu	Serum	FDA approved	Prediction/prognosis

to treatment with chemotherapy. We predict that a panel of biomarkers with clinical utility for breast cancer diagnosis, recurrence, and response prediction can be assembled from what has been reported. Table 8.2 includes several of the most promising candidates.

8.5 Summary

The greatest advances in breast cancer biomarker development thus far are in tailoring treatment to individuals with newly diagnosed breast cancer, since the tumor can be phenotyped. Phenotyping is being done both for specific alterations as well as gene panels, to guide hormonal therapy, biologic therapy as well as chemotherapy. Tissue assessment is not as practical to screen at-risk individuals, to follow response to treatment, or to monitor for disease recurrence. For early detection, breast-specific and circulating body fluids are appropriate for biomarker development. Interventions on the breast with excisional biopsy followed by radiation or mastectomy make it less probable that breast-specific body fluid samples will be obtainable, and therefore these samples are not

practical for the evaluation of disease recurrence. Serum/plasma is therefore the logical body fluid for serial assessment of disease response and recurrence.

Biomarker panels are coming into increasing use, not only in the assessment of tissue, but also of body fluids. Multianalyte assessment is both a practical and a reasonable approach to optimize sensitivity and specificity of a biomarker panel. Validation of single biomarkers as well as biomarker panels is critical to confirm usefulness of the biomarker(s) prior to clinical use.

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9
Evaluation of Proteomic Data: From Profiling to Network Analysis by Way of Biomarker Discovery

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9.1
Introduction

Mass spectrometry coupled to liquid-chromatography (LC-MS) has emerged as the core tool for large-scale proteomic analysis. A great boost to this success was given by the availability of genomic databases that are even more complete and by continuous technological improvements, which have led to highly accurate, fast, and sensitive instruments [1, 2]. Simultaneously, the increasing utility of MS-based proteomic analysis for research and clinical purposes has driven a parallel growth of a specific bioinformatic area, whose contribution in data handling and improvement of the discovery processes represents a further key point [3, 4].

Current proteomic methodologies based on LC-MS may be grouped in two categories: shotgun and targeted proteomics for discovery and validation purposes, respectively. Recently, it has been possible to categorize them based on the mode of data acquisition by mass spectrometry [5]. In particular, data-dependent acquisition (DDA) is commonly used for identifying proteins by means of shotgun approaches and it preferentially selects high-abundance precursor ions for the following fragmentation. On the contrary, data-independent acquisition (DIA) ideally allows fragmentation of the entire set of precursor ions in the visible range of the mass spectrometer; in this way, undersampling resulting from the stochastic nature of DDA methods is reduced, and the detection of lower level components is improved. The last cutting edge proteomic technologies therefore show higher data-gathering capabilities to characterize the analyzed proteomes and to discover differentially expressed proteins by tissues, cell lines, or biofluids [5–12]; it is of importance to see deeper into analyzed samples to obtain a good snapshot of the investigated systems, and to better correlate protein levels to specific physiopathological states (Figure 9.1).

In addition to research purposes, a fast and accurate production and evaluation of proteomic data is of importance for clinical proteomic strategies, which ultimately aim at providing clinicians with new tools to accurately diagnose disease states and treat patients in an individualized manner [13–15]. For these goals, bioinformatics is beginning to provide both conceptual bases and practical

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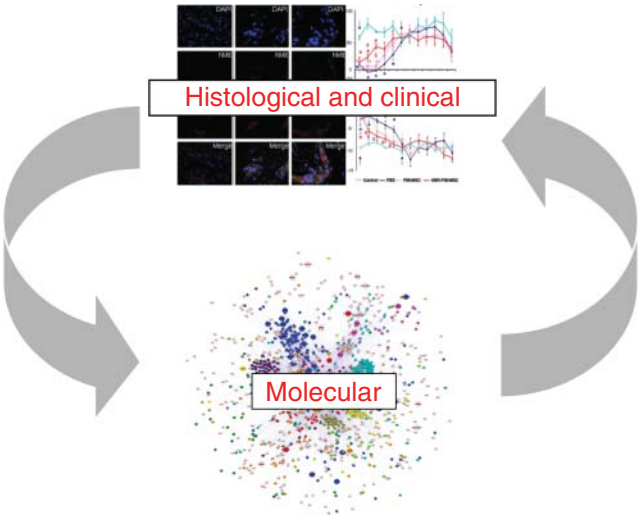

Figure 9.1 *Combination of clinical and molecular data*. Comparison and correlation between physiopathological states and proteomic data obtained by high-throughput proteomic analysis.

methods for systematically detecting functional behaviors of the analyzed systems. In this chapter, we propose a rapid overview of the most important high-throughput proteomic technologies based on LC-MS, focusing on statistical and computational evaluation of MS data to identify and quantify proteins, and finally to evaluate them at systems level by taking into consideration their functional relationship [16, 17]. To report a real example, data produced by a shotgun proteomic approach, such as multidimensional protein identification technology (MudPIT) [18], will be taken as reference. MudPIT is a methodology widely used for biomarker discovery and it allows the identification of thousands of spectra, peptides, and proteins per sample. This big amount of data will be used for describing a strategy of investigation that combines profiling and biomarker discovery with protein–protein interaction (PPI) network analysis [19]; we will show some aspects of this procedure, highlighting its utility for automatically extracting relevant results, such as sub-networks and biological and topological biomarkers, that underlines the emergence of specific phenotypes.

9.2 Proteomic Methodologies

Nowadays, one of the most interesting and exciting applications of MS concerns proteomic analysis [1]. Over the past two decades, multiple strategies have been developed to systematically and comprehensively profile the proteome of biological systems, and the main technological challenge in this context is still the

development of efficient methods that assure a deep and reproducible detection of as many components as possible, preferably in a quantitative manner [2].

Among technologies that allow analyzing proteins, MS has gained popularity thanks to its ability to handle the proteome complexity. Other techniques, such as two-dimensional gel electrophoresis (2DE) and protein microarrays, fail to achieve the depth of analysis seen with MS; for instance, the 2DE approach has been widely used over the years but it is limited by both identification and quantification capacities. In fact, it is difficult to resolve proteins with extreme pI and molecular weight (MW) or that are highly hydrophobic, and many of these limitations were solved using gel-free MS-based approaches [18].

The introduction of soft ionization methods, such as MALDI (matrix-assisted laser ionization desorption) and ESI (electrospray ionization), was one of the major turning points in MS-based proteomic studies [20]; in particular, ESI allowed the coupling of liquid chromatography (LC) with MS. In addition, recent advances in LC-MS, such as the introduction of low-flow high-performance liquid chromatography (HPLC) (micro- and nanofluidic systems) coupled to high-resolution mass spectrometers through nano-ESI ion sources allows reaching high sensitivity, reproducibility, and accuracy in protein detection [21]. In this scenario, instruments with hybrid mass analyzers have been introduced (e.g., LTQ-Orbitrap, TOF-TOF (time of flight), Q-TOF (quadrupole-time of flight)) to further improve performances of proteomic analysis. Choosing the appropriate technologies, or a combination of them, is essential to achieve the maximum proteome coverage and to reach the specific research objective. Nowadays, bottom-up strategies [22], in which peptide detection is used to infer protein presence, represent the standard to systematically and comprehensively profile biological systems, for both biomarker discovery and their validation (Figure 9.2).

9.3 Shotgun Proteomics

Shotgun proteomics have demonstrated, over the years, a great capability to characterize hundreds/thousands of proteins per sample, delivering a good representation of the analyzed complex biological systems [7, 12, 23]. In a typical experiment, proteins are enzymatically digested and the resulting peptide mixture is fractionated by LC, typically reverse phase (RP) chromatography, and analyzed by tandem mass spectrometry (MS/MS), according to which precursor peptide ions are subjected to fragmentation by CID (*Collision Induced Dissociation*) into the analyzer. Other fragmentation techniques, such as ETD (*Electron-Transfer Dissociation*) and HCD (*Higher Energy Collisional Dissociation*), may be used as alternatives to CID to improve identification of long, highly charged peptides containing highly labile post-translational modifications (PTMs) [24].

In shotgun proteomics, the selection of peptides to be fragmented is controlled by the data themselves (DDA) during the analysis [5]. The instrument selects the most abundant precursor ions from the first MS scan, acquiring their tandem

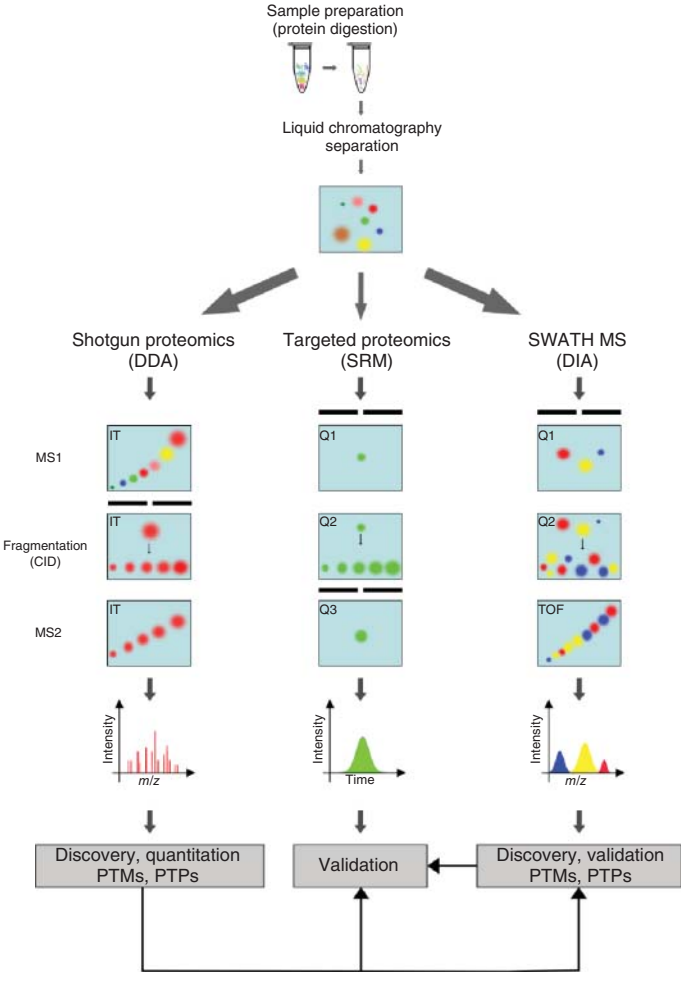

Figure 9.2 *Mass spectrometry-based proteomic approaches*. In shotgun proteomics, such as MuPIT, precursor ions are selected and fragmented by applying signal intensity and charge state filters. Data acquisition is performed in iterative cycles and fragment ion scans. The method is most frequently used on complex peptide samples generated by sample digestion. In targeted proteomics, such as SRM/MRM, precursor ions of predetermined peptides are selected (by mass filter) and gas-phase fragmented. The signal intensities of predetermined fragment ions unique to the targeted peptide(s) (transitions) are recorded over time; it is used for validation purposes. The instrument cycles through the list of predetermined peptide ion lists and their transitions. In DIA analysis, such as SWATH MS, many precursor ions are concurrently selected and fragmented, and the composite fragment ion spectra are recorded and then deconvoluted. SWATH MS, in which precursor ions are sequentially isolated in smaller mass windows (typically 25 *m*/*z*), is used for high-throughput discovery and validation purposes. DDA (data dependent acquisition), DIA (data independent acquisition), SRM (selected reaction monitoring), IT (ion trap), Q (quandrupole), TOF (time of flight), PTMs (post translational modifications), and PTPs (proteotypic peptides).

Table 9.1 Main computational tools (software and algorithms) involved in database searching methods (A), de novo sequencing (B), SRM/MRM (C), and DIA data processing (D).

(A)	Database-dependent search engines	MASCOT [26]
		SEQUEST [27]
		X!TANDEM [28]
		PEAKS [29]
(B)	De novo sequencing	PEAKS DB [30]
		PepNovo [31]
		SHERENGA [32]
(C)	Tools for a SRM/MRM	SRMCollider [33]
		MaRiMba [34]
		MRMaid [35]
		MRMer [36]
		Skyline [37]
(D)	Tools for processing DIA data	MProphet [38]
		Skyline [37]
		PinPoint [39]
		Spectronaut [40]

mass spectra in sequential MS/MS scans. Moreover, ions selected for fragmentation are dynamically excluded in the following scans, over a determined time, to improve the number of distinct MS/MS spectra and, consequently, the number of identified peptides [25]. Peptide identification is achieved by specific algorithms (Table 9.1(A)) that compare experimental MS/MS spectra to theoretical ones calculated *in silico* by specific protein sequence databases [41]. Alternatively, the identification of peptides may be carried out without the use of a sequence database through "de novo sequencing" [42]. Although database searching is extremely powerful and versatile, de novo sequencing (Table 9.1(B)) has provided a complementary and alternative unbiased means for identifying peptide sequences, particularly for organisms whose genomes are unsequenced. However, these two methods are often used in combination for improving speed and sensitivity of database searches and the confidence of de novo sequenced peptides [30].

The main limitation of DDA methods is that they preferentially fragment high-abundance precursor ions, while low-abundance ions could be never sampled; however, by performing technical replicate analysis this phenomenon may be overcome [43]. A further innovation introduced in shotgun proteomics, to increase analysis performances, is represented by the MudPIT approach, which is based on two-dimensional liquid chromatographic separation coupled to tandem mass spectrometry (2DC-MS/MS) [23]. It highly increases the resolving power of peptide chromatographic separations, minimizing MS undersampling. The whole process is completely automated and it allows the analysis of a wide range of samples without limits of pI, Mw, or hydrophobicity (Figure 9.3). For this reasons, nowadays MudPIT is widely used in the discovery phase for identifying, at a large scale level, informative protein profile (peptides, proteins, PTMs), and

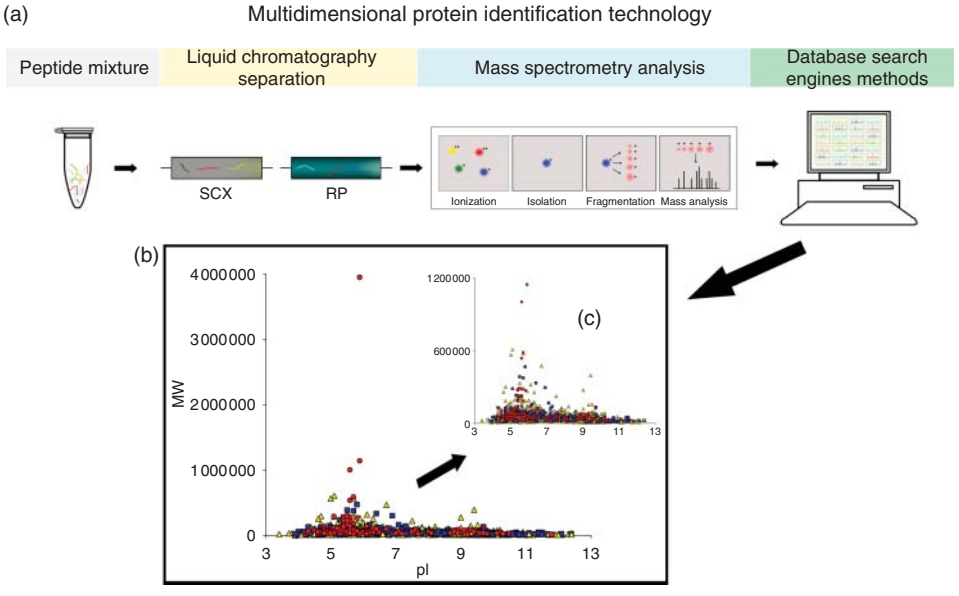

Figure 9.3 *Multidimensional protein identification technology (MudPIT).* (a) Main steps involved in MudPIT approach. (b) Virtual 2D map (MW vs pI) of proteins identified, by a single MudPIT experiment, in cardiomyocytes of *Mus musculus*, $n = 1120$. It was obtained by MAProMa software [44]. Color and shape codes are related to confidence of identification; specifically, yellow/triangles → SpC ≤ 2, blue/square → 2 < SpC ≤ 5, red/circle → SpC > 5. (c) 2D map zoom.

for identifying differentially expressed proteins through label-free quantitative approaches.

9.3.1 Targeted Proteomics

Conventional approaches based on immunoassays (e.g., enzyme-linked immunosorbent assay (ELISA)) are currently the gold standard for biomarker validation in body fluids. Despite their specificity and sensitivity, they have shown some limitations due to the use of antibodies [45]. However, the ability to detect and quantify with higher precision proteins, whose expression may be indicative of disease states, remains an essential task in biomedical and clinical research [46]. In this context, high-throughput proteomics based on the shotgun approach have accelerated the discovery of differentially expressed proteins, ideal candidates to be biomarkers, that need to be validated prior to being routinely used by clinicians. For this purpose, "targeted" proteomics have emerged as an effective approach because it is well suited for the reproducible and accurate quantification of specific proteins in many samples [47, 48]. Targeted proteomics are based on SRM (Selected Reaction Monitoring) acquisition data. An SRM experiment is usually performed on triple quadrupole mass spectrometer (QQQ): precursor

ions of a specific analyte are selected in the first quadrupole and fragmented in the second. Subsequently, specific fragment ions are selected in the third quadrupole and guided to the detector (Figure 9.1). Combinations of precursor ion and its fragment ion are monitored, and they represent the "transitions" that specifically target proteins of interest. To monitor target proteins, it is mandatory to know which "transition" optimally describes a specific protein, and therefore the targeted proteotypic peptides [49, 50]; to solve this need several softwares have been developed and some of them are listed in Table 9.1(C). Transitions are counted over time, resulting in a chromatogram "retention time" *versus* "signal intensity." The signal intensity of a specific transition provides a direct measure of the analyte concentration in the sample. Because several precursor–fragment pairs may be detected, the concurrent quantification of multiple analytes is allowed. This multiplexing capability has been defined as multiple reaction monitoring (MRM), which is frequently used as a synonym of SRM. In this case, interfered transitions are the main cause of erroneous detection and incorrect quantification of peptides, but high-resolution and accurate-mass analyzers, such as hybrid fast Q-TOF and quadrupole-Orbitrap (Q-Orbitrap) mass spectrometers can overcome this limitation [51, 52]. However, the limited number of target proteins, about a hundred, which may be measured per run, remains another current limitation of SRM [5, 10].

9.3.2
Data-Independent Acquisition (DIA) MS

Data-independent Acquisition (DIA) analysis has recently emerged in proteomics with the aim of addressing some limitations of data-dependent acquisition (DDA) analysis and SRM, employed in shotgun and targeted proteomics, respectively (Figure 9.2). Specifically, shotgun proteomics have limited capabilities on very large sample sets, mainly due to undersampling [53]. In contrast, targeted proteomics is limited to the measurements of a few hundred proteins per LC-MS/MS run, not sufficient to routinely quantify large fractions of a proteome [5–10].

The DIA method systematically scans samples by acquiring fragmentation spectra of all precursor ions within isolation windows, also named SWATH, cyclically repeated over a desired m/z range. Various implementations of DIA methods have been described using isolation windows of various widths, ranging from the complete m/z range to a few Daltons. DIA methods are obviously strictly related to features of the mass spectrometer; the two main methods described in literature consist of 32 swaths of 25 Da in a quadrupole–quadrupole-time-of-flight (QqTOF) instrument [5, 8] and 20 swaths of 10–20 Da on ion trap mass spectrometers [54, 55]. Data acquired by DIA can thus be described as successive MS2 maps consisting of the fragment ion spectra from all the precursors fragmented in each swath. SWATH MS data processing is mainly based on targeted data extraction from MS2 maps. Some available softwares for this purpose are shown in Table 9.1(D), but their systematic description is far from the object of this chapter. However, it is at least important to highlight that DIA allows extending

the discovery capacity of shotgun proteomics as well as the degree of multiplexing achieved by SRM, recording an enormous number of transition from possibly all peptides of the complex biological system.

9.4 Biomarker Discovery

In parallel with the progress achieved in MS-based proteomics, multiple MS-based quantification strategies have been developed in these years. They represent an important key point for clinical applications [9, 56, 57]. In fact, biomarkers should be introduced in routine clinical tests for screening high-risk individuals and detect disease at early stages, facilitating the prognosis prediction as well as the monitoring of treatment response. However, it is not always possible to realize the full potential of well-established markers [57–59]. It is more and more evident that many biological functions are only rarely attributed to an individual molecule. As a consequence, the use of a single biomarker to realize prediction models may be considered incomplete, and studies are now growing about the discovery of multiple biomarkers that contain a higher level of discriminatory information [14].

By now, it is well established that MS-based quantitative proteomic approaches may be categorized in stable isotope-labeling and label-free methodologies. [60] Labeling ones consist in introducing isotopes into peptides to create a specific mass tag recognized by MS; in this way, the ratio of the signal intensities is measured between the unlabeled peptide and its identical counterpart enriched with isotopes (Figure 9.4a). The main methodologies of this category may be grouped depending on how the isotopes are attached to peptides, for example, covalently in isotope-coded affinity tag (ICAT) [61] or Isobaric tags for relative and absolute quantitation (iTRAQ) [62], by enzymatic reactions for incorporating ^{18}O [63], or metabolically in stable isotope labeling by amino acids in cell culture (SILAC) approach [64]. Although they provide excellent results, allowing highly reproducible and accurate quantification of proteins, some limitations, including the complexity of sample preparation, have been documented [65]. On the other hand, label-free approaches represent a simpler and low-cost alternative. Some of them evaluate the chromatographic peak area of the identified peptides, while others take advantage of the direct relationship between protein abundance and sampling parameters [66], such as spectral count (SpC) (Figure 9.4b). Even if these methods are less accurate, due to, for instance, systematic and non-systematic experimental variations, over the years their efficiency and utility in discovering reliable differentially expressed proteins has been demonstrated [67, 68], and different statistical indices for this purpose have been published [3]. Finally, concerning the absolute measurements of protein concentration, the use of strategies that rely on the isotope dilution of spiked internal standard peptides is interesting [69]. It may be reached by methods, such as AQUA (Absolute Quantification), where synthetic peptides can be spiked into the samples after the proteolysis step, QconCAT, where concatamers are chimerical proteins composed of different

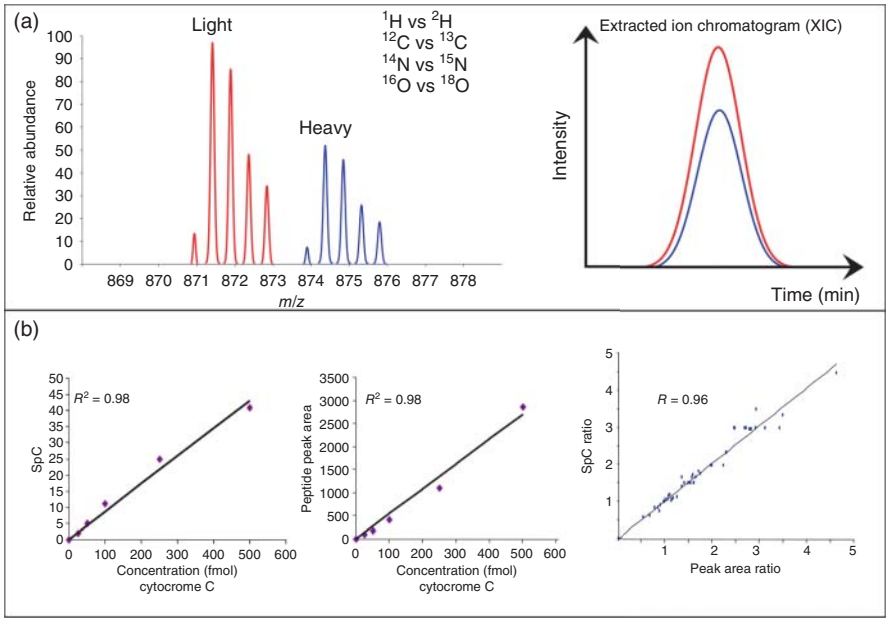

Figure 9.4 *MS-based quantitative proteomic.* Label approaches (a); unlabeled and labeled peptide counterparts are identified and their intensity ratio is calculated by XIC. (b) Label-free approaches (b); relation between sample parameters, such as SpC and protein abundance (cytocrome C).

proteotypic peptides, or protein standard absolute quantification (PSAQ), where the used standards are full-length proteins matching the biochemical properties of the target proteins.

Several algorithms and software for the semi-quantitative evaluation of proteins have been developed and published in the literature. Among the commercial software available for label-free proteomics, Nonlinear Dynamics Progenesis LC-MS (*http://www.nonlinear.com/*) and Elucidator (*http://www.rosettabio.com/*) quantify peak intensities and also provide SpC evaluation, while SIEVE (*http://www.thermo.com/*) and ProteinLynx (*http://www.waters.com/*) rely only the first one. In the same way, a good number of open-source software, including MapQuant [70], MsInspect [71], OpenMs [72], SuperHirn [73], PEPPeR [74], MSQuant [75], MaxQuant [76], ProteinQuant Suite [77], ProtQuant [78], and many others, have been developed. Among them, Census [79] and PatternLab [80] software are of interest for shotgun proteomics experiments; in particular, *Census* allows protein quantitation by MS and MS/MS spectra, and it is compatible with label and label-free analysis as well as with high and low-resolution MS data, while PatternLab implements a variety of strategies of normalization and feature selection to efficiently pinpoint differences between profiles. Finally, *MAProM*a software [81] is a simple tool, based on SEQUEST Score/SpC evaluation, which allows a label-free pairwise comparison up to 125 protein lists.

9.4.1 MudPIT Data Processing

Proteomics experiments performed by the MudPIT approach generate large amounts of data (thousand of raw spectra, peptides, and proteins) that need to be properly handled. Before statistically valid results can be reached, for biomarker discovery as well as future selection and classification algorithms, different steps of analysis must be implemented. Firstly, experimental data have to be pre-processed for adjusting experimental errors that may lead to incorrect conclusions. Standard procedures are applied to remove instrumental noise and to make measurements comparable; for instance, mass spectral profiles may be affected by baseline offsets, shifts in mass-to-charge ratio or alignment problem that may be corrected by software such as MZmine [44], MsInspect [71], or OpenMs [72]. Variations of sampling parameters, such as SpC, are adjusted by using strategies of data normalization [80], and a few examples are reported in Figure 9.5. Data outlier may be identified by various standard statistical methods, such as Chauvenet's criterion, Grubbs' test for outliers, and Peirce's criterion. However, a decision must be made establishing the outliers as experimental "glitch" or as potentially important biological phenomena.

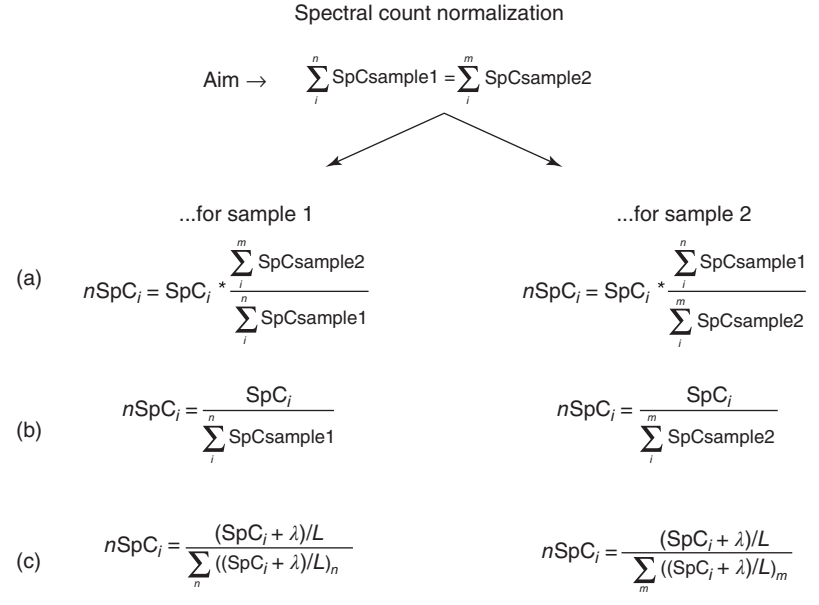

Figure 9.5 *Spectral count normalization*. (a,b) Total signal normalization approaches and (c) SpC normalization, based on protein length, for comparing protein within the same sample. SpC = un-normalized spectral count, *n*SpC = normalized spectral count, L = protein length, λ = measure to add to the spectral count of each protein, to avoid taking logarithm on zeros.

When dealing with the analysis of high-dimensional data, such as those derived by MudPIT, the most notable problem is the so-called "*curse of dimensionality,*" where the number of observations (n) is often far smaller than the number of variables (p) (thousands proteins, peptides or spectra) ($n \ll p$). To obtain a more informative lower dimensional space and to reduce computationally intensive models, dimensional reduction of data is a step required for both biomarkers extraction and for not affecting prediction rate and the efficiency of classification problem [82]. Analysts might choose to use a combination of dimension reduction and variable selection strategies to produce an informative set of biomarkers, specifically related to the problem it is addressing. A number of methods, including support vector machines (SVMs), Principal Components Analysis (PCA) artificial neural networks (ANNs), random forests (RFs), Partial Least Squares (PLSs), and Linear discriminant analysis (LDA) have been used for the analysis of proteomic data [14, 82, 83].

As shown in Figure 9.6, MudPIT data may be combined into a sparse matrix. Concerning characterized protein profiles, each row is relative to a specific protein and each cell shows the SpC value obtained in the relative analyzed sample (columns). *LDA* is used to find the linear combination of features that best separates two or more classes of samples. It assures data reproducibility and allows automatically extracting, from high-dimensional proteomic data, a number of proteins whose variations are repeated in all considered samples. For

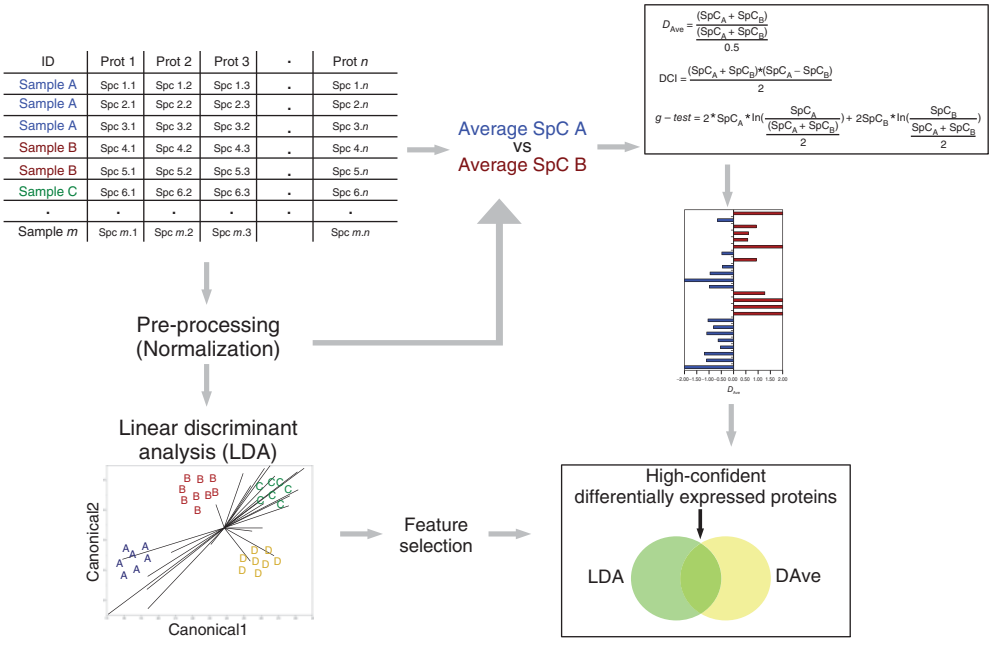

Figure 9.6 Processing of high-dimensional MudPIT data for identifying differentially expressed proteins.

this reason, the use of LDA assures a good extraction of meaningful data when SpC fluctuations between experiments are minimized. Concerning, for example, proteomic analysis of different patients, it might correctly indicate different levels of proteins and therefore subjective mechanisms of disease development and therapeutic response, leading to personalized proteomics [15]. In this cases, samples may be individually considered, or those representing the same condition may be grouped and average value of SpC processed by several indices, such as G-test [84], D_{Ave}, and DCI algorithms [81] and many others [3] able to select differentially expressed proteins; in any case, the use of specific statistical tests or a combination of them is strictly related to many variables, including sample origin, study purposes, and data structure, which time to time shape the best data processing procedure to be used.

9.5 Protein–Protein Interaction Network Analysis

As reported above, it is more and more evident that many biological functions are only rarely attributed to an individual molecule, and in many cases they emerge as a result of a complex network of interactions between different cellular components [16]. To solve this question, systems biology approaches embrace the complexity found in biological networks by taking a holistic view of the cell [17]. In this context, an increasing number of studies is combining experimental proteomic data with the PPI interaction networks analysis [12, 85, 86]. In fact, investigating the proteome in all its complexity is critical to elucidating the molecular mechanisms related to healthy or pathological states as well as to individual subjects [15].

At abstract level, a proteome can be reduced to a series of nodes that are connected to each other by links called edges, which represent the kind of interaction between components; data abstraction is the natural result of the desire to rationalize knowledge of complex systems. Physical interactions between molecules, such as PPI, can be easily represented and conceptualized using the node-link nomenclature, and more complex details, such as *direction* (edge direction), *sign* (activation or inhibition), and *mode* (such as phosphorylation or ubiquitination) may also be considered within this representation; in this way, it is possible to integrate computational modeling and experimental biology to characterize and predict properties of the investigated biological systems [12, 86–88].

Although the accessibility to interaction networks is continuously increasing, consistent data are at the moment available only for most investigated organisms, including *Homo sapiens, Mus musculus, E. coli,* and so on. In the last years, PPI interactions have been collected in specialized biological databases, including String [89], human protein reference database (HPRD) [90], Reactome [91], IntAct [92], and many others, that are continuously updated in order to provide complete interactomes. An excellent collection of these resources is listed in Pathguide repository (*http://www.pathguide.org/*), which contains information

about 547 biological pathway-related resources and molecular interaction-related resources; they may be subdivided into primary databases that collect information about published PPIs proven to exist via experimental methods, meta-databases that normally result from the integration of primary databases information and some original data, and prediction databases that include PPIs predicted using several techniques.

A wide set of bioinformatic tools is also available for visualizing and analyzing biological networks. Among the most used computational platforms useful for these purposes, Cytoscape [93], VisANT [94], and Ingenuity Pathway Analysis (*http://www.ingenuity.com*) are some of those worth mentioning. Cytoscape is probably the most famous. It is an open-source software for integration, visualization, and analysis of biological networks, with an accessible application programming interface (API) using the Java programming language. In particular, software developers may write extensions called plugins that link Cytoscape with new code and provide access to new and alternative features [95].

In addition to plugins that automatically allow importing networks from main specialized repositories, many others have been developed to handle and analyze the big amount of data associated with large-scale proteomic analysis. Plugins such as Bingo2.44 [96], Mosaic [97], network ontology analysis (NOA) [98], and ClueGO [99] are used for performing gene ontology analysis, while others, including MCODE [100] and ClusterMaker [101], allow weight nodes with local neighborhood density for finally graphically displaying extracted modules and associated information.

Once a network is generated by combining experimental data and PPI interactions, it is very useful to map and display onto defined functional modules/clusters of differentially expressed proteins, such as those derived by both label- and label-free proteomics (Figure 9.7). In this way, it is possible to visualize and interpret by a single image hundreds/thousands of proteins experimentally identified and functionally connected; in addition, pathways and sub-networks up- and downregulated in the investigated systems are easily identified. In this scenario, it is interesting to further evaluate the analyzed systems by taking as reference the topological properties of the corresponding PPI interaction network. For this purpose, the CentiScaPe plugin Cytoscape [19] computes several centrality parameters, including average distance, diameter, degree, stress, betweenness, radiality, closeness, centroid value, and eccentricity, for identifying nodes having a relevant position in the overall network architecture. In particular, stress and betweenness indices allow the extraction of proteins functionally capable of holding together communicating node, and that maintain functionality and coherence of signaling mechanisms. Finally, the combination of topological analysis and differentially expressed proteins permits the selection of nodes relevant from both biological and topological viewpoints; in fact, it allows characterizing proteins relevant for network organization and in that role are furthermore changing their expression, representing therefore putative key molecules that modulate the responses to an altered biological system.

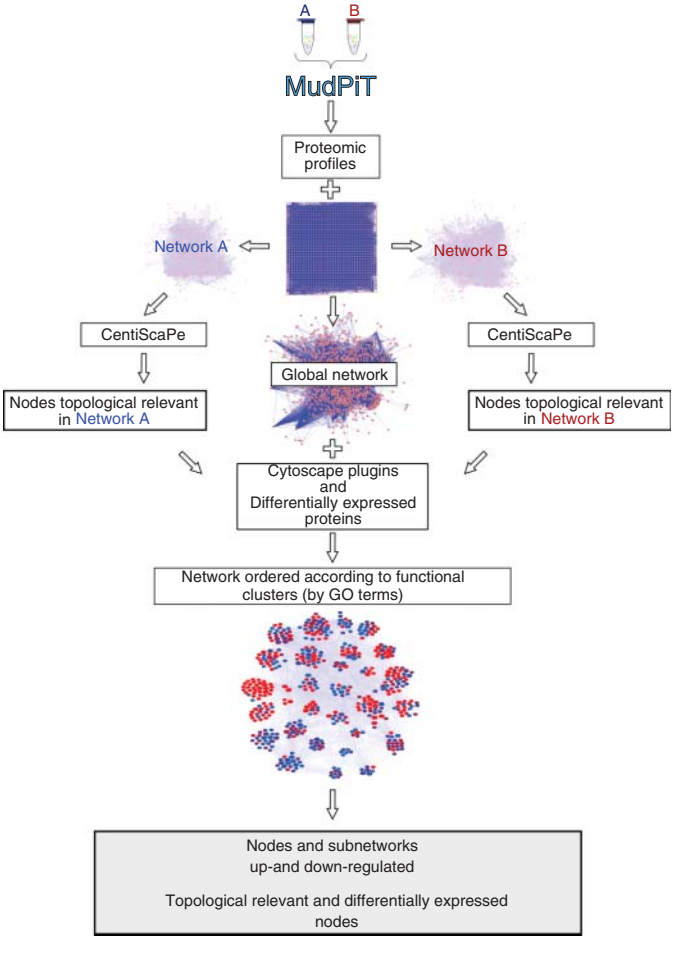

Figure 9.7 *Protein–protein interaction network analysis.* Main computational steps for integrating experimental proteomic data and protein–protein interaction networks. It aims to identify nodes and sub-networks up- or downregulated, as well as topologically relevant nodes whose expression changes between the investigated conditions.

9.6 Conclusion

As shown in this chapter, advances in MS instrumentation increase even more the amount and quality of data produced by proteomic experiments. To evaluate them, researchers need to integrate these large sets of experimental data into models for generating the hypothesis to be tested. In the last years, systems biology has provided powerful strategies for linking protein profiles and biomarkers with biological processes that can be segmented and linked to disease presentation. In fact, by combining experimental data and network-based approaches it is

now possible to automatically identify groups of functional related proteins that emerge in the investigated phenotypes. In addition to improving and speeding up data interpretation procedures, it is of great importance for characterizing large scale level multiple biomarkers, which for diagnosis purposes may allow a higher level of discriminatory information. In this context, spectrometry-based proteomics are emerging also as powerful tool to face clinical questions, but data reproducibility as well as lack of standardization of methods needs to be faced. However, as widely seen in the literature, computational and statistical tools support biologists and clinicians at various levels of sophistication, improving both data interpretation and the handling of data themselves. As consequence, constant advances in instrumentation and computational tools may represent a good starting point for tackling challenges in clinical proteomics in the near future, and to face its unrealized potential, the production of valuable data should go in step with a strict cooperation between biologists, clinicians, and bioinformaticians.

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10
Biomarkers: From Discovery to Commercialization

Sebastian Hoppe and Henry Memczak

The potential value – both commercially and academically – of biomarker discovery is undoubtedly immense. While the scientific efforts to elicit novel biomarkers of importance have been vast, the ramifications have been meager at best. As biomarkers are initially discovered via large-scale screens or multiplex assays, these results are often associated with a method-dependent bias. This bias might be the result of poorly validated methods in the first place, as most discoveries are performed in a scientific environment. Fluctuating detection reagents and neglect of co-recognition/cross-reactivity of other markers or substances are critical obstacles in successfully unveiling new biomarkers that satisfy the requirements for clinical applications. Consequently, research has adapted a method of cross-platform validation. Once a potential biomarker is discovered, it undergoes a sequence of rigorous trials before it is accepted as suitable for clinical diagnostics. These ordeals involve the independent analysis of the said biomarker on different platforms. A simplified scheme of the different stages of biomarker discovery and validation is shown in Figure 10.1. After a biomarker has been selected, it is experimentally verified using a single appropriate method. Next, the chosen detection method is validated, for example, by incubating with standard sera to analyze cross-reactivity. Only when both the biomarker and the detection method stand the proof, a cross-platform validation is performed by examining the biomarker on several different platforms. If the biomarker turns out positive results on all platforms, it is considered a suitable biomarker and passed to clinical studies.

While this approach to biomarker validation has some merit, it also comes with a number of pitfalls. If the individual methods for biomarker discovery and analysis are flawed, as indicated, the onus of confirming the results with different, in-itself also flawed, methods is exuberant. Might it only be a matter of choosing the "right" platform to validate a biomarker? Or does the choice of platforms used for cross-validation significantly influence the outcome?

This chapter addresses some of these issues. It provides information on some of the "en vogue" biomarker platforms and deals with cross-platform validation and transferring biomarkers between platforms. In addition, multiplex approaches are mentioned and their importance in the field of biomarker discovery and

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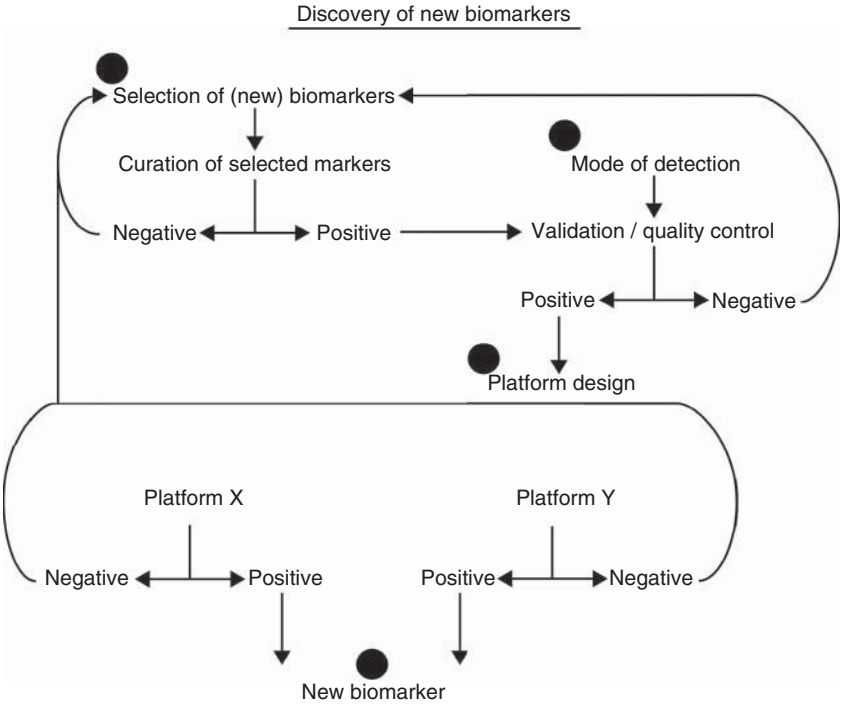

Figure 10.1 Schematic roadmap to the discovery of new biomarkers. After selection of potential biomarkers, these are first curated by a single established method (1). Next, an appropriate mode of detection needs to be selected and validated. If the validation is positive, platform design ensues leading to a set of multiple platforms suitable for cross-platform validation of the potential biomarker. Only if each platform individually recognizes the potential marker, it is deemed a suitable new biomarker, which will then undergo further studies in a clinical environment (clinical trials) to ascertain its capability for clinical applications.

validation. Furthermore, the chapter elaborates on the currently low success rate in commercialization of biomarkers. Finally, examples of some successful commercialized biomarkers are included as well.

10.1 Comparison of Different Platforms

There is a plethora of different platforms and technologies used for biomarker identification and analysis. As each platform harbors its own inherent advantages and drawbacks, a combined approach appears more suited to efficiently validate a biomarker. However, transferring a biomarker assay from one method to another might not be a trivial task. Certain limitations exist that demand thorough planning and careful consideration when choosing different platforms for biomarker analysis. Therefore, one has to possess a profound knowledge of

the independent methods, their potential as well as their limitations, and the expected results. Furthermore, one has to choose a suitable method from a panel according to the biomarker studied. As diverse as biomarkers are, the choice of a suitable method vastly influences the outcome of the analysis. Consequently, this section focuses on five different technologies that have been widely used in biomarker identification and analysis: mass spectrometry (MS), enzyme-linked immunosorbent assay (ELISA), surface plasmon resonance imaging (SPRi), reverse phase protein microarrays (RPMAs), and next-generation sequencing (NGS).

10.2 Mass Spectrometry

MS has long been a versatile and reliable platform for analytical tasks. It has significantly risen in prominence over the last two decades with the advent of electron-spray ionization (ESI) [1] and matrix-assisted laser desorption ionization (MALDI) [2], enabling researchers to couple liquid separation techniques, for example, high-performance liquid chromatography (HPLC) to MS. Owing to these soft ionization techniques, analysis of proteins and peptides by MS has been rendered possible. In the wake of these scientific advances and the deciphering of the human genome, a new field of science has emerged: the study of the entirety of the proteins in an individual, fittingly termed proteomics. In recent years MS-based proteomics has skyrocketed in importance, as indicated by the ever-increasing number of publications related to this particular topic.

MS consists of ionization and mass analysis, where ions are separated according to the mass-to-charge ratio. For ionization of proteins ESI and MALDI are available. The former technique produces ions directly from solution; thus it is easily coupled with liquid-based separation techniques such as capillary electrophoresis or HPLC. In ESI, ionization is induced by high voltage that is applied between the emitter at the end of separation and the entry to the mass analyzer. Consequently, an electrically charged spray is formed in a so-called Taylor cone [3] and multiple charged ions are released due to Coulomb explosion. Advances in ESI include micro- and nano-ESI in which flow rates are significantly reduced, thereby improving sensitivity. In contrast, MALDI requires a suitable matrix into which the analyte is embedded. Energy is transferred from a pulsed laser source to the matrix and onto the acidified analyte. The heat input of the laser causes desorption of matrix and $[M+H]^+$ ions of the analyte into the gas phase. The ions created by MALDI are usually single charged. MALDI is very dependent on accurate sample preparation [4]. Analogous to ESI, MALDI techniques have progressed in recent years, for example, matrix-free alternatives SALDI (surface-assisted laser desorption/ionisation) [5], DIOS (desorption ionization on silicon) [6] as well as atmospheric MALDI [7].

Apart from ionization, the heart of each MS instrument is the mass analyzer. The most important analyzers are time-of-flight (TOF), ion trap (IT),

ion cyclotron resonance (ICR), and quadrupoles (Q). Generally, these can be separated into two categories, scanning and ion-beam mass spectrometers such as Q and TOF on the one hand, and IT, Orbi-trap, and ICR as trapping mass spectrometers on the other. In addition, for specific uses, many of these mass analyzers may be combined in different ways leading to a broad spectrum of instruments such as QIT (quadrupole ion trap), Q/TOF (quadrupole-time of flight), TQ (triple quadrupole), and LTQ-FTICR (linear triple quadrapole-Fourier-transform ion cyclotron resonance) among others. An in-depth presentation of all different types of mass analyzers is clearly beyond the scope of this section; however, for more information and a detailed insight, readers ought to refer to [8].

Proteomics and biomarker research are closely linked as many important biomarkers are either cell-surface associated or membrane-bound proteins, secreted proteins in body fluids or glycosylated proteins. Therefore, MS plays an outstanding role in biomarker discovery and validation today. In general, two distinct approaches exist as to the proteomic analysis via MS. These approaches are called bottom-up [9, 10] and top-down [11]. The former has been the more prominently used approach since the implementation of MS-based proteomics, yet the top-down approach is gaining in popularity. In bottom-up proteomics the proteins are first digested by proteolytic enzymes prior to MS analysis, whereas a top-down approach omits the digestion and directly analyzes the proteins via MS. As additional fragmentation occurs during MS/MS analysis, the former approach detects ions of fragmented peptides, while the latter studies ions of fragmented proteins. In the bottom-up approach proteins may first be separated by gel electrophoresis or other suitable methods to reduce the number of proteins within a sample prior to digestion. Alternatively, crude protein extracts may be separated via liquid chromatography and subsequently analyzed, a method referred to as shotgun proteomics. In general, this offers higher sensitivity than a top-down approach as front-end separation is improved due to shorter peptides as compared to large proteins. However, labile post-translational modifications (PTMs) might be lost during proteolytic treatment [12]. Furthermore, redundant peptide sequences are somewhat difficult to map correctly to the corresponding protein. Nevertheless, bottom-up approaches have been extensively used in a variety of applications. Rouillon *et al*. [13] have revealed specific titin fragments as biomarkers of Duchenne muscular dystrophy, and Darville and Sokolowski [14] identified a comprehensive cochlear proteome. Furthermore, Mehus *et al*. [15] have developed a bottom-up MALDI-TOF/TOF-MS-based method to quantify and distinguish different isoforms of human metallothioneins, important regulators of metal homeostasis and believed to be potential biomarkers for different forms of cancer. Moreover, Planque *et al*. [16] have identified a number of lung cancer biomarkers by using a bottom-up approach. In contrast, the top-down method is mainly performed using ESI with FTICR or other ion trapping mass analyzers. In a study published in 2010 by Shen *et al*. [17] the blood peptidome-degradome profile of breast cancer was successfully analyzed via a top-down approach. Other intriguing examples include the structural analysis of intact monoclonal antibodies [18], quantitative analysis of human salivary

gland-derived proteins [19], examination of urine retinol-binding protein 4 [20], a biomarker of the proximal renal tube, and differentiation of autoantibody biomarkers to distinguish ovarian from non-ovarian cancer [21]. Recent studies have combined top-down and bottom-up approaches successfully. Hung *et al*. [22] have applied this to determine disulfide linkages of macin, a family of antimicrobial peptides, while Dekker *et al*. [23] have used it to characterize the antigen-binding fragments of antibodies.

Another important aspect of MS-based biomarker analysis is the emergence of glycoproteomics. Glycosylation of proteins is one of the most frequently encountered PTMs of proteins. Many glycosylated proteins play a pivotal role in diseases, for example, cancer, and inflammatory and degenerative diseases. In fact, metastasis and tumor progression have been linked to altered glycosylation patterns for some time [24, 25]. Thus, glycoproteins have risen to prominence in biomarker discovery and a thorough and adequate analysis is mandatory. In principle, glycoproteomics follows the same venue as regular proteomics and mainly consist of four stages: glycoprotein or glycopeptide enrichment, separation, tandem MS, and data analysis. Proteolytic cleavage is usually applied, as bottom-up approaches are still the predominantly used technique. Cleavage may be performed either prior to or after glycan removal. If quantitative measurements are desired, isotope labeling is needed. One of the most challenging aspects of glycoproteomics research has been the limited number of glycoproteins investigated in parallel. Generally, studies have reported on 20–150 non-redundant glycopeptides derived from no more than 60 glycoproteins [26]; however, in 2013 Medzihradszky and coworkers were able to describe the N- and O-glycosylation in the murine synaptosome leading to a total of 463 linked O-glycopeptides on 122 O-glycoproteins [27]. In a different study, Parker *et al*. [28] examined the global rat brain membrane *N*-glycoproteome, identifying 863 non-redundant *N*-glycopeptides derived from 161 *N*-glycoproteins. These recent studies show the potential and ongoing improvement of MS-based methods. Therefore, it is safe to assume that MS will remain a key technology in discovering and validating biomarkers.

10.3
Enzyme-Linked Immunosorbent Assay

ELISA has long been a standard tool for scientific and diagnostic laboratories worldwide. In principle, ELISA works by immobilizing an analyte from a sample to a surface and applying a specific binding partner, usually an antibody, to the reaction cavity. The binding partner is coupled to an enzyme. This enzyme allows for a detectable signal once the detection solution is added to the cavities. If the enzyme is present, an enzymatic reaction takes place and leads, for instance, to a color change. Over the years, this basic principle has been modified and expanded to create other ELISA approaches, such as sandwich ELISA, indirect ELISA, or competitive ELISA. Owing to its simple handling, low cost and easy read-out it is

still one of the most popular methods used today. However, ELISA has some limitations, especially regarding high-throughput screening or multiplexing. In addition, detection in ELISA relies on the presence of a specific antibody. Although common antigens are easily detected, disease biomarkers are often more complex and more difficult to detect. As Buhimschi *et al*. [29] have shown ELISA failed to identify biomarkers of intra-amniotic inflammation. In fact, it was only possible to distinguish these isoforms using SELDI-MS, an MS-based approach. Moreover, in a study by Wind *et al*. [30] two different commercial ELISA kits were examined. While one allowed for detection of carbonic anhydrase IX, a hypoxia biomarker, the other ELISA kit failed due to influences of metal ions. Despite these shortcomings, ELISA remains an important platform for biomarker validation. As Matsui and colleagues have shown, ELISA is quite capable of detecting the gamma-isoform of 14-3-3 proteins in the cerebrospinal fluid of patients with Creutzfeld – Jacob disease and displays a higher sensitivity than Western Blot, which was used in comparison [31]. ELISA has been successfully established as a platform to detect a broad range of biomarkers including but not limited to the following: 3-phenoxybenzoic acid for exposure to pyrethroid insecticides [32], graft-versus-host disease-associated biomarkers [33], Tau protein for Alzheimer's disease [34], and anti-centromere antibodies for systemic sclerosis [35].

10.4 SPR Imaging

Surface plasmon resonance (SPR) has evolved to be the gold standard for quantitative interaction analysis in the last 20 years. By label-free observation of binding events in real-time, SPR allows characterization of the dynamics, that is, association/dissociation rate constants, of an interaction beyond the parameters obtained by steady-state analysis (e.g., ELISA). In addition, it has played an important role in the examination of thermodynamic parameters since commercially available devices with the ability to accurately modify the temperature during the measurement have appeared. Besides the classical SPR to analyze a single interaction, modern SPRi techniques that combine SPR and microarrays have become available.

While the emergence of SPR is directly and solely related with Biacore, the company who first sold SPR devices, there are several devices for SPRi on the market. They allow detection of the interaction of analytes with several hundred (Biacore FlexChip, HORIBA SPRi-Plex, IBIS *i*SPR) to several thousand (Lumera Proteomic Processor, Graffinity Plasmon Imager) ligands immobilized within the microarray spots. These systems are equipped with only one flow cell for the injection of one analyte per run. BIO-RAD offers another system (ProteOn XPR36) featuring six flow cells and six lines of different ligands immobilized, enabling the characterization of up to 36 interactions simultaneously. Beside the advantages of classical SPR, SPRi facilitates ligand-saving by immobilization in small spots (diameter 50 – 500 µm) and analyte-saving by detecting the interaction with

hundreds or thousands of ligands within one injection. It was also proved for the characterization of all relevant interaction partners, including ions, oligosaccharides, peptides, proteins, DNA, and whole cells. For the purpose of biomarker characterization and identification, SPRi opens up to multiparametric analysis, fast and efficient ligand screening, recognition of binding patterns, and the extension of analysis criteria due to the accession of binding kinetics.

In an outstanding example for the use of SPRi for the detection of biomarkers, Brakha and coworkers developed an SPRi peptide array, consisting of 56 peptides covering the whole hepatitis C virus (HCV) proteome. This renders the identification of anti-HCV antibodies in sera and the correlation of binding patterns to the different peptides with the disease state possible (DSP) [36]. Ladd *et al*. [37] established an SPRi-based antibody array for the detection of different cancer biomarkers, for example, the activated leukocyte cell adhesion molecule ALCAM and transgelin-2 (TAGLN-2). The same approach was followed by Piliarik *et al*., who demonstrated an antibody array capable of parallel detection of cancer markers, namely, human chorionic gonadotropin (hCG) and ALCAM [38].

In order to identify new protein biomarkers, the combination of SPRi and MALDI-MS, called Supra-MS, is a promising tool. Depending on the ligands, different biomarkers can be caught on the surfaces, their binding parameters characterized using SPRi, and subsequently identified using MS. The proof-of-concept for this approach was shown for a breast cancer marker LAG3 [39].

One major drawback in biomarker detection by SPRi is the appearance of nonspecific binding, although some studies have successfully used undiluted sera or blood samples [40]. Several efforts to enhance the detection limit of SPRi were performed, which is intrinsically restricted due to the label-free measurement and missing signal amplification. The implementation of SPRi into microsystems allowed for an intrinsic calibration and reduction of the limit of detection for the ovarian cancer marker r-PAX8 [41]. In addition, there are methods to increase the amount of specifically adsorbed mass corresponding to a signal enhancement, for example, by additional reductive gold deposition [42], the use of quantum dots [43], or on-spot polymerization [44].

Nevertheless, the most promising applications for SPRi within the identification of new biomarkers are still under development. The most important challenges so far are ligand design and the implementation of bioinformatic analytical tools.

10.5
Reverse Phase Protein Microarrays

RPMAs [45] represent a versatile tool in researching protein–protein interactions. Therefore, they form a useful method for biomarker discovery and validation. The general setup of RPMAs is simple. Cells of interest are cultivated, harvested, and lyzed. Next, the cell lysate is applied to a microarray surface in a predetermined pattern. Proteins stick to the surface via adsorption to

nitrocellulose or PVDF (polyvinylidene difluoride) membranes. After blocking the microarray to reduce nonspecific binding, primary antibody reactive to, for example, a biomarker of interest, is added. Subsequently, fluorescently labeled secondary antibodies are applied and fluorescent signals detected. Thus, specific biomarkers can be detected from various samples. One of the main advantages of RPMAs is their high-throughput capability allowing for hundreds and thousands of samples to be processed simultaneously. In addition, sensitivity of microarrays is high, especially in comparison to ELISA, which often requires micrograms of protein, whereas picograms or less suffices for microarray detection. Another advantage of RPMAs compared to more traditional forward phase microarrays is the reduction of antibodies needed. The latter approach is designed as a "sandwich," thus demanding a first antibody to capture the antigen on the microarray surface and a second antibody binding to a different epitope of the said antigen as a detection antibody. In RPMAs only one specific antibody is needed. Finally, as RPMAs focus on proteins rather than nucleic acids, it is suitable for analyzing PTMs [46]. In contrast, RPMAs also feature some drawbacks and limitations that need to be carefully considered when applying the technique to biomarker validation. First and foremost, RPMAs rely predominantly on suitable antibodies. While more and more antibodies are generated, the general limitation remains as monoclonal antibody production is a time-consuming step. Furthermore, the mere existence of an antibody reactive to a desired target biomarker is not sufficient. Instead, thorough validation of this antibody needs to be performed prior to microarray analysis. This is usually achieved by Western Blot. Another issue normally encountered with microarrays is inter- and intra-slide reproducibility that needs to be carefully observed in order to provide the highest level of confidence and reliability. Despite these limitations, once a validated system is in place, it may be successfully used for high-throughput screenings of a vast range of samples. In recent years, RPMAs have been transferred successfully from a mere scientific tool to an integral part of preclinical trials including the assessment of heterogeneous tissue samples [47–49], cells [50–52], and serum [53–55]. Furthermore, RPMAs have been included in several clinical trials, for example, [56].

Owing to this widespread applicability of RPMAs in biomarker validation procedures within a clinical environment, efforts have been intensified to develop more improved data assessment tools and strategies for microarray normalization and analysis [57].

10.6 Next-Generation Sequencing (NGS)

DNA sequencing has taken a giant leap forward in recent years. Thirty years after Sanger [58, 59] and the cooperative effort of Maxam and Gilbert [60], DNA sequencing has taken the next step in what was adequately termed second-generation or next-generation sequencing. Different technologies and

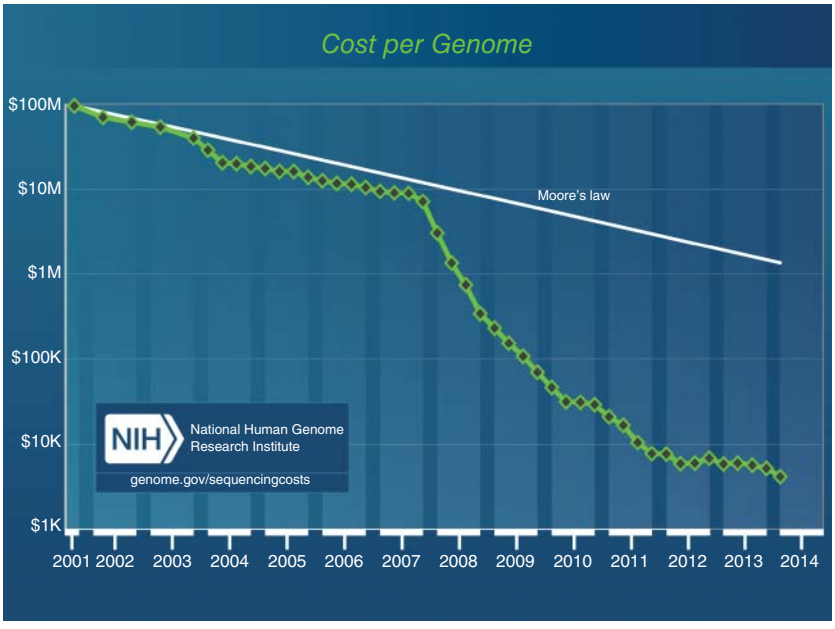

Figure 10.2 Sequencing cost per genome. The green line depicts the average sequencing cost per genome. From 2007 onward, costs per genome has significantly dropped below the projected cost according to Moore's law. Consequently, in 2014, the cost per genome equals less than $10 000. Figure taken with kind permission from Ref. [61]

approaches have been developed in recent years. The advent of single molecule sequencing technologies has heralded the third generation of DNA sequencing. The performance of NGS is outstanding, consequently magnifying the number of reads possible as compared to the traditional DNA sequencing techniques. Combined with a more potent computer technology to assess the huge amount of data, prices for whole genome sequencing projects have plummeted. In fact, in 2014, the average cost for a whole genome sequencing is less than $10 000, a significant drop from that in 2001, when sequencing an entire genome cost approximately $100 million; see Figure 10.2 [61].

Owing to its overwhelming success, a lot of research is performed to continuously enhance NGS and to create novel techniques. Currently, there are four to five main solutions available: 454 Pyrosequencing [62], Illumina sequencing [63], SoLiD sequencing [64], Ion Torrent [65], and single molecule sequencing [66]. Pyrosequencing, first described by Ronaghi and Nyren in 1996, is a method based on sequencing by synthesis [67]. During synthesis of a DNA strand the incorporation of a new deoxynucleotide occurs in conjunction with the release of pyrophosphate. This pyrophosphate is a substrate for ATP (adenosine triphosphate) sulfurylase, which converts the pyrophosphate into ATP. Consequently, the produced ATP drives the enzymatic reaction of luciferase converting luciferin to oxyluciferin under emission of light. This emitted light can be detected and

is directly proportional to the incorporation of the nucleotide. Finally, apyrase degrades ATP and unincorporated nucleotides to reset the reaction, so that it may be repeated with a new nucleotide.

Another example of sequencing by synthesis is Ion Torrent. In this approach, it is not the pyrophosphate but rather hydrogen release that is measured during the synthesis of a nascent DNA strand by addition of one deoxynucleotide. In contrast to other methods, Ion Torrent does not require modified nucleotides or complex optics in order to sequence DNA. Rather, a pH change is detected by the hydrogen release if the nucleotide is complementary to the template strand. In the case of homopolymeric repeats, a higher electronic signal, as more hydrogen is released, is detected. This may, however, impair detection and differentiation of long homopolymeric repeats as it is difficult to distinguish signals derived from eight or nine identical nucleotides.

Illumina dye sequencing is a third approach and an example of sequencing by synthesis. In this case, DNA molecules are attached to primers on slides and amplified to create local colonies, termed DNA clusters, by addition of the corresponding nucleotide. Reactions are performed with a mixture of all four nucleotides, each modified by a different fluorescent dye and containing a blocking group. Having all four nucleotides present at the same time maintains a competition for the binding, thus reducing a sequencing bias. Once a nucleotide has successfully bound to the nascent DNA strand and the unbound fractions have been removed, the base is detected by its specific dye and the blocking group subsequently removed to initiate the next sequencing cycle. The Illumina sequencing technology has proved to be very reliable and accurate. Therefore, the method has been applied to a plethora of sequencing tasks.

Apart from sequencing by synthesis, the sequencing by oligonucleotide ligation and detection, abbreviated as SoLiD, follows a different path in which DNA ligase and not polymerase is pivotal. Clonal bead populations are created via initial library construction and emulsion PCR (polymerase chain reaction). These clonal beads harbor multiple copies of a single template DNA per bead. The DNA is attached to the beads by an adapter sequence. Afterwards, beads are deposited via a 3′ modification enabling covalent attachment to a slide surface. For sequencing, primers hybridize to the adapter sequence and four fluorescently labeled dibase probes are added to the reaction. These probes will compete for the ligation reaction. Specificity is achieved by interrogating the first two bases. After cleavage of the fluorophore, a new dibase probe attaches and two more bases are analyzed. Upon completion of several sequencing cycles, the product is removed and the reaction is repeated with a different primer, complementary to the $n-1$ position. Following five rounds of primer resetting, the information from each sequencing reaction is collected and combined. Because of this approach, each base is read at least twice by two independent ligation reactions. Consequently, SoLiD offers high accuracy.

Finally, Single-Molecule Real-Time Sequencing (SMRT) was developed by Pacific Biosciences and is usually considered to be a third-generation sequencing technology. It is based on zero-mode waveguide (ZMW) [68], which is an optical

waveguide capable of directing light into a volume that is smaller than the wavelength of the light. Consequently, ZMW is applied to zeptoliter volumes. In SMRT, a single DNA polymerase molecule is immobilized at the bottom of a ZMW. A single DNA molecule is used as a template. Owing to the ZMW, it is possible to detect the incorporation of a single nucleotide or base into the DNA strand. Each nucleotide is labeled by a different fluorescent dye. The dye is cleaved off during incorporation of the base and subsequently leaves the ZMW. This loss of fluorescence can be detected. One of the key advantages of SMRT is its exceptional read length covering several thousand bases [69, 70]. Throughput of SMRT chips, which contain hundreds of thousands of ZMWs, is approximately 400 Mb.

Compared to the other methods of biomarker discovery and validation in this section, NGS focus on detection of DNA rather than proteins, which are often important sought-after biomarkers. Nevertheless, the performance and capability of NGS justifies mentioning of this method in detail. A number of intriguing biomarkers have been successfully discovered and studied by NGS. Foremost, microRNA (miRNA) has been acknowledged as an important disease-associated biomarker. Thus miRNA profiling has become an important task in understanding certain diseases and in assessing the status of a disease. This has been especially true in detecting certain types of cancer, as Zhang *et al*. [71] have shown by profiling gene mutations in a colorectal tumor by NGS, whereas Wu and colleagues have used this technology to examine breast cancer [72]. Furthermore, breast ductal carcinoma has been studied by Kaur *et al*. [73] and important molecular markers unveiled by NGS. Other forms of cancer discovery might also benefit from NGS [74]. Not only miRNA might be of interest, as a recent study has focused also on small nucleolar RNA (snoRNA) and tRNA [75]. Despite the supposed limitation of NGS to nucleic acids, Darmanis and coworkers have just recently adapted NGS as a detection technique for multiplex proximity ligation assays, essentially enabling NGS to detect proteins rather than nucleic acids [76].This is an outstanding example of the ongoing development and improvement of this fairly new technique. Therefore, more advances and new applications will continue to appear rendering NGS as one of the most important tools in molecular biology and potentially in biomarker discovery.

10.7
Still a Struggle: Achieving Clinical Trial Status

The prevalent issue in biomarker discovery is still the transfer from the laboratory to the clinic. While many biomarkers have been initially discovered through some of the methods mentioned here, only a select few have ever reached the state of clinical trials. And once they reach that stage, there is no guarantee for a commercial product. Rather, another high percentage of biomarker candidates fail the clinical trials leading to a very small number of commercially successful biomarkers. What is the reason for this discrepancy? Are so many of the initially identified

biomarkers bogus? Probably not! Still, the challenge to validate a biomarker that was discovered using one particular method is difficult at best. While transferring the biomarker to a different platform seems to be a good and valid approach to verify the capability of this potential biomarker, the devil is in the details. In most cases, pre-analytical variations occur abundantly. This affects sample collection, processing, and storage in numerous ways. The collection device for a certain type of sample, for example, blood, may differ. Moreover, sample processing is a crucial step that is often overlooked. Factors such as intrinsic proteolysis, autolysis, the timely addition of protease inhibitors, the overall handling time and experimental parameters, correct dilution of samples, uniform centrifugation conditions, and many more may have a lasting effect on the outcome of the biomarker experiments [77]. Consequently, it is essential for cross-platform validation to standardize these operations and reduce variations caused by sample processing to an absolute minimum. This also applies to shipping and storage conditions such as temperature, time elapsed prior to freezing, or number of freeze/thaw cycles, as the biomarker under scrutiny may well be negatively affected by variations of these parameters [78]. In order to reduce the influence of these factors, standard operating procedures (SOPs) need to be implemented and utmost attention has to be paid to the specific details prior to biomarker testing. Hence, a number of standardized platforms such as MIAME (Minimum Information About a Microarray Experiment) [79], HUPO-PSI (Human Proteome Organisation-Proteomics Standards Initiative) [80], and MIAPE (Minimum Information About a Proteomics Experiment) [81] have been initiated in recent years. Although differences in sample treatment might adversely affect cross-platform validation, it is far from the only issue that needs to be dealt with. Instead, inherent biological diversity and disease heterogeneity pose a formidable challenge to biomarker validation. Therefore, it is of utmost importance to choose an adequate cohort size and to carefully monitor the sociodemographic background of the participants, for example, age, gender, diet, lifestyle, ethnicity, and so on. Those parameters are paramount in assessing the suitability of a potential biomarker. Hence, a well-designed control system with rigorous inclusion and exclusion criteria is mandatory. In addition, diseases may present different clinical manifestations in individuals. This disease heterogeneity needs to be considered when dealing with biomarker validation. Neglecting genetic and proteomic variation of individuals is a major pitfall in cross-platform and biomarker validation. This necessitates a collective study of disease heterogeneity and improved data sharing and bioinformatic analysis of population-associated parameters. It is hard to fathom a way to standardize these variations; however, by gaining a deeper insight, it may be possible to estimate the influence and consequently to improve prediction of potential ramifications. Still, other issues prevail that may be easily reduced if appropriate care is taken. Data analysis is another crucial step in biomarker validation. Even if all the prerequisites are identical, the results will differ, presumably significantly, if inadequate data acquisition and analysis is performed [82]. Consequently, many biomarkers fail to stand the test of clinical trials despite being considered significant in initial discovery phase. Hernandéz *et al*. [83] have pointed out that the abundance of this

phenomenon might well be associated with model overfitting, excessive prefiltering, and incorrect cross-validation. One of the immanent issues with prefiltering, which is predominantly performed by ANOVA (analysis of variance), is as follows: the underlying data set or sample cohort is used twice, once to choose a prefiltered subset and then again to build the classification model. Consequently, if a different sample cohort is applied to the model, the results differ and will not give a realistic prediction of the accuracy of the said model. It has also been shown in other instances that prefiltering implies a bias regarding the predictive ability of classification algorithms [84, 85], caused by the advantage of the prefiltered variables to appear strongly associated with the response [86]. Therefore, Hernandéz *et al*. draw the conclusion that larger sample sizes in proteomic biomarker discovery are essential for future success.

In addition, variations caused by different platforms need to be treated with extreme caution as platforms vary in sensitivity and dynamic range. Furthermore, approaches implemented in biomarker discovery are often not suited for validation [87]. In order to reduce these errors, copious sharing of scientific data acquired from research groups around the globe is integral, and robust MS-based methods are beneficial in comparing results.

Despite various obstacles for a quick and efficient biomarker discovery and validation route, a number of commercially available tests have sprung up in recent years. The next part of this chapter gives some examples of successful commercialization of biomarker assays.

10.8 Commercial Biomarker Assays

While initial biomarker discovery has blossomed in recent years, only a select few have actually reached the clinical stage. Apart from the potential reasons elaborated on earlier in this chapter, researchers often lack the knowledge about analytical, diagnostic, and regulatory requirements associated with biomarker implementation in clinics [88]. As an example of the difficulties in biomarker implementation into the clinics, only five single immunohistochemical biomarkers have been approved by the FDA with the latest approval occurring in 2005, roughly a decade ago. These biomarkers – p63 protein, c-Kit (CD117), estrogen receptor (ER), progesterone receptor (PR), and HER-2/neu – are used in single biomarker detection devices. Since then, a plethora of potential single cancer biomarkers have been discovered, yet none has garnered FDA approval. Nowadays, modern assays and commercially available tests preferably use a biomarker panel rather than a single one. MammaPrint, Oncotype DX, Mammostrat, and IHC4 are examples of multiparameter assays for breast cancer. MammaPrint is a microarray-based multigene assay that offers a prognostic evaluation of recurrence of breast cancer in women with ER-positive or -negative, lymph-node-negative breast cancer. It uses a panel of 70 genes to assess a risk of recurrence score to help determine whether chemotherapy is advisable to

reduce the risk of cancer recurrence. In a similar approach, the Oncotype DX uses 21 genes to assess the risk of early-stage breast cancer recurring in patients. Both tests use gene expression to calculate a risk score. Oncotype DX has been used with over 300 000 patients worldwide and has led to a change of therapy in roughly 30% of all cases. In contrast, Mammostrat and IHC4 are based on immunohistochemical biomarkers detecting a panel of biomarkers each. While Mammostrat focuses on p53, HTF9C, CEACAM5, NDRG1, and SLC7A5, IHC4 as the name suggests uses four different biomarkers – ER, PR, HER2, and Ki-67. Acs *et al*. have compared a genomic assay, Oncotype DX, with an IHC-based approach, Mammostrat. In this study, the latter assay revealed a better estimation of tumor behavior within low-grade breast carcinomas [89].

Apart from biomarker assays targeting breast carcinomas and their recurrence risk, other biomarker assays have received FDA approval. These include more traditional antigen assays to assist in cancer diagnosis such as prostate-specific antigen (PSA) for prostate cancer, carcinoembryonic antigen (CEA) for colon cancer, and alphafetoprotein (AFP) for testicular and liver cancer. In addition, an assay directed at detecting cervical cancer caused by human papillomavirus has been sanctioned by the FDA.

In recent years, especially in the wake of microarrays, qPCR (quantitative polymerase chain reaction), and next-generation-sequencing technologies, the number of multigene assays has risen dramatically. A vast variety of biomarker assays is available that utilizes the specific detection of genes or gene alterations for cancer prognosis, metabolic diseases, sexually transmitted diseases, diabetes, sepsis, diseases of vital organs, for example, central nervous system (CNS), liver, heart, kidney, respiratory tract, and more. Qiagen's GeneRead DNAseq Targeted Panels V2 for cancer diagnostics, Roche's Tina-quant® Cystatin C Gen.2 for diagnosis of chronic kidney disease, and Qiagen's QIAsymphony DSP Virus/Pathogen Kit for detection of viruses and bacteria are only a short list of many biomarker assays that are currently available. Thus, all global players in diagnostic assay development, for example, GE Healthcare, Merck, Thermo Fischer, Roche, Bayer Healthcare, Pfizer, Sanofi, Novartis, and others have a substantial number of biomarker assays within their portfolio. In the coming years, this trend will likely continue to slope upward. The major driving force for biomarker assay development and transfer of novel biomarkers to the market is the expected huge growth in revenue generated by biomarker assays.

In Western Europe alone, the market for drug discovery and development is expected to rise significantly; see Figure 10.3. The growth rate is expected to climb to 17.8% by 2020, resulting in annual revenue of $3.5 billion compared to $1.3 billion in 2010. Thus, the compound annual growth rate from 2013 to 2020 is 13.8%.

The major market share (42%) is held by genomics-based approaches including NGS followed by proteomics with 34% as summarized in Figure 10.4. Unsurprisingly, the resulting compound annual growth rates of genomics and proteomics-based methods is somewhat above the overall average number of 13.8% with 16 and 14.4%, respectively [90].

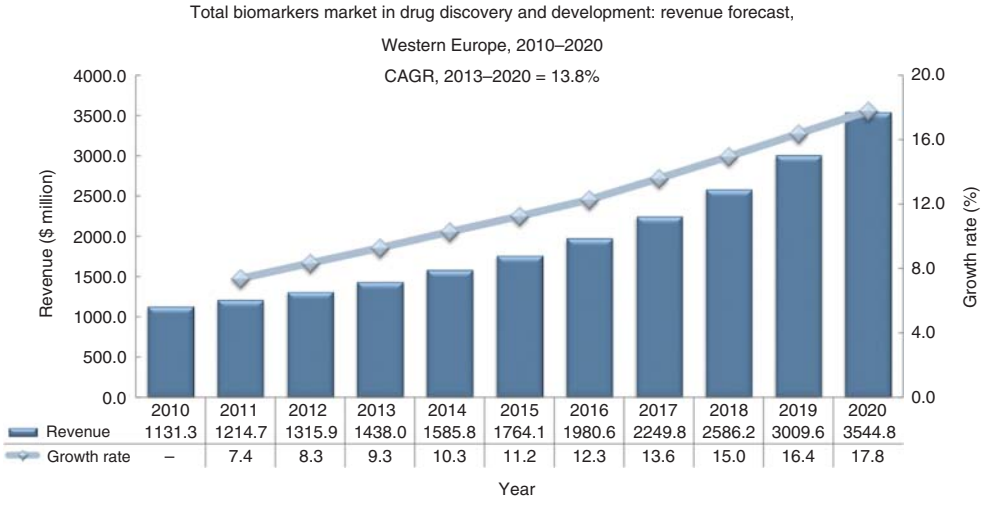

Figure 10.3 Total biomarkers market in drug discovery and development, revenue forecast, Western Europe, 2010–2020. The revenue will rise from $1.4 billion in 2013 to more than $3.5 billion in 2020, equal to a compound annual growth rate of 13.8%. Figure taken with kind permission from Ref. [90].

10.9 Quo Vadis, Biomarker Assays?

The outlook for biomarker research, discovery, and validation is favorable. Despite the issues raised in this chapter regarding biomarker discovery, the inherent

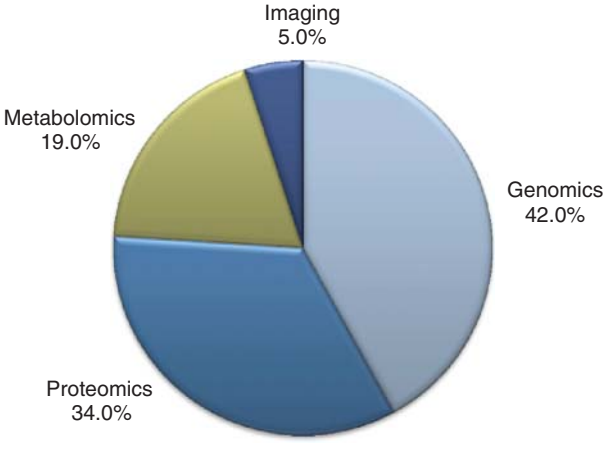

Figure 10.4 Total biomarkers market in drug discovery and development – percentage sales breakdown, Western Europe, 2013. Genomics holds the major share with 42%, followed by proteomics with 34%. So far, imaging accounts for only 5% of sales. Figure taken with kind permission from Ref. [90].

issues with platform comparison and the inconsistencies in validation, biomarker discovery will remain a preferable research area. As new techniques emerge that allow widening the spectrum of biomarkers accessible for identification, this research will continue to be a prominent player in the current landscape of life sciences and medicine. Especially, NGS technologies will continue to grow in prominence. Furthermore, proteomics-based biomarker assays, while not redundant, will face the challenge of competing with an ever-increasing spectrum of multigene and multiparameteric assays that are available by sequencing. Nevertheless, MS and other proteomic technologies ought to remain important pillars in biomarker discovery. Still, more traditional assays, such as ELISA and microarrays, will face even tougher competition and might be reduced to laboratory and scientific applications, while playing only second fiddle in clinical and commercial assays. Another key aspect to the future of biomarker assays is the growing awareness of personalized medicine. This calls for even more specific assays, adapted to meet the optimal requirements for each individual patient. While this might still be a few years off and raises many questions, not only scientific but of ethical, religious, and social nature, that need to be

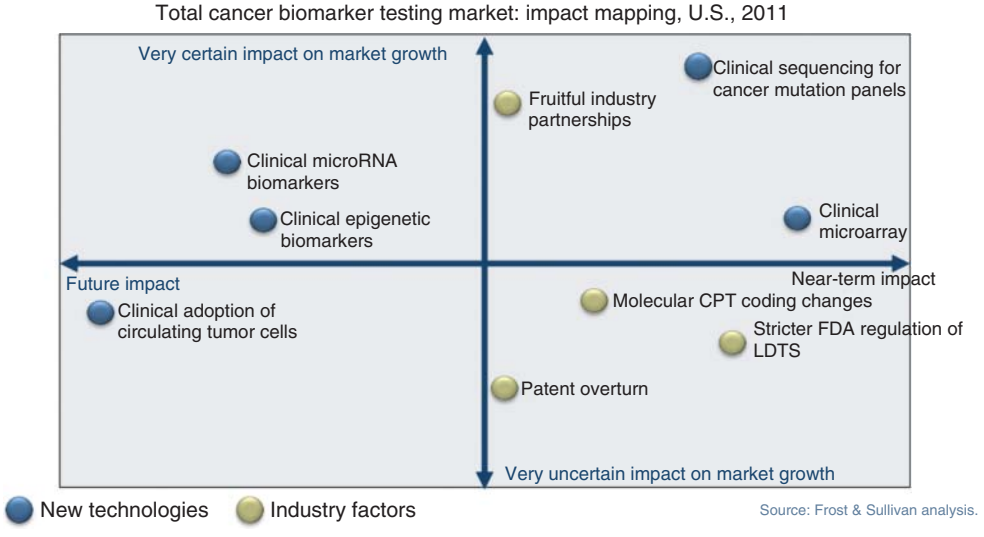

Figure 10.5 Impact mapping of the total cancer biomarker testing market in the United States in 2011. Technologies are depicted as blue circles; brown circles indicate industry factors. Circles to the left are predicted to have a future impact, while those at the very right have a more immediate impact. The probability of impact on market growth is indicated by the horizontal position. Circles near the bottom are highly unlikely to garner market growth, whereas circles on the top of the figure are extremely certain to impact market growth. Regarding new technologies, sequencing ought to have a high impact in the near future, while miRNA, epigenetics, and circulating tumor cells are predicted to have an impact in the future. Figure taken with kind permission from Ref. [91].

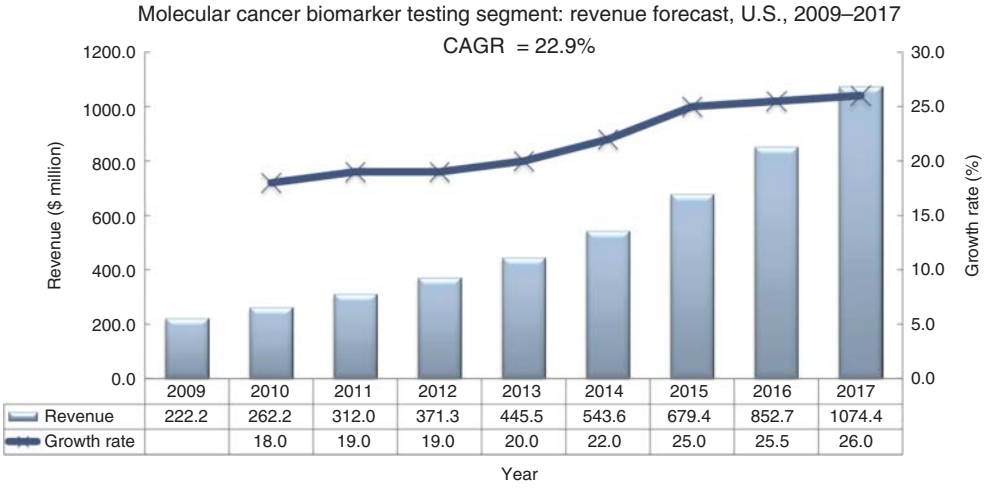

Figure 10.6 Molecular cancer biomarker testing segment, revenue forecast, United States, 2009–2017. The compound annual growth rate is 22.9%, resulting in an increase of revenue from $222 million in 2009 to more than $1 billion in 2017. Figure taken with kind permission from Ref. [91].

answered, sequencing technologies, molecular diagnostics, circulating tumor cells, miRNA biomarkers, and epigenetic assays are prime candidates for personalized assays. Therefore, it is highly plausible to envision their rising importance in the biomarker market; see Figure 10.5.

Moreover, double-digit growth of the molecular diagnostic biomarker market for cancer testing in the United States in the coming years is expected; see Figure 10.6. Consequently, molecular cancer biomarker testing will conquer a much larger overall market share in cancer testing, rising from 3.1% in 2009 to approximately 10% of expected overall revenue of close to $11.5 billion in 2017.

Owing to this potential commercial success and the exorbitant growth rates in the market of biomarker assays, pharmaceutical and biotechnology companies will continue to actively pursue biomarker research. Furthermore, for many scientists the potential to "strike oil" with a successful biomarker discovery will remain a driving force in this research area. The chance at one's own bonanza will keep biomarker research as vivid as ever.

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11
Clinical Validation

Mads Almose Røpke

11.1
Introduction

Drug candidates entering clinical testing only have a 10% chance of resulting in a marketed product [1] and only 5% of basic science discoveries of clinical relevance are successfully translated into approved agents [2]. This is primarily a result of a poor success rate of clinical candidates, especially in phase 2 and 3 of development [3]. The objective of experimental medicine and the use of biomarkers is to increase efficiency of the drug development process by providing information on mechanisms of action, dose response, and early reads of efficacy and safety, thereby improving the quality and speed of decision-making (de-prioritizing failures and accelerating winners). With the ever-increasing mechanistic insight into disease processes and treatment responses, the potential also increases for the measurement of specific biological corollaries of the disease or therapy. For example, blood and urine glucose have been measurable for centuries and are the only markers of an underlying disease, that is, diabetes. These days, most diseases have known biological readouts and some of them may be useful as biomarkers for drug discovery and development purposes. During drug development, biomarkers may be utilized as discovery tools, as pharmacodynamic (PD) markers of drug mechanism or efficacy both preclinically and in early phase trials, and as predictive indices of patient response in late phase trials [4, 5]. As biomarkers reflect the biology or the progression of disease and/or the effect of drug treatment, information provided by properly selected biomarkers can greatly influence go/no go decisions. Biomarkers may also provide a diagnostic readout or be prognostic or predictive of disease or therapeutic outcome [6]. The challenge is to identify relevant biomarkers early enough to implement them for decision-making at the critical stages of the R&D process. Assay characterization remains a critical component in biomarker qualification and often biomarkers can fail not because of the underlying science, but because of poor choice of assay and lack of validation [7]. Nevertheless, proper incorporation of biomarkers into clinical trials has the potential to guide and accelerate the pace of development of new drugs.

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11.2 Classification of Biomarkers

Biomarkers, broadly defined, can be a variety of quantitatively measured indicators of biological or pathophysiological processes, or the response to therapeutic intervention, including molecular entities, images, or other measured activities or properties, alone or in combination. Different types of biomarkers are briefly described below.

- *Target biomarkers* specifically demonstrate the interaction between the drug and its molecular target. This can be in the form of binding to the target or a very proximal molecular consequence to the target induced by the drug.
- *Pharmacodynamic (PD) biomarkers* are markers that predict the molecular consequences of target engagement or modulation by the candidate drug. PD biomarkers may measure effects that are proximal in the biochemical pathway modified by the manipulated drug target or more distant consequences such effects *in vivo*. Effect on the PD marker suggests that the drug target is modulated by the drug to an extent sufficient to generate a biological response. However, PD biomarkers do not need to be linked to the intended mechanism of action and as such effect of a drug on a PD marker is no guarantee that the drug target is involved in the pathogenesis of the disease or that the drug will produce the desired therapeutic response.
- *Disease biomarkers* are markers that correlate the disease phenotype for which the drug is developed. Disease marker expression levels should reflect the disease state (initiation, progression, regression, or relapse) and thus respond to effective therapy. Ideally, disease markers may be translational and also be sensitive to the corresponding pathologic phenotype in the preclinical animal models. Disease biomarkers are specific to the indication but not to the target. Therefore, they can be relevant for development of different classes of drugs within the same indication.
- *Toxicology biomarkers* are biomarkers that predict or translate into a clinically relevant adverse outcome and are used to monitor drug-induced toxicity. The use of toxicology biomarkers in preclinical toxicological studies can shift attrition of failing molecules upstream in discovery and avoid spending resources on molecules with development-limiting liabilities. It should be noted that inclusion of exploratory tox markers presents a risk of generating false positive data that may raise concern about the safety of a lead candidate.
- *Surrogate endpoints* are biomarkers that are intended to substitute for, or be a supplement to, a clinical endpoint. A surrogate endpoint is expected to predict clinical response, based on epidemiologic, therapeutic, pathophysiologic, or other scientific evidence. The use of surrogate markers is especially useful in cases where documentation of treatment effect on the clinical endpoint will require a very long and expensive clinical study. An example of this is the use of bone density measurements as a surrogate endpoint for fracture rate.
- *Stratification biomarkers* can be divided into predictive and prognostic biomarkers. Predictive biomarkers are biomarkers that predict the response to

treatment and therefore can be used to select patients who will respond and exclude those who will not. Such a biomarker could be the expression level of the drug target, functional variants in the gene that encodes the drug target (or its receptor if the target is a ligand), or a gene or protein expression profile that correlates to treatment outcome of the specific treatment. Prognostic biomarkers classify patients treated with standard therapies (including no treatment if that is standard) into subgroups with distinct expected clinical outcomes, and this information can have therapeutic implications. For example, if a prognostic biomarker can identify a group of patients with very low risk of recurrence, additional treatment might not be considered, whereas higher risk patients would be treated.

11.3 Translational Use of Biomarkers

An important feature of biomarkers is their role in characterization and validation of preclinical models of human disease. Disease models should manifest the major aspects of human disease and this should be reflected in the development of the key clinical signs that occur in humans and also in the pathogenesis that leads to these clinical signs. In addition to the clinical characteristics of a particular animal model the molecular signature of the disease phenotype should correlate with that of human samples obtained from patients. Once such a translation has been established, key markers from such a profile can then be used at the discovery stage for drug candidate selection and modeling of pharmacokinetic (PK) and PD data and later transferred into the human clinical setting. As an example, the cytokine IL-6 has been identified in preclinical animal models as a major driver in the pathogenesis of autoimmune diseases such as rheumatoid arthritis (RA) [8, 9]. The finding that increased levels of these cytokines circulate in mice with RA and that these levels correlate with the severity of disease in these animals identified TNFa and IL-6 as potential biomarkers for human disease. Furthermore, the fact that these cytokines decrease on immunosuppressive therapies confirmed their potential as biomarkers for the testing of novel drugs for RA in the clinic [10].

Biomarkers typically have a time course that is different from that of the clinical endpoints but more directly related to the time course of drug concentration. After establishing a correlation between the drug concentration and biomarker in the preclinical model, PK/PD modeling may be performed to gain insights into the effect of drug concentrations on the biomarker as a function of time. Although this concept is relatively easily applied to blood-based markers it is more challenging to implement when the relevant markers are only expressed in other organs or solid tumors where biopsies and tissue extractions are required to generate such data. Another benefit of developing biomarkers based on animal studies is that the controlled laboratory environment provides relatively constant conditions and the animals are typically homogenous and free of many of the complicating comorbidities observed in human patients. Also, in this setting there are more options

regarding the assays available, and the lower variation in matrix allows analytes to be detected with high precision and sensitivity. The drawback of this approach is that many of the biomarkers discovered in animal models cannot be translated into humans. This can be the result of the fact that the animal model does not reflect human disease or that the biomarker does not perform acceptably in heterogeneous patient groups [11, 12].

A way to avoid some of the interspecies issues of preclinical biomarker research is to use xenograft models. Human diseased tissue is transferred to an immune-compromised host where it can be maintained for several weeks during which it can be exposed to a drug, and the treatment response monitored. Human tumor transplant models have been used extensively for cancer drug development, but inflamed tissue from autoimmune diseases can also be transplanted into such models [13, 14].

11.4 Biomarkers in Clinical Studies

During the clinical development phases of a new drug the objectives for using biomarkers changes. In the exploratory phases (phase 1–2a) the biomarkers are primarily used for internal decision-making to confirm target engagement in humans, correlate PK and PD, aid in dose finding, and establish an early link between PD and clinical response. This is particularly useful to build confidence in the project early, help the project progress faster to larger patient studies, and support preclinical programs in the same area (back-translation). As the output will generally not be subject to regulatory review, the extent of method validation can be limited to a few basic components. After clinical proof of concept is achieved, the aim of using biomarkers changes. In the confirmatory phase (2b/3) biomarkers are used for disease monitoring (by linking the biomarker more strongly to efficacy and safety and thus clinical outcome) or for patient stratification.

11.4.1 Healthy Volunteer Studies

First-in-man studies in healthy volunteers are traditionally used to determine safety and tolerability and evaluate PK of a new drug. However, if the drug target is expressed, or can be induced in non-diseased individuals, target engagement/PD effects may be demonstrated already in phase 1. A positive PD marker response may be sufficient to make a go/no go decision in the program, and linkage to disease or clinical outcome may not be necessary. Furthermore, biomarker data can support dose selection for the subsequent phase 2 study after modeling of biomarker responses with pharmacokinetic data. In some cases, the drug induces a perturbation of the biomarker from its baseline level in healthy subjects (e.g., cytokine or growth factor agonists). An example of this

is the increase in neutrophil counts used to evaluate the pharmacodynamics of a novel recombinant G-CSF molecule [15]. When evaluating the PD effects of antagonistic compounds in healthy subjects it will often be necessary to activate the targeted pathway to induce a relevant biomarker signal to be able to observe an effect of the drug. This can be done *in vivo*, for example, by using small and local doses of pro-inflammatory agents. Lipopolysaccharide challenges of the lung of healthy volunteers via inhalation have been used as a model to test the anti-inflammatory activity of investigational new drugs early in development [16, 17]. Experimentally induced skin inflammation is also used as a model to evaluate the effect of anti-inflammatory agents as reactions are easily monitored and samples for biomarker analysis readily obtained from the skin [18, 19]. Alternatively, target activation can be induced *ex vivo* in blood samples from subjects dosed with the experimental compound in a phase 1 study. The pharmacodynamics of the Janus kinase (JAK) inhibitor INCB 18424 was evaluated in phase 1 by measuring the level of phosphorylated signal transducer and activator of transcription (STAT)3 in peripheral blood cells after stimulation *ex vivo* with IL-6 to activate the JAK/STAT pathway [20]. Compared to inducing inflammation in healthy subjects the *ex vivo* approach is less inconvenient for the subjects and easier to control. The drawback is that it merely confirms the presence and activity of the compound in the central compartment and does not demonstrate distribution to other target organs.

The number of subjects to be enrolled in such experimental clinical studies driven by biomarker readouts in healthy volunteers will be based on several factors. These include expected response in the biomarker(s), expression level, and dynamic range of the marker, assay performance (precision), comparator, budget, and the required level of significance. For example, detecting the positive effect of a compound on a biomarker in a placebo-controlled study will typically require fewer subjects than are needed to identify the most active compound in a group of drug candidates tested.

11.4.2 Early Patient Studies

For projects in early development, lack of efficacy in phase 2 is a major contributor to the overall failure rate [21]. Efficacy failures often occur from one of two major reasons: either the investigational agent did not achieve the required pharmacology or the mechanism targeted by the investigational agent did not significantly contribute to the disease in the tested patient population. The use of biomarkers in the early trials in patients addresses both these problems by providing early evidence of the pharmacological effect as well as the effect on disease parameters. Furthermore, the first studies in patients play a key role in the identification and validation of clinical biomarkers. Early studies of an exploratory nature often offer a possibility to collect biological samples more readily than larger trials and are therefore well suited to analyze biomarker responses to drug and to compare these to the clinical phenotype. Positive correlation between target biomarker response

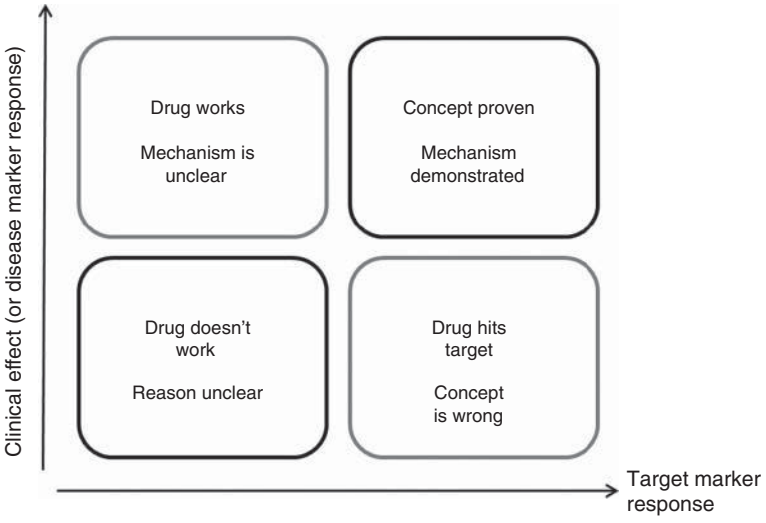

Figure 11.1 Outcome scenarios from a clinical study incorporating target biomarker and clinical response (or disease biomarker assessment). Traditionally, proof-of-concept studies are successful when the experimental drug has shown clinical effect in patients. However, only when target engagement is demonstrated can the mechanism of action be confirmed. With the lack of clinical effect in a patient study does not occur the project is typically stopped. However, if modulation of the target the project concept can be discarded and further studies on the same target in this indication avoided.

and disease activity is part of proving the project concept. Furthermore, it can be used to generate a translation of drug response between clinical and preclinical studies, which is very useful information for later studies in the same program as well as other programs with the compounds of the same class. In fact, much greater investment can be supported on the basis of multiple uses of biomarkers, particularly those directed at identifying drug candidates that are predestined to fail at earlier time points and lower cost than with conventional endpoints.

Lack of effect on a target marker can explain the lack of clinical effect of the drug whereas clinical effectiveness in the absence of target engagement questions the project concept (Figure 11.1). Although modulation of target markers by the drug does not require changes in the disease, target marker levels are often influenced by changes in disease severity. Sampling for target marker expression assessments should therefore be done sufficiently early after dosing to confirm direct action of the drug on the target. Target marker expression analysis at the end of the study, after clinical effect is observed, does not necessarily confirm the drug's mode of action (Figure 11.2).

The use of disease biomarkers in small patient studies increases the likelihood of obtaining proof of concept as they support the clinical score by providing additional efficacy parameters. These markers may even provide earlier signs of effect than the clinical signs. An example is the PoC study of a neutralizing anti-interleukin 17 antibody (Ixekizumab) in plaque psoriasis. The data from this

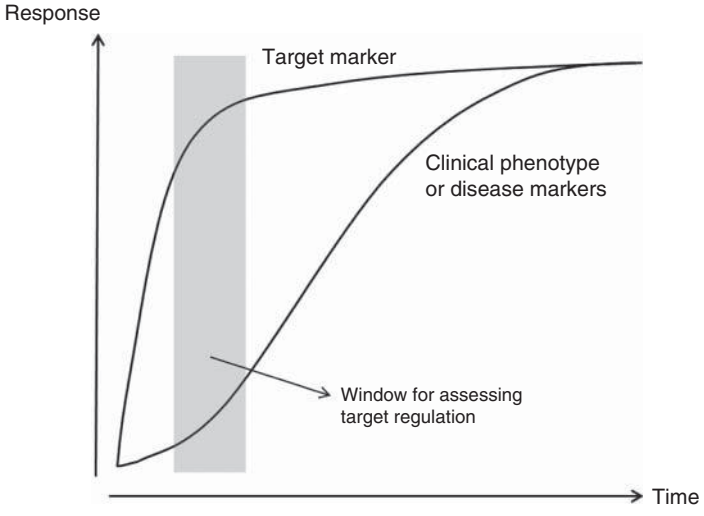

Figure 11.2 Example of response profiles of a target biomarker and the corresponding clinical phenotype or disease biomarker in the same patients. There is typically a time delay between when target regulation and disease modulation can be detected. If biosamples are collected at the appropriate time target engagement can be confirmed without interference from a changed disease phenotype.

biomarker arm of this study showed a clear dose effect, which supported dose selection for subsequent studies. Furthermore, it provided the molecular basis for understanding the surprisingly fast clinical effect of this drug in psoriasis by demonstrating very rapid modulation of key cytokines and epidermal markers in the skin of psoriasis patients [22].

While the search for predictive biomarkers often starts already in the discovery phase the hypotheses on these biomarkers can be tested in phase 2a studies. The criteria for selecting predictive biomarkers can be derived from a hypothesis based on a prospective analysis or from a post hoc evaluation of other treatment studies. Biomarker studies can be conducted where material from randomized clinical phase 3 trials is used in order to conduct a so-called prospective-retrospective biomarker evaluation according to a stringent analysis plan [23]. On the other hand, Beckman *et al.* [24] have suggested casting out a broad net early on for possibly predictive biomarkers, followed by continuous integration of biomarker data into new or ongoing trials and continuous correlation of clinical benefit and biomarker data. Using this data-adaptive approach, by the time the clinical studies reach the phase 2b stage, the best predictive biomarker hypothesis can be chosen and validated in a subsequent study against accepted endpoints for clinical benefit. Within oncology, Carden *et al.* recently suggested a restructuring of the patient selection process to incorporate predictive biomarker assessment as part of the initial workup of the patient before entry into the trial based on molecular evaluation of archival tissue, serum markers, fresh tumor biopsies, circulating tumor cells, or functional imaging [7].

In the case of crizotinib, a small molecule inhibitor of the protein encoded by the anaplastic lymphoma kinase (ALK) gene, the hypothesis behind patient selection in phase 2 was based on a publication identifying a novel fusion between the EML4 and ALK genes that is present in approximately 5% of non-small cell lung cancer (NSCLC) patients [25]. The discovery was published during the early clinical phase of crizotinib and led to selection of patients harboring the fusion gene for the following studies where the drug demonstrated impressive clinical effect. Within 4 years of the initial publication by Soda *et al.* crizotinib was approved for treatment of certain late-stage NSCLC patients whose tumors have ALK fusion genes [26].

Another strategy for patient selection has been employed by Selventa, who has developed a strategy for identifying likely responders for a targeted therapy within a population of patients based on a systems biology approach using molecular profiling data from diseased patients at baseline [27]. The strategy relies upon the hypothesis the target mechanism signalling strength in individual patients determines their response to treatment with that targeted therapeutic. The strength of this method was demonstrated in a group of ulcerative colitis patients where the clinical response to infliximab was predicted by analyzing TNF pathway activation in individual baseline blood samples [27].

11.4.3 Confirmatory Clinical Studies

In the confirmatory phase, biomarkers are used for disease monitoring (by linking the biomarker more strongly to efficacy and safety and thus clinical outcome) or for patient stratification. However, at present, there are few fully qualified biomarkers available to utilize as surrogate markers of efficacy in clinical trials and mainly in diseases where clinical assessment is complicated and requires long studies to achieve traditional clinical endpoints [28–30].

Prognostic biomarkers may or may not be useful for guiding therapy, in contrast to predictive biomarkers, which, by definition, are intended for use in selecting among treatments. If a prognostic biomarker separates patients into subgroups with increasing expected failure risk, and if it can be demonstrated that this separation can improve outcome by indicating more aggressive treatment strategies for the higher risk group (or less aggressive treatment for the lower-risk group), then the prognostic marker has clinical utility in guiding therapy. However, if there are no effective alternative treatment strategies for the high-risk group (or less aggressive treatment strategies for the low-risk group), then the prognostic information is of limited clinical use.

When considering developing a predictive biomarker test to guide therapy decisions, three key factors are necessary: a biological characteristic with the potential to induce differential patient responses to a therapy, multiple therapeutic options with different responses, and an appropriate clinical biomarker that can link therapies to a subset of patients that are more likely to show that different response. Furthermore, the project team should be aware of the large

challenges related to the development of a companion diagnostic test for patient selection. These include more complex (and potentially larger) clinical studies to validate the tool, a complex regulatory approval process, and additional commercialization requirements to market both drug and diagnostic. In general, retrospective analyses of data from clinical studies with randomized parallel group designs can be useful to identify biomarkers that include patients with both high and low values or levels of the biomarkers. However, conventional randomized trial designs only allow for estimation of the average treatment effect in the overall study population, and therefore, alternative designs must be considered to evaluate biomarker-guided therapy. Fundamentally, there are two types of phase 3 designs involving biomarkers: the biomarker-enrichment design and the biomarker-stratified design [31].

11.4.4 Enrichment Design

The choice of appropriate randomized clinical trial design depends on the strength of the preliminary data for the biomarker. If there is compelling evidence that the potential benefit from a new therapy is limited to the biomarker-positive subgroup, and that an accurate and reproducible assay is available, then the most efficient way to evaluate the new therapy is with an enrichment design in which the biomarker is assessed in all patients, but randomization is restricted to the biomarker-positive patients [31, 32].

Patients who test negative are excluded from the study whereas biomarker-positive patients are randomized to one of the treatment groups. This can result in smaller studies when the effect of treatment is greater in the positive group. An example of a single-arm phase 2 study leading to an enrichment phase 3 design is the development of vemurafenib for patients with melanoma harboring BRAF mutations. A dose-escalation phase 1 trial was conducted in an unselected patient population, with a phase 2 extension restricted to patients with BRAF-mutation-positive melanoma. The results of this study showed that responses occurred in 11 of 16 BRAF-mutation-positive patients, but none of the five mutation-negative patients, who received $\geq$240 mg of vemurafenib twice daily. Moreover, responses occurred in 26 of the 32 BRAF-mutation-positive patients in the phase 2 expansion cohort, who received the recommended dose of 960 mg twice daily [33]. On the basis of these data (and the results of preclinical studies), a phase 3 enrichment trial, which compared vemurafenib to standard chemotherapy, was conducted in previously untreated patients with BRAF-mutation-positive metastatic melanoma [34].

An issue with the enrichment design is that it does not address efficacy in the biomarker-negative subgroup and therefore does not validate the biomarker to guide treatment selection. With the enrichment design one must be confident that the biomarker can identify the subpopulation of patients who benefit with reasonable accuracy. If the targeted therapy actually benefits all patients equally regardless of biomarker status, then enrolling only biomarker-positive patients

will slow down trial recruitment, increase expense, and unnecessarily limit the size of the indicated patient population [35]. For example, adjuvant use of trastuzumab (a monoclonal antibody targeting the HER2 receptor) in breast cancer was tested in two randomized controlled trials that restricted eligibility to approximately 20% of patients defined as HER2 positive. While the studies established significant benefit in the enriched HER2-positive population, questions about the possible trastuzumab benefit in a wider population remained open [36].

11.4.5 Biomarker-Stratified Design

If there are two or more existing treatment options with no definitive evidence for one being preferred in a given population, the most efficient trial design for evaluating biomarker utility is the biomarker-stratified design. All patients are randomly assigned to treatment regardless of biomarker status, but the analysis plan is centered on testing treatment effect dependence on biomarker status. The biomarker-stratified design maximizes the advantage of randomization by providing unbiased estimates of benefit-to-risk ratios across different biomarker-defined subgroups and for the entire randomly assigned population [35]. The precision with which treatment effects can be assessed in each of the biomarker-defined subgroups depends on the number of patients in each subgroup. The biomarker-stratified design can assess whether the marker is useful in selecting the best among two or more treatments for a given patient as this design gives more complete knowledge as information on the treatment effect in the marker-negative group. This allows parameters such as sensitivity and specificity of the biomarker assay to be estimated [32].

Recently, a staggered drug approval scheme has been proposed where initial drug development would focus on marker-positive patients before it is studied in biomarker-negative patients if there is a reasonably good chance of identifying a high benefit–risk subgroup [37]. Following from this, the European Medicines Agency is inviting companies to participate in its adaptive licensing pilot project where medicines are initially approved for a restricted patient population. This is followed by iterative phases of evidence gathering and adaptations of the marketing authorization to expand access to the medicine to broader patient populations [38].

11.5 Safety Markers in Clinical Development

Translational safety biomarkers can improve drug candidate selection, dose selection, and monitorability of potential toxicities. However, the panel of validated safety biomarkers that is currently used in drug development has not changed in decades and there are significant deficiencies in the sensitivity, specificity, and predictive abilities of currently used biomarkers [39]. In addition,

these biomarkers provide little, if any, mechanistic understanding of underlying tissue effects [40].

Traditional safety biomarkers and measures of organ toxicities have significant limitations, and for some organ toxicities, there are no biomarkers available. The development of reliable safety biomarkers is challenging because prospective clinical studies evaluating performance of safety biomarkers are ethically not feasible. As an alternative, the utilization of clinical samples from subjects with a variety of liver damage etiologies via retrospective study design significantly facilitates clinical evaluation of candidate biomarkers [41]. Furthermore, employing samples from cases of accidental poisonings and/or cases from clinical trials will build confidence in identified biomarkers. For example, typical standards to measure renal toxicity, which include serum creatinine (sCr) and blood urea nitrogen (BUN), only show changes when at least 50% of kidney function is lost due to significant renal reserve [42, 43]. In addition, sCr is influenced by several nonrenal factors such as body weight, race, age, gender, total body volume, drugs, muscle metabolism, and protein intake [44]. A panel of seven rat urinary renal safety biomarkers was qualified by FDA and EMA in 2008 [45]. This was the first-ever regulatory biomarker qualification decision under the FDA's and EMA's joint pilot *Biomarker Qualification Program.* The FDA qualification stated that the urinary kidney biomarkers (KIM-1, albumin, total protein, b2-microglobulin, cystatin C, clusterin, and trefoil factor-3) are acceptable biomarkers for the detection of acute drug-induced nephrotoxicity in rats and can be used in addition to traditional clinical chemistry markers and histopathology in toxicology studies [46]. Although these novel biomarkers can potentially be used clinically to monitor for kidney injury initially in healthy volunteer studies they are not yet qualified for clinical use and sponsors are required to discuss these with the appropriate regulatory review groups before they can be included in clinical studies.

Compared to markers of efficacy, sponsors have been reluctant to include novel safety biomarkers into their drug development programs due to concerns about broader regulatory agency awareness and receptivity versus established biomarkers. With the safety of human subjects being the primary concern for regulators it is likely that safety biomarkers that demonstrate the presence of a potential safety problem are accepted with less evidence and rigor as the consequences of a false decision based on the markers will be stopping development of a drug that was in actuality safe, which is considered less serious than letting a harmful drug through. The QTc interval prolongation is a marker for predicting cardiac arrhythmias, which can occur as a drug side effect and which can lead to sudden cardiac death [47]. The measurement is now required by regulatory authorities worldwide to be prospectively applied in every drug development program although there are a number of problems with this measurement, including assay sensitivity and thresholds [11]. This is an example of how regulatory acceptance and implementation of poorly qualified safety biomarkers can severely impact the pharmaceutical industry by potential discontinuation of a number of safe and effective drugs. In response to this, a consortium between

academia, industry, and regulators suggested that the thorough QT (TQT) study can be replaced by robust ECG monitoring and exposure–response (ER) analysis of data generated from first-in-man studies [48].

11.6 Statistical Considerations

Statistical testing of a hypothesis is a cornerstone of clinical research. However, in contrast to confirmatory trials, the objectives of exploratory trials may not always lead to simple statistical tests of predefined hypotheses, and statistical significance is not as meaningful in exploratory studies as it is in confirmatory trials [49]. An exploratory study that is intended to guide the design of subsequent larger studies can be successful if clinically significant changes in a set of biomarkers are observed even if these changes are not statistically significant. The magnitude and direction of the effect must be considered. Thus, confidence interval is more helpful than statistical test to assess the presence or absence of the clinical significance. Furthermore, the objective of exploratory studies is often to investigate the modulation of multiple exploratory endpoints where there is limited knowledge about biological and technical (assay) variability. In this case, it can be difficult to power the study for a certain level of statistical significance. In addition, the size of exploratory trials is often determined by practical and financial aspects rather than statistical considerations. To obtain fast proof-of-principle data on a new drug in a patient population using novel biomarker techniques, it will likely be most optimal to conduct the study at a single center to reduce variability in the assessments and in a number of patients that can be recruited by this clinic within a reasonable time. By using subjects as their own control (e.g., by comparing values before and after treatment, by comparing effects of several simultaneous locally administered doses, or by using a cross-over design) the size of the clinical study can also be reduced compared to traditional parallel group designs.

Detailed descriptions of statistical design of biomarker-based confirmatory studies are made elsewhere [50–52].

11.7 Validation

Before a biomarker is used in the clinic it should be subjected to both clinical validation and method validation. Clinical validation, or qualification, is the process of demonstrating the link of a biomarker with a biological process and clinical endpoints [53]. Clinical biomarkers are qualified in retrospective or prospective analyses as they progress from discovery to the clinic. The link between the marker, the disease, and the drug effect is established through literature review, analysis of expression in archival clinical material, preclinical testing in animal models, and eventually prospective clinical trials. It should be considered whether the

proposed biomarkers may be used to validate animal models such as mechanistic models or disease models. For most projects, the entire pathway from target to disease may not be well described, and it may be necessary to use animal disease models to identify steps in the pathogenesis that links the target to the disease. A predictive biomarker may be clinically validated using data from a single arm phase 2 study of patients who received that regimen. Clinical validation of a test is often accompanied by calculating the sensitivity of the test for identifying responders and the specificity for identifying non-responders. Sensitivity and specificity are computed based on a specified cut-point of positivity for the test.

Method validation is the process of assessing the assay and its measurement performance characteristics and determining the range of conditions under which the assay will give reproducible and accurate data [54]. In contrast to bioanalytical methods for pharmacokinetic analysis the diverse nature and purpose of biomarker research has led to confusion and inconsistency in the method validation process for biomarkers. FDA guidance offers a detailed description on bioanalytical method validation [55, 56]. According to the latest update of FDA's guidance on the subject, method validation for biomarker assays should address the same questions as method validation for PK assays although FDA realizes that some characteristics may not apply or that different considerations may need to be addressed [56]. Because of the diverse purposes of biomarker research (particularly for biomarker discovery and exploratory biomarker use), current regulations and guidance fail to meet adequately the needs of novel biomarker study purposes [57]. Despite this lack of specific regulatory guidance on biomarker method validation there is general consensus in the biomarker research community that the basis of the validation is to ensure that the biomarker assays are reliable for their intended purpose [4], a principle commonly referred to as fit-for-purpose validation [54]. According to this principle, the rigor of method validation depends on the purpose and should increase from the initial validation for exploratory purposes to more advanced validation dependent on the status of the biomarker. Firstly, the robustness, sensitivity, and dynamic range should be sufficient to ensure that the expected exposure in humans will lead to measurable perturbations of the biomarker. Sample characteristics are influenced by biological variations in the population as well as by the sample handling. The sources of variation should be considered in the context of the study and the assay be characterized at a level that appropriately addresses these risks. Samples from healthy persons and patients can be used to establish expected ranges of biological variations for the biomarker. Secondly, the clinical/operational feasibility of taking the sample at the planned time point should be confirmed. Thirdly, it should be evaluated if the assay in its current format should be transferred to a bioanalytical CRO for analysis. Phase 1 studies are typically run at a specialized clinical center with experience and capability in bioanalytical assays either in house or via closely associated laboratories. However, transfer of an assay requires considerable time and effort and is not always possible in the early stages. If the biological samples are shipped before analysis either right after

sampling or at the end of the study the relevant stability of the samples should be confirmed.

As an example, a biomarker used for a purely exploratory objective in a phase 2a study may only be subject to exploratory method validation that will generate sufficient confidence in the quality of the result to make the required decision. If the role of the biomarker is to confirm the mechanism of action of a new drug candidate the level of required method validation is lower than if the marker is used for making critical decisions (dose selection, patient stratification, and demonstration of safety or efficacy). In general, as biomarkers are more often used for internal decision-making in exploratory development rather than to support regulatory submissions, the level of validation is mainly determined by the sponsor [58]. Detailed descriptions of method validation of biomarkers are made elsewhere [4].

Advances in gene-array technologies has made it possible to measure gene expression levels for tens of thousands of genes. They may provide a set of biomarker candidates that need to be verified individually by a conventional analytical method or a classifier consisting, for example, of a set of genes, which together provide a good discrimination [59]. The components of expression classifiers need not be mechanistically understood and accepted as markers of disease activity. It is, of course, desirable to understand the mechanistic relationship of the components of an expression signature, but the classifier can be validated without such understanding [60]. Although the availability of gene arrays and other "-omics" technology platforms allow more scientific questions to be posed they represent additional analytical challenges in terms of assay validation. As the classifier is developed, there is a risk of overfitting of the model because the number of candidate genes available for use in the classifier is much larger than the number of cases available for analysis. The model will fit the original data but may predict poorly for independent data, and consequently some kind of validation on data not used for developing the model is necessary [61]. The simplest way for clinical validation of a classifier is to use the split-sample method where available samples are divided to ensure that the data used to develop the classifier are not used to test it [62]. Recently, Simon introduced the concept of predictive analysis of clinical trials, which permits classifier development and evaluation to be validly performed in the same clinical trial [63].

A standard method of analyzing biomarker performance, for a defined set of samples, is the ROC (receiver operating characteristic) plot [64]. It plots the true positive (correctly identified subjects with cancer) rate (sensitivity) against the false positive rate (subjects without cancer, incorrectly identified as having cancer) or 1 – specificity (Figure 11.3). The corresponding AUC is a measure of the diagnostic performance of the biomarker; an AUC of 0.5 indicates performance no better than chance, while an AUC of 1.0 indicates a perfect classifier. The AUC allows a comparison of the performance (when the analysis is done on the same sample set) of different biomarkers or biomarker panels. The method presupposes a separate method (e.g., gold standard analysis, such as histopathology for organ toxicity) for accurately determining true positives. For specific contexts of use, the

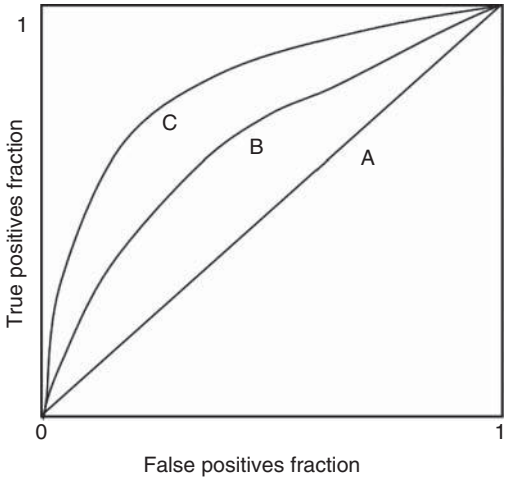

Figure 11.3 Receiver operating characteristic (ROC) plot of true positive rate vs. false positive rate. A perfect model of biomarker data has an AUC of 1.0, while model A, which separates positives from negatives no better than a random assortment, has an AUC of 0.5. The performance of model C is better than for B, which is better than A.

tradeoff between sensitivity and specificity, obtained from the ROC plot, will need to be explicitly defined.

11.8 Regulatory Considerations for Implementation of Biomarkers in Clinical Studies

Since the FDA's Critical Path Initiative, launched in 2004, recognized the need for new tools to support drug development, (CPI) biomarkers have been discussed within Regulatory Authorities [65]. A decade later, the guidance on qualification process for biomarkers was recently published [56]. Since then, the guidelines have been adopted both at the national [66] and at a more global [67, 68] levels. It is FDA's position that the use of biomarkers in exploratory phase 1 and 2 studies can provide mechanistic support and be applied in the design or analysis of later trials, potentially improving their efficiency or likelihood of success [69]. ICH guidelines E15 (Definitions For Genomic Biomarkers, Pharmacogenomics, Pharmacogenetics, Genomic Data And Sample Coding Categories) and E16 (Biomarkers Related To Drug Or Biotechnology Product Development: Context, Structure And Format Of Qualification Submissions) were recently adopted and will be used in EU, United States, Japan, and other regions. Apart from the harmonized format of qualification submission, it is worth mentioning that both the EMA and the FDA encourage applicants to apply in parallel to the EMEA and FDA for advice on biomarkers [70, 71]. The agencies will then communicate the assessment and meet with the applicant together. This must be considered an opportunity for increased dialog, which will maximize the chance for scientific consensus – each agency will

still provide their independent advice to the applicant. Submission in the EU will be assessed by the Committee for Medical Products for Human use (CHMP)'s Scientific Advice Working Party, and in the United States the submissions will be assessed by the Interdisciplinary Pharmacogenomics Review Group (IPRG) [72]. The mission of IPRG is to establish a scientific and regulatory framework for reviewing genomic data and IPRG is the primary review body for Voluntary Exploratory Data Submissions (VXDS). Upon request, the IPRG also consults with FDA review staff on the review of required submissions containing genomic data [72]. It is emphasized that validation of biomarkers takes place on a voluntary basis. In case biomarkers will only be used for internal decisions, there is no clear need for qualification by regulatory authorities. However, in case biomarkers are used in essential parts of regulatory submissions (for instance as endpoints in preclinical or clinical trials), it is highly recommended to engage in a discussion with authorities as early as possible. It is the FDA's position that if a companion diagnostic is required for therapeutic selection, an FDA-approved or -cleared test will be required at the same time that the drug is approved [73]. An *in vitro* pharmacogenomic test would be considered a companion diagnostic device if it will provide information that is essential for the safe and effective use of a therapeutic product as directed in labeling. In general, a universal and efficient process for biomarker qualification is urgently needed. Such a process could be developed from the ICH E16 harmonization document [68] that describes the first harmonization for biomarker qualification across the FDA, EMA, and PMDA. The contents and structure in this document may be developed into an effective regulatory qualification process in the future [71].

11.9 Biorepositories and Ethics

The discovery and validation of biomarkers in drug development are highly dependent on the availability of stored patient material. In the discovery phase such samples are used to evaluate the expression of various types of biomarkers for assessing the relevance and performance of the biomarker assays. During the clinical phases it will also allow investigation of hypotheses emerging from ongoing clinical research without conducting a new prospective study. Furthermore, representative sample sets make it possible to identify subpopulations of responders within a trial and to ensure that retrospective analysis results are broadly applicable to individuals within subpopulations of responders or nonresponders [74]. According to the Industry Pharmacogenomics Working Group (I-PWG) the collection of DNA samples from subjects participating in pharmaceutical clinical trials provides a vital resource for companies investigating intersubject variability in treatment response [75]. This view is shared by regulatory authorities and a recently published FDA guidance on pharmacogenomics states that "An important prerequisite to successful use of genetic information in drug development is the appropriate collection and storage of DNA samples from

a large enough number of participants in clinical trials, both exploratory studies and the adequate and well-controlled trials intended to demonstrate effectiveness and safety. Ideally, baseline DNA samples should be collected from all patients in all arms of clinical trials in all phases of drug development" [69]. In spite of this, most companies collect DNA through optional subject participation. In contrast, RNA samples are collected by the majority of companies for trial-related planned analysis in a mandatory manner [76]. This difference is likely driven by the perceived greater privacy risk associated with DNA data and the broader applications of RNA for analysis of response prediction, pharmacodynamics measurements, target engagement, and disease modulation [76].

For meaningful storage of biospecimens it must be ensured that the quality of the samples is maintained for the intended purpose over time. The quality of a sample on the molecular data obtained from its analysis depends on the class of biomolecule analyzed (DNA, RNA, protein, metabolite) as well as the type, specificity, sensitivity, and robustness of the analytical method [77, 78]. Biobanking method validation requires both knowledge of the preanalytical variables that need to be controlled and identification of those factors that do not affect the quality of the biospecimen for a given type of research. In many clinical settings the possibilities for controlling conditions that influence biomolecule integrity are limited but efforts should be made to optimize sampling in order to preserve the sample. In addition, appropriate tests to assess the integrity of the biospecimens should be implemented [79]. There are several guidelines for the management of biobanks, such as the International Society for Biological and Environmental Repositories (ISBER) guideline [80] and Organization for Economic Cooperation and Development (OECD) guideline [81]. ISBER is the largest international forum that addresses the technical, legal, ethical, and managerial issues relevant to repositories of biological and environmental specimens.

The most important ethical issues emerging from biomarker research are related to the collection, storage, and use of human biological samples and associated data for research purposes. To identify new biomarkers of exposure, effect, and risk of disease, studies require access to samples of annotated human blood and tissues and associated medical and lifestyle data. Ethical and social concerns related to biomarker research on human material often stem from uncertainty about the outcome of the research and how new technological advances in research technologies will impact extraction of biological information from human samples. As in other parts of medical research, the use of informed consent in biomarker studies ensures that research participants are informed about the risks and benefit of the study and that they participate voluntarily. Sampling of human material during clinical studies to support drug development is a well regulated and transparent process with limited ethical concern. The intended use of the samples is specified in the clinical protocol to which the participants consent and the samples are destroyed after the trial is completed. However, when research participants provide tissue and information to prospective biobanks they cannot give informed consent to future research projects that have yet to be specified. To avoid the need to contact participants to give

consent for each new research project using their samples, European guidelines consider broad consent for unknown future uses to be acceptable as long as such future projects gain approval from research ethics boards and participants retain the right to withdraw samples at any time [82]. Broad consent is generally considered the preferred model both for ethical and practical reasons [83, 84]. As an alternative solution, anonymization of samples has been seen as protection of privacy in biomarker research. The Declaration of Helsinki states that "Medical research involving human subjects includes research on identifiable human material or identifiable data" [85]. The US Office for Human Research Protection has therefore proposed a broadening of the definition of "non-identifiable" [86]. It follows that any research using non-identifiable samples does not create an obligation to obtain informed consent and approval of the protocol from an IRB or a research ethics commission. However, if samples contain any trace of DNA, they are not truly anonymous, because it is always possible to identify the donor through DNA fingerprinting. Comparing DNA sequences at only 30–80 statistically independent single nucleotide polymorphisms will uniquely define a single person [87]. Moreover, the size of online genotype databases has made re-identification of anonymized genotype data a real threat [88]. Finally, in order to most optimally use the samples for developing predictive biomarkers it should be possible to match historical samples to the subjects in future studies, which requires as much information on the stored samples as possible [89]. Consequently, the use of broad consent is considered the most appropriate.

11.10 Conclusion

Biomarkers play an important role in biomedical research and can be used in all phases of drug development. Appropriately characterized and qualified biomarkers can drive translation of compounds from the preclinical setting into the clinic. In early exploratory clinical studies they can be used to explore the mechanism in humans and for making go/no go decisions before larger clinical studies are initiated. In confirmatory studies the key roles of biomarkers are to act as surrogate endpoints and to define subgroups of patients with particular benefits or risks in relation to a particular therapeutic intervention. The value of biomarkers has long been realized by industry, academia, and regulators, and high throughput technologies such as proteomics and microarrays have led to hundreds of thousands of papers claiming thousands of biomarkers. However, only a few have been accepted for routine clinical practice mainly due to the lack of appropriate validation of the markers [90]. Most pharmaceutical companies lack the resources to adequately develop the evidence to support a biomarker qualification and such projects require multiteam collaborations through consortia of multiple laboratories and organizations [91, 92]. This has led to numerous collaborations and public–private partnerships on biomarker development since the Critical Path Initiative introduced the concept a decade ago. The next decade

will hopefully witness a further increase in these collaborations, resulting in a more efficient process of bringing new biomarkers into clinical use.

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12
Genomics and Proteomics for Biomarker Validation

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12.1
Introduction

Despite the tremendous advances in the understanding of the molecular basis of diseases such as cancer, substantial gaps remain both in our understanding of disease pathogenesis and in the development of effective strategies for early diagnosis and treatment [1].

The current interest in proteomics is due, in part, to the prospects that a proteomic approach for disease investigations will overcome some of the limitations of other approaches [2]. In particular, the areas of research include deciphering the altered protein expression in different levels (tissue, cells, subcellular structures, body fluids, protein complexes, etc.); the development of novel biomarkers for diagnosis or early detection of disease; the identification of new targets for therapeutics; and accelerating drug development through more effective strategies to evaluate therapeutic effect and toxicity [1, 3, 4].

The dynamic nature of the proteome of a cell provides sample justification for studying gene expression in disease directly at the proteomic level, taking into consideration that capturing this dynamic state is a technological challenge. Undoubtedly, tackling all the aspects of disease proteomics requires implementation of multiple strategies and technology platforms, most of them with high-throughput capacity and high sensitivity; thus no available proteomics technology (from functional to expression proteomics) emerges as the unique platform for biomarker discovery [3–5].

There is a substantial interest in applying proteomics to the identification of disease markers. Mainly based on the proteome, alterations in disease may occur in many different ways that are not predictable from genomic analysis, and it is clear that a better understanding from genomic analysis together with a better understanding of these alterations will have a substantial impact in medicine.

A useful repertoire of proteomics technologies is currently available for disease-related applications, although further technological innovations would be beneficial to increase sensitivity, reduce sample requirement, increase throughput and, more effectively, uncover several types of protein alterations such as

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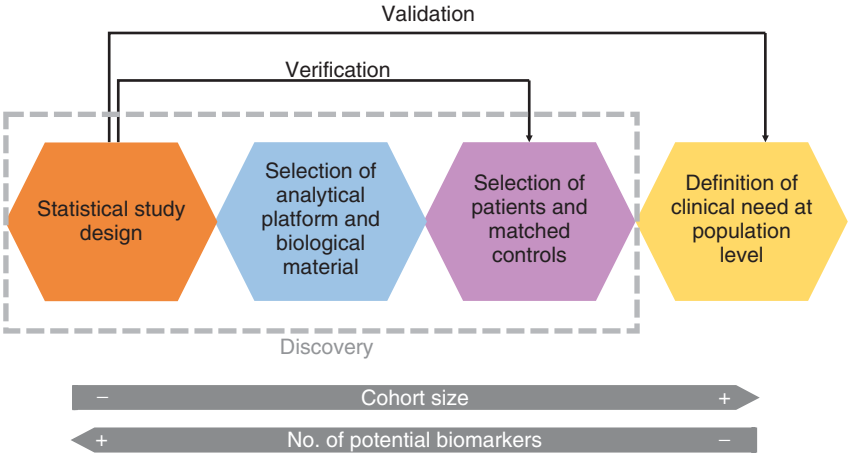

Figure 12.1 *Biomarker discovery-validation pipeline*. Different steps are followed in order to validate the potential biomarkers. As the number of samples is increased, the number of definitive biomarkers decreases.

post-translational modifications. The employment of these technologies would likely expand substantially, particularly to meet the need for better diagnostics and to shorten the path for developing effective therapy [1].

The biomarker pipeline is commonly viewed as a series of preclinical phases: discovery, verification, and clinical evaluation [6–9]. The relationship between the number of samples and the number of proteins is considered inverse because the number of proteins is decreased as the number of samples is increased (Figure 12.1). In fact, the actual clinical validation of the final biomarkers is done by quantitating a small number of proteins on fifties to hundreds of samples [10].

A typical proteomics discovery experiment employs a non-targeted approach (*shot-gun proteomics*) for the relative quantitation of thousands of proteins in a small number of samples. The comparative analysis results in a list of hundreds of proteins that are differentially expressed between healthy and diseased samples. After the discovery phase, these potential biomarker proteins are narrowed down by performing studies on additional patients or at more time points, and/or by using another technique. Then, these potential biomarkers are verified on a set of 10–50 patients. In the final step, a small number of biomarkers are "validated" on 100–500 samples [10].

Although the initial findings at the discovery phase of potential biomarkers are very promising, unfortunately their ultimate clinical translation is found to be very rare. Thus, a plethora of biomarkers for a number of diseases have emerged; however, only a few of them have received approval for clinical use by the Food and Drug Administration (FDA).

In this chapter, several key aspects in biomarker verification/validation phases have been analyzed, which could be relevant in these important steps for bridging

the gap between translational research and unmet clinical needs (from bench to bedside).

12.1.1 Biomarker Discovery

A biomarker (biological marker) was defined as "A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention" (*www.cancer.gov/*).

Depending of the intended use, biomarkers can be distinguished on the following categories: diagnostic biomarkers (which are related to disease detection); prognostic biomarkers (the ones which are related to prediction of the course of a particular disease such as, recurrence, progression, and survival); predictive biomarkers (which allow prediction of the response to treatment that could be subsequently applied in patient assessment) [11, 12].

In general, biomarkers that would enable early disease diagnosis are required, together with those that would provide prognostic values in disease status and predict an outcome of an illness prior to any treatment designed.

Over the last decades, the emergence of *-omics* techniques has accelerated biomarker research by providing platforms suitable for screening a large number of biomolecules in a rapid manner (high-throughput and high-density methodologies), facilitating the identification of valuable biomarkers in a short period of time without the requirement of any prior in-depth knowledge into the mechanism of disease progression.

Identification of new biomarkers most often commences with a discovery phase, during which an initial and large set of proteins is measured in relevant clinical material.

In general, biomarker discovery phase currently relies on untargeted approaches resulting in identification of a vast number of potential biomarkers. Untargeted approaches are also referred to as unbiased since discovery of new putative biomarkers is performed without any prior assumptions as to constitute promising markers (Figure 12.2) [11].

However, methods used for untargeted analysis tend to be relatively intensive and cannot handle the immense complexity of the blood proteome, in practice, limiting analysis to more abundant proteins that represent only a small fraction of the proteome.

Accordingly, untargeted analyses may be more meaningful using materials other than blood, such as fluids near the affected tissues (proximal fluids) or tissue homogenates, in order to establish a first set of protein targets that may be further evaluated in blood using more sensitive methods.

Any interesting protein biomarker candidates that are identified can then be further validated in subsequent steps. In general, targeted approaches are adopted for

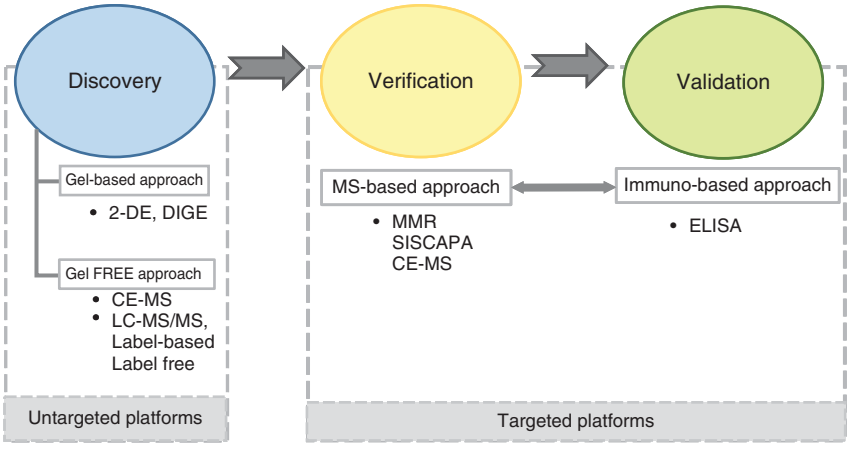

Figure 12.2 *Proteomics platforms for biomarker validation*. Targeted and untargeted approaches are employed depending on the phase of the pipeline.

validation stages, with the major goal to identify targets with promising characteristics as biomarkers are selected for further assays in independent and larger sample collections, using assays developed for that purpose.

12.2 Challenges in Biomarker Discovery/Verification Phases

There are numerous obstacles that lie along the biomarker discovery stage, which can have a strong influence in the translation of biomarkers in clinical settings. Therefore, they need to be addressed for a successful biomarker discovery stage. A good example is performing experimental designs including inclusion/exclusion criteria for subject selection, type of sample, collection and handling procedure, storage, accurate data acquisition, analysis, and documentation (FDA guidelines).

Herein, some of the challenges or limitations and the proposed solutions or recommendations are briefly described.

1) *Pre-analytical variations*: Mostly of them are caused because there is a lack of standard operational procedures (SOPs) that could generate differences in sample collection (body fluids, plasma, serum); sample processing (i.e., addition of proteases/inhibitors/stabilizers, processing time, intrinsic proteolysis, processing conditions such as centrifugation or dilution); and sample storage (i.e., storage temperature, shipping conditions, numbers of freeze/thaw cycles).

   The possible solutions suggested include the establishment of SOPs and quality assurance protocols; well-planned and standardized experimental designs; uniform sample collection/handling/storage procedures; Minimum

Information About a Proteomics Experiment (MIAPE); and/or initiatives for standardization procedures from Human Proteome Organization (HUPO).

2) *Biological diversities*: Social demographic background (i.e., age, gender, ethnic, lifestyle, diet); clinic pathological background (i.e., stage of infection, medication, pregnancy, existence of co-infections, hormonally related variables, circadian cycles, exposure environmental risks).
   The recommendations of possible solutions suggested include detailed documentation of the background of subjects, analysis of bigger clinical cohorts, analysis of suitable controls, cross-validation with different cohorts, implementation of stringent inclusion, and exclusion criteria for specimens in the study.
3) *Ethical and social issues*: Maintenance of confidentiality of the subjects, biosafety, and quality control.

12.3 Verification of Biomarkers

12.3.1 Protein Binding Assays

Enzyme-linked immunosorbent assays (ELISAs) are best suited for projects where only a few biomarkers need to be verified/validated on a large number of samples. However, ELISAs require antibodies against each targeted protein/peptide. Thus, high-quality ELISA assays are often unavailable for proteins of interest [13].

The development of an ELISA could be a very tedious and expensive process, and time consuming (involving development times of 1–2 years). In addition, ELISA assays also have limited multiplex capability, and the cross-reactivity is always dangerous in these assays. In fact, ELISA is not well-suited for quantitating a large number of candidates biomarker proteins/peptides, and in many cases high-density and high-throughput assays (Figure 12.3) are required for the biomarker validation phase [11, 14].

The lack of a technique to verify around hundreds to thousands of potential biomarkers in a rapid and simultaneous manner has been identified as the *bottleneck* in the biomarker pipeline [6, 10].

Hence, protein microarrays seem an optimal methodological approach for biomarker verification. Depending on the kind of array, three different features must be taken into account: type of surface, molecules of study, and detection techniques.

Concerning the surface, the usual materials include glass, plastic, metal, or polymer membranes. In addition, the surface can be functionalized with different chemistries (aldehyde, epoxysilane, gold, etc.). Surfaces must preserve the function, structure, and binding capacity. Related to the structural dimension, microarrays can be planar (2D) or bead based (3D).

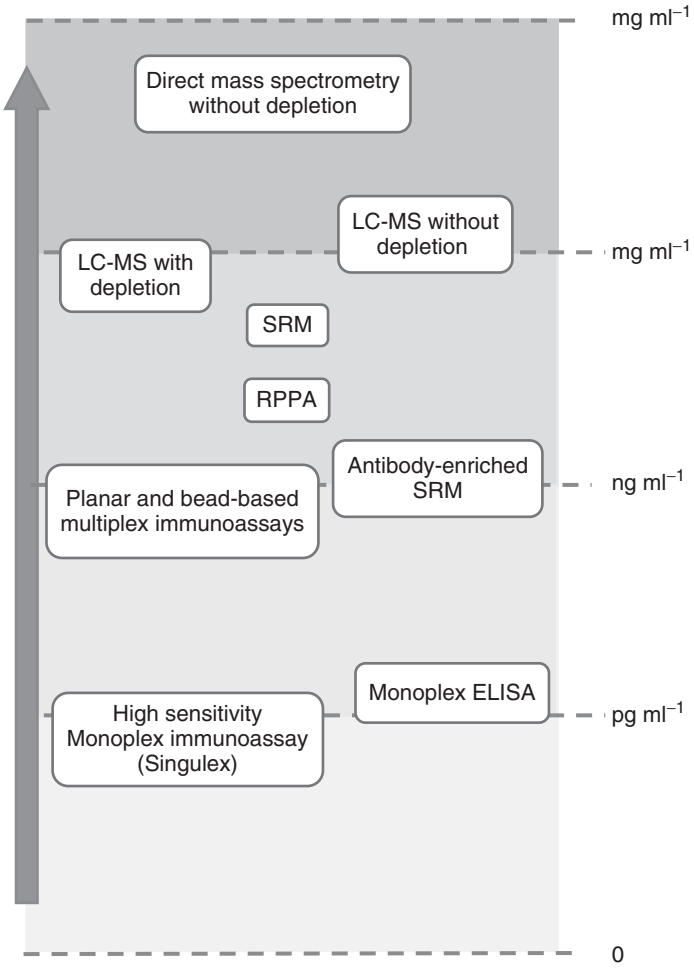

Figure 12.3 *Proteomics strategies and protein levels*. Depending on the sample and, thus, on the relative quantity of the protein biomarker, distinct methods can be selected to improve the identification.

Analytes are also an important point in the design – not only the capture agents printed onto the slide but also the molecules of interest. Depending on this, proteins, antibodies, cell extracts, tissues, or DNA, among others, can be printed. Achieving high-affinity bindings and avoiding cross-contamination are the main challenges.

Finally, detection techniques play an important role in protein microarrays analysis. There are two major groups: label-based and label-free detection strategies. For the first approach, a fluorochrome or radioisotope tag molecule is necessary [15].

12.3.2 Targeted Proteomics

Multiple reaction monitoring (MRM) or selected ion monitoring (SRM) has emerged as the method of choice for performing validation with large numbers of biomarkers as candidates for validation/verification.

MRM is a tandem mass spectrometric technique (MS/MS) that is performed on triple-quadrupole mass spectrometers. This technique involves selection of a precursor ion, a peptide that acts as a surrogate for the protein of interest, which is selected by the first quadrupole. The precursor ion (protonated intact peptide) is fragmented in the second quadrupole, and one of the fragments is selected by the third quadrupole and reaches the detector. This precursor/product ion pair is referred to as a *transition* and the quantitation of the protein is based on the signal that reaches the detector. This fragmentation occurs on the millisecond time scale and, in a LC/MRM-MS experiment; different transitions can be selected as a function of the retention time. This type of analysis allows the quantification of hundreds to thousands of peptides/proteins, and, as a consequence, the protein presented in the sample [16].

It should be highlighted that multiplex-MRM assays can be employed as biomarker discovery platforms due to the feasibility of quantitative identification of several to hundreds of proteins in a single LC/MRM-MS analysis; hence, this platform could be compared with protein microarrays. Several biomarker studies have been performed using this approach [17–19].

12.3.3 Correlation Between MRM and ELISA

The correlation between MRM and ELISA assays plays a critical role because most of the large-scale clinical evaluation is commonly performed in ELISA format.

Végvári *et al.* [20] detected a previously undetected PSA variant coded by SNP-L132I (rs 2003783), which is specifically detected by using an MRM method particularly designed for specific peptides from PSA in 72 samples. The results suggest that only nine samples (out of 72 samples) were positive, which also correlates with a commercial immunoassay, but with a concentration value 34–60% lower than that obtained by ELISA assay.

Another representative example is the study led by Pan and collaborators [21], in which five proteins were determined as easily overexpressed in pancreatic cancer tissue or pancreatic cell culture secretome, by both ELISA and MRM.

In general, MRM performed at least as well as ELISA in several other pathologies (non-cancer-related studies) – for example, C-reactive protein in rheumatoid arthritis [22], biomarkers related to acute rejection in heart transplantation [23], and 13 potential biomarkers for Down syndrome [24].

12.3.4 MRM and Biomarker Pipeline

Whiteaker and collaborators [25] described the complementary role of *shotgun* and MRM proteomics assays for biomarker discovery and verification, respectively, in breast cancer samples. The combination of MS techniques proposed in this study has become the general approach to biomarker discovery and verification.

One aspect to keep in mind is that there is a difference between biomarkers that fail during the verification process and those that cannot even be evaluated, mostly due to lack of suitable antibodies or affinity reagents. In fact, commonly both situations are considered as *biomarkers not possible to verify.*

Another important aspect is the cost evaluation of a complete *discovery-to-verification study.* Anderson [26] discussed the challenges and costs of performing a complete *discovery-to-verification study.* In this review, Anderson presented the progression from a large number of proteins (more than one thousand), considered as potential biomarkers, to a small numbers (from ten to hundreds) of well-characterized and, afterwards, well-verified biomarkers. In summary, a total of 121 proteins were found to be differentially expressed and around 20 proteins could be evaluated in a verification study (6 by ELISA, 7 by Western blot, and 7 by MRM with isotopic peptides). Then, only 40% of the potential candidate proteins could actually be quantitated. At the end, six proteins were analyzed by ELISA in 100 patients (verification step). All in all, this $2.2 million study did reveal important findings about plasma biomarkers of the disease.

Based on this study, and another one about breast cancer, Anderson predicted a cost of $1.5 million and 1 year to develop MRM assays for 50–100 good candidates for biomarker proteins, and a cost of $4 million and 4 years for a full pipeline.

12.4 Role of Biobanking in Biomarkers Validation

To develop research finding into a clinical tool with diagnostic or prognostic value, a large number of biological samples and/or tissue specimens is required. Prerequisites include not only biological material resources but also a very well-organized preservation domain to be retained, the so-called biobank.

Ideally, a biobank should retain maximum quality of the biological material stored (following standardized protocols of sample handling), and of associated clinical and demographical data, and it should be easily accessible and open to the scientific community.

The current interest in translational research has generated a large demand for clinical samples (blood, tissue, etc.) that find use in a broad variety of research including genomics, proteomics, and metabolomics. Hence, hundreds and thousands of millions of dollars have been invested on the collection, storage, and distribution of samples. Nevertheless, many biomedical researchers complain about

their inability to obtain relevant and/or useful samples for translational research projects. In general, there are some common problems or drawbacks (such as lack of access to samples, storage conditions of the samples, non-control samples, etc.) that have slowed down most of the studies focused on biomarker discovery.

Bearing this in mind, a list of challenges can be described: (i) Defining biobanking with specific experiments; (ii) establishing well-described SOPs; (iii) evaluating interobserver differences in diagnoses; (iv) identifying internal protein controls appropriately; and (v) redefining the clinical sample paradigm by building partnerships with the public.

In this regard, Prof. Joshua LaBaer suggests [27] that there are tools available to achieve these objectives in short periods of time (approximately 5 years); however, it is important to keep in mind that the success of biomarker validation relies on access to high-quality clinical samples, collected under standardized conditions, accurately annotated, and shared under conditions that promote the research we need to do.

12.4.1 Biobanking Challenges Associated with Biomarker Discovery and Validation

The challenges highlighted above are described.

12.4.1.1 Preanalytical Variations and Lack of SOPs

Well-established and globally accepted SOPs are required for sample collection (tube additives, coagulation process, etc.); sample processing (protease inhibitors, processing time and environment, conditions of incubation, fractionation and centrifugation, etc.); sample storage (number of freeze/thaw cycles, shipping conditions, storage devices (temperature ramping, time elapsed before freezing, etc.); and storage duration.

The recommendation and suggested solutions, among others, are the following: (i) well-planned and standardized experimental design; (ii) uniform sample collection, handling, and storage procedure; (iii) minimum information about a proteomics experiments (MIAPE); and (iv) establishment of SOPs and quality assurance protocols.

12.4.1.2 Biological Diversities

It is important to pay special attention to sociodemographical background (i.e., age, gender, lifestyle, diet, ethnicity, etc.), clinical background, and sample size.

In this case, amenable solutions are based on implementation of strict inclusion and exclusion criteria, analysis of specimens from different populations for cross-validation and big clinical cohorts, and detailed and complete clinical background of each sample.

12.4.1.3 Disease Heterogeneity

One of the more commons challenges is the lack of consideration of genetic and proteomic variation while profiling and researching a disease (i.e., in many studies intratumoral heterogeneity is neglected). This challenge is shared with the lack of

global initiatives for diseases predominant in developed and developing countries. In addition, there is a common lack of consideration of genetic and proteomic variations while profiling and researching disease.

To overcome these challenges, Gupta *et al.* [28] suggest a global incentive to collaboratively study disease heterogeneity of diseases, together with the establishment of consortia for data sharing and bioinformatics analysis of population-wide variables.

12.4.1.4 Technical Limitations

One of the major challenges is linked to the insufficient dynamic range of the detection technology, variation of findings among different technological platforms, and lack of reproducibility. In fact, most of the low reproducibility could be explained by lack of uniformity and standardization in the data acquisition and analysis process. In addition, in many cases the experimental procedures imply a long process and are very expensive. To overcome these challenges, next-generation high-throughput technologies are required to be applied in prefractionation samples (to reduce complexity). It would be very useful to design and establish networks between researchers and clinicians to share scientific data across the world.

12.4.1.5 Validation and Clinical Trials

One of the most important challenges is that biorepositories should be also compatible for validation, because most of the currently available collections are only suitable for the discovery phase and are not properly designed for validation approaches, which are based on immunoassays. In this case, it is very important to design in advance the biorepository according to the future requirements in the biomarker pipeline.

12.4.1.6 Lack of Stable Biorepository

Currently, there are no well-established biobanks in developing countries. Besides biobanks or biorepositories, one remaining challenge is related to ethical issues regarding long-term use of biospecimens, confidentiality, and social implications. For all these reasons, the establishment of national and international societies to address the technical, legal, ethical, uniformity, and quality assurance issues is extremely essential.

12.5 Conclusions

Proteomics appears to be a revolutionary approach for biomarker discovery. Although huge progress has been made thanks to genomics, proteins are the final molecules in charge of the development of a disease. The ideal biomarker discoveryo pipeline includes three main phases: discovery, verification, and validation. As the number of samples is increased, the number of proteins decreases due to its validation.

Several -omics techniques can be employed, distinguishing between the targeted and the untargeted. Regardless of their nature, these approaches are able to screen biomarkers in a rapid manner, reducing the phase times, due to their high throughput and high density. The principal points to take into account include the establishment of inclusion/exclusion criteria for subject selection, type of sample, collection and handling protocol, storage conditions, accurate acquisition and analysis, and documentation, following the FDA guidelines.

Traditionally, ELISA has been the technique of choice for proteomics studies. Nowadays, it is the main validation strategy in proteomics. New approaches, such as MRM or SRM, are good alternatives as deduced by the high number of clinical studies performed.

Finally, we highlight the importance of biobanking and standard procedures for obtaining reliable results. In this way, proteomics will be able to accomplish the biomarker discovery pipeline, creating the basis for a large database for the relationship between biomarkers and diseases.

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