Clinical Protein Science and Bioanalytical Mass Spectrometry with an Emphasis on Lung Cancer

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1. Introduction

There is a considerable shift in the demand and expectations on the health care systems of the future worldwide due to drift to chronic illnesses and aging demographics. This creates novel opportunities and challenges to the medical research community to drive a patient-centric and technology driven research strategy. Biological mass spectrometry plays a major role in the development of new medicines, as well as the understanding of diseases and disease mechanisms, where new drug developments play a key role. Lung cancer is a major target area within the health care system, with an ever-increasing cost to society.[1,2] Chronic obstructive pulmonary disease (COPD) is the fastest growing lethal disease, especially in Asia, with no effective drugs available on the market. COPD patients are also at risk to develop lung cancer, where these causes and phenotype reasons are still unknown, as of today. Lung cancer and COPD are the leading causes of smoking related mortality worldwide, whereas lung cancer continues to be the most common cause of cancer death in men in the EU, with 178,400 deaths estimated in 2004 (27.3% of all cancer deaths).[3] With new developments of biomarkers, the disease associations will lead to new routines, delivering earlier diagnosis of COPD, emphysema, and lung cancer. Furthermore, wider mass spectrometry applications of these quantitative methodologies in current practice would aid in the differentiation of patients with high and low turnover states at disease, and differing needs, in regard to clinical intervention and treatment strategies and subsequent health care costs to society.

The development of new diagnostic biomarkers has a great potential, where both industry and the academic field are

Ákos Végvári was born in Siklós, Hungary, in 1968. He got his degree in Biology in 1996 at the University of Pécs, Hungary, with a thesis on the bioanalytical applications of capillary electrophoresis. During the period of 1997−2001 and 1999−2003, he did his Ph.D. in analytical chemistry at the University of Pécs and in biochemistry at Uppsala University, Sweden, respectively, working on analytical methodology development for separation of low-molecular-weight compounds, peptides, proteins, and nucleic acids. Soon after, he joined a biotechnology company in Stockholm, working on the analytical evaluation of a newly developed AIDS drug. While at the Uppsala University, he was also working on other bioanalytical research as a postdoctoral fellow. In 2007 he moved to Lund, Sweden, where he is currently working at the Biomedical Center of Lund University. His main research interests focus on disease linked, mass spectrometry based proteome analysis, including targeted cancer proteomics as well as fundamental method development for localization of drug compounds in tissue sections by MALDI imaging mass spectrometry.

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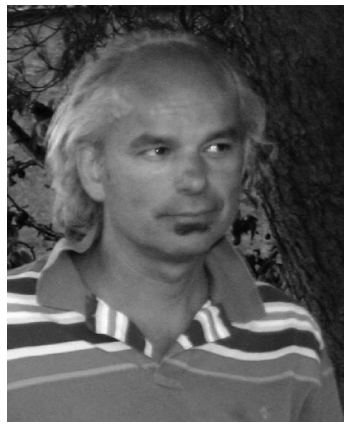

György Marko-Varga has been working as a senior Drug, Discovery/ Development scientist at Astra, and AstraZeneca for a period of 17 years. Marko-Varga started in 1993 at Astra as a Lead Scientist coordinating a collaboration on inflammatory mechanisms and pathway biology with Bengt Samuelsson, the Nobel Price Lauriate at the Karolinska Institute. He was heading the AstraZeneca Global Proteomics Network and was responsible for developing clinical biomarker platforms, used in clinical studies. In 2006, Marko-Varga established "Nietorp AB", a MicroTechnology company within AstraZeneca. He had responsibilites in several "Gefitinib" (-IRESSA-), Protein Biomarker Discovery studies in Japan (2004−2009), working with 52 Lung Cancer Clinical Centres throughout Japan. This has been the biggest biomarker study initiative in the industry, with more than 6,000 patients. Developing novel diagnostic assays and platforms, interfacing high resolution separation with mass spectrometry applied to lung cancer and COPD disease areas, and building an understanding of mode of drug action in disease mechanisms are currently research areas that he is building new research groups around in collaborations with pharma, academics, and clinical hospitals. György Marko-Varga has filed 17 patents worldwide, published 200 scientific papers in international peer reviewed journals, edited 2 books, and coauthored 7 books. In 2010, Marko-Varga became the Head of the Division of Clinical Protein Science & Imaging at the Biomedical Center, Lund University, and also became Professor at Department of Surgery, Tokyo Medical University, Tokyo, Japan. One of his major challenges will be responsibility for the "Big Three" study, *Lung Cancer, CardioVascular disease, and COPD*, with 400,000 patients (2010−2014) in Sweden.

investing and exploring approaches to harness technology to make innovative discoveries.[4−9] There are currently large numbers of putative diagnostic markers to be assessed where validations in clinical studies are needed to determine which combination of markers has the greatest diagnostic and prognostic power.

In clinical protein science, new and future complementary technologies, such as protein shotgun sequencing and quantitative mass spectrometry, have been instrumental in these advancements.[10−14] With at least 20,000 gene products and their multiple structural variations involved in disease pathobiology, this has become a tremendous challenge.[15] No bioanalytical technology today can measure such high numbers of proteins, being expressed in clinical samples.[16,17] Lack of sensitivity is another major challenge, and even today, there is no PCR for proteins to address this problem.

Today's mass spectrometry instrumentation and its merits was pioneered by Fenn's group on electrospray instrumentation,[18] while MALDI developments by Karas and Hillenkamp,[19] and Tanaka,[20] and their innovations were honored with a Nobel Prize in 2002. These were seen as the inventions that would revolutionize and change our way of working with Clinical Protein Science.

There is an ever-increasing interest in the area of clinical understanding of disease and malfunctions of patients and a focus on optimal drug treatment for the patients. Smoking constitutes a unique global public health problem with increasing burden on the health care system due to smoking related comorbidities. In addition, increased life expectancy is leading to geriatric and chronic illnesses. The predominant diseases in this regard are obesity, diabetes type 2, neurodegenerative disease, cardiovascular disease, cancer in general, and pulmonary diseases, such as COPD and lung cancer.[21] Though considerable progress is being made, there is still an unmet need for the "holy grail" of how to manage and address these health care challenges.

Clearly, these shortcomings are well-known to governments and health care institutions on a global scale. In Europe, for instance, the European Commission has dedicated large-scale research programs to address the developments of disease mechanism research within dedicated research areas,[22] and so has the National Institutes of Health (NIH) in the USA, as well as other sponsoring bodies in the world. In a joint effort between Europe and the U.S., there have been common strategies of how systems biology can be useful in cancer research.[23]

Globally, there is an increasing interest and need to support research areas that can help to solve disease understanding and improve on patient care, including novel medicines such as "Personalized Medicine" and alternative treatment technologies and early indications of disease diagnosis utilizing both imaging techniques and biomarker diagnostics. Ultimately it is the patients who are suffering and experiencing these limitations in the treatments of today. The combination of imaging techniques and their relation to biological changes that can be measured on a molecular level is currently one front line research field attracting a lot of attention. Combining Computer Tomography with protein expression[24,25] will, for instance, provide a landmark of tissue changes that could be identified in rheumatoid arthritis or lung disorders such as emphysema and bronchial wall changes, that has a link to inflammation of the pulmonary tracts.[26,27] Classical histology in combination with mass spectrometry has also been shown to be useful in finding common morphological changes, that can be correlated with protein expressions.[28,29] Lately, there was a combination of quantitative mass spectrometry and cryoelectron tomography that was able to localize protein complexes of pathogens in human cytoplasm,[30] as well as imaging mass spectrometry with matrix assisted laser desorption ionization (MALDI,)[31,32] that recently also proved to be readily compatible with archived formalin fixed tissues.[33] As the resolving power of imaging techniques is improved with better optics and computer power, we are approaching microenvironments that scale down to <1 mm regions and in some cases to single cells, striving for intracellular organelles. The very latest developments within the area of mass spectrometry imaging allow drug localization at 300−500 μm resolution,[34−36] and the compound imaging process steps are outlined in Figure 1. Complementary to MALDI-MS imaging, secondary ion mass spectrometry (SIMS) has a higher resolving power, down to <10 nm. SIMS recently improved its ability to overcome its low sensitivity for high-mass molecular weight analytes, by using a MALDI matrix (matrix enhanced SIMS[37]), gold coatings,[38] or polyatomic ion,[39] providing improved signals for a broad range of analytes, including peptides and proteins.[40] The application of SIMS imaging on breast cancer cell lines of MCF-7, T47D, and MDA-MB-231 allowed the differentiation and identification of these cell types in homogenates.[41] Furthermore, recently, SIMS demonstrated

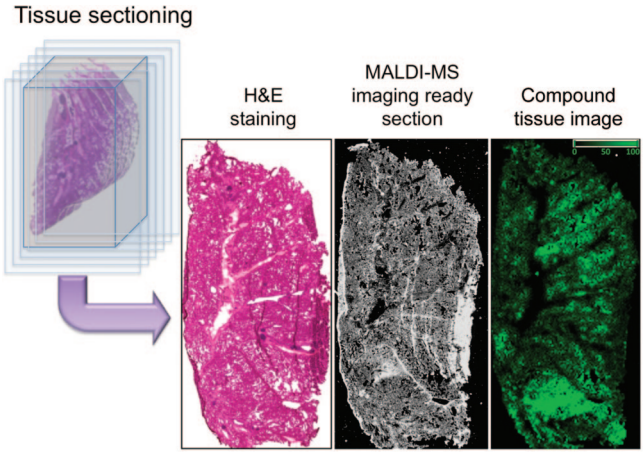

Figure 1. Illustration of the molecular imaging process: including tissue sectioning, hisological staining, tissue section preparation, readiness for tissue imaging, and localization of drug compound by MALDI-imaging mass spectrometry.

its applicability with formalin-fixed paraffin-embedded sample analysis.[42] All these combinations can provide future understanding of the complex biology ongoing in diseases and disease evolvements. We know that proteins have cellular roles as primary effectors that are impacting biological functions. Successes in these areas will be mandatory, in order to outline both mechanisms and markers of disease for future clinical use.

2. General Overview

Protein science, with a focus on bioanalytical assays used in the health care area, with qualitative and quantitative measurements, helps in early indication of disease and disease progression. This focus also includes the understanding of the disease link of any given target protein, protein alteration upon drug treatment, and general human safety measures. Patient safety and toxicity are areas of expansion with a high priority in today's clinical and biomedical developments.[24,43] The usefulness and interest in developing methodologies and assays for diagnostic application of protein analysis is a priority and is increasing. Advancing protein analysis for clinical use is aimed toward diagnostics and biomarkers, where proteins have been used as markers of disease for more than 150 years.[44] The fast development within this field is owed to the improvements in technology that have been made within the mass spectrometry field, along with new enabling tools and methods for quantitative proteome analysis.[8,15,45,46] The high resolution nanochromatographic separation being interfaced with sequencing mass spectrometry, as part of the technology platform, is currently the most powerful protein analysis engine, that became the global "work horse" in biological protein science.[44] The ability to work with methodologies that provide a quantitative description of proteins being expressed in a disease state, with the levels of possible association with drug action, is central in front line bioanalytical protein measurements.[47−49] Today, there is a high unmet need in the medical area for methods, instrumentation, and diagnostic capabilities that can meet the demand for improvements within the clinical health care area. These demands extend from early indicators of disease, through disease severity, to the evolvement phases of disease, on to therapeutic efficacy. Furthermore, these demands extend through to the next generation of personalized drugs, as well as to protein-based biopharmaceuticals. Within all these clinical stages, correlations to biomarkers are critical and highly important victories that science must win. This is the case in order to improve medical efficiency in an elderly and growing population, as well as to give hope and relief for suffering patients.[50]

Post-translational modifications of proteins are of central importance in disease and clinical analysis. Biological activation and initiation within diseases are very much related to smaller modifications such as phosphorylation,[51] acetylation, methylation, sulfatation, and glycosylation of proteins.[52,53] Phosphorylation of proteins has been shown to affect an approximation of one-third of all proteins. Phosphorylation is the most widely studied post-translational modification.[54] Within proteins, there are a large number of modifications occurring as metabolic activity within cells, organs, and biofluids. The challenge of today is to use advanced bioanalytical technology to identify the key regulatory position where on−off modifications occur, and that is linked to protein functional events.[55]

Currently, today in clinical chemistry, more than 200 proteins originating from 211 genes are being analyzed in blood plasma and serum with an addition of a large number of protein markers that are used for flow cytometry, as red cell antigens, and as tissue antigens.[50] Interestingly, none of these clinical targets have been generated from the proteomics or biological mass spectrometry research fields.[50] In an extensive survey, Anderson outlined the challenges and opportunities of new protein markers that have been approved for diagnostic use in clinical laboratories for cardiovascular diseases.[44,56]

As of today, there are no guidelines from the Food and Drug Administration (FDA) or European Medicines Agency (EMEA) for the Protein Analysis field. However, there are ongoing biomarker projects, where the FDA in collaboration with the pharmaceutical industry is looking into standardization procedures for future use. The pharmagenomics area, on the other hand, has been given a guideline for industry to use upon submission of data to the FDA (http://www.fda.gov/downloads/RegulatoryInformation/Guidances/ucm126957.pdf). This document is an important milestone and a collaborative effort between the U.S. Department of Health and Human Services, FDA, the Center for Drug Evaluation and Research (CDER), the Center for Biologics Evaluation and Research (CBER), and the Center for Devices and Radiological Health (CDRH). A similar guideline is to be expected in the not too far future that will regulate the data quality and format required to be used in drug development and clinical biomarker and diagnostic developments. The FDA also has a program on protein based multiplex assays.[57]

Biomarkers are targeted biomolecules, used to define a state of presence or a state of change in a given biology. These markers are often of Protein or peptide origin but can also be DNA (genetic), RNA (transcript), or other endogenous small molecules such as fatty acids and metabolites (metabolomics). Biomarkers are defined as "*a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention*" (Biomarker Definitions Working Group FDA−1998).

The biomarker field has taken its time to evolve. While even Wall Street[58] was valuing the area as highly profitable and futuristic, the quick pay back did not happen, as

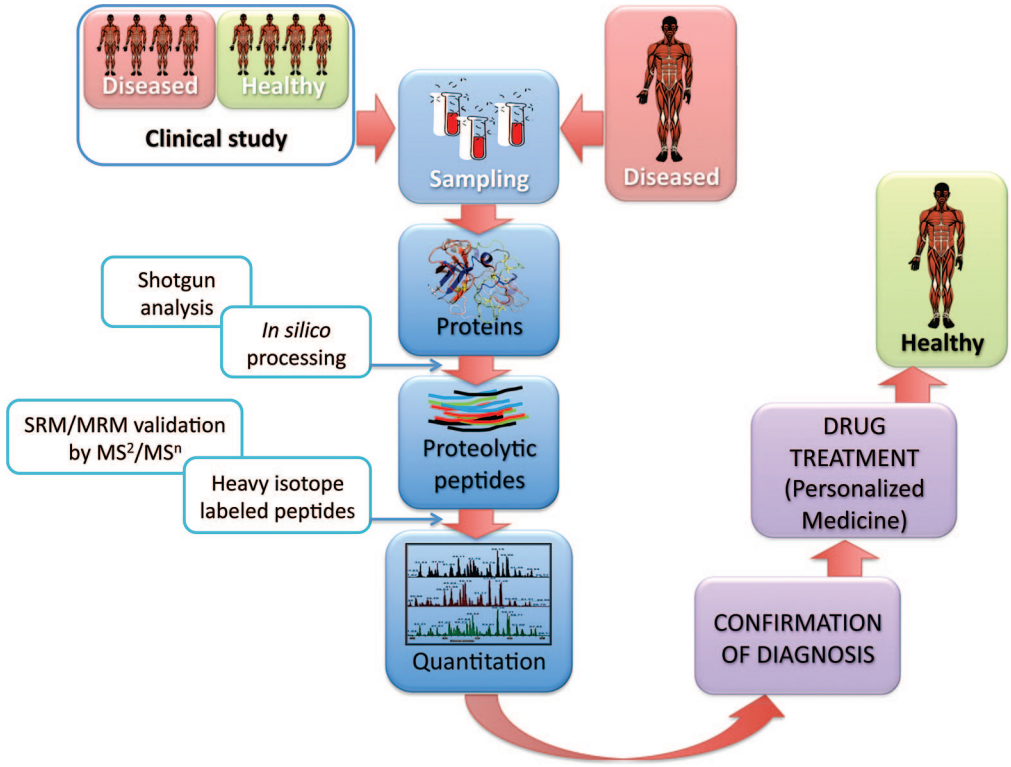

Figure 2. Protein assay development process from discovery to targeted MRM assay developments.

expected. The "Hype Cycle" theoretical model (http://www.gartner.com/technology/research/methodologies/research_hype.jsp), introduced for the adoption and business application by Gartner (Stamford, CT), explains this phenomenon. According to the "Hype Cycle", we have passed the technology trigger phase, passing through disillusionment, and are at an increasing phase of enlightenment, within the Biomarker technology area. We are at a stage when the biomarker field, after a build up phase, is developing at a fast speed. One reason for this change is the value for the pharmaceutical industry to use biomarkers in decision making, on candidate drugs that are being tested in the clinic.

The biomarker research field of today covers the pharmaceutical and diagnostic pipeline, including safety and efficacy assessment in drug discovery, biomarker translation from preclinical to clinical studies, and biomarker applications in clinical trials, including patient selections. Biomarkers are today a concept and tool within clinical studies that are used to ensure the quality of drug effects. Conceptually they will be used in relation to the clinical phase of testing (see Figure 2).

3. Protein Expression Analysis—Proteomics

Within the area of bioanalytical analysis of proteins, there has been extensive progress made in the past decade. Proteomics, the area of protein expression analysis, is the research field where most of the technology currently used today has been developed.[59−61] In retrospect, one of the first reports that was presented on the ability to map protein expression in human blood plasma was made with 2D gel electrophoresis with intact proteins[16] instead of the protein digest protocol,[62] followed by LC-MS, that is the most commonly used separation and mass spectrometry platform of today.[63] In fact, the idea to map the entire human proteome was already presented by Anderson and Anderson in the 1980s.[64] The "Human Protein Index" was the definition and science direction at the time of the agenda for postproteomic biology that Anderson and Anderson proclaimed.[64] This task was somewhat ambitious and more difficult than anyone had foreseen. Nevertheless, this task was still under heavy debate at the first HUPO World Congress that was held in Versailles, France, in 2002 (http://www.hupo.org/meetings/congress/?1). The developments within the Proteomics field of today were highlighted by the GenomeWeb Daily News Articles for 2009, where biomarker developments for Lou Gehrig's disease (amyotrophic lateral sclerosis, "ALS") were No. 3 of the top 10 most-read articles of the year.

The major protein analysis principles that have progressed over the years have been divided into the following:

Global expression analysis, where thousands of proteins can be analyzed and sequenced in a minute amount of sample.

Targeted analysis, where a specific and smaller set of proteins are measured in dedicated assays. In the last few years, MRM multiplex assays have become very popular due to their generic concept and the ability to generate multiplex quantitations[29] (for more details, see section 8.6 below).

Global expression studies are generally used in a "Discovery phase", including a wide scale analysis of many thousands of peptide sequences that originates from digested proteins. This global protein expression analysis field is the area of proteomics research.[65] Proteomics of today holds the postgenomic research activities, in one big research community, where many of the successes are and, it will be expected to deliver on the understanding of the complex pathophysiology.[66] In one respect, the proteomics field has played a significant role in opening up a number of doors that have been important for other researchers, such as the clinical field. The current status is an extensive discovery phase protein candidate delivery, that has been reported on by the proteomics society, where still the extended validation of candidate proteins still remains to be pursued.

A number of books covering various clinical and biomedical areas have recently been published, presenting the latest technology developments within separation science and mass spectrometry. These disease areas are as follows: cancer,[67] renal and urinary,[68] cardiovascular,[69] neurology and CNS,[70] biotechnology and industry,[71] and general.[72−74]

The marriage and the interface between high-resolution chromatographic separation and mass spectrometry have been a critical part of the platform developments. As the samples are more complex and contain many more proteins than we are able to analyze, the high resolving chromatographic separation will need to deliver multiple-peptide fractions of protein sequences into the mass spectrometer. No separation technology of today manages to resolve all proteins in a clinical sample such as blood plasma with millions of proteins. Correspondingly, the identified proteins of interest, that the system is going to determine, will need to be identified, where the assay needs to be optimized accordingly. The separation of this high number of peptide sequences can be performed utilizing one separation mechanism, that in most cases is a hydrophobic separation step utilizing an organic modifier/aqueous mixture of mobile phases. Multidimensional separations are being used when high separation power is required. This approach, when interfaced to mass spectrometry, was first reported by Yates' group.[75] There have been additional reports on the combination of liquid phase separation mechanisms[76] and the resolving power achieved in both plasma fractions,[63,77] as well as quantitative profiling in urine.[78−80]

3.1. Post-translational Modification

Post-translational modification is most often interconnected with protein function within disease biology. In this respect, phospho-speciation of protein activation has been extensively studied. MIDAS is one system, being a method that is monitoring the initiated detection and sequencing, with a highly sensitive approach to determine protein phosphorylation.[81] Most commonly, isolation of phosphorylated proteins from complex clinical samples has been made in a sample preparation step,[82] prior to analysis by affinity capture to lectin[83,84] and TiO_2 supports.[85,86]

An interesting approach recently reported was the verification of associated proteins' phosphorylation and gene expression. In this study, the group quantified the phosphopeptide expression and identified a total of more than 4,000 proteins.[81,87] In another investigation, the influence of cigarette smoking on endothelial cells was studied by monitoring the protein phosphorylation. Following *in vitro* exposure of human microvascular endothelial cells to extracts of cigarette smoke total particulate matter, 94 proteins were putatively identified as differentially phosphorylated.[88] Phosphorylated proteins proved to be linked with ovarian cancer in comparisons of late stage cancer and pooled control groups, idenfying the phosphorylated fibrinogen-α-chain isoform upregulated in cancer patients' plasma.[89]

Glycoproteins similarly undergo a high number of modifications within biological processing. The glycosylation can be very high within a glycoprotein, in many cases >50%. A very recent report presented a differential labeling technique, using [$^{12}C_6$]-glucose and [$^{13}C_6$]-glucose that considered all possible glycations.[90] These ^{13}C-reducing sugars were applied to quantitative analysis of glycated proteins in human plasma. Nonenzymatically generated modifications were quantified, where the positional amino groups were located in the N-terminal position or in the lysine and arginine residues.

The protein separation, using different fractionation techniques in blood serum, was summarized for all N-linked glycopeptides. These fractionation schemes were developed and interfaced to mass spectrometry platforms.[91] One general conclusion was that the incorporation of an immunoaffinity isolation step was found to be the most effective means for protein target isolation.

Another study on congenital disorders of glycosylation (CDG) was performed with the objective to quantify the extent of underglycosylation in healthy control and patient serum samples.[92] It was found that among glycoproteins there was 98−100% occupancy for all *N*-glycosylation sites of transferrin and α-1-antitrypsin. The study also presented a correlation of transferrin glycosylation site occupancy versus clinical presentation using MRM technology.[93]

Proteomics as a research area will not be able to make successes and deliveries on its own. The collaborative effort and cross sections science activities are a general trend we already experience today where we see a fast expansion and growth within many science fields. One such example is the natural link where the medical field itself is collaborating in closely designed clinical studies with the protein expression research field. As a natural progressive step, statisticians with new algorithms are mining the large data masses that are being generated. The clinical role that these new proteins found, and their functional role, is also of major importance in order to be able to make a *Disease Link* to the bioanalytical discoveries. The bioinformatics scientists play a very important role in the collaborative proteomic studies.[94,95]

4. Protein Biomarkers and Targeted Analysis

4.1. Targeted Approaches and Concepts

Diseases are very often multifactorial and are driven by more than one biological mechanism. Diseases are also in many cases not a one-point position of a treatment event. On the contrary, it is a situation where multifold biologies are cooperatively making out the disease effects. These are reasons why the diagnosis of the disease is a highly important part of the health care process. The diagnosis as such will appear in the very beginning when the patient first meets the doctor, the responsible physician, but the diagnosis will be repeated several times throughout the treatment of the disease.

Currently, protein quantitation is preferably performed with an immunoreagent based assay technique, where there are a large number of methodologies available. Currently, ELISA kits and reagent clinical flow injection assays are being used in highly automated analysis platforms where robotic instrumentation is state-of-the-art. However, modern mass spectrometry developments have already brought sufficient power together with supplementary methodologies, that prove as a strong complement to immunoreaction based technologies in biomarker research.[96]

As there is an interest in trying to understand complex biology such as the stratification in disease, the systems biology approach is gaining more and more interest.[97−99] Systems biology will help us to link the different parts of the ongoing events and build them into one common view, explaining how the system works. This will make it possible to open up new and efficient future treatments. In this context, the information that is needed in the clinical field

for improved medical treatments is increasing. We see the rise of high-density protein arrays,[100−103] as well as tissue microarrays.[104,105] The methods with zeptomole detection limits for the analysis of low-abundance proteins were already demonstrated.[106] These techniques and methods often give semiquantitative data qualities with a read-out value that will be based on the number and amount of antigen and antigenlike analytes within the assay. High quality antibodies are not easy to get hold of, although the human protein resource (HPR) project, with the aim to systematically map the human proteome is progressing strongly.[107,108]

Another development line that is gaining much attention is the use of mass spectrometry based multiplex assay technologies that will be described in detail below. In these assay formats, both selected reaction monitoring (SRM) and multiple reaction monitoring (MRM) techniques, where absolute quantification is generated, are used.[107,109,110] The SRM/MRM area is quickly taking hold and developing to simultaneously measure 10−100 analytes in one assay-cycle.[111] The assay development work has progressed in two steps. The discovery phase was initiated by a global expression study where the most interesting biomarker candidates were differentially expressed. This discovery phase was followed up by quantitative assay development in the second phase, where a selected list of biomarkers was analyzed in a SRM or MRM assay (see section 8).

5. Biomarkers

Biomarkers are probably one of the areas within biomedical research that gathers scientists into a cross-functional work relationship. Here, clinicians that meet patients every day collaborate with high tech science groups, and statistics/informatics groups. Most of the new technology platforms generate multitudes of data from a biology of interest, where the informatics and mathematics groups are needed to help elucidate the value and the interpretation of these results. The health care area and clinical organizations are utilizing these developments and are expecting pay back, by means of new and useful protein assays, in the treatment of patients.[44] In addition, there are extensive developments ongoing, where ELISA tests[112] are being challenged by new mass spectrometry based assay principles.[113−117] Biomarker perspectives and overviews, where new technology progress plays a central role in cancer and other disease areas, were recently presented by joint research initiatives.[118,119]

Biomarkers can be divided into several classes:

Primary biomarkers, *e.g.*, EGF signaling kinases (*low abundant*).

Secondary biomarkers (*low−medium abundant*), indirect biomarkers that are a resulting outcome of the signaling pathway biology.

Tertiary biomarkers (*medium−high abundant*), proteins that are associated with functional changes in disease, e.g., tissue degradation in chronic obstructive pulmonary disease (COPD) and asthma, measuring elastin and collagen connective lung tissue proteins.[120,121]

Disease biomarkers *can be low−medium−high abundant*—directly related to the disease; both to be used as diagnostic markers, e.g., PSA and HERB2, and S100, KL6 in prostate, breast, and lung cancer, respectively (see Figure 3).

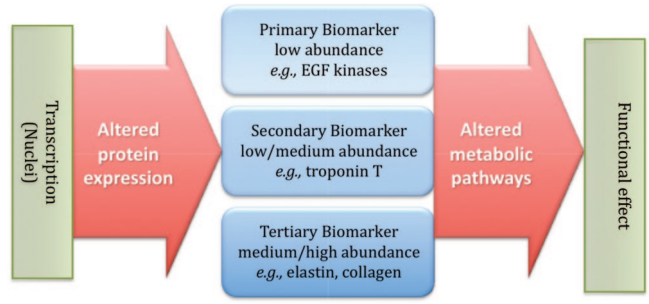

Figure 3. Illustration of biomarkers identified within a given clinical disease study.

5.1. Biomarker Classifications for Clinical Drug Studies

Biomarkers for "Proof of Mechanism"

A biomarker demonstrates an effect, which results in a functional change related to the proposed mechanism of action. The proof of mechanism effects can be measured, for instance, with an in vivo assay, where an effect is measured following an appropriate stimulus.

Biomarkers for "Proof of Principle"

A biomarker demonstrates an effect that results in a biological change, closely related to the proposed mechanism of action and known to be associated with disease activity in patients. The proof of principle biomarker readouts are proven in a dedicated patient study. It can be a measure of, for example, an acute phase marker regulation in patient studies after drug intervention.

Biomarkers for "Proof of Concept"

The biomarkers used in clinical studies which relate to the proof of concept will be measuring a study end point that demonstrates an effect on a clinical end point. Proof of concept biomarker evaluation needs to be carried out in patients with the disease in question. In cancer studies, for instance, a tumor reduction would be a positive effect where the biomarker quantitation provides a positive effect that has been achieved.

Another area of intense development is the health care field, where disease diagnosis is a great burden to hospitals. Within the drug development process, there is a successive use of biomarkers, such as the following:

- biomarkers in translational medicine
- safety and toxicity biomarkers
- biomarkers for clinical pharmacology
- patient stratification biomarkers
- monitoring of response to therapy
- molecular diagnostics biomarkers

Biomarkers can be acting as primary molecular agents, where the protein is found to be directly related to the biological event. These proteins are often low abundant with few copies expressed per cell, and in the low, or subpicogram to nanogram per milliliter concentration region. Receptor ligands, cytokines, chemokines, and growth factors are examples of primary biomarkers. Secondary biomarkers are proteins that are a response to an effect driven by a primary biomarker, such as a target interaction or a growth factor stimulation. Usually these protein markers are medium abundant proteins within the nanogram to microgram per

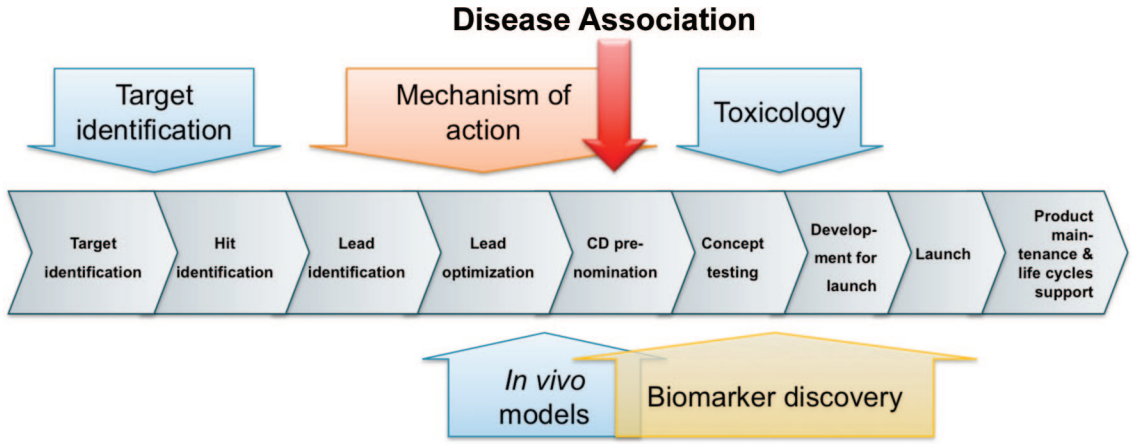

Figure 4. Illustration of the biomarker relationship of target to disease.

milliliter concentration region. Tertiary biomarkers are expressed at medium to high abundance, which can be the outcome of a functional effect.

Remodeling of an organ, such as the lung, may occur as a functional change within asthma, fibrosis, and other pulmonary diseases.[122,123] In these cases, connective tissue proteins within the cellular network, are exchanged, thereby altering the organ effects and the morphology within the lung structure.[124] These biomarkers are usually in the microgram to milligram per milliliter concentration levels. Another group of biomarkers that play an important role in the clinical field are the markers used to identify disease or changes of disease.

Most of the biomarker discovery studies have been made within the cancer area (Figure 4). An extensive candidate cancer biomarker list with 1,261 targeted proteins differentially expressed in human cancer was presented.[66] Interestingly, this report found that only 9 out of the 1,261 proteins have been approved as "tumor associated antigens" by the FDA. This initiative was also aimed at closing the gap that currently exists between basic research and clinical use of advanced diagnostics. As a global initiative within cancer research, the "Candidate List of your Biomarkers" initiative has been made. A public web-based database was developed for cancer biomarker research, and all the comprehensive data have been reported by the research community.[125]

Current ongoing clinical drug studies make use of the proof of principle, proof of mechanism, and proof of concept biomarkers and use them for decision making. Looking into the future generation of drugs, it is anticipated that an increasing number of "Personalized Medicine" drugs will be made available for patients. Personalized Medicine can be defined as the link of disease and patient to a drug through an accompanying biomarker test. The goal is to have very selective drugs with high specificity for a given patient group.[126,127] Upon use of these drugs, the specific patient group needs to be defined, and this is where the biomarker analysis step comes in, helping the patient segregation to be made. These drugs have already been introduced and are currently used by an ever-increasing number of patients globally.

6. Tissue Degradation Biomarkers

Tissue degrading products such as small proteins and/or peptides have been investigated in biofluids such as blood, urine, and sputum, bronchial and bronchialveolar lavage (BAL). As an example, the protein breakdown from the connective tissue of the lung originates from both elastin and collagens. Correspondingly, the degradation product discovery studies have focused on desmosine, and its respective variants, and hydroxyproline, correspondingly. The two pyridinium amino acids, desmosine (D) and isodesmosine (I), are positional isomers which have been found in pulmonary tissue to serve as cross-linking agents that bind the polymeric chains of amino acids into a 3D-network of elastin.[128,129] The degradation of elastin-containing tissues that occurs in several widely prevalent diseases, such as pulmonary emphysema, COPD, cystic fibrosis, atherosclerosis, aortic aneurysm, etc., has been associated with the biomarker excretion in the urine. Until now, these biomarkers with $M_w = 526$ were found to occur in urine only as higher molecular weight ($M_w = 1{,}000-1{,}500$) peptides.

In most cases, a sample preparation protocol includes acid hydrolysis of the urine with elevated temperature. The quantitative bioanalytical method applied LC-MS with electrospray ionization. Lower limits of quantitations were at the 100 pg level. Applications were made in study subjects of both healthy controls and COPD patients.[130]

6.1. Elastin and Collagen Degradation Biomarkers

Hydroxyproline is a useful biomarker where assays have been worked out by several groups over two decades. Liquid chromatography separation interfaced with mass spectrometry using internal standards is the basic bioanalytical platform that has been developed.[28,131,132] These quantitative assays have been related to chronic obstructive pulmonary disease and lung cancer, for emphysema developments. The collagen and elastin degradation rate is the clinical measure that these assays have been developed to analyze.[133]

The role of elastin and collagen degradation in the urine of smokers with and without chronic obstructive pulmonary disease was reported on with quantitative methods.[132,134−136] These platforms have also been used for *in vivo* translational developments with smoking disease models where both neutrophils and macrophage metalloelastase correlations have been accomplished.

Desmosine, which is a degradation product from pulmonary elastin, is an additional disease biomarker that is related to emphysema in inflammatory lung diseases. Molecular forms of elastin and its value as a biomarker were already worked on in 1976.[137] Desmosine quantitation has also been quantified in cystic fibrosis of the lung.[138] Very recent quantitative biomarker methodologies using MS-identifica-

tion were developed, utilizing both electrokinetic capillary chromatography and liquid chromatography.[139−143] Most of these assays have been developed measuring total desmosine. Recently, a more specific analysis was developed where both free desmosine and isodesmosine in human urine and their peptide-bound forms in sputum were quantified.[130,144] In order to enhance the sensitivity of the assay, NanoLC was interfaced with MS/MS for the quantification of urinary desmosine and isodesmosine.[145] Desmosine has also been used in drug effect studies, and a tiotropium therapy effects study has also been investigated.[146] In summary, interesting protein biomarker analysis developments are ongoing that can be useful for patients who significantly benefit from epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs).[147] At present, the scientific development focus is directed toward the ability to discover novel protein efficacy biomarkers with 1,217 nonsmall cell lung cancer (NSCLC) patients selected for adenocarcinoma and smoking status in Asia.[147]

7. Cancer

Cancer is the disease area where most of the progress in biomarker discovery science has been made. The reason is clear, which also reflects the overall health care priority that is practiced in the world today. Cancer is quickly becoming more of a global burden, with increasing incidence in the developing world as well as in the developed world. Lung cancer is the most common cancer worldwide, and it accounts for 1.2 million new cases annually, followed by cancer of the breast, just over 1 million cases, while the three leading cancer killers are different than the three most common forms, with lung cancer responsible for 17.8% of all cancer deaths, stomach, 10.4%, and liver, 8.8%. Males have a predominance of lung, liver, stomach, esophageal, and bladder cancer, and these differences derive from patterns of exposure to the causes of the cancers, and to a smaller extent, they reflect intrinsic gender differences in susceptibility. Screening and genetic based assays offer the best hope to prevent the cancers and also to target specific treatments against each cancer type. Biomarkers are expected to be an even more valuable tool in future cancer treatments.[148−150] This was recently outlined for European infrastructure, following the Stockholm Declaration,[151] as well as for FDA perspectives.[152]

7.1. Lung Cancer

Smoking is the largest cause of disease within the lung cancer patient group, although organic cooking, occupational health, and air pollution are additional causes. Only in the U.S., close to 1.5 million patients are identified each year, and the mortality currently targeted for 2009 is 562,000. It is well-known that cigarette smoke is the major cause of lung cancer, as well as the leading cause of cancer deaths. In addition, COPD has become the fourth leading cause of death in the United States. Within the lung cancer patient group, the dominating phenotype is NSCLC, with a frequency as high as approximately 80%. The prognosis for this patient group is still very poor, with only 15% surviving 5 years.[153] Within the field of NSCLC, there are recent findings that look interesting and promising, where new pathways and key regulating proteins are identified and further progressed for validation.[154]

Specific molecular targeted therapy in lung cancer with a new clinical rationale and biology was introduced less than a decade ago.[147,155−157] Interestingly, the last half-decade has brought forward a shift in paradigm toward personalized medicine in lung cancer therapy, and in this respect, both genetic and protein biomarker discovery activities have played a role.

7.2. Lung Cancer and Epidermal Growth Factor

In an early phase, epidermal growth factor receptor (EGFR) was identified as a target for anticancer drugs due to the high frequency of overexpression in cancers. Unexpectedly, it was discovered that Asian mutations in the EGFR are found to be as high as 40−60%, while the same level is at 10−15% in the western population, a very interesting observation that is proving to have medical consequences for cancer frequency, based on a genetic background probably, but certainly a molecular difference, that is presenting in a variation of EGF-pathway signaling. Secondary and tertiary effects in protein expression differences are expected to open up opportunities to identify new biomarker candidates. Correlation studies with epidermal growth factor receptors in nonsmall-cell lung carcinomas proved the correlation between protein expression and the gene copy number, with an impact on prognosis.[158] These are evidences that the current development is directed toward the developments of biomarker diagnosis.

Already today, NSCLC patients are treated based upon biomarker indications on assays confirming apparent EGFR mutations,[159] where commercial kits are available. Future trials are expected to include protein biomarker treatment of molecular targeted agents. Big efforts have been made where the molecular mechanisms of receptor tyrosine kinase (RTK) function has been the heart of research developments, by understanding RTKs role in the signaling pathways governing fundamental cellular processes, especially when EGFR is mutated or structurally altered. These findings have been very important in order to develop new lung cancer drugs. Interestingly, a group at Genentech identified another biomarker candidate, HER2, that showed a high level of homology to human EGFR.[160]

The overexpression of HER2 in tumors and reduced patient survival and time to relapse were milestone findings, and the role as a prognostic factor could later be developed into a biomarker for breast and ovarian cancer.[161]

It is evident in Asian populations that the majority of the NSCLC treated patients with activated mutations achieved a durable and effective response with EGFR TKI-treatment, such as Gefitinib (IRESSA)[162] and Erlotinib (TARCEVA).[163]

Today, the pathological grading by histology remains the golden standard for the determination of the disease progression and characterization, in clinical science. The bioanalytical technology and methodology developments will have to make the link to these pathohistological measures in order to prove their value. As there occurs molecular tissue leakages from the lung through the vascular system, these proteins that are engaged in the active biology of the lung pathology will eventually be entering into the bloodstream. These are reasons why biofluids such as blood plasma and serum are the most common sample type in clinical chemistry. The additional biofluids most often used in clinical studies are bronchial lavage, bronchialveolar lavage, sputum, exhaled breath condensate, and isolated cells from pulmonary tracts and compartments.[164,165]

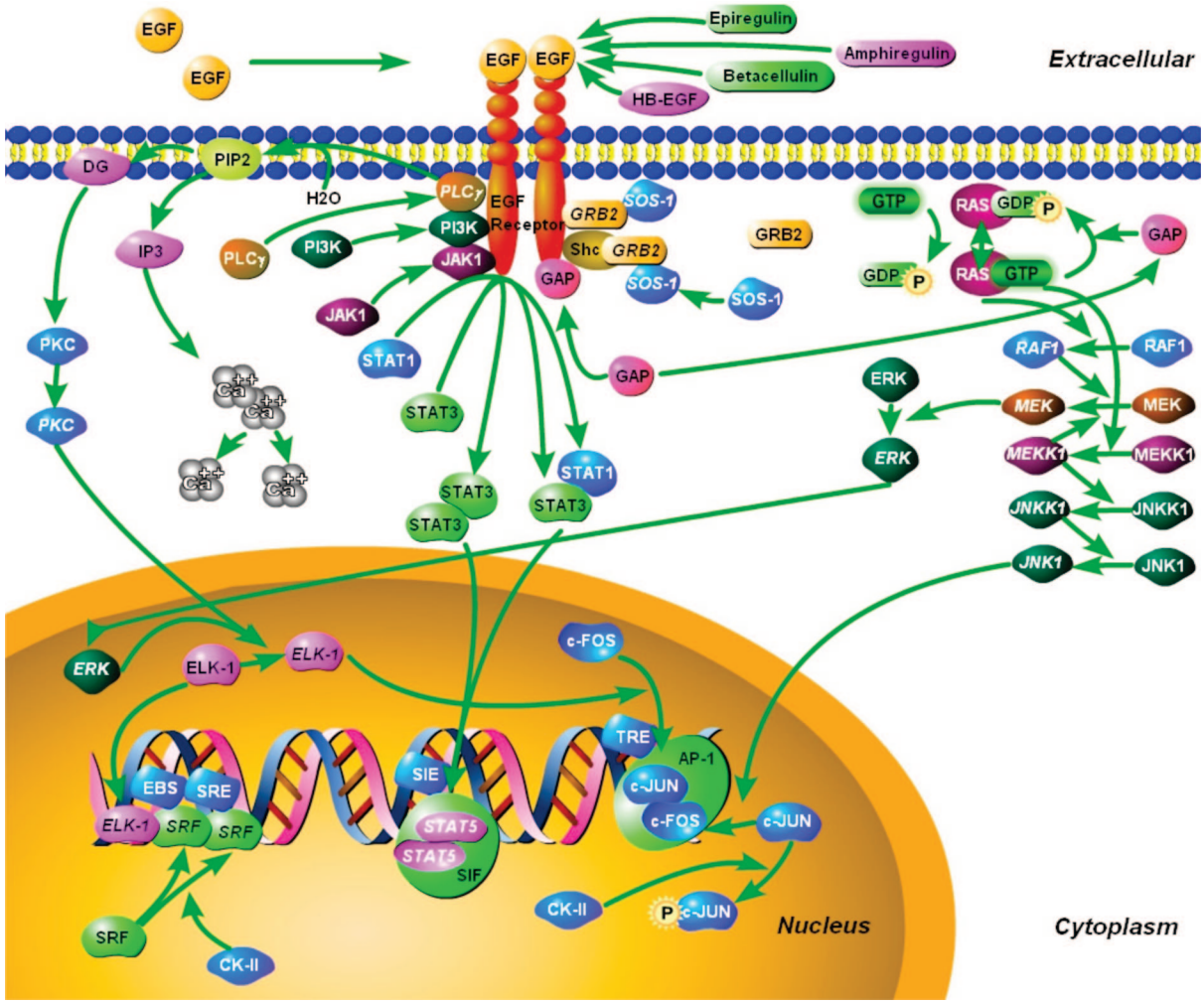

Figure 5. Schematic illustration of the EGFR signaling pathway.

The discovery of the epidermal growth factor protein earned Stanley Cohen a Nobel Prize in Physiology and Medicine in 1986.[166,167] The pioneering work by Cohen on cellular growth factors has been shown to be fundamental for the understanding of the development of cancer as well as in the development and design of anticancer drugs. The EGFR is a multifactorial protein that is a member of the HER family of receptor tyrosine kinases: HER1/EGFR, HER2/ERBB2/NEU, HER3/ERBB3, and HER4/ERBB4. The EGFR is a membrane glycoprotein with an observed molecular weight of 170-kDa in plasma. The receptor contains an extracellular ligand-binding domain, with a single transmembrane region and an intracellular domain. In addition, there is a C-terminal tail with multiple phosphorylation.[168] The extracellular domain is composed of four subdomains that contain two cysteine-rich domains and comprise the ligand-binding domain. The other parts of the extracellular domain mediate receptor dimerization and interactions with other membrane proteins. The EGFR-trans membrane domain mainly is a site for feedback attenuation by protein kinase C ERK and MAP kinases.[169]

The EGFR has been identified as one target of lung cancer, with proven associations, where EGFR has been shown to be clearly up-regulated in disease.[170] In the last few years, there has been a shift of paradigm toward targeted treatment where small molecule drugs or monoclonal antibodies are being used for patient treatment.[171]

Upon ligand binding and subsequent signaling, there is an induction occurring whereby dimerization through a receptor-mediated mechanism occurs. Next, the dimerization induces the kinase activity, which will lead to an autophosphorylation of the carboxyl-terminal tail and association with various adaptor proteins. The downstream signaling is mediated by two signaling pathways: the Ras-Raf mitogen-activated kinases (ERK1 and ERK2) and the phosphatidylinositol 3-kinase/AKT pathway.[172,173] Besides the EGF, other ligands are also available for the EGF receptor, to induce kinase signaling. The receptor−ligand binding interactions and affinities for the ligands and their modified forms will have an impact on the affinity for the receptor.[174] The subsequent pathway signaling is depicted in Figure 5.

Mann's group has in a number of papers presented the ability to follow the signaling mechanism, which includes phosphorylation mechanisms when ligand−receptor binding occurs.[51,175] A number of protein expression papers have recently been published on the regulatory role of EGFR and its role in lung cancer.[155,176−179]

7.3. Epidermal Growth Factor and Drug Treatment

There has been an early indication of somatic mutation appearances of EGFR and an increased frequency, especially in Asia.[147] The EGF mutation opened up for a new molecular mechanism of targets, to be designed with specific inhibitory

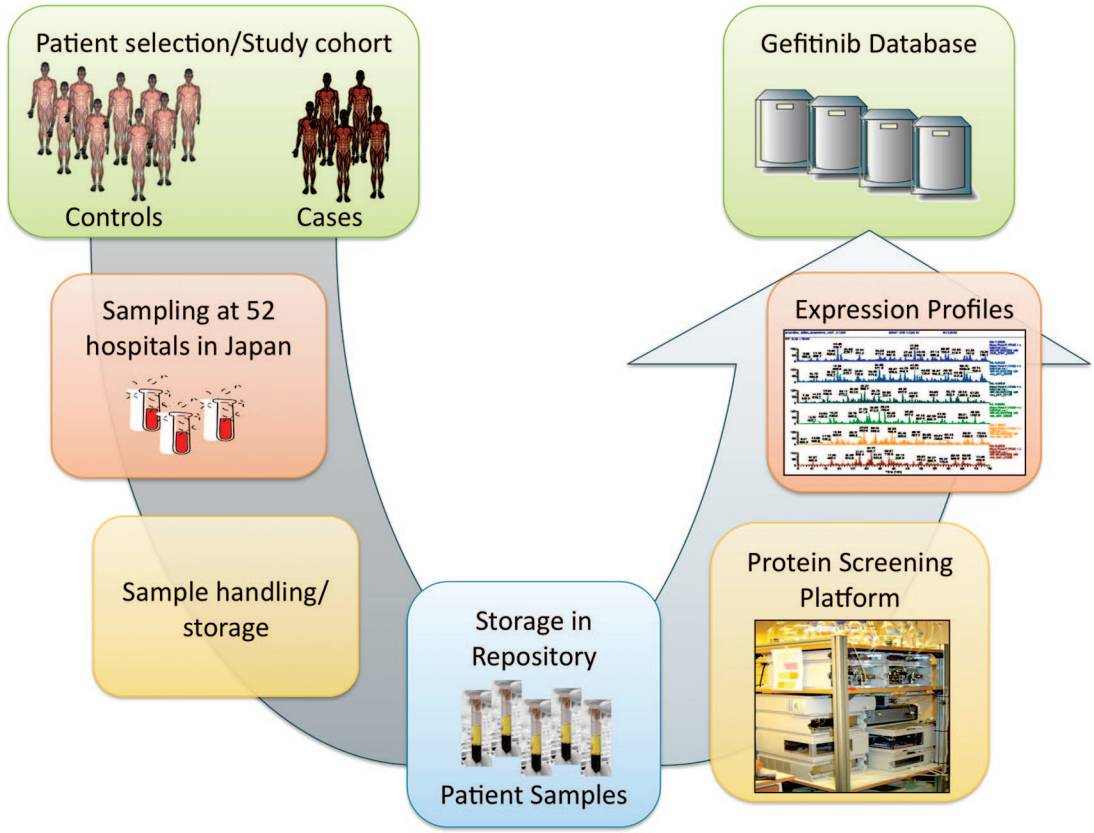

Figure 6. IRESSA clinical analysis process.

drugs that seemed to work better with mutations within the EGF receptor. Since the epidermal growth factor receptor is associated with resistance to chemotherapy as well as radiation therapy, novel drugs such as Gefitinib and Erlotinib have been developed as specific EGFR-TKIs, with good efficacy and fewer side effects. This has also been presented recently to be the situation in Europe.[180] Biopharmaceuticals such as monoclonal antibodies are also becoming more important in health care treatment.

Both Gefitinib and Erlotinib have been shown to confer hypersensitivity on patients with somatic mutations in the epidermal growth factor receptor.[180,181] A Biomarker test for the EGFR mutation in cancer patients is available. This may predict who will respond to tyrosinase kinase inhibitors. These changes in protein sequences have also been investigated where the correlation of biomarker and clinical benefit has been identified.[158,182,183] Protein biomarker studies have also been conducted in order to provide biomarkers to be used for drug safety.[157] The shotgun sequencing platform was applied within this CASE/CONTROL study in a Japanese study with close to 4,000 patients[155,179] where interstitial lung disease was investigated upon Gefitinib administration.[184−186] The Japanese CASE/CONTROL study was run with 52 clinical centers throughout Japan (Figure 6). This probably makes it the biggest clinical biomarker discovery study ever undertaken within the industry with drug treatment that has been performed with the objective to discover novel biomarkers, to be used upon drug treatment.

This clinical study and sample processing are similar in nature to several other biomarker studies that have been published. An additional Gefitinib efficacy study was conducted: the "IPASS" study.[147,187] Within the study 1,217 NSCLC patients were enrolled. Part of this adenocarcinoma cohort has been enrolled for biomarker discovery with Japanese patients alone.[147] Through the experience of the biomarker discovery study, methods were developed to stabilize variances using quality control of LC-MS measurement within the large-scale process conducted. Targeted protein markers was reported on, providing evidence for mode-of-drug-action by Gefitinib, exploring possible kinase signaling partners within the EGF pathway.[179] Rikova et al. provided evidence of Gefitinib mechanisms by identifying the tyrosine kinases that drive the disease and localizing the phosphorylation stoichiometry.[188]

Signaling by phosphotyrosines was identified within 150 NSCLC tumors and 41 NSCLC cell lines. The signaling mechanisms of EGFR, by c-Met, and other novel findings such as ALK, ROS fusion proteins, and activated tyrosine kinases such as PDGFRa and DDR were identified. PDGFRa and DDR have not previously been linked to the genesis of NSCLC, and these findings were the first on a large scale that provided evidence of tyrosine kinase activity linked to phosphorus speciation of over 50 various tyrosines and >2,500 downstream substrates.

The effects of cigarette smoke on the patient airway compartments have been studied by Spira et al.[189] Epithelial cells were investigated in this clinical study with 93 subjects. The transcriptome identified a number of biomarker candidates that have been the major focus of discussion for the screening of clinical samples at the protein level. A follow up study was made recently where Spira's group focused on how to determine which changes in gene expression are and are not reversible when smoking is discontinued. In addition, these reports have had a major impact on the field in order to search for diagnostic evaluators and candidates that can explore the normal biology of specific isolated human epithelial cells within a complex organ across a broad spectrum of health and to define the reversible and irrevers-

ible genetic effects of cigarette smoke,[190] airway epithelial gene expression in the diagnostic evaluation of smokers with suspect lung cancer.

Drug effects and mode of drug actions are fundamental in successful drug developments. In this respect, a number of EGFR-signaling tyrosine kinase inhibitors are globally used within the cancer field. The efficacy has been especially good within lung cancer and those patients with somatic mutations in the EGFR.[156,191] Gefitinib treated patients have been studied in very large patient cohorts in several studies in Asia,[147,157] the USA,[191−193] and Europe.[180] Biomarker studies have been undertaken in the Asian studies, the CASE/CONTROL study that was conducted in Japan, and the IPASS study that was run in several Asian countries. The CASE/CONTROL was probably the largest study ever performed, and it included the discovery of genetic[183] and protein biomarkers upon drug treatment.[157,179,184] The study was outlined so that biomarker regulations could be followed over the treatment period. Clinical diagnosis and blood sampling of patients were made both before and after drug administration. Patients who had progressed tumor development were treated with Gefitinib doses of 200 mg, daily.

It was made obvious at an early stage that the overexpression of EGFR in lung cancer and the signaling mechanisms were key molecular phosphorylation occurrences of central importance.[194] The EGF pathway kinases play an important role in lung cancer, where ErbB1 and its mechanistic role upon Gefitinib treatment were investigated. Computational modeling was applied, and a central role of the ErbB family proteins was predicted. The Erb protein members and the signaling interactions were outlined, and a hypothesis was presented at an early stage.[195] This is an example of collaborative efforts, where clinicians, protein scientists, and computational biologists identified central areas of disease pathology at a molecular level. Initially, a theoretical pathway model was developed that later was followed up by experimental wet laboratory assays.[196] During this period of scientific interactions, it became obvious that the EGF signaling also had a role in the protein kinase C mechanisms that were linked to cancer cell recruitments.[197] These studies were later followed up with findings made in the previous developments.[198] All of these pathway mapping biology elucidations were made in collaboration between Academia and the Pharma industry to understand the mechanisms involved in EGF signaling after Gefitinib drug treatments. As there was evidence of the somatic mutations of the EGF receptor and the role that it would have on patient response, as well as side effects,[181] this made it a major research area of interest. These specific findings became evident in order to try to find the signaling pathway alteration in EGFR mutated patients. The EGF signaling protein phosphorylation identities that Mann's group published around this time period were an important addition to the ongoing clinical analysis work at the time, and all of these data were generated by front line LC-tandem mass spectrometry instrumentation at that time.[12,175]

Lauffenberg's group, who had been involved in these developments, also came up with a targeted MRM assay that quantitatively could determine some of the interesting EGFR signaling network.[199] The MRM assays were directed toward the high-resolution temporal dynamics of the phosphorylation stoichiometry of selected nodes within the network. To undertake the study required the *a priori* knowledge of the proteins and phosphorylation sites that was previously outlined by the group.[199]

These quantitative LC-MS data showed that the phosphorylation of c-Cbl at position pY552 and of MARVEL D2 at position pY23 reached a maximal phosphorylation within 2 min after EGF stimulation. The kinetic investigation showed that ERK1 at position pY204 reached a phosphorylation maximum between 4 and 5 min and that the pY1148 position of EGFR increased quickly within a minute and then plateaued within 8 min of stimulation.

There are some concerns with lung cancer therapy, using Gefitinib or Erlotinib drugs. For some patients, the resistance to Gefitinib treatment in late stage lung cancer was observed and studied in some detail.[200,201] A target protein that was regulated in these studies was the Epithelial membrane protein-1, also classified as a biomarker for Gefitinib resistance.[178] Extension of patient survival, another indication of treatment, has been presented by association of biomarker candidates, with survival in lung adenocarcinoma.[202]

Initially, drug treatment was showing good efficacy; eventually these patients develop drug resistance. It was observed that the resistance coincides with mutations in the EGF-receptor or as changes observed in some specific proteins.[203] About half of the cases resulted in a second site point mutation. This mutation appeared in the kinase domain of EGFR, where methionine will be substituted for threonine at position 790. Consequently, the positional role of threonine 790 was to act as the "gate keeping residue" in the EGF receptor. Within the 3D structure, the location was shown in the near vicinity of the entrance to a hydrophobic pocket in the ATP binding cleft.

Other studies which address the effect of kinase inhibitors targets, such as the MAPK inhibitors, were recently presented.[204] They found in this study that, out of the thousands of phosphopeptides quantified in the study, <10% had a response pattern indicative of the targets. The impact of pathway biology and chemical proteomics techniques for translational research in patient tissue was presented in a study where the mechanistic insights in the EGFR signaling were presented.[205] In addition, other groups have also presented mechanistic data on protein signaling networks and the phosphorylation dynamics within the EGF pathway.[51,206−208]

7.4. Additional Studies on Lung Cancer

Formalin-fixed paraffin-embedded tissue (FFPE) studies have lately been accessed extensively for protein expression studies.[28,29,204,209,210] The purpose of these studies is to make use of archived material, where clinical records from patients are available, and subsequent pathophysiology correlations and learnings can be made.[211,212] Nishimura's group presents a retrospective global proteomic study with the aim to identify biomarkers for drug treated adenocarcinoma grading in tissue,[213] similar to a pulmonary shotgun sequencing study.[214]

Patient samples from grade I with primary lung lesions and grade III with spreading to the lymph nodes were chosen as tissue subjects for the biomarker discovery investigation. They used laser-microdissection as a sample isolation technique, with the FFPE material, isolating approximately 30,000 cells. Dedicated sample solubilization protocols were applied, that including stepwise organic solvent preparations, using both xylene and ethanol. This study was aimed at identifying biomarkers that could be used to grade the patient

tumor tissues, with a comparative analysis to classical histology staining and grading. The statistically significant biomarker candidates from grade I lesions were as follows: carcino-embryonic antigen-related cell adhesion molecules, Napsin-A, S100-A9, and the anterior gradient protein 2 homologue (hAG-2). These candidates were found to be useful for IA and IIIA patients. A follow up study was made with a MRM assay for quantitative determination of six biomarker candidates.[215] These studies present biomarker applicability where the prediction of long-term survivors could be made with access to information concerning diagnosis and clinical outcome. As an extension to these developments, the group has presented preliminary data on the grading of lung cancer patients where similar expression maps are identifying NSCLC, small cell lung cancer (SCLC), and neuroendocrine lung cancer (LCNEC).[216]

Another FFPE biomarker study with 34 patients with advanced adenocarcinomas that underwent neoadjuvant chemotherapy over a three-year period was presented. These patients were treated with cisplatin, in combination with folinic acid and 5-fluorouracil.[216] This study had data available from PET imaging, made 1 week before the start of chemotherapy, and could be used as a complementary biomarker correlation supplement. The associated proteins discovered were as follows: heatshock protein (HSP) 27, HSP60, glucose-regulated protein (GRP) 94, GRP78, and a number of cytoskeletal proteins whose pretherapeutic abundance was significantly different with $P < 0.001$. Rikova et al. identified by LC-MS extensive mutations in receptor tyrosine kinases (Met, ALK, DDR1, ROS, VEGFR-2, IGF1R, PDGFRa, EGFR, and Axl) and nonreceptor tyrosine kinases (FAK, LYN, FYN, HCK, FRK, and BRK) in the tissue study.[188]

One objective was to map the drug responses and activation mechanisms with Imatinib (GLEEVEC) and the EGFR inhibitor Gefitinib.[188] In more detail, the Ser473 position in Akt could be blocked by Imatinib but not by Gefitinib. An almost complete inhibition of PDGFR was reached at 100 nM levels of the drug. Another interesting outcome was that the drug effects on p44/42MAPK phosphorylation were found to be weak.

8. Absolute Quantitation of Proteins

8.1. Assay Principles

Quantitative analysis of single or multiple proteins (multiplex) using LC-MS technology in the SRM/MRM mode, an attractive direction, has gained extensive attention recently.[217] The SRM/MRM mass spectrometry principle has been used extensively in the past[218−220] and was showing impressive power for small molecules,[221−223] where triple quadrupole instruments were developed as the real "high capacity and qualitative work horses" in laboratories around the world.[224−227] This was especially recognized within the pharma industry,[223] where drug compounds and metabolites were quantified, in the discovery and developments of new drug entities. This became a standard in the pharma industry in the 1990s, where the FDA was welcoming these developments ensuring high quality data from both animal studies and also the translation into human clinical trials. It's not an understatement to add that the mass spectrometry developments in terms of instrument numbers, MS technology developments, and progress to the state and performance of today can be linked back to these early developments of SRM/MRM.

As a consequence of these successes, Barr et al. first proved the applicability for protein quantitation in an attempt to develop complementary methodologies to immunoassays, introducing the apolipoprotein A1 assay.[218] In 2001, Stemmann et al. continued building on this strategy to quantify separase, a mitosis-regulating protein, in HeLa cell extracts.[228] By using pairs of unphosphorylated/phosphorylated isotope-labeled peptides, they showed that separase was totally phosphorylated at its inhibitory site when cells were pharmacologically arrested in metaphase. This development opened up for phospho-stoiciometry studies, that are key areas in the disease understanding of kinase signaling, as well as drug development for drugs targeted toward kinases.[229] A couple of years later, Gerber et al. applied this strategy, under the acronym AQUA, for "absolute quantification", to quantitatively determine the cell cycle-dependent phosphorylations of human separase.[230] This acronym has become the trade name under which this strategy came to be known to the proteomic community.

Absolute quantification methods for the analysis of large polypeptides, or small proteins (MW > 15 kDa), which rely on isotopic dilution, require an initial analysis (experimental or predictive) to identify signature peptide(s) for each target protein(s). These signatures, or so-called "proteotypic" peptides, are distinguished by their sequence uniqueness in the context of a specific (predicted) proteome and their detection using LC-MS platforms.[231] Second, an internal standardization is performed with stable isotope-labeled analogs of these proteotypic peptides which are spiked into the samples by known amounts, before the LC-MS analysis.

Similarly, Beynon et al. presented an alternative priciple, where they designed and constructed artificial gene encoding "concatenation" of tryptic peptides (QCAT protein).[232] These synthetic peptides of concatamer proteins provide the ability to quantify up to 100 proteins in a single construct.

Absolute quantification of "heteroatom-tagged" proteins, e.g., metalloproteinases, can be accomplished, thanks to different isotope dilution standards. In these cases, heteroatoms with altered isotopic abundances have been reported on,[233] as well as heteroatom isotope-labeled peptides[234] or proteins.[235]

SMR and MRM technology, which are two abbreviations of the same MS-principle, is a mass spectrometric scan type with the highest duty cycle that can monitor one or more specific ion transition(s) at high sensitivity. The MRM/SRM mass spectrometry technique identifies and quantifies specific peptides within digested samples that are complex mixtures. MRM/SRM offers high sensitivity and speed, which is a future requirement for high throughput screening of clinical samples for candidate biomarkers within the clinical study area.

These assays use electrospray ionization followed by two stages of mass selection. Typically, the modern triple quadrupole and hybrid ion trap instruments are capable of measuring many such transitions in a single experiment, and this technique is hence referred to as multiple reaction monitoring, during which only specified transitions will be made from precursor to product ions.[6,236−239] At the initial step of the analysis, the peptides have been introduced into the ion source and ionized, and these "precursors" are selected, based on their respective mass-to-charge ratio (m/z) by the first mass filter quadrupole 1 (Q1 is not

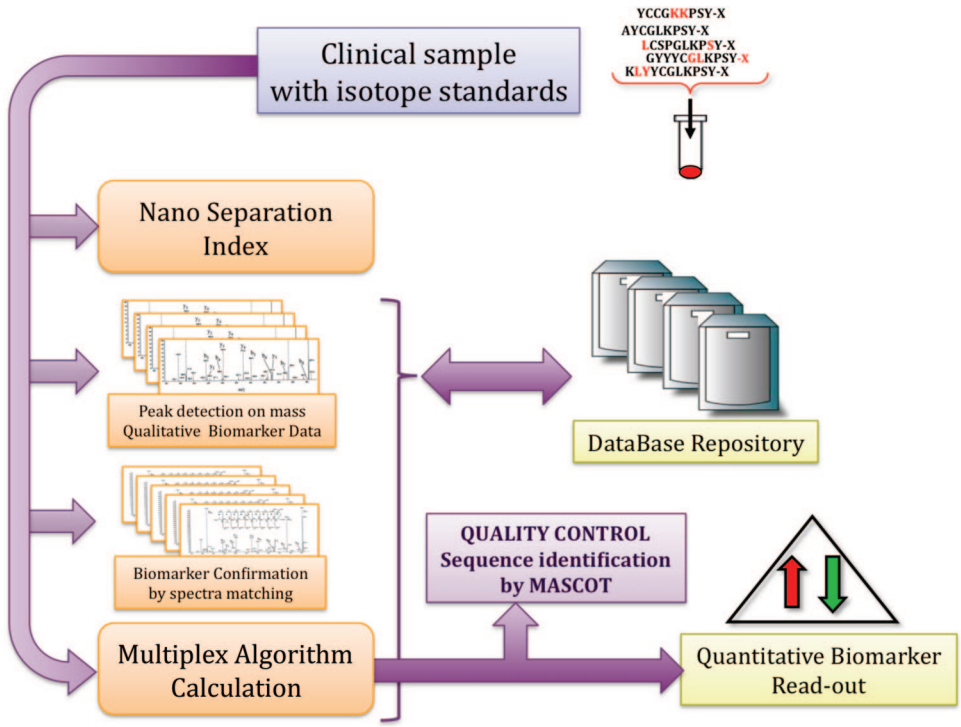

Figure 7. MRM quantitation protein platform and the process steps.

scanning) for further processing. In the next step, the selected precursor ion is fragmented by collision-induced dissociation in quadrupole 2 (Q2), where the collision energy is set to produce the optimal diagnostic charged fragment of the parent ion. Finally, quadrupole 3 (Q3) is set to the specific *m/z* of that generated fragment, as the product ion is now being transferred through the second mass filter Q3. Only ions with a given exact transition will be detected. In this step, selective detection is made on one or a number of fragment ions that will have specified mass-to-charge ratios.[240,241] The advantage of the MRM mass spectrometry methodology principle is that it offers a high selectivity with long dwelling times given by the nonscanning nature.

8.2. Assay Design

Historically, MRM has been used to quantify small molecules, such as drug metabolites, but the same principle can be applied to peptides,[219] either endogenous moieties or those produced from enzymatic digestion of proteins[218] and their post-translational modifications.[242] When it is combined with the appropriate stable isotope-labeled internal standards, the MRM approach provides absolute quantitation of the analyte concentration.[218,242,243] Technological advances now permit multiplexing of biomarker candidates to accurately measure peptide levels in a range of fluid and tissue samples.[66]

Establishing MRM assays builds on the use of one or several target peptides for each protein within the assay. Upon digestion, the resulting enzymatic products or the endogenous peptides will be determined by LC-MS, where the respective isotope labeled synthetic peptide will be added as an internal standard. The time intensive part is to identify the target peptides of the assay. Many groups have been developing tools that help in the process of making these optimizations by picking out the most sensitive peptides within the protein sequence. The difficulties, pitfalls, and solutions have been presented by several groups, providing important recommendations and guidance in the development of MRM assays and platforms (Figure 7). In addition, one of the challenges brought forward is that more than one protein can serve as the precursors of a single peptide; depending on the peptide(s) selected, this would result in the protein levels possibly being significantly under- or overestimated.[244,245]

8.3. *In Silico* Processing

An *in silico* methodology can be used in the selection process,[246,247] followed by blast searching in protein databases where identified proteins in biological samples can verify the utility of target peptides, identified as candidates. Since this is a time-consuming part of the assay development, precursors and the *m/z* of the products are key to identify at an early stage. Tools to be use for these steps for rapid optimization of MRM-MS instrumentation were presented recently.[248] Peptide libraries are very useful in order to funnel the large number of peptide candidates in the *in silico* processing step that helps to make judgments of the most useful candidates for the assay.[249]

Additional tools have been developed, such as MRM software such as the "MRMer", for management of the data generated in highly complex MRM-MS experiments. The "MRMer" is an open source product with an interactive software platform that can handle quantitative analyses with heavy/light isotopic peptide pairs. Extraction is made from the data generated in the experiments and infers precursor−product ion transition pairings and computes integrated ion intensities. The ability of rapid visual inspection, which can exceed 1,000 precursor−product pairs, makes it a useful tool to process large data sets from study samples.[241]

Further software tools have been presented, with algorithms for MRM assay, to predict calculated presentations of spectral counts of the most representative fragment ions in the experiment, for each target peptide of high importance.[213,216,248,250−251] New ways to identifying target precur-

sors from full-range MS2 spectra were developed, using the multiple products monitoring (MpM) methodology. MpM was built on a methodology that made use of a scoring system, which considers both the absolute intensities of product ions and the similarities between the query MS2 spectrum and the reference MS2 spectrum of the target peptide. In a recent report, it was concluded from a 48 protein mixture assay that great improvements in sensitivity and selectivity of peptide quantification, using an ion-trap mass spectrometer, could be achieved.[252]

Another way to identify targeted peptides for MRM assay development is the unique mass spectrometry Ion Signatures (UIS) methodology. UIS uses MS/MS spectra, which are based on data and information content, that previously have been collected by proteomics technologies. The peptide identification method captures unique mass spectrometry Ion Signatures that allow the assignment of the peptide identity.[253]

A recent web-based system has also been presented that facilitates the design of MRM transitions.[254] Walsh et al. presented a data repository driven approach, the Global Proteome Machine database (GPMDB) for MRM assay developments.[255] The GBMDB holds approximately 70,000,000 peptide tandem mass spectra. The newly developed worksheet for the design of MRM assay transitions can be found at http://gpmdb.thegpm.org. Furthermore, the current version of the "Peptide Atlas" developed by Aebersold's group holds tens of thousands of peptides from human and various species (http://www.peptideatlas.org/). It is indeed a very useful resource that is essential for target selection upon developing MRM-assays and other protein biomarker workflows. This database is publicly accessible and holds a library of peptides identified in many tandem mass spectrometry proteomics studies.[256]

In another report, a human cancer-specific peptide library with 9,677 peptides ($p < 0.001$), that makes up approximately 1,572 proteins from human breast cancer using linear ion trap instrumentation, was presented.[249] An additional improvement in this peptide library construction was the use of 2-dimensional liquid chromatography, utilizing both an electrostatic mechanism by cation exchange and hydrophobic portioning, by reversed-phase separation in the second dimension.[249]

One initiative as a response to the shortcoming within the area of the new versatile disease proteome database was the launch of the "BiomarkerDigger" database (http://biomarkerdigger.org).[257] This new system contains data from cancer biomarkers identification in plasma, as well as other biosources, and also a survey of biological studies. It is also possible to make an expression analysis survey, with the identification of patterns and expression distributions using KO pathways, GO terms, protein homology domains, and tissue expression patterns.

8.4. Isotope Labeled Internal Standard Peptides

The synthesis of chemically stable isotope-labeled peptides that are used for MS-based absolute quantification of proteins is the preferred methodology that most groups use in the multiplex assay developments with MRM platforms. The "AQUA" peptide concept is well-known, with high quality chemically stable isotope-labeled peptides with purities of >95%. The concept of chemically stable isotope-labeled peptides for absolute quantitation builds on a generic principle. The AQUA peptides are used as internal standards, where known concentrations are spiked into biological samples. As ^{13}C or ^{15}N isotopes are built into the amino acids of the synthetic peptide, the chemical and physical properties of these internal standards will be close to identical to the sequence of the endogenous protein targets.

The isotope labeled internal standard methodology has been combined with immunoaffinity techniques, to give the "SISCAPA" methodology.[258] Physiology levels of hemopexin, α-1-antichymotrypsin, interleukin-6, and tumor necrosis factor-α were determined. Somewhat later, a follow up to the SISCAPA technology was presented, where 53 high and medium abundance proteins in human plasma were quantified.[259] CVs ranging between 2 and 22% could be generated with this approach.

An alternative to this approach utilizes *in vitro*-synthesized isotope-labeled full-length proteins as standards for absolute quantification.[260] This approach allows the standard addition of the protein to be made to the sample, whereby the digestion step is also included in the processing within the methodology. That will offer a more accurate solution for bioanalytical quantitation, since the isotope-labeled full-length proteins used as the internal standards will be following the exact same conditions as the endogenous biomarkers. This is not the case using the isotope labeled peptides. The current protocols were applied to urine samples that were contaminated with *Staphylococcus aureus* superantigenic toxins as typical biomarkers of public health interest and to their absolute quantification.

Besides the isotope labeled peptide-based quantification of proteins, a newly developed approach for robust quantitation of glycans, the isotopic detection of aminosugars with glutamine (IDAWG), is worth mentioning as useful for comparative glycomic studies in cell cultures.[261] The technique is principally similar to SILAC, incorporating differential mass tags into the glycans in the presence of amide-^{15}N-glutamine, presenting further information about the isotopically heavy monosaccharides to the interpretation of glycan fragmentation and quantification.[261]

8.5. Bioanalytical Assays for Candidate Biomarker Validation

After global expression analysis studies, the follow up step is to evaluate those proteins that have been discovered to have high significance in differential expressions, typically between the control group and the patient group. These transition developments, moving from biomarker discovery to clinical validation, were recently presented.[262,263]

A targeted approach utilizing multiple reaction monitoring is recently the preferable choice. Within MRM assays, the triple quadrupole mass spec-based technique enables researchers to simultaneously analyze a high number of proteins of interest with absolute quantitation. In a paper, Anderson et al. showed that about 50 proteins could be monitored at the same time using an MRM assay.[6] Mass spectrometry-based MRM assays may serve as a good intermediate step to push proteomic biomarkers beyond the discovery phase and into the validation phase. Immunoassay-based validation approaches, such as antibody arrays and ELISA tests, are relatively easy to perform, but they are expensive and may take a long time to develop, especially if antibodies for the candidate biomarkers are not immediately available.[264]

MRM delivers a unique signal that can be monitored and quantified in the midst of a very complicated matrix. The

mass spectra plots are simple, usually containing only a single peak for each MRM. This characteristic makes the assay especially suitable for sensitive and specific quantitation. Recently, absolute quantification by MRM detection was developed for selected MFGM proteins using stable isotope dilution to provide health benefits of two MFGM-enriched future nutritional applications.[265,266]

The advantage of performing large-scale protein expression studies using tandem mass spectrometry is the ability to build a database on annotated potential biomarker sequences. This becomes very handy when specific biomarker studies are being designed, allowing an *in silico* prediction to be blast searched within the database where all previously identified biological samples containing proteins are available. Maed et al. just recently provided a useful software tool where the specificity of a precursor and its transitions is predicted.[254] The prediction of the signal intensity of the resulting peptides and fragmentation patterns is a challenging process step in MRM assay designs. In addition, a collection was made of published SRM transitions which provides functionalities such as searching and submitting new entries. In comparison to the databases we have today, many species are being covered, providing useful interfaces for users to investigate new SRM transitions. These SRM transitions were recently compiled into the "MRMaid-DB".[267]

8.6. The MRM Technology Platform of Biomarkers

Protein biomarkers are identified as differentially expressed in clinical samples; comparing for instance the clinical status of disease and health, there is a follow up step of validations that is highly important. MRM seems to be a very good choice to make these validations, whereby a list of biomarkers can be processed and quantitatively verified in clinical samples.[236,253,268]

By utilizing stable isotope standards with a sample preparation step (fractionation or antibody-capture), MRM platforms are becoming a complementary protein assay technology to ELISA and other antibody based assay techniques. A multitude of research groups in laboratories globally is picking up on the developments and utility of MRM platforms and assays. The relative ease of multifold reagent (stable isotope peptide) procurement is an attractive feature for MRM assays when compared with ELISAs.[269] Comparative studies have pointed out that isotope dilution together with the MRM methodology could provide more detailed information about molecular forms of circulating biomarkers than the routinely used ELISA or chemically nonspecific point-of-care tests.[270] Most of the MRM assays developed for absolute quantitations make use of a digestion step followed by spiking of isotope labeled heavy peptides.[271] An additional advantage, using MRM in relation, is that specific biomarker MS/MS fragments and sequence information are governed by the mass spectrometer, which is not the case with ELISA. Quantitation, based upon these fragment ions, is also an added advantage that links the specificity of the biomarker with the concentration calculations. ELISA techniques do not offer these features; rather, the epitope binding specificity, when performing at its best, circumvents unspecific binding.

The standards used in ELISA assays are generally recombinant proteins. Producing high quality recombinant protein standards along with the antibodies of the ELISA is far more expensive and time-consuming in comparison to MRM requirements.[264] One of the big advantages with MRM assays is the multiplexing capability. It allows large panels of protein measurements to be made in a single pass. This ability, as well as the generic concept of the technology, is that in principle any protein that can become an analyte within the assay is far superior to the ELISA-based assays of today.

In SRM or MRM assays, a series of transitions are made with target peptides that are the precursors being ionized after LC-separation and interfacing to MS. These precursor ions are then fragmented ion pairs. A multitude of peptides are quantified during a single LC-MS experiment. There seems to be a multiplex number around 50 that is preferred by many groups working on MRM assays.[117,272] One of the first reports presented the opportunities as well as the challenges by applying a 53-multiplex assay with high and medium abundant proteins in human plasma.[259] Most targeted multiplex assays developed have utilized the early experiences from Anderson and his group.[6,44]

8.7. MRM Applications

Looking back to decades of biological mass spectrometry and the recent decade with proteomics studies, it is obvious that all the generated protein sequence data, compiled and built within databases, is a great resource.[273−275] These experimental reports provide in many instances not only sequences of importance but also, in numerous cases, quantitative information regarding specific biology of clinical relevance and interest. These clinical analysis data have been generated both within academia, where they are made public, and within the pharma and biotech industries, where they are kept within the firewall of these companies. Nonetheless, the value of all this information, also highlighted by HUPO in the standardization program, Proteomics Standards Initiative (http://www.HUPO.org/research/psi/), is a strong basis for MRM/SRM assay developments.

MRM assay developments have been proven to be a highly useful technology principle for clinical protein and peptide analysis. In recent years, there has been a constant increase in reports on the assay developments that can be made by MRM platforms. They offer robust and reliable quantitations of proteins and peptides with good accuracy and precision. MRM applications are currently the fastest growing targeted protein analysis area, with multiplex assays for absolute quantitation in clinical disease areas. The cardiovascular area[276] is, besides cancer,[277] the most challenging disease area worldwide for the health care system to handle at present, as well as in this new decade.

The cardiovascular effect of symptomatic hypertrophic obstructive cardiomyopathy was investigated in a study where Troponin I, Troponin T, IL-33, CRP, BNP-32, MRP14, NT-proBNP, and MPO levels were quantified in the nanogram per milliliter regions in blood samples.[276] Multidimensional separations were applied, combining electrostatic and reversed phase separation mechanisms. The MRM technology is able to quantify proteins down to low nanogram levels and higher concentration regions, which means high and medium abundant, and upper low abundant areas. These are the applicable expression regions for proteins of interest. Importantly, the precision as well as stability of these platforms needs to be met for practical use. The report demonstrated limits of CVs of 10−20% in the 1−20 pg/mL (LOQ) range in plasma.[278] Another group presented recent data on quantitation of cardiac plasma biomarkers at 2−25

ng/mL concentrations. The intra- and interassay coefficients of variation were predominantly <10% and 25%, respectively.[276]

By the use of uniformly ^{15}N-labeled blood plasma, levels of 100 ng/mL calreticulin could be quantified. The accurate inclusion mass screening (AIMS) methodology was used that made it possible to build a targeted assay, from data generated from an unbiased discovery study.[279] An additional fractionation step was included using strong cation exchange separations of digested plasma samples to reach these levels.

A first report came out very recently where a magnetic bead-based immunoaffinity sample preparation method could reach relevant medium abundant concentration levels at nanogram per milliliter sensitivities. By automation of the peptide antibody capturing process step, high throughput capacities were reached with low statistical variations (median CV 12.6%) for quantifying biomarkers using only 10 μL of plasma. Interestingly, by increasing the sample volumes to 1 mL, an improvement of the detection to the low picogram per milliliter range was reached.[264]

Still, the low abundant region, i.e., picogram per milliliter, with proteins with a few copies per cell, such as interleukins, chemokines, and other growth factors, is still better analyzed by ELISAs.[43] In order to improve the LOQ-limits even further using current MRM technology platforms, one way forward would be to increase the sample volumes. This will require a sample preparation methodology, which is able to enrich larger sample volumes and to elute into an enriched and smaller sample volume, ready for analysis.

A recent paper by Kuzyk et al. presented a MRM-assay with 45 plasma proteins and absolute quantitation.[272] The assay development presented makes a selection of clinically interesting markers and develops an assay that has a fast development time and is generic in its choice of candidate proteins. The multiplex protein assay was chosen to optimize the usability of these proteins. The plasma protein expression profiling of 31 out of the 45 selected proteins shows well-known biomarkers of cardiovascular disease. Aebersold's group worked out a survey of MRM methodologies, to cover a full dynamic range, being able to measure high, medium, and low abundant regions of protein expression, as low as 40 protein copies/cell level defined in a yeast system.[276,280] Interestingly, this group could achieve signal amplification by fractionation, resulting in a 10-fold sensitivity improvement. The group reports an average maximum signal gain of a factor 80. The fractionation was achieved by a tryptic digest using isoelectric focusing.[281] This study could demonstrate that expression of proteins at a single-digit number of copies/cell can be detected by SRM coupled to a simple, fast, and predictable sample fractionation step. More than 1,500 yeast proteins, including complete cellular pathways, are processed during a 1 h assay run.[280] Additional studies have presented applicational approaches. Kuster et al. reported on an MRM protocol where, for each protein, five proteotypic peptides (unique peptides preferentially detectable by MS [PTPs][282,283]) were selected. PTPs were derived by screening a large yeast proteomic data repository, PeptideAtlas (>36,000 unique peptides observed in an array of shotgun proteomic experiments[284]) for the most frequently observed peptides for each target protein. For proteins for which fewer than five PTPs could be extracted from PeptideAtlas, additional peptides with favorable MS properties were derived by bioinformatic prediction using the tool PeptideSieve.[231,216]

Addona et al. presented data made from a multisite study, where the assessment, precision, and reproducibility of MRM-based analysis of proteins in plasma was demonstrated. This study presented a good correlation between respective laboratories and is a landmark of MRM-multiplex assays in clinical biofluids. The assay and methodology transfer are key elements in future successes and developments within the multiplex assay to be extensively used in clinical analysis. This multilaboratory study provided comparisons on reproducibility, recovery, linear dynamic range, and limits of detection and quantification of multiplexed, MRM-based assays. The study was conducted by the NCI Clinical Proteomic Technology Assessment for Cancer initiative (NCI-CPTAC). Information on the initiative of the National Cancer Institute can be found at http://proteomics.cancer.gov/programs/CPTAC/. The new initiative provides data on applying new technologies for biomarker verification in plasma.[285]

Prostate cancer is another major disease problem for men throughout the world. The prostate specific antigen (PSA) is a biomarker that is used in many countries as a routine clinical measure. Its increased level indicates a potential problem of early onset stages of prostate cancer. There are a number of ELISA test kits that are used in everyday diagnosis.[286,287] However, a number of shortcomings remain regarding the PSA variants that are being measured in an ELISA, which is the reason why developments of new assays are of mandatory importance.[288] Sample preparation of blood samples is key in assay development.[280,289] Quantitation was achieved by an MS3 fragment using a Q-TRAP instrument.[243] Furthermore, the isotope dilution mass spectrometry principle was also reported on absolute quantification of PSA in blood serum, achieving good linearity and reproducibility that can pave the way for protein sequence based detection of this biomarker.[290]

There is a huge unmet need of rapidly increasing elderly patient groups around the world, to get improved treatments for neurodegenerative diseases and brain tumors. Biomarkers for Alzheimer and Parkinson disease are well underway. Ferritin expression in the neurons of the human *substantia nigra pars compacta* from post-mortem human brain tissue was investigated by a MRM tandem mass spectroscopy assay.[291] The targeted protein Ferritin that is a 24 protein was investigated in relation to its ability to bind Fe(III) atoms and form inorganic complexes.

9. Concluding Remarks and Outlook

It is envisaged that multiplex platforms and assays will gain much attention and that they will become a cornerstone in future clinical science. The ability to screen patient samples with panels of hundreds of protein biomarkers with absolute quantitation provides immense value. The future generation of personalized medicine drugs where the patient will receive the right medicine and/or dose of drug for the specific disease or phenotype by the first visit at the doctor's clinic will drive the entire diagnosis field forward.

As an example, an average ELISA kit cost of $500 for a 96 well format allows the analysis of 60 samples, if QC samples and calibration samples are added to the plate. With a panel of 50 biomarkers, this will be a substantial cost of $250,000. As an alternative to immunoassays, using MRM assays, especially for decreasing the number of candidate biomarkers from many tens to a panel of about 10 biomarkers, would be reasonable to deal with during validation. It

is anticipated that bioanalytical developments within the clinical research area will play a major role in the future. Implementation of new platform and technology developments is expected for a future world where the number of patients or consumers will increase and will be an ever-increasing elderly population. These are forthcoming global changes that will enforce solutions where clinical protein science with mass spectrometry based technology platforms will have its place.

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