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Recent developments in proteomic methods and disease biomarkers

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Proteomic methodologies for identification and analysis of biomarkers have gained more attention during recent years, and has evolved rapidly. Identification and detection of disease biomarkers are important to foresee outbreaks of certain diseases and thereby avoiding surgery and other invasive and expensive medical treatments for patients. Thus, more research into discovering new biomarkers, and new methods for faster and more accurate detection is needed. It is often difficult to detect and measure biomarkers because of their low concentrations and the complexity of their respective matrices. Therefore it is hard to find and validate methods for accurate screening methods suitable for clinical use. The most recent developments during the last three years and also some historical considerations in proteomic methodologies for identification and validation of disease biomarkers are presented in this review.

Introduction

Recently, more focus within the study of proteins, usually referred to as proteomics, have been searching for new biomarkers for diseases. Meanwhile, less focus have been on the methodologies. The terminology "biomarker" or a "biological marker" was first defined in 1989 [1] as a medical subject heading term: "measurable and quantifiable biological parameters (e.g., specific enzyme concentration, specific hormone concentration, specific gene phenotype distribution in a population, presence of biological substances) which serve as indices for health- and physiology-related assessments, such as disease risk, psychiatric disorders, environmental exposure and its effects, disease diagnosis, metabolic processes, substance abuse, pregnancy, cell line development, epidemiological studies, etc." We also refer to Good *et al.* [2] for a discussion about the term biomarker.

In other words, biomarkers are indicators of which state we are in and if we have or are in the risk zone of developing some type of disease, for example by monitoring of clinical responses [3–5]. Characteristics that are desirable for a biomarker includes that it should be both specifically and quantitatively correlated with the state or disease in question and that it can be measured with specific and sensitive methods that provide reproducible results. Moreover, the biomarker should be chemically stable, not only when analyzed but also during storage, and not be susceptible to changes from outside sources. Ideally, the biomarker is also available by non-invasive assessment and the levels of the biomarker should be fairly constant in each individual [6,7]. Different proteomic methods can be used effectively for analysis of biomarkers, and an advantage would be fast and accurate screening for diseases using biomarkers in, e.g., blood serum, which is the easiest accessible source of biomarkers [8] and is more safe and less expensive than a biopsy [9]. In 2012, there were more than 14 million new cancer cases according to the International Agency for Research on Cancer [10]. The discovery of biomarkers is thus of importance for the future, since many different diseases, e.g., cancer types, may be detected in an earlier more treatable stage, making the patients suffering less, and it would also be interesting economically, since it will lead to easier treatment, and surgery may be avoided.

To identify and detect potential disease biomarkers in proteomics is difficult and many biomarkers are seldom verified or clinically validated [11]. High throughput, fast, simple, low-cost, accurate, and sensitive methods are needed [12,13], and often separation methods in one dimension do not have enough resolution for the complex samples [14]. Large amounts of data are also necessary to handle. Moreover, there is a plenitude of different methods for analysis of disease biomarkers. These methods are typically based on mass spectrometry (MS) in combination with different separation techniques like liquid chromatography (LC), electrophoresis, two dimensional gel electrophoresis (2-DE), two dimensional polyacrylamid gel electrophoresis (2D-PAGE), 2D differential gel electrophoresis (2D-DIGE), matrix assisted laser desorption/ionization (MALDI-TOF-MS), surface-enhanced laser desorption/ionization (SELDI-TOF-MS), liquid chromatography tandem mass spectrometry (LC-MS/MS) and high resolution MS such as Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) or Orbitrap-MS. LC has the advantage of flexibility due to the large abundance of stationary and mobile phases to choose from [15]. Validation methods such as e.g., enzyme-linked immunosorbent assay (ELISA) can then be used. Most focus have

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not been on the different methodologies, therefore we find it essential to highlight what has happened during the most recent years within identification and validation of disease biomarkers and the methodologies used for this purpose.

The proteomic methods can be based on either a "top down" approach, where the intact protein is analyzed, or a "bottom up" approach[16], where digested proteins are analyzed with, e.g., LC-MS/MS. A schematic picture can be seen in Figure 1. Furthermore, three types of labeling are common when analyzing biomarkers: isobaric tags for relative and absolute quantification (ITRAQ), tandem mass tag (TMT), and stable isotope labeling by amino acids in cell culture (SILAC).

In a review by Craft *et al.*[17], recent developments in quantitative neuroproteomics are considered, and newer MS-based approaches are discussed, including label-free, labeled, and quantitative methods, and moreover, how these apply to both brain tissue and cerebrospinal fluid (CSF). Moreover, substantial reviews have previously been written by Megger *et al.*[18] concerning label-free quantification in clinical proteomics, and Shukla and co-workers[19] have also written a review about membrane proteomics, and the discovery and importance of biomarkers. Other current trends in biomarker research worth mentioning in particular includes companion diagnostics and personalised medicin[20].

Proteomic methods already play an important role and this role will only be expanded in the future. However, the focus has previously not been on the methods used for discovery and validation of biomarkers. Therefore it is essential to underline the importance of the research using the different methods leading to, e.g., faster diagnosis and easier treatment by finding biomarkers. This is the aim of this article. Many of the proteomic methods that are currently in use are described, compared, and discussed. Moreover, some novel approaches that very likely will be used in the future are also considered.

Sample Preparations in Proteomics

One common method for sample preparation in proteomics is electrophoresis. There are different types of electrophoresis, e.g., those based on separation in capillaries and those in gels. Historically, 2-DE has been one of the most common techniques for quantitative analysis and separation of proteins. The separation by charge occurs by isoelectric focusing, and separation by size by e.g., sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The method is straightforward and the fractionated proteins are then analyzed by mass spectrometry. Capillary electrophoresis is a technique that depends on the migration of the analytes exposed to an electric field. The analytes will be separated depending on their mobility in a solution in a capillary column without stationary phase, influenced by the electric field. They will be separated according to factors that influence the mobility such as size and charge. WB or immunoblot is another type of gel electrophoresis that is used for analysis of biological samples. These methods separate the proteins similar to 2-DE, but only by size, which can be an advantage. The proteins are stained with specific antibodies after the gel separation, which makes it possible to detect the proteins by e.g., chemiluminescence, and images can be taken by a cooled CCD camera. WB is commonly used for absolute quantification, or for quantification relative to a control sample. Furthermore, WB is commonly used during the verification step of a biomarker[21].

Tung and co-workers[22] have analyzed biomarkers from the aggressive cancer type oral squamous cell carcinoma (OSCC) in plasma samples, related to the chewing of beetle nut. Analysis was made by fluorescence 2D-DIGE and MALDI-TOF-MS. They successfully identified vitamin-D binding protein which is a marker for OSCC. Moreover, other potential biomarkers such as fibrinogen (alpha/beta/gamma) chain, haptoglobin and S6 kinase alpha-3 (RSK2) were detected.

In India, a large part of the population has an endemic infection, a latent tuberculosis infection (LTBI). Methods for differentiating between LTBI and the active disease is important, but many methods of analysis does not differentiate between the two. Anbarasu *et al.*[23] found that seven biomarkers in blood samples could potentially be used for differentiation. Proteins were separated with two dimensional liquid phase electrophoresis (2D-LPE) and SDS-PAGE before analysis by MALDI-TOF-TOF and multiplex cytokine assay.

A biomarker for the complicated Fabry disease have been studied by Cigna *et al.*[24]. The only method used to detect the disease today is by mutation analysis by sequencing α-galactosidase A gene. Therefore it would be advantageous to find a method for screening using biomarkers. They compared peripheral blood mononuclear cells (PBMC) in healthy patients to those with Fabry disease by 2-DE and detected differentially expressed proteins using MALDI-TOF-MS. Preliminary results show that further investigations need to be made on larger patient groups, and other methods such as WB may be used for validation of the proteins they have found.

In plasma, cellular and membrane proteins have posttranslational modifications (PTMs) that are important — even if they occur in low concentrations — in the analysis of tumors and can be separated by electrophoresis. In 2013, Megger *et al.*[18] used label-free quantification in clinical proteomics, and in 2006, Wu *et al.*[15] considered the three quantitative methods DIGE, cICAT, and iTRAQ using 2-DE or MALDI-TOF-TOF in proteomic analysis.

In a notable work by Uchida *et al.*[25], they discovered ten new potential biomarkers for microscopic polyangiitis (MPA) and granulomatosis with polyangiitis (GPA) by 2D-DIGE and mass spectrometry from samples originating from blood samples.

Li *et al.*[26] have used mass spectrometry for detection of proteins in serum samples, and ELISA measurements for quan-

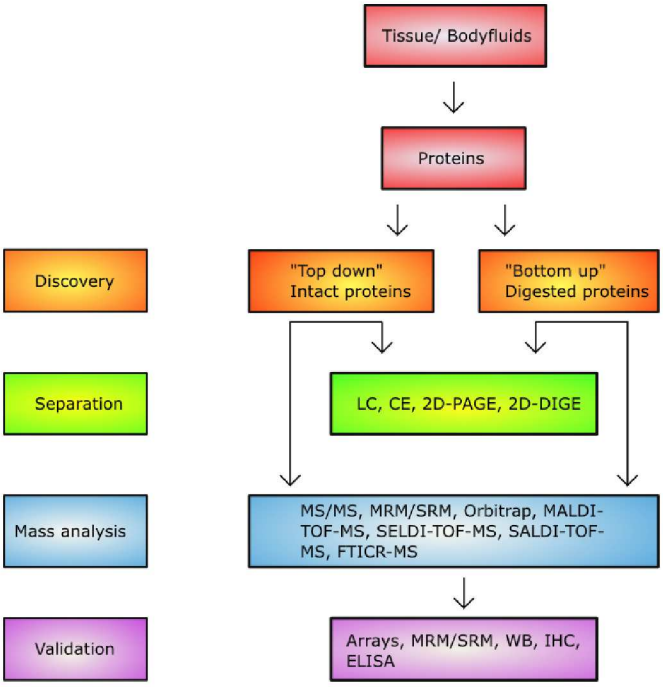

Fig. 1 Processes involved from discovery to validation of biomarkers. During the discovery, the proteins are either analyzed by a "top down" or "bottom up" approach. During the separation step, liquid chromatography, capillary electrophoresis, two dimensional polyacrylamid gel electrophoresis, or 2D differential gel electrophoresis can be used. The mass analysis step can consist of tandem mass spectrometry, Orbitrap, matrix assisted laser desorption/ionization, surface assisted laser desorption/ionization, or surface enhanced laser desorption/ionization mass spectrometry, for example. In the validation, enzyme-linked immunosorbent assay, multiple reaction monitoring/selected reaction monitoring, western blotting, immunohistochemistry, or arrays, are examples that can be used.

tification. They detected the biomarker SAA. This biomarker is also found in other diseases as well as an acute phase protein. The SAA peak could be found in untreated epileptic patients, and when treated the peak decreased. The peak could not be found in healthy control groups or after treatment of patients for three months. The authors suggest that CSF might be used instead of serum in clinical trials in the future.

Li *et al.*[27] used two-dimensional gel electrophoresis and LC-MS/MS to analyze peroxiredoxin 1 (PRDX1) as biomarker in tissue interstitial fluid (TIF) for non-small cell lung cancer (NSCLC). To verify the expression of PRDX1, ELISA was used. Results show that PRDX1 may be used as a biomarker for NSCLC because of the elevated expression in TIF.

Conti *et al.*[28] have analyzed skeletal muscle biopsies to find biomarkers for amyotrophic lateral sclerosis (ALS). They have found that myosin binding protein H (MyBP-H) is a potential biomarker that might be used for the purpose of differential diagnosis. The methods employed were 2-DE and SDS-PAGE, imaging, MS and WB.

HCC is a common form of liver cancer and biomarkers are important both for early diagnosis and treatment progression. Megger *et al.*[29] have used a combined 2D-DIGE and LC-MS approach to find two proteins in liver tissue that may have potential as biomarkers for diagnosing HCC, namely major vault protein and betaine-homocysteine S-methyltransferase.

Dong *et al.*[30 27] have identified HSP90 as a potential biomarker of biliary atresia. 2-DE and MALDI-TOF-MS was used to compare protein profiles, and HSP90 was identified by LC-MS/MS. Immunoblotting analysis was employed for verification. Their results showed that HSP90 was most significant and possibly used as a biomarker.

Katakura *et al.*[31] have used peptide-mass fingerprinting (PMF) combined with 2-DE to analyze proteins for OSCC in saliva samples. To confirm their results they have used tissue samples and made immunohistochemical staining. Results showed that enolase 1 can be a biomarker for OSCC in salivary samples and that it may originate from OSCC tissue.

Furthermore, Xiao *et al.*[32] have used gel electrophoresis and LC-MS/MS to study microvesciles (MVs) for diseases in saliva samples. Their results show 63 proteins to be identified in human MVs, and that salivary MVs could be used for discovery of diseases.

Roscioni *et al.*[33] have employed CE-MS to analyze urine samples, and discovered 14 peptides that could potentially differentiate between normoalbuminuric controls and patients who have developed microalbuminuria. However, the small sample size limits the statistical power for the results. Moreover, Kistler *et al.*[34,35] have previously found polypeptide patterns for autosomal dominant polycystic kidney disease (ADPKD) in urine samples by CE-MS. The disease is caused by mutations in PKD1 or PKD2. They have continued their research and verified their findings by radiolic imaging studies.

Additionally, Dawson *et al.*[36] have used CE-MS coupled to a micro-TOF-MS for analysis of urine samples, to develop a method for screening of biomarkers of ischaemic stroke. They used a vector machine based software to find potential disease-specific peptides, and the potential biomarker candidates were sequenced by LC-MS/MS. Their results were successful, and they developed a two biomarker-based classifiers, one containing 14 biomarkers, and another containing 35 biomarkers. This method can be useful for patients with mild symptoms of acute stroke, and they are currently working on exploring the clinical utility in larger tests.

Shimwell *et al.*[37] have used shotgun proteomics and ELISA to make a combined proteome and transcriptome analysis for bladder cancer in urine samples. They managed to identify tumor secreted proteins such as midkine and HA-1, that were significantly higher in those patients with bladder cancer than the control group.

Current Proteomic Approaches

As has been stated above, proteomics can play an important role in the discovery of potential biomarkers. In this section, we summarize some of the different proteomic methods currently employed in the search for biomarkers.

Untargeted Label-Free Proteomics

Liquid chromatography is a separation technique based on the equilibrium between a mobile phase and stationary phase, and depending on the physical and chemical properties of the analytes and phases, compounds are more or less retained, thus separation occurs. The technique is often used together with MS. There is an alternative method that can provide high throughput information, called "shotgun proteomics" or "shotgun mass spectrometry"[38]. This method relies on digestion of the proteins and LC coupled to online mass spectrometry. Shotgun mass spectrometry can be used e.g., for identification and localization of proteins, and have some disadvantages compared to targeted proteomic mass spectrometry methods including multiple reaction monitoring (MRM) or selected reaction monitoring (SRM), such as lower sensitivity and reproducibility, thus targeted mass spectrometry can be used instead to avoid problems with shotgun mass spectrometry[39]. Addona and co-workers[40] developed a pipeline for both discovery and verification of possible biomarkers in plasma. Using LC-MS/MS for analysis of blood sampled from the hearts of patients directly before, during, and after a planned myocardial infarction, resulted in 121 proteins expressed highly differentially in the different groups. To choose a suitable subset of possible biomarkers, accurate inclusion mass screening (AIMS) was employed. Analysis by MRM or immunoassays of controls and patients with myocardial infarction indicates that the

chosen proteins may be biomarkers of myocardial infarction.

Plasma samples have also been analyzed by Kim *et al.*[41] where they used LC-MS/MS and found 86 candidate biomarkers, among them plasma Complement C3 that can be used in measuring the state of neuroblastoma patients.

Jaros *et al.*[42] have used the methods immobilized metal ion affinity chromatography (IMAC) for enrichment, and label-free LC-MS^E for identification. This was made to investigate both proteomic and a post-translational modification (PTMs) phosphoproteomic analysis in blood serum samples, where 20 antipsycotic-naïve schizophrenia patients were compared to 20 healthy individuals. Analysis resulted in the identification of 35 proteins that had higher levels than in the healthy control group. The enriched samples resulted in 72 phosphoproteins of which 59 only showed changes in phosphorylation, and no overall protein change. Further studies need to be made of the phosphorylation-specific changes, and might lead to the investigation of biomarkers for schizophrenia.

Ozaki[43] have by mass spectrometry discovered the new serum biomarker 13 C-terminal amino acid residue of apolipoprotein A-I called AC13, for microscopic polyangiitis (MPA) that may be helpful in future treatment.

Abdominal aortic aneurysm (AAA) is a common cause of death for e.g., elderly men. A difficulty with AAA is that it is often asymptomatic, and thus, potential biomarkers are interesting. Ramos-Mozo *et al.*[44] have used 2D DIGE and MALDI-TOF-TOF to analyze polymorphonuclear neutrophils in blood samples, and found several potentially interesting proteins. Moreover, connections between progression of AAA and oxidative stress are also considered.

Gastrointestinal stromal tumor (GIST) is a common malignancy. Predicting poor prognosis for patients with GIST is important, and Kikuta *et al.*[45] have identified ATP-dependent RNA helicase DDX39 as a novel biomarker. The technique used was 2D-DIGE for profiling, LC-MS/MS with a linear ion trap for protein identification, and Western blotting (WB) and immunohistochemical staining for validation.

Plasma and serum are ideal to screen for biomarkers in, however, Zhang *et al.*[46] have written a notable review regarding salivary proteomics. Analysis of salivary samples would be more safe, economical, and easier than e.g., blood samples. Salivary contain many proteins, about 20% of the amount found in plasma samples, thus biomarkers are possible to detect. By using a less expensive and easier sampling method, diseases could be possible to detect earlier.

Border *et al.*[47] have used salivary samples in a preliminary study where they have analyzed biomarkers for diabetes type 2 by 2D-LC-MS/MS. They found that glyceraldehyde-3-phosphate dehydrogenase (G3P) and serum amyloid A (SAA) increased in samples from patients with diabetes, while other biomarkers such as amylase, palate, lung and nasal epithelium associated protein (PLUNC) and serotransferrin (TRFE) were found to decrease. Previously, carbonic anhydrase 6 and alpha-2-macroglubin have been found to increase in samples from diabetic patients. However, in this study they decreased instead. To further investigate the results from the decreased biomarkers, WB was used. Further validations are currently made with e.g., larger sample populations.

Analysis of pancreatic fluid has been made by Paulo *et al.*[48] where they have used LC-MS/MS after fractionation by SDS-PAGE to find differences in posttranslational modifications (PTMs) in proteins. They determined several PTM proteins that differed between patients with chronic pancreatis and the control group without. These PTMs may serve as biomarker candidates for chronic pancreatis.

Glycosilation is a common protein modification, but unfortunately, glycosilated peptides are more difficult to analyze by mass spectrometry compared to the non-glycosilated counterparts. Kuo *et al.*[49] presents a variation of hydrophilic interaction liquid chromatography, where amine derivatized Fe_3O_4 nanoparticles are used for enrichment. This approach enabled direct analysis by both MALDI-MS and LC-MS/MS after elution.

Moon and co-workers[50] have employed a gel-assisted protocol with nano-UPLC-MS/MS in MS^E-mode to study urinary exosomes for patients with IgA nephropathy (IgAN) and thin basement membrane nephropathy (TBMN). Four biomarker candidates were identified, aminopeptidase N, vasorin precursor, α-1-antitrypsin, and ceruloplasmin, that could potentially differentiate between early IgAN and TBMN. Validation of exosomes purity in urine was carried out by WB analysis.

Urinary exosomes in diabetic rats have been studied by Raimondo and co-workers[51]. In their study they used nano-LC-MS/MS and validated their findings with electrophoresis and immunoblotting. Their conclusion is that label-free LC-MS/MS can be a reliable approach for analysis of urinary exosomes in diabetic rats.

Feig *et al.*[52] have identified 11,562 peptides that represented 2013 proteins in earwax for discovery of otorhinolaryngological diseases. Three different methods were used for validation: 1D PAGE with LC-MS/MS, online SCX-fractionation with LC-MS/MS, and shotgun LC-MS/MS. They also made a comparison between earwax and other biofluids by uploading it to Ingenuity Pathway Analysis (IPA) where 1955 of the 2013 proteins were mapped to known genes. By this discovery, the authors suggest further studies to discover biomarkers, and that earwax is an advantageous biofluid since it is e.g., easily accessible.

Another interesting and easily accessible medium is exhaled breath condensate (EBC), and for the exploration of the pathophysiological role of the leukotrienes (LTs), possibly as potential biomarkers for respiratory diseases, LC-MS/MS have been used by Montuschi *et al.*[53] to analyze EBC.

Shotgun proteomics has been used by Sjödin *et al.*[54] to an-

alyze potential biomarkers for patients with traumatic brain injury (TBI) in CSF. For enrichment of the potential biomarkers hexapeptide ligand libraries were used. They also used isoelectric focusing and nano-LC-MS/MS. Their study resulted in detection of neuron specific enolase, glial fibrillary acidic protein, S100-β, and creatine kinase B-type.

Investigations of proteins in CSF from patients with familial Alzheimer's disease (FAD) by Ringman *et al.*[55] identified 56 proteins that differed significantly from related noncarriers. The tryptic peptides were quantified by LC with ESI coupled to TOF-MS. The peptide identification was carried out using a linear ion-trap mass spectrometer. Furthermore, Choi *et al.*[56] have reported analysis of CSF for validation of several possible biomarkers for Alzheimer's disease. They monitored 24 different peptides as biomarkers. Good linearity and reproducibility were obtained when they used a nano-LC-MRM/MS assay. Their method also showed good correlation with results obtained by ELISA. Using MRM is advantageous for the good selectivity and specificity.

Schutzer and co-workers[57] have analyzed CSF to establish the proteome of normal CSF using 2D-LC-MS/MS with immunoaffinity depletion, and also use this type of analysis for identifying proteins in first attack MS patients[58]. Moreover, the same method has been used to investigate[59] the possibility to differentiate between Neurologic Post Treatment Lyme disease (nPTLS) and Chronic Fatigue (CFS) using CSF. Several proteins were identified, but future validation studies are necessary. Further results for early detection of Lyme disease can also be found in Eshoo *et al.*[60].

Salmon *et al.*[61] have used nano-LC-MS/MS and LTQ-FT to analyze alveolar bone, and a bone like tissue called dental cementum. By comparing which proteins they have in common they could identify new biomarkers for treatments for periodontal regeneration.

One approach towards using label-free LC-MS/MS analysis for quantitation of proteins associated with colon cancer (CC) in tissue samples have been reported by Zougman *et al.*[62]. Results showed that retinoic acid-induced protein 3 (RAI3) was overexpressed in patients with CC. They also used WB and immunohistochemistry (IHC) for confirmation.

Untargeted Labeled Proteomics

A technique frequently used in proteomics in combination with mass spectrometry is "isobaric tag for relative and absolute quantitation" (iTRAQ). This is a method based on derivatization by an isobaric designed tag of the amino groups of the proteins, causing the proteins to have the same mass. This method makes it possible to simultaneously compare several samples[63], called multiplexing, and quantitative information can only be found in MS/MS-mode[64]. By using the iTRAQ method the accuracy will be higher, and the statistical relevance can also be increased[8]. Another method that can be used is differential tagging of proteins with an isotope labeling, e.g., isotope-coded affinity tag (ICAT), cleavable isotope-coded affinity tag (cICAT)[15], stable isotope labeling by amino acids in cell culture (SILAC)[65], or tandem mass tag (TMT). However, label-free LC-MS/MS analysis is attractive since it allows for identification and quantification at the same time without the need for chemical derivatizations and specific knowledge of the analytes.

The iTRAQ method was recently used for its high specificity and sensitivity by Lv *et al.*[66] for identification and absolute quantitation of plasma biomarkers for acute graft-versus-host-disease (aGvHD). They used ELISA for validation of four of the target proteins, namely ceruloplasmin (CP), myeloperoxidase (MPO), compliment factor H (CFH) and alpha-1-acid glycoprotein (AGP). Results showed CP to be the most promising biomarker for aGvHD.

Huang *et al.*[67] found a predictive biomarker in sera for microvascular invasion (MVI) in HCC by iTRAQ and 2D LC-MS/MS. By investigation of different stages they found paraoxonase 1 (PON 1) as the biomarker candidate for vascular invasion. For validation of these results they used WB and ELISA. Detection of this biomarker will be helpful to chose the best therapy methods for patients in early stages of metastasis of HCC, which is one of the most common aggressive types of cancer.

Altered levels of proteins have been found by Gautam *et al.*[68] in plasma samples using an iTRAQ based LC-MS/MS method in glioblastroma (GBM) patients. Results from the study showed several proteins as the authors claim to be useful in further studies of biomarkers for GBM.

Wetterhall *et al.*[69] have studied the performance of affinity and immunoaffinity based spin columns to be able to reach low enough levels for analysis of e.g., biomarkers in CSF. We evaluated the columns by 1D gel electrophoresis and LC combined with MALDI. The reproducibility for one of the columns was tested using iTRAQ. Moreover, Lehnert and co-workers[70] have employed iTRAQ and MALDI-TOF-TOF for identification and MRM and LC-MS/MS for validation to find biomarkers of Parkinson's disease dementia in CSF. The findings indicates that Tyrosine-kinase non-receptor-type 13 and Netrin G1 are potential biomarkers of Parkinson's disease dementia.

A combined iTRAQ and SRM/MRM method for discovery and certification of biomarkers was proposed by Muraoka *et al.*[71]. For discovery, iTRAQ labeling and LC-MS/MS is used to identify proteins followed by SRM/MRM analysis for verification of the chosen proteins in individual patients. Potential biomarkers in tissue samples from patients with limited breast cancer were obtained in MFAP4 and GP2, which were also additionally verified by WB and IHC.

Thézénas *et al.*[72] used shotgun mass spectrometry which makes it possible for high throughput[73]. They used se-

lected reaction monitoring (SRM) and targeted proteomic strategies to find protein candidate biomarkers for acute plasmodium falciparum, that causes malaria. They found four *P. falciparum* proteins PF10_0121 (hypoxanthine phosphoribosyltransferase, pHPRT), PF11_0208 (phosphoglycerate mutase, pPGM), PF13_0141 (lactate dehydrogenase, pLDH), and PF14_0425 (fructose biphosphate aldolase, pFBPA). Both pLDH and pFBA were significantly correlated with higher levels of *P. falciparum* proteins, therefore further studies should be made using pHPRT as a candidate biomarker.

Tsuchida *et al.*[74] have identified proteins in gingival crevicular fluid (GCF) that tracks progression of Periodontal disease. They employed tandem mass tag (TMT) labeling to label peptides and analyzed by LC-MS/MS using a LTQ-Orbitrap XL. WB and ELISA were used to specifically verify two biomarker proteins: matrix metalloproteinase (MMP)-9 and neutrophil gelatinase-associated lipocalin (LCN2).

Label-free quantification (LFQ) and SILAC combined with shotgun mass spectrometry have been used by Qing Liu *et al.*[75] to quantify biomarkers related to breast cancer in microdissected tissue samples. Their results showed that LFQ in combination with shotgun mass spectrometry was the best method for quantification and validation for the biomarkers. Furthermore, Rivera-Torres *et al.*[76] have used SILAC to analyze fibroblasts from patients with Hutchinson-Gilford progeria syndrome and healthy patients. Results indicated that a mitochondrial dysfunction contributes to both aging and premature organ decline.

Isotope-coded protein labeling (ICPL) and nanoflow-HPLC-MS/MS have been used by Biniossek and co-workers[77] to find biomarkers for the effects on telomere dysfunction on the proteome of tumor cells. In their study they found a list of 59 potential biomarkers.

Another type of labeling have been made by Ping *et al.*[78], where dimethyl-labeling-based protein quantification was used. This method is simple, fast, cheap, and can be used for the analysis of proteins from small central nervous system samples.

Targeted Proteomics

Tandem mass spectrometry is used fairly frequently in clinical applications, and have advantages such as that a larger amount of analytes can be quantified and allowing good robustness and sensitivity[79]. Furthermore, there are MS methods based on targeted proteomic methods such as MRM or SRM that often provide higher specificity[79]. In this technique, only certain ions are monitored instead of all ions. For instance, a chosen precursor ion at a certain m/z value and after a collision induced dissociation (CID) in a collision cell, specific product ions are monitored at their respective m/z values. Mass spectrometry is often combined with proteolytic digestion and a separation technique in more than one dimension before mass spectrometric analysis[14,38]. MRM, or SRM is a targeted method using MS, the method is, e.g., used to selectively detect and quantify peptides specific for certain proteins by using a precursor ion and fragment ion that are known in advance[39]. The method can be an alternative to antibody-based methods because of the high selectivity, sensitivity, and high throughput, for verification of biomarkers[80]. These methods combined with databases and software such as MRMaid and PRIDE can produce very efficient methods[81–83].

Chen *et al.*[84] are trying to find cancer biomarkers in chromosome 4, which is the fourth largest chromosome containing 757 protein coding genes. Previously, several marker genes have been found in this chromosome, and Chen *et al.* have made a pilot study using SRM or MRM assays, for the upcoming chromosome 4-centric human proteome project (CHR 4HPP). In their study they tried to find cancer biomarkers in these 757 proteins for analysis of body fluids. They found 141 encoded proteins as cancer cell-secretable or shedable proteins and 54 of them have been classified as cancer-associated proteins.

Moreover, Percy and co-workers[85] proposed an anti-body free method for extending the multiplexing ability of MRM. Utilizing stable isotope labeled peptides, 142 proteins common in plasma for many non-communicable diseases were analyzed, where the concentrations also spanned a large range (mg/mL to ng/mL). Results showed strong linearity in the response curves.

MRM was also used by Ahn *et al.*[86] for diagnosis of hepatocellular carcinoma HCC to target proteins related to the disease. The authors claim that their method can be applied for fast verification of biomarkers for HCC.

Cawthorn *et al.*[87] made a study for identification of biomarkers to obtain more knowledge of how to treat breast cancer patients by more tailor made treatments. This was made by differential proteomic methods such as LC-MS/MS offline with iTRAQ labeling and label-free SRM-MS, and validation using tissue microarrays and IHC. Their findings identified that decorin (DCN) and heat shock protein 90 kDa beta (Grp94) member 1 (HSP90B1) are potential biomarkers that can lead to better prognostics in the future.

Dried blood spot samples (DBS) are another highly interesting choice of medium to screen for biomarkers in. It has many advantages compared to, e.g., blood or serum. For instance, it is less invasive and the risk for getting infected with a blood borne virus is negligible. Moreover, this method allows for very good sample stability and samples can be shipped at room temperature without any special treatment[88,89]. Chambers *et al.* have employed LC-MRM-MS to analyze DBS with the purpose of proving that screening for biomarkers in DBS is feasible. They monitored 60 proteotypic tryptic peptides and found good linearity for the majority of them and also obtained reproducible results. Stability tests showed that the DBS samples were stable for 80% of the proteins when stored under different temperatures for at least ten days.

Current Mass Spectrometry Techniques in Proteomics

The majority of the methods used to screen for biomarkers employ some form of mass spectrometry. These are powerful techniques based on ionizing analytes and examining the resulting mass-to-charge ratio m/z. This examination can be done by different techniques, and in this section some of the more popular methods for ionization and detection in proteomics are discussed.

LDI Techniques

Laser desorption/ionization (LDI) techniques have several intrinsic properties which makes them interesting such as simplicity, fast analysis, high throughput, small analyte volumes, tolerance towards salts and impurities, and no or little fragmentations. Most commonly, LDI is used with some form of compound or surface that assists the desorption/ionization process. In MALDI, the sample is mixed with an organic matrix compound which during the ionization process absorbs the laser radiation and supports the desorption/ionization process of the analyte molecules. MALDI is a robust technique that is easy to use, has high selectivity and sensitivity, and provides fast analysis. In contrast, SALDI is a technique that is matrix free in the sense that there is no organic matrix involved. The ionization is helped by thermal desorption of the analyte by either the surface of the target plate, or a nanostructure surface on particles mixed with the analyte. The SELDI method also involves the use of a surface that selectively retain certain proteins and peptides[90].

Giribaldi *et al.*[91] have used 2-DE and MALDI-TOF to analyze 18 differentially expressed proteins for early diagnosis of renal cell carcinoma (RCC) in tissue samples. Results indicate that one of the 18 proteins, RCN1, was over-expressed. They used WB and IHC to verify the over-expression, and results from WB show that this occurs in 21 of 24 tumor tissue specimens, while with IHC there was a diffuse expression in all 24 tissue samples.

Employing a nanoporous silica-based assay, Fan *et al.*[92] have devised a method for separating proteins based on weight to support detection of low mass peptides by mass spectrometry. Tests were carried out on sera samples from the B16 mouse model for pulmonary metastatic melanoma using MALDI-TOF-MS after enrichment by the assay. Following biostatistical analysis, 27 peptides were identified as potential biomarkers.

Dong *et al.*[93] have studied and recently identified a potential predictor for acute coronary syndrome (ACS) in blood samples by a SELDI protein chip technology. The aim was to forecast a negative outcome. This was made to find a better prediction possibility due to the lack of both sensitivity and specificity of the biomarkers currently used. They identified one peak that may correspond to prediction of long term outcome.

Li *et al.*[94] have studied human nasopharyngeal carcinoma (NPC) to find which markers that are resistant to radiotherapy. They used two different carcinoma cell lines: CNE-2R and the parental cell line CNE-2. The methods used were 2-DE and MALDI-TOF-MS. The candidate proteins were validated by WB and sixteen differentially expressed proteins were found. Among these, the marker Nm23 H1 increased and the marker Annexin A3 decreased in CNE-2R cells in comparison to the CNE-2 cells. Conclusions were that further research have to be made on these two potential markers.

Ahmad-Tajudin *et al.*[95] have used MALDI with an integrated selective enrichment target (ISET). Their method can capture biomarkers by immunoaffinity capture and also includes digestion and sample cleanup. They have studied prostate specific antigen (PSA) in plasma samples.

Pastor *et al.*[96] have analyzed biomarkers related to oxidative stress for lung cancer and chronic obstructive pulmonary disease (COPD) in bronchoalveolar lavage (BAL). They separated the proteins by 2D-PAGE and MALDI-TOF-TOF. Sixteen oxidative stress proteins were differentially expressed in patients with lung cancer or COPD compared to their control group. They selected four proteins for validation by WB: TXN, GSR, GSTA1, and CAT. Results showed that TXN and GSR were expressed increasingly in a similar way in patients with lung cancer or COPD, while GSTA1 decreased compared to the control group.

Adipocytokine zinc α_2 glycoprotein (ZAG) has been found to be a biomarker for normo-albuminuric diabetic nephropathy found in urine. Lim *et al.*[97] has used 2D-DIGE and MALDI-TOF-TOF to identify and WB to validate ZAG as a biomarker.

Martinez-Pinna *et al.*[98] identified peroxiredoxin-1 as biomarker for AAA. Samples of the intraluminal thrombus were analyzed by 2D-DIGE and proteins were identified by mass spectrometry using MALDI-TOF-TOF. For verification, WB and ELISA were employed. An early sign of kidney dysfunction is microalbuminuria, and therefore, biomarkers could prove to be a fruitful tool for detection of patients in need of therapeutic intervention.

To analyze biomarkers in serum samples for early diagnosis of tuberculosis (TB), SELDI-TOF-MS have been used by Liu *et al.*[99]. They combined their SELDI-TOF-MS method with magnetic beads of weak cation exchange properties. Their results showed 35 peaks related to TB, and fibrinogen was identified as a potential biomarker. Furthermore, SELDI-TOF-MS has also been used by Clarke and co-workers[100] to measure protein biomarkers in serum of patients with epithelial ovarian cancer. Seven proteins were chosen in combination with CA125 to enhance the sensitivity of early stage detection. In a validation set, the combination of these seven biomarkers and CA125 had 84% sensitivity and 98% specificity, which was significantly better compared to only CA125. Furthermore,

Xiao *et al.*[101] have written a review about SELDI and disease biomarkers.

Momeni *et al.*[102] have reported a novel set of biomarkers for autism spectrum disorders (ASD) in blood plasma samples, where SELDI-TOF-MS was used. The results were then verified by MALDI-TOF-MS and nano-LC-ESI FTICR MS. Threee diferentially expressed peptides were found that were significantly changed in those with ASD compared to a control group.

Orbitrap

The Orbitrap is a mass analyzer that use Fourier transforms to obtain a mass spectra. The ions are injected and trapped between an outer and an inner electrode by the balance between electrostatic attraction to the inner electrode and centrifugal forces.

Kosanam *et al.*[103] have with Orbitrap mass spectrometry made proteomic analysis of four tissue samples with pancreatic ductal adenocarcinoma (PDAC). Results show that they have identified a new putative PDAC biomarker called Laminin gamma 2 (LAMC2).

Salazar and co-workers[104] have found that 20 proteins in human saliva had 1.5 times higher concentration in patients with periodontitis compared to controls. The method of analysis was based on proteolytic digestion with trypsin followed by nano-UPLC-MS/MS with Orbitrap Velos-MS detection. The authors confirmed eight previously identified biomarkers for periodontitis. The conclusions drawn includes that this type of label-free proteomic analysis can be used to characterize and differentiate between healthy and patients with a periodontal disease.

Atagi *et al.*[105] have analyzed serum samples for risk factors for the discovery of interstitial lung disease. They made a case control study where they used patients with non-small-cell lung cancer treated with erlotinib. These patients were studied since the drug erlotinib give rise to the drug induced condition. Sera samples were taken before and after drug intake, and were analyzed by LC-MS/MS. They found three proteins, C3, C4A/C4B and APOA1, that were confirmed to be associated with interstitial lung disease by LC-MS/MS analysis (LTQ-Orbitrap). Unfortunately they could not find any statistical proof that there were any predictive serum proteins and they suggest that this should be studied more in the future.

Shang *et al.*[106] have identified osteopontin (OPN) as a novel marker in plasma for early HCC. After separation, peptide samples were analyzed by LC-MS/MS with ESI and 2D nano-HPLC coupled to LTQ-Orbitrap. OPN levels were measured by ELISA. The conclusion was that OPN was more sensitive compared to alpha-fetoprotein (AFP).

Webhofer *et al.*[107] have for the first time managed to shed light on the *in vivo* effects of antidepressants at the cellular level. They identified possible biomarkers for the effect of antidepressant treatment in plasma samples, and how the metabolites changed by time in hippocampus and plasma samples. They compared protein levels of chronic paroxedine and vehicle treated animals, by in vivo ^{15}N metabolic labeling with mass spectrometry using LC-MS/MS coupled online with an LTQ-Orbitrap. Another result they revealed was that the effects of the paroxetine were that the energy metabolism changes, and they also identified myo-inositol as a biomarker candidate.

DBS can be advantageous when screening for disease biomarkers in infants due to the fairly non-invasive techniques available for obtaining the sample. Ostermann *et al.*[108] used a combination of MALDI and an LTQ-Orbitrap for high resolution to screen for inborn errors in metabolism. The (acyl)carnitine levels were measured and could be both differentiated and quantified. Moreover, since Orbitrap offers high resolution the authors could demonstrate both the identification and quantification of several disease-specific organic acids.

FTICR-MS

FTICR-MS is a high resolution method that is based on trapping ions in a magnetic field and exciting them at the so-called cyclotron frequency. The ions then separate into "packets" that have the same m/z-value, and by Fourier transform it is possible to examine the spectrum. Since a fixed magnetic field is employed, high resolution is in part obtained due to the stable super conducting magnets typically used.

Street and coworkers[109] have investigated exosomes content in human CSF to discover, e.g., Alzheimer's disease. They used WB, isopycnic centrifugation using a sucrose density gradient, and electron microscopy of the antibody treated exosomal fraction. The protein profiling was carried out with HPLC coupled to FT-ICR mass spectrometry. Exosomes were identified in CSF, but concentration techniques for exosomes need to be refined.

FTICR has also been employed by Paulo *et al.*[110], where a nanoflow reversed-phase ultra-HPLC with a linear trap quadrupole-FTICR mass spectrometer (LTQ-FTICR) was used to analyze PTMs in pancreatic fluid from patients with chronic pancreatitis and healthy patients as controls. The PTMs that are different in frequency between the controls and the patients with chronic pancreatitis and may serve as biomarker candidates for chronic pancreatitis.

In Shevchenko *et al.*[111], a longitudinal study is done of the Tg2576 mice model to investigate abnormal protein accumulation and amyloid plaque deposition. This is important for research of, e.g., Alzheimer's disease. The approach used was "shotgun" based and label-free. In the analysis, a nanoLC-MS/MS with LTQ-FTICR was used, and the findings identified a set of 10 proteins that were significantly upregulated.

Moreover, in Shevchenko *et al.*[112], a comparison of different extractions techniques for mouse brain proteome. Similarly, a "shotgun" approach is used and nanoLC-MS/MS with LTQ-FTICR employed.

Validation Methods

When a potential biomarker has been found, the next step is usually to validate it. The methods employed for this purpose are often based on immunoblotting, arrays, ELISA or imunohistochemistry. However e.g., ELISA and WB represents older techniques, and are not proteomic methods unless they are multiplexed. Therefore we have chosen to focus on arrays and imunohistochemistry techniques mainly. ELISA is a method that is based on the labeling of antibodies or antigens with an enzyme, that reacts with a substrate and produce fluorescence that can be detected. Today many ELISA techniques are automated in medical laboratories[113].

Arrays

Protein microarrays or protein chips is a high throughput multiplexed immunoassay method, where an array of proteins are captured to a special surface. It was first introduced by Ekins *et al.*[114,115]. The surface can be made by printing molecular recognition molecules on a substrate and it can be of different kinds. Those where the antigen is attached to a substrate and those where the antigen competes with the target to bind with the antibody that is in a certain concentration. Depending on which amount of antibodies that have bonded to the antigen attached to the surface, the target can be quantified[116].

A major issue for patients with COPD and acute exacerbations (AECOPD) is the complex systematic inflammation. Chen *et al.*[117] have made a preliminary study to find biomarkers in plasma samples for these inflammatory immediator diseases. They used a chemokine multiplex antibody array for analysis. The preliminary results showed thirteen potential disease biomarkers to be different between healthy control groups, patients with COPD, and patients with AECOPD such as BTC, IL-9, IL-18Bpa, CCL22, CCL23, CCL25, CCL28, CTACK, LIGHT, MSPa, MCP-3, MCP-4, and OPN.

Additionally, Genini *et al.*[118] have used protein chip arrays and SELDI-TOF-MS for analysis of serum samples in large white piglets with and without porcine reproductive and respiratory syndrome (PRRS). They found 200 peaks that were significantly different and among those, 47 peaks were confirmed during validation.

Jiang and coworkers[119] have employed multiplexed antibody arrays for profiling protein expressions and compared with paired serum samples. The correlation was good and the authors claims that their results indicates that DBS is a good alternative to, e.g., using blood samples.

Another approach has been made by Heslop *et al.*[120] using arrayed primer extension micro-arrays (APEX) where they analyzed the effects of oxidative stress in coronary angiography patients. They analyzed the biomarkers myeloperoxidase (MPO), oxidized low-density lipoprotein, nitrotyrosine and antioxidant capacity.

Notable work have been made by DeGroote *et al.*[121], where they have made a large scale proteomic target method that should be unbiased. The method is called SOMAscan and is a highly multiplexed proteomic technology. It is an assay able to measure many proteins at the same time. After 8 weeks of intense tuberculosis therapy many proteins were measured and the changes in the proteins during the therapy were investigated to find biomarkers for tuberculosis. The top proteins found that changed from the start of the treatment and at the end of the treatment were TSP4, TIMP-2, SEPR, MRC-2, Antithrombin III, SAA, CRP, NPS-PLA2, LEAP-1 and LBP. Their discovery makes it possible for deeper knowledge and understanding of the treatment and healing of tuberculosis as a response to therapy.

Ostroff *et al.*[122] have studied malignant pleural mesothelioma, which is a rare and asbestos related type of cancer. When patients are diagnosed it is often in one of the late stages of cancer, and possibilities for a cure are small. To improve the ability to discover this type of cancer early, series of both case-control and multi-center studies were made. They used a SOMAmer proteomic technology able to measure over 1000 proteins simultaneously in unfractionated biological samples and they found 64 biomarker candidates during the study, including both inflammatory and proleterative proteins. Statistic evaluation of these 64 biomarker candidates resulted in the construction of a 13 biomarker panel containing APOA1, C9, CCL23, CDK5/CDK5R1, CXCL13, F9, FCN2, FN1, ICAM2, KIT, MDK, SERPINA4, and TNFRSF8. Accuracy was 92% and detection of 88% of stage I and II of the disease. This made it possible to detect the disease and a provides a chance for early treatment.

Immunoassays has successfully been used by Hsich *et al.*[123] where the brain protein 14-3-3 was analyzed in CSF from patients with dementia. This was made for the diagnosis of Creutzfeldt-Jakob disease. Results proved to strongly support the diagnosis of the disease.

Kim *et al.*[124] have investigated other potential biomarkers for HCC than the most commonly used biomarker alpha-fetoprotein (AFP). The need is due to the lack of sensitivity and specificity. To find more biomarkers for HCC and to screen for biomarker proteins, the methods cDNA micro array, copy number variation, somativ mutation, apigenetic, and quantitative proteomics data were used. By comparisons between three patient groups, four biomarkers were obtained: anilin (ANLN), filmin-B (FLNB) complementary C4-A (C4A) and AFP. The combination of two of these markers, ALNL and

FLNB, was used which improved discrimination of before treatment of HCC from a healthy control group compared with AFP. Global datamining was used to identify candidates for clinical verification, to find potential biomarkers from databases and MRM. They combined global datamining, MRM with a triple quadropole, and an antibody based verification method.

Farina *et al.*[125] used immunoblotting and found the protein carcinoembryonic cell adhesion molecule 6 (CEAM6) — which is overexpressed in many human cancer types — to be a potential biomarker in bile samples to distinguish between malignant and nonmalignant causes of biliary stenoses. They confirmed the overexpression with ELISA.

A novel on-line chamber was used by Otieno *et al.*[13] to capture cancer biomarkers on the surface of a modular microfluidic immunoarray with a surface of multilabeled magnetic beads. This method provided simultaneous assays and detection limits at interleukin-6 (IL-6) 5fg/mL IL-6 and 7fg/mL IL-8 in serum samples.

Fage display technology can be a tool for protein identification with specific binding properties. Zayakin *et al.*[126] successfully used a 1,150-feature microarray testing sera from patients with gastric cancer (GC) and cancer free controls to find antibodies with the highest diagnostic value. With a 45-autoantibody signature they could discriminate GC and healthy controls with higher sensitivity and specificity then currently known serological GC biomarkers. Furthermore, by the usage of this test both early GC and advanced GC could be detected with equal sensitivity.

Furthermore, Principe *et al.*[127] have studied the proteome of urinary expressed prostatic secretion (EPS-urine), identifying 1022 unique proteins by multidimensional protein identification technology (MudPIT). Combining with publicly available proteomic data, transcriptomic data, and immunohistochemistry images, a 49 protein panel was defined for EPS-urine. WB was used to validate that 7 of these potentially originate from the prostate.

Nolen *et al.*[128] have made a preliminary study of several biomarkers in urine samples from healthy donors for clinical development. Methods used were multiplexed bead-based immunoassays of 211 proteins, and WB for confirmation. They analyzed urine samples because of its proteomic stability, and it is a low protein matrix compared to other biological fluids. Results showed that over 200 distinct proteins were present in varying concentrations, and almost one third of the biomarkers analyzed were in concentrations over 1ng/ml.

Hu *et al.*[116] have investigated an immunoassay microarray, where antigens were spotted on a non-fouling polymer brush. Detection of several mycotoxins reached detection limits as low as a couple of picograms per milliliter and the technique had good dynamic range. The authors claim that this is comparable or better than what is obtained by ELISA. Moreover, spiked samples of finely ground peanuts produced results with high recovery for concentrations as low as 50 pg/ml.

Immunohistochemistry

Martinez-Perdiguero *et al.*[129] have studied TNF-α protein as biomarker in human serum. This was made after sandwich immunoassay with nanoparticles by a surface plasmon resonance biosensor. After optimization of the assay parameters they achieved LOD at 11.6 pg/mL in spiked buffer and 54.4pg/mL in serum.

Kubota *et al.*[130–133] have previously made several studies where they for example found the potential biomarker pfetin. Recently, they have used WB and IHC to analyze gastrointestinal stromal tumors (GIST) using both a commercial pocyclonal antibody against pfetin, and a monoclonal antibody. The results from WB revealed that both antibodies recognized multiple post-translationally modified pfetin isoforms. The immunohistochemical study revealed that the disease free survival rate was 88% for pfetin positive and 56% for pfetin negative. Additionally both univariate and multivariate analysis were made, and their conclusions suggests that pfetin expression level with the commercial antibody can be beneficial as a possible biomarker for GIST in the future.

The protein Ki-67 has been used as a biomarker for the response to treatment with chemotherapy for breast cancer. However, there has been controversies regarding the methods of measurement. Brown *et al.*[134] have made a quantitative analysis using immunoflourescence automated quantitative analysis (AQUA). The authors demonstrates that the AQUA scoring was comparable to conventional calculations.

Patani *et al.*[135] used "proliferation markers" that made it possible to access information to be able to make the decision about systemic therapy in breast cancer. The methodology used was immunohistochemical assessment. This information will have both importance for patient evaluations in the future, as in prospective clinical trials. Currently, oesterogene and progestorene receptors, and human epidermal growth factor receptor 2 (HER2), are established biomarkers. Including this new proliferation marker into the regular screening process would be advantageous. Examples of proliferation markers are KI67, cyclin D1 and cyclin E, oestrogen receptor β, topoisomeraseIIα, urokinase-type plasminogen activator and inhibitor, cerculated and dissaminated tumor cells, and tumor specific DNA. However, breast cancer and the response of the treatment are different for each patient. Therefore it would be an advantage to have several biomarkers. Moreover, Kim *et al.*[136] have developed a novel proteomics-based assay, which they call Collaborative Enzyme Enhanced Reactive immunoassay (CEER), that is based on a microarray surface where capture-antibodies and additional detector-Abs form an immuno-complex. This assay was then used successfully to, among other things, detect single-cell level expression of HER2 in breast cancer systems.

CEER can be multiplexed with very high sensitivity and specificity and can be useful for, e.g., companion diagnostics.

Alternative Methods

Different research groups have used other alternative methods than those mentioned above. For example, Wang *et al.*[137] have made an electrochemical assay by the use of square wave voltammetry to analyze S100B, a serum biomarker of melanoma. They reached detection limits of 0.1nM.

Recently, Medina-Sánchez *et al.*[138] have used square wave anodic stripping voltammetry to analyze apolopoprotein E (ApoE) — a potential biomarker for Alzheimer's disease — by using quantum dots as labels. Results show high accuracy, LOD of approximately ng/mL, and a linear range between 10 to 200 ng/mL in plasma.

Tabakman and co-workers[139] used a nanostructured plasmonic gold surface, and developed a protein microarray that extended the dynamic range of detection down to femtomolars. They showed that this array is compatible with the usual techniques at least for the cancer biomarker carcinoembryonic antigen (CEA) in mouse sera. This protein microarray also allows for multiplexed detection of human autoantibodies, something the authors believe will expand the possibilities of proteomics.

Another rather novel technique is flow cytometry, which is a method based on florescence analysis. It makes it possible to examine and assort mixed cell populations in a stream of fluid. Flow cytometry is used in clinical applications and can be advantageous in biomarker research[140]. Mourino-Alvarez and co-workers[141] have used flow cytometry. For acute coronary syndrome (ACS) there is no method to prevent today, the information we know is that circulating endothelial cells (CECs) and endothelial progenbator cells (EPCs) are important for vasculatization in tissue, and they have therefore tried to analyze them as biomarkers for ACS. However, there is a need for more studies to investigate if it works, since the statistical method did not show any differences between the patient group with ACS and one without using these as biomarkers.

Recently, Mohd Azmi *et al.*[142] have used antibody-functionalized silicon nanowire biosensor devices to detect a biomarker for oxidative stress, 8-hydroxydeoxyguanosine (8-OHdG), where results show that concentrations at 1ng/mL can be reached.

In a study by Zhou *et al.*[143], a strategy for naked eye detection of the cancer biomarker carcinoembryonic antigen (CEA) is described. The idea is to use a biobarcode and a form of DNA recycling amplifications that are assisted by enzymes. If the biomarker is present, the enzymes change the color of the product, thus enabling the detection by the naked eye. Levels of CEA as low as in the pM range can be detected. The authors also claim that this detection strategy can be used for samples in a complex serum matrix. The obvious advantage of a naked eye detection strategy is of course that this type of method is applicable at the point-of-care without the need for expensive and sensitive equipment that requires specially trained personnel to operate.

Concluding remarks

Considering the rammifications for a patient with a serious illness, it is of utmost importance to detect the development of a disease in an early stage, or perhaps even prevent the disease from breaking out. Moreover, there is also a clear economical aspect which makes early detection or prevention highly desirable since expensive and invasive treatments can be avoided. For this purpose, we have in this paper considered many of the proteomic methods currently used. We believe that this will assist future researchers when choosing a method for analysis. In Table 1, an overview of selected techniques and results is provided.

It is true that most of the applied proteomic techniques are based on MS or MS/MS, either just for the detection of possible biomarkers, but in some instances also for verification. Historically, LC has been used frequently and it is still one of the most common methods. It is a very versatile process due to the multiple choices of stationary and mobile phases that are available. However, this also makes LC a rather complex method with several pitfalls and experienced personnel is often necessary. One alternative that has been used frequently is different variations of electrophoresis. This can be advantageous when handling small sample volumes in CE, compared to LC, and it is also a good separation technique for, e.g., proteins using gel based electrophoresis. Moreover, different LDI techniques such as MALDI, SALDI, and SELDI are other methods often used for proteomic analysis of biomarkers, and these methods have advantages such as easier and faster analysis compared to both LC and electrophoresis.

Of course, the choice of method can be limited to what type of equipment that is available. An Orbitrap or FTICR-MS instrument can incur a quite substantial cost for a laboratory, so in some instances it is better to develop a method for the instrumentation available.

Another interesting aspect is that analysis of biomarkers in different biological fluids is rapidly evolving. For instance, easily accessible biological materials such as salivary samples or even earwax has been analysed successfully. Other types of highly interesting biological materials include dried blood spotting and analysis of breath condensate. There have been several studies where DBS has proved to be comparable to analysis of conventional blood samples, and with the obvious advantages of this non-invasive technique of extraction and the stable samples obtained, this is a method we believe will be used more frequently in the future.

Table 1 Overview of selected methods used for analysis of disease biomarkers in different sample matrices

Methods	Sample	Disease	Potential biomarkers	Ref.
LC-MS/MS	Plasma	Neuroblastoma	Complement C3	41
iTRAQ, ELISA	Plasma	aGvHD	CP, MPO, CFH, AGP	66
iTRAQ, LC-MS/MS	Plasma	GBM	FTL, S100A9, CNDP1	68
iTRAQ, MALDI-TOF-TOF, MRM, LC-MS/MS	Plasma	Parkinson's disease	Tyrosine-kinase non-receptor-type 13, Netrin G1	70
LC-MS/MS with ESI, LTQ-Orbitrap	Plasma	HCC	OPN	106
2D-DIGE, MALDI-TOF-MS	Plasma	OSCC	vitamin-D binding protein, fibrinogen (alpha/beta/gamma) chain, haptoglobin, S6 kinase alpha-3	22
Chemokine multiplex antibody array	Plasma	COPD and AECOPD	BTC, IL-9, IL-18Bpa, CCL22, CCL23, CCL25, CCL28, CTACK, LIGHT, MSPa, MCP-3, MCP-4, OPN	117
Square wave anodic stripping voltammetry, quantum dot labeling	Plasma	AD	ApoE	138
IMAC, label-free LC-MS^E	Serum	Schizophrenia	35 proteins with higher values than control groups	42
iTRAQ, 2D-LC-MS/MS	Serum	MVI in HCC	PON 1	67
2D-DIGE, MALDI-TOF-TOF, WB	Urine	Normo-albuminuric diabetic nephropathy	ZAG	97
MS, ELISA	Serum	Epilepsia	SAA	26
Square wave voltammetry	Serum	Melanoma	S100B	30
CE-MS	Urine	Microalbuminuria	14 peptides	33
CE-MS	Urine	ADPKD	PKD1, PKD2	34,35
Shotgun proteomics, ELISA	Urine	Bladder cancer	midkine, HA-1	37
2D-LC-MS/MS, WB	Saliva	Diabetes type 2	G3P, SAA, PLUNC, TRFE	47
Shotgun proteomics, isoelectric focusing, nano-LC-MS/MS	CSF	TBI	neuron specific enolase, glial fibrillary acidic protein, S100-β, and creatine kinase B-type	54
LC-ESI-TOF-MS, linear ion-trap MS	CSF	Familial AD	56 proteins	55
nano-LC-MRM/MS, ELISA	CSF	AD	24 peptides	56
HPLC-FT-ICR MS	CSF	AD	Exosomes	109
Immunoassays	CSF	Creutzfeldt-Jakob disease	Brain protein 14-3-3	123
2D-DIGE, LC-MS/MS with linear iontrap, WB, IHC	Tissue	GIST	DDX39	45
Label-free LC-MS/MS, WB, IHC	Tissue	CC	RAI3	62
LC-MS/MS offline with iTRAQ labeling, label-free SRM-MS, IHC	Tissue	Breast cancer	DCN, Grp94, HSP90B1	87
iTRAQ, SRM/MRM, LC-MS/MS, WB, IHC	Tissue	Breast cancer	GP2, MFAP4	71
Orbitrap and MS	Tissue	PDAC	LAMC2	103
2-DE, MALDI-TOF, WB, IHC	Tissue	RCC	RCN1	91
2-DE, MALDI-TOF-MS, LC-MS/MS, IB	Tissue	Bilary artresia	HSP90	30
IHC	Tissue	PDAC	SIX1	144
2D-GE, LC-MS/MS, ELISA	TIF	NSCLC	PRDX1	27
LC-MS/MS, SDS-PAGE	PF	Chronic pancreatis	Specific PTMs	48
1D PAGE, LC-MS/MS, online SCX-fractionation with LC-MS/MS, shotgun LC-MS/MS	Earwax	Otorhinolaryngological diseases	2013 proteins	52
2D-PAGE, MALDI-TOF-TOF, WB	BAL	Lung caner, COPD	TXN, GSR, GSTA1, CAT	96

After the discovery of a potential biomarker it is necessary to validate that this protein is indeed a biomarker, and for this process validation methods such as arrays, where ELISA, WB or immunohistochemistry are used, are still the most common approach.

Further projects that are current and relevant in this field of future developments are, e.g., the human proteome atlas and recent work using high throughput genomics technologies to inform and analyze proteomics [81,145,146].

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