Chapter 4

Clinical proteomics in chronic inflammatory diseases, a review

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Abstract

There is a need for better markers for the diagnosis and prognosis of chronic inflammatory diseases. Proteomic strategies can be helpful to detect new biomarkers. Proteomic analyses are performed to characterize the behavior of the system rather than the behavior of any single component. Since the genome has been unraveled, the proteome received more attention. Aim of this review is to focus on the use of different proteomics techniques to detect potential and/or common biomarkers in chronic inflammatory diseases. The identified and validated proteins detected in the different studies are compared and discussed to conclude if there are some common markers which can be used in the diagnosis and prognosis of the three chronic inflammatory diseases described in this study; multiple sclerosis, rheumatic diseases and lung inflammatory diseases. The heat shock protein family were entitled as biomarkers with potential for further research in multiple sclerosis. Myeloid-Related protein 8 was found in three different rheumatoid arthritis studies with different sample materials and could be a potential marker for rheumatoid arthritis. Alpha1-antitrypsin was validated in two studies as a marker for sarcoidosis and α1-antitrypsin was also found to be a marker for cystic fibrosis, together with myeloperoxidase and immunoglobulin G.

Introduction

Proteomics studies biological systems at the protein level. Proteomics is the study of the proteome, the protein complement of the genome. Proteomics can be viewed as an experimental approach to explain the information contained in genomic sequences in terms of the structure, function, and control of biological processes and pathways[1]. Until the mid-1990s scientists studied individual genes and proteins or a handful of biologically related genes and proteins. With proteomics multiprotein systems, the interplay of multiple, distinct proteins in their roles as part of a larger system or network are studied. The proteome is dynamic and in constant flux due to a combination of factors. These factors include posttranslational modifications and functional regulation of gene expression[2]. Analyses are directed at complex mixtures and identification is not performed by complete sequence analysis, but instead by partial sequence analysis with the aid of database matching tools. The point of proteomics is to characterize the behavior of the system rather than the behavior of any single component.

On the basis of mRNA processing and post-translational modifications, the estimated number of different human proteins is about 500,000, more than 15 times higher than the estimated number of coding genes in humans. Although only a fraction of these proteins is present at any given time in any particular cell, it will be a special challenge to catalogue their structures and functions and also to describe their actual concentrations differing by orders of magnitude, and to detect their activities in complexes. Identifying, cataloging and functionally describing all human proteins will be much more challenging than it was to sequence the human genome[3].

The true scientific goal of serum proteomic pattern analysis is in fact biomarker discovery. Biomarkers are biological molecules that are indicators of the physiological state and also of changes during a disease process. There is a great need to discover novel biomarkers and translate them to routine clinical use[4]. Mass spectroscopic serum proteomic pattern analysis can sort through tens of thousands of potential biomarkers. The utility of biomarkers lies in their ability to provide an early indication of the disease, to monitor disease progression, to provide ease of detection, and to provide a factor measurable across populations[5].

There are different techniques for expression analysis of proteins, like two-dimensional gel electrophoresis (2-DE), Matrix Assisted Laser Desorption/ Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS), Surface-Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (SELDI-TOF-MS), Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS), and the isotope-coded affinity tags (ICAT), and isotope tags for relative and absolute quantification (iTRAQ) technologies which are used for quantitative analysis. This review is focused on the use of different proteomics techniques to detect potential and/or common biomarkers in chronic inflammatory diseases. In our review we will summarize the proteomics studies in multiple sclerosis, rheumatic diseases and

lung inflammatory diseases. We have chosen chronic inflammatory diseases because of existing expertise on these chronic inflammatory diseases in our own and in other hospital research settings. The identified proteins detected in the different studies will be compared and discussed in this review.

Proteomics strategies

Until recently, 2-DE has dominated the field of proteomics. The principle of 2-DE is separation on a gel of the protein content of a sample in two dimensions according to charge and mass (Figure 4.1). The gels are stained and spot intensities in samples are compared among different gels. The advantage of the 2-DE is the high separation, which can be achieved, and the fact that post-translational modifications can be detected with this technology. The disadvantages are its lack of real high throughput capability (only one sample per gel). The method is very time-consuming and difficult to automate for routine laboratory. Other limitations are that you have to deal with between-gel irreproducibility, a poor resolution for extreme high molecular weight proteins and extremely acidic or basic proteins, and hydrophobic proteins often do not enter the gel. There is also a lot of starting material needed compared with other techniques and you cannot perform direct protein identification.

Other techniques used for the expression analysis of proteins are MALDI-TOF-MS, SELDI-TOF-MS and LC-MS/MS. With MALDI-TOF-MS, protein mixtures are spotted on a gold plate and by using an energy-absorbing matrix, proteins are ionized and the protein masses and peak intensities can be measured with the time-of-flight principle. The peak intensities will be compared between samples. The SELDI-TOF-MS is a variant of MALDI-TOF-MS. Different surfaces (like hydrophobic, hydrophilic, ionic and metal affinity binding) are used to make a selection of proteins that will bind to these surfaces and the rest is washed away. An extensive overview of this ProteinChip technology has been described by Wiesner[3]. Both MALDI-TOF-MS and SELDI-TOF-MS have a high throughput capability. The disadvantage of the MALDI-TOF-MS is the need for sample fractionation of complex samples and the disadvantage of the SELDI-TOF-MS is the lower resolution and mass accuracy compared to MALDI-TOF-MS.

Most analytical proteomics problems begin with a protein mixture. Before peptide sequences can be obtained the proteins must be cleaved to peptides. Cleavage of proteins in peptides can be performed with trypsin digest. It is also possible to separate the proteins by 1-DE, 2-DE, MALDI-TOF-MS or SELDI-TOF-MS and then cleave them into peptides. By using database search algorithms, the peptide data can be matched with known proteins. This general principle of proteomic analysis is illustrated in Figure 4.2.

With LC-MS/MS peptide mixtures can be separated either after trypsin digestion by LC and measurement of peptide masses by MS/MS or directly through fragmentation. The advantage of this technique is the direct identification of a lot

of proteins per sample by MS/MS of peptides. However, this technique is very time consuming, one sample can be running at a time. The MALDI-TOF-MS, SELDI-TOF-MS and LC-MS/MS techniques are particularly important for the low molecular weight fraction of the proteome because, in this part of the proteome, the use of immunological assays such as ELISA is limited owing to difficult antibody production for low molecular weight proteins[6].

Other more quantitative techniques, which are used for the expression analysis of proteins, are ICAT and iTRAQ. With ICAT, protein samples from healthy and diseased (or perturbed) sources are denatured, reduced and labeled. All cysteines in the healthy sample are modified with one isotopic version of the tag, and the cysteines in the perturbed sample are tagged with the opposite isotopic reagent. The two labeled protein mixtures are combined, and proteolyzed to peptides with trypsin. Cysteine-tagged peptides are enriched by affinity chromatography and are subsequently chromatographed by Reverse Phase High Performance Liquid Chromatography (RP-HPLC), alone or in combination with ion exchange liquid chromatography. Both samples are analyzed concomitantly by LC-MS/MS. The advantage of the ICAT technology is the direct identification of peptides by MS/MS. The results are relative quantities. The ICAT technology has expanded greatly the range of proteins that can be analyzed (such as low-abundance, hydrophobic or highly charged proteins), and allows accurate quantification and concurrent sequence identification of individual proteins in complex mixtures. The disadvantage is the low throughput (only two samples per run). Another disadvantage is that other possible post-translational modifications can be missed, because only the cysteine-containing peptides are tagged[6,7].

Recently, an improved approach analogous to ICAT has been developed called iTRAQ**.** The technique is based upon chemically tagging the N-terminus of peptides. The labeled samples of patient and control groups are then combined, fractionated by nanoLC and analyzed by tandem mass spectrometry. Database searching of the fragmentation data of the peptides results in the identification of the labeled peptides and hence the corresponding proteins. Measurement of the intensity of these reporter ions, enables relative quantification of the peptides in each digest and hence the proteins from where they originate. There are four tags available enabling four different conditions to be multiplexed together in one experiment. The advantage of the iTRAQ technology is the direct identification of peptides by tandem mass spectrometry (MS/MS). One can select MS/MS of the same peptide in the four conditions in the same single run. The disadvantage, is just like the ICAT technology, the low throughput (four samples per run). Each and every peptide must be subjected to tandem MS analysis, making iTRAQ both time consuming and sample-intensive for biomarker discovery applications. Furthermore, any untagged isobaric chemical noise may confound tandem-MS sequencing of the iTRAQ labeled peptides. Another disadvantage is the high sample complexity and the limited resolution of LC[6,7]. More technical information about these proteomics

technologies are reviewed in the recent studies of Verills et al.[8] and Engwegen et al.[6].

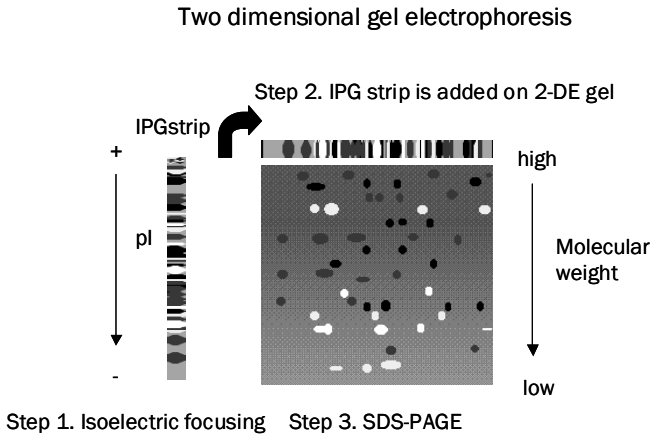

Figure 4.1 The proteins in the samples are separated in two dimensions.
By using IPGstrips, the proteins are first separated on base of their charge (pI value) and subsequently the strip is added on the gel and after isoelectric focusing the proteins are also separated according to mass. The proteins with a lower molecular weight migrate faster through the gel compared to the proteins with a higher molecular weight.

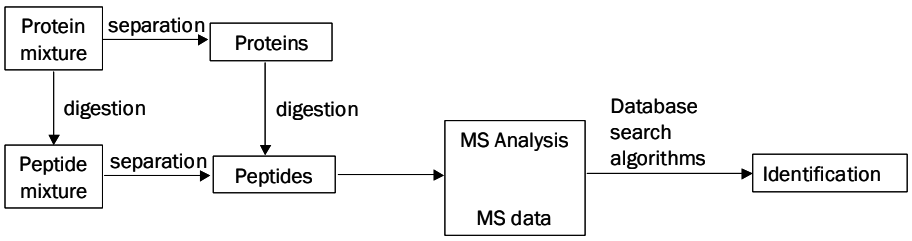

Figure 4.2 General principle of proteomic analysis.
Cleavage of proteins in peptides can be performed directly with trypsin digest. It is also possible to separate the proteins first by 1-DE, 2-DE, MALDI-TOF-MS or SELDI-TOF-MS and then cleave them into peptides. By using database search algorithms, the peptide data can be matched with known proteins.

Multiple sclerosis

Multiple sclerosis is an autoimmune inflammatory demyelinating disease of the central nervous system. Disease mechanisms in multiple sclerosis at the molecular level remain poorly understood and no reliable proteinaceous disease markers are available yet[9]. A definitive diagnosis is still difficult to ascertain, especially at disease onset, due to the lack of reliable molecular markers[10].

The most common clinical symptoms include paralysis, sensory disturbances, reduced coordination and visual impairment. The diagnosis is based on clinical examination, magnetic resonance imaging (MRI) and detection of oligoclonal immunoglobulins in cerebrospinal fluid (CSF)[11]. Although reliable serological tests are available for most autoimmune diseases, no such assay is available for the diagnosis of multiple sclerosis in part because no single antigen has been specifically associated with the disease. Nevertheless, the availability of effective immunomodulatory therapy makes it important to identify biological markers that reliably distinguish multiple sclerosis from other neurological diseases[12].

Rheumatic diseases

Rheumatoid arthritis (RA) is a common human autoimmune disease. It is characterized by an inflammatory process in the synovium resulting in progressive destruction of the affected joints due to an infiltration of blood-derived cells, mainly memory T cells, macrophages, and plasma cells[13,14]. Although the cause of this disease remains elusive, considerable advances have been made in the past few years towards identifying factors involved in the pathogenesis of RA such as auto antigens, which are recognized by autogen reactive T cells[15]. However, we are still far from understanding of the basic molecular grounds of the disease.
Up to now, routine clinical examination of synovial fluid (SF) including evaluation of physicochemical features, such as transparency, viscosity, glucose and protein concentrations, as well as determination of quantity and morphology of appearing cells turns out to be insufficient for the precise description of particular disease states[16].

Lung inflammatory diseases

In this review we focus on two chronic lung inflammatory diseases: sarcoidosis and cystic fibrosis. Sarcoidosis is a systemic granulomatous disorder of unknown cause characterized by its pathological hallmark, the noncaseating granuloma[17,18]. The clinical presentation of sarcoidosis is highly variable[19]. Involvement of the lungs or intrathoracic lymph nodes becomes clinically evident in 90% of the symptomatic patients during their disease and up to 30% show spontaneous remission[20]. A chronic course occurs in 10-30% of the patients, at times resulting in significant impairment of lung function[21,22]. Unfortunately, till now there is no good marker for both diagnosis and prognosis of sarcoidosis.
Cystic fibrosis (CF) is the most common lethal, autosomal, recessive, genetic disease among Caucasians. The gene, which when mutated is responsible for CF, encodes the CF-transmembrane conductance regulator (CFTR) protein. Progressive lung disease is the major cause of morbidity and mortality in patients with CF[23]. Although CF can be diagnosed in newborns by genetic screening, not all children will be screened for CF. Pilocarpine iontophoresis with quantitative chloride

analysis (the sweat test) remains the gold standard for the diagnosis of CF. Clinicians have been concerned about the ability to get accurate sweat chloride results when performing early sweat tests on infants primarily because of concerns that either insufficient sweat volumes would be generated or falsely high sweat chloride concentrations might be measured during the first days to weeks of life [24]. Therapy is directed by evaluation, which includes review of symptoms, lung function, and to a lesser extent radiologic changes, and is therefore likely to lag behind the occurrence of established lung pathology. Until now, therapy approaches for CF lung disease have been largely symptomatic, namely through antibiotic treatment against bacterial lung infection, and also with anti-inflammatory drugs[25]. The finding of novel therapeutic strategies that can efficiently overcome this pathology is even more complex as there is no straightforward correlation between CFTR genotype and CF lung disease severity[26]. Therefore, the identification of CF biomarkers may be of interest to help in the diagnosis, and perhaps prognosis and follow-up, of this disorder.

Results

The results of the different proteomics studies about multiple sclerosis, rheumatic diseases and sarcoidosis are illustrated in Table 4.1, 4.2, and 4.3, respectively.

Multiple sclerosis

The multiple sclerosis studies are summarized in Table 4.1. The SELDI-TOF-MS technique was used in the study of Irani et al.[12] to compare CSF samples from patients with multiple sclerosis or clinically isolated syndromes (CIS), transverse myelitis, human immunodeficiency virus (HIV) and other neurological diseases. A peak around *m/z* 12,540, corresponding to a 12.5 kDa protein was found in two thirds of multiple sclerosis and CIS samples and was absent in the controls with transverse myelitis or other neurological diseases. Tandem mass spectroscopy of a tryptic digest of this 12.5 kDa protein identified it as a cleavage product of full-length cystatin C (13.4 kDa). Although total cystatin C levels in the multiple sclerosis patients were not different compared with controls, the patients with the highest 12.5/13.4 peak ratios also had the greatest cathepsin B inhibitory activity.

Cystatin C is an inhibitor of cathepsin B. A significant inverse correlation between the cystatin C levels and cathepsin B activity was found. A greater 12.5 to 13.4 kDa ratio resulted in a greater inhibition of cathepsin B. They suggested that the cleavage of the carboxy terminus might lead to enhanced activity of this protein. They also tried to confirm their results with immunodepletion of cystatin C. Both the 12.5 kDa and the 13.4 kDa proteins were selectively removed by the anticystatin antisera, but a new peak at 12.1 kDa was seen after the removal of cystatin C.
According to Del Boccio et al.[10] is the cleavage product of cystatin C, found by Irani et al.[12], a result of long-term storage at -20ºC. In the article of Del Boccio et al. was illustrated that the N-terminal eight-amino acid-truncated form of cystatin C at *m/z* 12,536 was always present in CSF samples which were stored more than ten days at -20ºC, while this peak was absent in fresh samples or in samples stored at -80ºC. This truncation at -20ºC occurred in all the CSF samples analyzed regardless of the underlying neurological status, indicating a storage-related phenomenon rather than physiological or pathological processing of the protein. So they suggested that the degradation is not specific in the CSF of multiple sclerosis, but rather is given by inappropriate sample storage at -20ºC. These data are in agreement with the results of Carrette et al.[27]. They studied patients with dementia of various origin and they also showed an N-terminal truncated cystatin C in human CSF as a result of long-term storage at -20ºC.
Dumont et al.[9] used the 2-DE technique to construct a database of 2-DE separated CSF proteins from multiple sclerosis patients. With LC-MS/MS 65 different proteins were identified from 300 spots. Eighteen of these proteins have not been reported previously on 2-DE gels of CSF. Three of these were potential contaminants, and the other 15 newly identified proteins (see Table 4.1) were repeatedly identified in the CSF samples studied, providing supporting evidence for their presence in CSF. To detect multiple sclerosis biomarkers, the CSF samples from patients and controls need to be compared with the LC-MS/MS technique.
In the study of Noben et al.[28] pooled and ultra filtered CSF of multiple sclerosis and non-multiple sclerosis patients was digested with trypsin and analyzed by off-line strong cation-exchange chromatography (SCX) coupled to on-line reversed-phase LC-ESI-MS/MS. In an alternative approach, the trypsin-treated subproteomes were analyzed directly by LC-ESI-MS/MS and gas-phase fractionation in the mass spectrometer. In total 148 proteins were identified and sixty proteins were identified in CSF for the first time. Eighty proteins were common in the multiple sclerosis and non-multiple sclerosis group, 24 proteins were only detected in the non-multiple sclerosis group, and 44 proteins were only detected in the multiple sclerosis group. The most important proteins which were only encountered in the multiple sclerosis group were: complement C3, peptidyl-glycine α-amidating monoxygenase and members of the heat shock protein family (hsp) hsp90, glucose-related protein 78, endoplasmatic reticulum protein 29, serotransferrin. Hsp proteins were entitled as biomarkers with potential for further research since dysregulation in the hsp system was found to be the most prominent and

consistent result of gene expression studies in multiple sclerosis and other autoimmune diseases. Western blot analysis was performed to check if serotransferrin was converted to toxiferrin. The immuno-reactive bands in both groups gave no substantial difference. They suggested that the reason that there were no serotransferrin peptides detected in the non-multiple sclerosis group could be caused by the ultrafiltration device itself. The other proteins were not validated.

Rheumatic diseases

The rheumatic disease studies are summarized in Table 4.2. To detect new rheumatoid arthritis biomarkers, the group of de Seny et al.[29] used the SELDI-TOF-MS technique. Serum samples from patients with rheumatoid arthritis (RA), psoriatic arthritis, asthma, Crohn's disease, knee osteoarthritis and healthy controls were compared. The following peaks were highlighted in the article as potential biomarkers: *m/z* 2924 (RA versus controls on hydrophobic (H4) ProteinChip arrays), 10,832 and 11,632 (RA versus controls on weak cation-exchange (CM10) ProteinChip arrays), 4824 (RA versus psoriatic arthritis on H4 arrays), and 4666 (RA versus psoriatic arthritis on CM10 arrays). Sensitivities of 79-91% on CM10 and 88-97% on H4 arrays and specificities of 75-85.5% on CM10 and 87-91% on H4 arrays were achieved. According to the results obtained in previous studies, the 10,832 peak was suspected to be Myeloid-Related protein 8 (MRP-8). To confirm this hypothesis, Western blot analysis was performed. MRP-8 was detected in the RA patient samples and not in the control samples. Depletion of MRP-8 from RA serum using IDM affinity beads coated with MRP-8 monoclonal antibody resulted in a decreased peak at *m/z* 10,832. The definitive identification of the potential biomarkers will be obtained by MS/MS analysis in the future.

In the paper of Sinz et al.[16] the 2-DE technique was combined with MALDI-TOF-MS for the analysis of proteins present in plasma and SF of patients suffering from RA, reactive arthritis or osteoarthritis. The most important findings were as follows: Calgranulin B was exclusively identified in SF samples from RA patients. Calgranulin B was not observed in SF from osteoarthritis patients, nor in plasma samples from either patient group. Serum amyloid A (SAA) protein spots were determined in plasma and SF from patients with RA, but not in patients with osteoarthritis. Fibrinogen β-chain degradation products, presumably plasmin-derived, appeared solely in SF and not in plasma. The biomarkers were not validated with other tests.

A reference map of the human normal articular chondrocyte was obtained by Ruiz-Romero et al.[30]. Cells were isolated from cartilages and the cultured cells were used to obtain protein extracts, which were resolved, by 2-DE and visualized by silver nitrate or CBB staining. Ninety-three different proteins were identified. A significant proportion of proteins are involved in cell organization (26%), energy (16%), protein fate (14%), metabolism (12%), and cell stress (12%).

From all the identified proteins, annexins, vimentin, transgelin, destrin, cathepsin D, heat schock protein 47, and mitochondrial superoxide dismutase were more abundant in chondrocytes than in other types of mesenchymal cells.
Tilleman et al.[31] investigated the cytosolic proteome of inflamed synovial tissue of patients with RA, spondyloarthropathy (SpA), and osteoarthritis. Cytosolic proteins were extracted from the tissue and subjected to 2-DE. Proteins of interest were independently identified by MALDI-TOF-MS and electrospray ionization-mass-spectrometry. Calgranulin A, also known as MRP-8, was up-regulated in RA and SpA compared to osteoarthritis. A real-time RT-PCR experiment was conducted. Statistically significant higher mRNA expressions levels of MRP-8 were found in inflamed synovial tissue of RA in comparison to osteoarthritis patients. Vimentin, protein disulfide isomerase A3 precursor, triosephosphate isomerase, a-enolase, glutamate dehydrogenase 1, fructose biphosphate aldolase A, endoplasmin precursor, ceruloplasmin, creatine kinase M chain were up-regulated in SpA versus osteoarthritis. These proteins were not validated with other tests.
The aim of the study of Liao et al.[32] was to identify a panel of candidate protein biomarkers of RA, that can predict which patients will develop erosive, disabling disease. 2-DE was combined with LC/LC-MS/MS to generate protein profiles of SF from patients with either RA or nonerosive RA and healthy controls. Thirty-three prospective candidate biomarkers were selected from a total of 418 identified spots. Among the proteins that were elevated in the SF of patients with erosive RA were C-reactive protein (CRP) and six members of the S100 protein family of calcium-binding proteins. These markers were also measured in serum. Among the six members of the S100 protein family, only calgranulin A (MRP-8), B and C were increased in erosive serum versus non-erosive RA serum, although there was no significant difference. CRP levels in non-erosive RA samples were 2-12 fold higher than in samples from healthy individuals, while in patients with erosive RA, CRP levels were 47-142 fold higher than in healthy controls (see Table 4.2).
Dotzlaw et al.[33] compared the expression levels of proteins in peripheral blood mononuclear cells of healthy control individuals and RA patients. With 2-DE they detected 18 proteins that were two-fold or more highly expressed in patients versus controls, and 11 proteins that were two-fold or more highly expressed in controls versus patients. Eight spots could be identified. Beta actin, DNA –type molecular chaperone HSPA 5 precursor, and fibrinogen-gamma chain were up-regulated in the patient group. Heterogenous ribonucleoprotein K isoforms b, swiprosin 1, heat shock 60 kDa protein 1 (Hsp60), and Ran-binding protein 1 were down-regulated in the patient group. No other tests were used to validate these potential biomarkers.

Lung inflammatory diseases

The sarcoidosis and cystic fibrosis studies are summarized in Table 4.3. To detect sarcoidosis biomarkers, bronchoalveolar lavage fluid (BALF) of sarcoidosis and

healthy controls were compared using SELDI-TOF-MS in the study of Kriegova et al.[34]. Forty differentially expressed protein entities were detected in patients with pulmonary sarcoidosis versus control subjects ($p<0.05$). Thirteen peaks were present across all chest X-ray stages (CXR) and 27 were specific for particular CXR stages. They were able to identify three proteins by peptide mapping and the results were confirmed by immunodepletion analysis. Human serum albumin, α1-antitrypsin and protocadherin-2 precursor were identified as sarcoidosis associated proteins. Albumin and protocadherin-2 precursor were up-regulated in the patients and α1-antitrypsin was down-regulated. The markers were confirmed with immunodepletion assays. A significant reduction of the peaks of interest was observed in immunodepletion assays. In the negative controls without the specific antibody the peaks were clearly detectable.

Magi et al.[35] studied the protein composition of BALF using 2-DE. BALF samples from patients with sarcoidosis and idiopathic pulmonary fibrosis were compared. Sarcoidosis and idiopathic pulmonary fibrosis are both interstitial lung diseases, but hey have a different cellular composition and cytokine profile in BALF. Comparison of the BALF protein maps of the two groups of patients showed 32 spots with statistically significant disease-related variations in relative abundance (see Table 4.3). Alpha1-antitrypsin, macrophage migration inhibitory factor (MIF), and translationally controlled tumor protein (TCTP) were proved by Western blotting. The other potential markers were not validated.

The 2-DE technique was also used by Sabounchi-Schütt et al.[36]. The BALF protein patterns from sarcoidosis patients and healthy controls were compared. Eleven significantly different proteins could be successfully identified (see Table 4.3). No other tests were used to prove these proteins. Sabounchi-Schütt et al.[37] also compared serum protein patterns using the 2-DE technique. Serum protein patterns from sarcoidosis patients and healthy controls were compared and 19 significantly different proteins were successfully identified (see Table 4.3). The total number of serum-protein spots was significantly increased in patients, although the total number of BALF-protein spots did not differ significantly[36]. When they compared the proteins found in BALF and serum , they found similar alterations of three identified proteins in BALF and serum, β2-microglobulin, immunoglobulin kappa, and protein β2-glycoprotein 1, which is a plasma lipid binding protein. All three proteins are increased in the patients versus the controls.

Roxo-Rosa et al.[38] identified potential biomarkers for CF lung disease by 2-DE of nasal cells from CF patients and non-CF controls. Thirteen proteins were significantly different in the CF patients versus non-CF controls (see Table 4.3). The independent validation of each protein identified will be performed in the future. The most important findings which fit with the clinical picture, are cathepsin D and Hsp 27.

In the study of Sloane et al.[39] the saline-induced sputum 2-DE protein profiles from adults with CF with an exacerbation and from children with CF with stable disease and preserved lung function were compared with profiles from adult and young controls. Three proteins were identified which were significantly different in the adults with CF with an exacerbation compared to control subjects. Myeloperoxidase and cleaved α1-antitrypsin were up-regulated in the CF patients. IgG degradation was present in the CF patients. Protein identifications were confirmed by Nanoflow liquid chromatography-MS with an LCQ DECA ion trap MS. Differential expression of myeloperoxidase in sputum between CF and control subjects was confirmed by ELISA. Western blotting and MALDI-TOF-MS analyses identified numerous IgG-γ1 heavy-chain fragments in sputum, molecular mass around 25 to 45 kDa, from all adults with CF with an exacerbation, but only full-length chains in adult controls. Non-cleaved α1-antitrypsin was measured and the CF patients had lower non-cleaved α1-antitrypsin. The relationship of each marker with FEV1 was assessed by statistical tests. Statistical correlation analyses indicated that changes in myeloperoxidase expression and IgG degradation were the strongest predictors of preserved lung function.

Discussion

The proteomics studies in multiple sclerosis, rheumatic diseases and lung inflammatory diseases are summarized in this review. A lot of proteins were identified and in some of the studies the identified proteins were also validated with other tests. The importance of pre-analytical strategies is also illustrated in this study. Del Boccio et al. [10] confirmed that the in vitro N-truncation of cystatin C, the marker which was found by Irani et al. [12] for the diagnosis of multiple sclerosis, occurred because the CSF samples were stored at –20ºC. This indicates that standard pre-analytical strategies need to be performed, like described in the review of Bons et al.[40]. The study of Noben et al. [28] illustrated that not only pre-analytical, but also technical problems can cause artefacts.

In this overview we could not find a common marker for multiple sclerosis which was detected and validated in more studies, however Noben et al.[28] described that the Hsp proteins were entitled as biomarkers with potential for further research in multiple sclerosis since dysregulation in the hsp system was found to be the most prominent and consistent results of gene expression studies in multiple sclerosis and other autoimmune diseases.

De Seny et al.[29] found a peak at *m/z* 10,832 which was upregulated in rheumatoid arthritis patients and was suspected to be MRP-8. Western blot analysis and immunodepletion gave some evidence that it could be MRP-8, but the definitive identification of the peak needs to be done. Tilleman et al.[31] also found MRP-8 as a potential marker for rheumatoid arthritis. In their study MRP-8 was also

up-regulated in the RA group versus SpA and OA. They also identified other markers, but only MRP-8 was validated with real-time RT-PCR experiments. They found significant higher mRNA expressions levels of MRP-8 in inflamed synovial tissue of RA patients compared to osteoarthritis patients. Liao et al.[32] identified some other markers in SF to differentiate between erosive and non-erosive rheumatoid arthritis. Four markers were validated in serum. Calgranulin A (MRP-8), B, and C were increased (not significant) in erosive serum versus non-erosive RA serum and CRP levels were absolutely higher in the erosive RA patients versus the non-erosive patients. MRP-8 was found in three studies described in this report in different sample materials: serum, cytosolic proteins from synovial tissue and SF. The group of Wu et al.[41] also discussed recent proteomic approaches, like two-dimensional gel electrophoresis, liquid chromatography-MS/MS, capillary electrophoresis-MS/MS, SELDI-TOF-MS, and a variety of targeted antibody-based protein arrays, which can also be used for rheumatoid arthritis.

Kriegova et al.[34] identified three potential markers for sarcoidosis, human serum albumin, α1-antitrypsin, and protocadherin-2 precursor. Albumin and proto-cadherin-2 precursor were up-regulated in the patients and α1-antitrypsin was down-regulated. All three markers were confirmed by immunodepletion assays. Magi et al.[35] identified three proteins which were also validated by Western blotting. Alpha1-antitrypsin was increased in the sarcoidosis patients versus the idiopathic pulmonary fibrosis patients. Kriegova et al.[34] also detected α1-antitrypsin, but in their study α1-antitrypsin was down-regulated in the patients versus healthy controls. MIF and TCTP were increased in the idiopathic pulmonary fibrosis patients versus the sarcoidosis patients.

According to Sloane et al.[39], Myeloperoxidase, α1-antitrypsin, and the formation of more IgG-γ1 heavy-chain fragments can be effective in monitoring pulmonary status in patients with CF. Total α1-antitrypsin was down-regulated in the patients, but the cleavage products of α1-antitrypsin were increased in the CF patients versus the controls. Roxo-Rosa et al.[38] also detected an up-regulation of cathepsin D in CF patients versus controls. Cathepsin is an enzyme which is involved in the inflammatory process of CF. They also found Hsp 27 as an important marker. Hsp27 is an ATP-independent chaperone that functions as an inhibitor of nuclear factor kappa-B pathway. Therefore, the reduced levels of this negative regulator might explain the increased activation of the nuclear factor κB and overproduction of Interleukin 8 reported to occur in CF bronchial gland cells and CF lungs[42,43].

In conclusion, the Hsp proteins were entitled as biomarkers with potential for further research in multiple sclerosis. MRP-8 was found in three different rheumatoid arthritis studies with different sample materials and could be a potential marker for rheumatoid arthritis. Alpha1-antitrypsin was validated in two studies as a marker for sarcoidosis, although in one study sarcoidosis patients were compared with idiopathic pulmonary fibrosis patients and in the other study the sarcoidosis patients were compared with healthy control samples. Alpha1-antitrypsin was also found as a marker for CF, together with

myeloperoxidase and IgG. The use of these markers should be confirmed by increasing the sample sets. In the coming years, the following proteomics technologies will probably dominate the clinical proteomics field; two-dimensional fluorescence difference gel electrophoresis (2-DIGE), and multi-dimensional liquid chromatography approaches. With 2-DIGE, Cy dye fluorophores are used for prelabeling the protein samples. The 2-DIGE technology adds a quantitative component to conventional 2-DE analyses allowing comparison of protein expression changes across multiple samples simultaneously without gel-to-gel variation[8]. Most multi-dimensional liquid chromatography approaches utilize a strong cation exchange followed by a reverse phase separation, and the chromatography columns can be physically attached on-line to the mass spectrometer. Such multidimensional protein identification technology (also referred to as MudPit) is an attractive approach for analyzing complex samples in a large scale manner[8]. These new advances will also be suitable for the detection of potential biomarkers in chronic inflammatory diseases.

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