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NEUROPROTEOMICS TOOLS IN CLINICAL PRACTICE

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ABSTRACT

Neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) are characterized by neuronal impairment that leads to disease-specific changes in the neuronal proteins. The early diagnosis of these disorders is difficult, thus, the need for identifying, developing and using valid clinically applicable biomarkers that meet the criteria of precision, specificity and repeatability is very vital. The application of rapidly emerging technology such as mass spectrometry (MS) in proteomics has opened new avenues to accelerate biomarker discovery, both for diagnostic as well as for prognostic purposes. This review summarizes the most recent advances in the mass spectrometry-based neuroproteomics and analyses the current and future directions in the biomarker discovery for the neurodegenerative diseases.

1. Introduction

The brain is the most fascinating and highly complex organ of the nervous system with the ability to control most of the body activities, and is responsible for perception, storage and process of memory, controlling psychological processes and behavioral aspects. The high diversity and heterogeneity of the brain pose a tremendous challenge for proteomic analysis. Proteomics is a relatively recent field of research currently undergoing rapid development and growth. It covers the large-scale study of protein functions, interactions, dynamics and structures, complementing the genomic and transcriptomic studies. Recently, there has been a growing interest in applying proteomics to the clinical diagnosis of the disease. Clinical proteomics is increasingly used for the discovery and verifications of new diagnostic and prognostic biomarkers, novel drug targets, as well as for unraveling novel biological mechanisms [1].

One of the important sub discipline of proteomics is "neuroproteomics" (established in 2003) [2]. The challenge of clinical neuroproteomics is to understand the protein-driven molecular mechanisms and functions of the central nervous system (CNS) and CNS disorders through the study of protein expression and the discovery of novel protein biomarkers in order to facilitate diagnosis, treatment monitoring and prognosis. Therefore clinical neuroproteomics is a very useful tool for meeting the needs of neurological disorders.

In general, the field of proteomics can be divided into two extensive areas based on the detection methods used: (1) approaches using mass spectrometry (MS) for the identification and quantitation of proteins and (2) approaches using antibody-based methods. MS-based proteomics has emerged as a powerful approach with the potential to accelerate biomarker discovery [3]. The possibility to analyze complex samples globally and to create comprehensive lists of possible candidate biomarkers makes mass spectrometers an indispensable tool for the discovery phase of biomarker research. Specificity, sensitivity, accuracy, acquisition speed and automation are all advantages offered by MS quantification.

As life expectancy is increasing, the number of elderly people at risk of developing dementia is growing rapidly. Dementia is one of the main causes of disability in later life. Age-related neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), multiple sclerosis (here abbreviation MSc to distinguish from mass spectrometry MS), amyotrophic lateral sclerosis (ALS) are growing public health problems. Recently, it has been

estimated that more than 36 million have dementia today, with 4.6 million new cases of dementia every year. Numbers of people affected will double every 20 years to more than 66 million in 2030 and 115 million by 2050 [4,5]. A failure to find a curative treatment, or even more challenging, to gain insight into the elusive context of understanding the disease's pathogenic mechanisms cause very large future costs to the national health care system. The main issue with these disorders is the difficulty in correctly clinical diagnosing the patient at an early phase of the disease. The clinical diagnosis of neurodegenerative disorders requires a medical history and a series of examinations including blood and cerebrospinal fluid (CSF) tests, psychological assessments, imaging technologies, and different radiological investigations [6]. Therefore, the identification and validation of novel biomarkers for diagnosing of different form of dementia are increasingly important.

This review will discuss some important aspects of high-mass resolution MS-based clinical neuroproteomics, with a focus on recently published works in the field of protein biomarker discovery in neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis and multiple sclerosis, with the aim to provide a prospective view on current and future research aspects in this field. In our laboratory we have been studying the neuroproteome since early 2000 developing and applying novel sample preparation and proteomic approaches in neurodegenerative diseases [7-17]. There are many other tools available for proteomic research including protein microarrays [18], proximity ligation assay [19], Luminex-based multiplex assay [20,21], Bio-Plex assay [22], etc. However description of them is beyond the frames of the current review. For more detailed information we refer the reader to specific reviews of each of these methodologies.

2. Biological samples – clinical specimen

In the search for protein biomarkers there are a few options of sample sources. Different tissues (brain biopsy, spinal cord, muscles, etc) or body fluids (blood, plasma, CSF, saliva, tears, urine) can be examined for protein biomarkers discovery. Choosing the appropriate biological sample is one of the most important steps in the discovery of biomarker candidates that might be suitable for clinical research and clinical use.

2.1. CNS tissue samples

Since most neurodegenerative disorders are region-specific, clinical studies of the affected CNS tissue and its surroundings during disease development is therefore very important, and the CNS tissue would be the ideal source for discovering biomarkers. Postmortem proteomics analysis of brain compartments or, alternatively, brain biopsies is very useful to understand the complexity of neurodegenerative disease and might allow screening for disease-related protein changes in the CNS tissue where the degenerative process occurs, but they are not practical for routine biomarker analysis. Brain tissue sampling is extremely invasive for the patient and is not ethical or, practical to perform the tissue collection is *praxis*. Brain tissue can serve as a good starting point for finding new potential biomarkers though. However, for actual clinical usefulness such biomarkers are also needed to be detectable in more available clinical matrices.

2.2. Cerebrospinal fluid

For practical purposes, analysis of protein expression in body fluids is likely to provide important information about how the CSN is functioning both in normal healthy conditions and in neurological disorders. Body fluids can be easily obtained by non-invasive or minimally invasive techniques.

Human CSF is a valuable and promising body fluid for differential proteome analyses that provides molecular insights into the pathogenesis of CNS diseases and potentially provide biomarkers that can help in diagnoses and monitoring of disease progressions. CSF circulates within the ventricles of the brain and surrounds the whole spinal cord. It is created by passive filtration of blood through the epithelial cells and by diffusion of interstitial fluid from the brain renewing about four times every 24 hours [23]. The most common way to obtain CSF is via lumbar puncture. Routinely, 12 mL CSF is collected for analysis from adults [24].

Since CSF is in a close proximity to the diseased brain tissue and in a close anatomical contact with the brain interstitial fluid, where biochemical changes related to CNS disease are reflected, it is an exceptional source of potential protein biomarkers for neurodegenerative disorders [25]. Therefore, the CSF analysis remains an important and practical tool to get closest to the pathology of CNS disorders. However, the major disadvantage of using CSF as the sample sources for clinical applications is the invasive nature of CSF sampling.

Additionally, the collection of CSF from healthy controls is still an ethical concern. Furthermore, depletion of highly abundant proteins, such as albumin, IgG, transferrin, etc, is often desired prior to the proteome analysis of CSF [26-28]. Though the CSF collection is quite invasive and tricky, CSF is still the best biological sample source to perform biochemical and clinical analyses to detect pathophysiological abnormality of the CNS [29] (besides brain or spinal cord tissue).

2.3. Blood and serum/plasma

Human blood is the most frequently used body fluids for measuring levels of proteins and other biomolecules in clinical research, since they can be reasonably easy to sample and technically easy to process. The blood proteome is one of the most complex components of the human proteome, which contains a large diversity of proteins [30,31]. Blood can be separated into two compartments, namely, plasma (cell-free content of the blood) and serum (plasma without the clotting factors, fibrinogens and includes all proteins not used for blood coagulation). Plasma and serum contain extremely informative proteomes that may present unique information from different tissues and organs in the body and may be an excellent sample for the discovery of potential clinical biomarkers and for their clinical quantification. However, despite the simplicity of sampling, biomarker discovery from these samples has been lacking. The tremendous analytical challenge in the development of diagnostic assays for the very low concentration of biomarker proteins is the broad dynamic range of its protein constituents and complexity. The estimated dynamic range of protein concentrations in human plasma may be up to 15 orders of magnitude [30,31].

Additionally, the use of blood is still limited for biomarker discovery for neurological disorders. Proteomic research using blood as a source of protein biomarkers still did not answer the question whether there is a linear relationship between changes of proteins in diseased CNS tissue and in blood.

2.4. Saliva, tears, urine

Saliva is a multi constituent biological fluid that reflects the general body health and disease. Being extremely readily accessible and containing about 30 % of blood proteins, saliva

represents an attractive diagnostic fluid for the early detection of various diseases including oral cancer, renal, and autoimmune diseases [32,33].

Tears are a complex fluid composed of many proteins, lipids, carbohydrates, electrolytes and other small organic molecules. The chemical composition of tears is significantly less complex as compared to plasma, CSF and urine. Additionally, the tear sample collection is non-invasive and pain free which makes tear fluid a good source for biomarker discovery studies of diseases such as, dry eye, visual disease, inflammatory disease, cancer, etc [34]. However, despite of simple sampling of tears, the tear proteome profiling is quite challenging due to the very small volume of tear fluid (less than 5 μ L) that can be collected under normal conditions [35]. Furthermore, different exogenous factors such as inflammatory or metabolic influences can easily effect the composition of saliva and tears leading to poor reproducible results in CNS diseases. The applicability of saliva in biomarker discovery for neurological disorders is extremely limited, to the best of our knowledge, only one study revealed novel alterations in saliva metabolomic profiles between various neurodegenerative disorders [36].

Urine is one of the most useful human body fluids for clinical proteomics. It is generated as a result of filtration of plasma by glomeruli in the kidneys, retaining the majority of the proteins[37]. Urine is an easily accessible in a big amount sample source that has the potential to provide various disease specific biomarkers related to urogenital and some of non-nephrological diseases. Changes in urine composition are becoming also promising in biomarker discovery for neurological disorders. Metabolic [38] and lipidomic [39] signatures of AD and PD urine samples are reported in a few studies. However, to the best of our knowledge, only in one paper AD urinary proteome was characterized using ELISA approach revealing up-regulation of novel neural thread protein, a membrane-associated phosphoprotein, in the urine of patients with AD [40].

3. Technologies for mass spectrometry-based neuroproteomic analysis

During the last 15 years, neuroproteomic technologies have been used to analyze various biological samples collected from patients with different neurological diseases. The field of neuroproteomics is very challenged by the heterogeneity and complex cellular and subcellular architecture of the CNS. However, by implementing the appropriate sample preparation protocols and proper liquid chromatography-tandem mass spectrometry (LC-MS/MS)

approaches, MS-based neuroproteomics analysis can provide wide information about the neurological tissues/body fluids, including identification, characterization, distribution and location of proteins, as well as determine changes in their abundance under various conditions [2].

Proteomic analysis is a multistep process comprising sample preparation, separation, quantification, identification of proteins and validation of protein biomarker candidates.

Figure 1. Proteomic workflow: Sample collection from different source (disease and healthy), sample preparation, protein or peptide separation, quantification, identification using mass spectrometry and validation of the biomarker candidates.

3.1. Sample preparation

With the advances in the instrumentation, mass spectrometry has emerged into a powerful tool for the screening and discovery of potential biomarker candidates in addition to the traditional protein identification and characterization applications. The proteins/peptides required to be analyzed by MS should be free of contaminants to reduce their interference during electrospray ionization (ESI) and to prevent their dominant appearance in the chromatogram. Therefore, sample preparation plays a key role to produce a MS-compatible end product for the analysis.

Neuronal tissues such as brain and spinal cord, body fluids such as CSF, plasma and primary cells were frequently employed to study the proteins involved in neurodegenerative disorders. The neuronal cells have unique lipid composition of glycolipids, sphingomyelin and cholesterol on the insulative myelin sheath that renders the extraction and analysis of proteins difficult. These issues can be solved by: (a) employing detergents, chaotropes and various mechanical tissue disruption methods for the efficient extraction of the proteins from cell compartment; (b) delipidation using various organic solvents to precipitate proteins or other protein purification techniques for the removing of lipids as well as other interfering components.

Mechanical disruption is achieved by grinding/homogenizing tissue with various devices mentioned in Table 1, which helps in disrupting the cell membrane and releasing the cell components into an appropriate lysis buffer. The presence of various additives such as detergents, chaotropes, salts, organic solvents in lysis buffer helps in solubilizing/denaturing the proteins and maintaining the pH and ionic strength.

Detergents are amphiphilic molecules and are classified as ionic, non-ionic, zwitterionic and bile acid salts, based on their structure (Table 1). Ionic detergents are either positively (cationic) or negatively (anionic) charged and are effective in disruption of cellular structures and denaturation of proteins. Ionic detergents act by disrupting the protein-protein and intra-protein interactions but are usually incompatible with the downstream analysis. Non-ionic detergents contain either polyoxyethylene or glycosidic groups. They act by disrupting lipid-lipid and lipid-protein interactions rather than protein-protein interactions and hence considered as relatively mild and non-denaturing compared to ionic detergents [41]. Zwitterionic detergents combine the properties of ionic and non-ionic detergents and do not alter the native charge of the protein molecules. Due to its mild nature, zwitterionic detergents can be employed in structural studies of membrane proteins (MPs). Bile acid salts are ionic detergents but differ in their structure due to presence of rigid steroidal group. Bile acid salts are mild in nature compared to linear ionic detergents and can be removed by precipitating at acidic pH.

There is no universal detergent that can serve all the needs of proteomic research, and the choice of detergent selection is determined experimentally to suit the type of work to be carried out. However, the usage of detergents compromises with the MS analysis step to some extent due to- their interference in ionization step causing the shifts in charge-state distribution in ESI-MS [42]. Hence their removal prior to MS-analysis is essential, which can be achieved by different techniques such as dialysis, hydrophobic absorption, ultrafiltration, precipitation, gel filtration or solid phase extraction (Table 1). Selection of the purification method should be based on the characteristics of tissue to be studied as well as the desired end product. For example, in case of neuroproteomic studies on brain tissue, the removal of lipids (delipidation) is the main priority due to presence of 60% fat content in the brain [43]. Delipidation can be done by using various organic solvents to precipitate the proteins and to remove the lipids [15].

Apart from the purification techniques, cleavable detergents such as 3-[3-(1,1-bisalkyloxyethyl)pyridin-1-yl]propane-1-sulfonate (PPS) [44], sodium 3-((1-(furan-2-yl)undecyloxy)carbonylamino)propane-1-sulfonate (ProteaseMax from Promega), sodium 3-[(2-methyl-2-undecyl-1,3-dioxolan-4-yl)methoxy]-1-propanesulfonate (Rapigest from Waters Corp) can be employed. Such detergents can be cleaved under acidic condition making them inactive and removable prior to the MS-analysis. Additionally, perfluorinated detergents [45] that do not interfere with ESI-MS can be used in proteomic analysis. Also organic solvents and organic acids can be used for the extraction of proteins. Organic solvents are compatible with trypsin digestion and can be removed easily by evaporation prior to LC-MS/MS analysis. Chaotropes disrupt the ordered water structure, thus promoting the solubility of hydrophobic MPs. Chaotropes alone cannot solubilize the lipids, for this reason a mixture of detergent-chaotrope is often employed to increase the solubility of MPs and to solubilize the lipids [46]

3.2. Protein/peptide separation

Separation of proteins/peptides is the pre-requisite prior to MS analysis of CNS sample (neuroproteomics) or any biological samples to reduce the complexity. Two-dimensional gel electrophoresis (2-DE) is a classical technique used in the separation of complex protein mixtures, to detect the qualitative and quantitative differences in the protein composition between two or more biological sample sets [47,48]. In 2-DE proteins are separated in two-dimensions by combining isoelectric focusing (IEF) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In the first dimension of IEF, separation is based on net charge at different pH. In the second dimension the separation is based on the molecular weight. Although 2DE served as a major workhorse in proteomic workflow, it has inherent limitations such as difficulty in reproducibility, poor resolving of low abundant, highly acidic/basic and hydrophobic proteins [48]. With the development in the MS instrumentation in 1990's, a huge leap in the proteomics and biomarker research was attained by replacing the time consuming chemical degradation methods with sensitive and reproducible MS technology.

The next step in sample preparation contains enzymatic cleavage of proteins into peptides. Two types of proteomic approaches, in-gel digestion [49] and in-solution digestion [50,51], have been employed in many laboratories on a routine basis. In-gel digestion consists of

separation of proteins by SDS-PAGE) or 2-DE followed by picking the individual bands/spots and cleaving proteins into peptides using enzymatic digestion. In comparison, in-solution digestion approach involves enzymatic digestion of proteins into peptides in an aqueous solution. Both approaches can be performed by various proteases such as trypsin, chymotrypsin or lys-C, which cleave amino acid-specific peptide bonds. After protein digestion, peptides can be separated by one- or two-dimensional liquid chromatography (LC) prior to ionization and mass spectrometry analysis.

3.3. Mass spectrometry identification

Mass spectrometry has become a versatile and indispensable tool for the analysis of proteins, to define protein-protein interactions and to identify the post-translational modifications (PTMs). The introduction of two soft ionization techniques ESI [52] and matrix-assisted laser desorption/ionization (MALDI) [53] has revolutionized the usage of MS for proteomics research.

ESI, introduced by Fenn and co-workers in 1980's [52] has emerged into a powerful technique in high-throughput proteomics workflow. In the ESI process, the protein/peptide solution is sprayed across a high potential difference to generate a mist of droplets carrying excess charge at the surface. The solvent is evaporated by passing through a heated capillary or by counter-flow of heated drying gas resulting in the closer movement of the droplets. Droplets explode due to the Coulombic repulsive forces, resulting in smaller droplets and the process goes on until solvent-free ions are formed. In addition to ESI, the introduction of microspray and nanospray has greatly benefited the proteomics and biomarker research. The principle of both microspray and nanospray is similar to ESI but differs in needle diameter and the flow rates. With the reduced flow rates to nl/min, nanospray provides the advantage of improved sensitivity and less sample consumption which is important for biological samples [54]. Finally, ionized peptides are transferred into mass spectrometer, where the mass-to-charge ratio of peptides is detected. State-of-the-art mass spectrometers involve multiple steps of mass detection combined with high-energy fragmentation of peptides to achieve high mass accuracy (Tandem-MS). Identification of proteins occurs in the last step by correlating identified masses with known peptide masses based on data base search algorithms.

In MALDI process, the sample is co-crystallized with a suitable organic, UV-absorbing matrix (e.g., *p*-cyano-4-hydroxy cinnamic acid, sinapinic acid or 2,5-dihydroxybenzoic acid, etc) on a conductive surface. When irradiated with the laser, energy is transferred to the matrix and ionizes the analyte into the gas phase. Another recent approach MALDI-imaging mass spectrometry (IMS) is becoming a valuable tool in neuroproteomics to identify the distribution of neuropeptides and proteins directly in thin brain tissue sections [55-57]. MALDI-IMS has advantage of creating three-dimensional images of brain to determine the evolution of sub-proteome [55,58].

3.4. Quantitative neuroproteomics

Quantitative neuroproteomics is frequently applied to measure the relative or absolute protein abundance between two or more conditions, e.g. normal versus disease state, to explore changes in the proteome and post-translational modifications based on neurological disorder or to discover novel protein biomarkers.

Over the last two decades, quantitative MS-based methods evolved tremendously based on technological improvements of mass spectrometry instrumentation and the development of methods for protein or peptide quantification including label and label-free techniques. Several quantitative MS-methods were successfully applied to neuroproteomics studies like classical techniques using two-dimensional gel electrophoresis and differential two-dimensional gel electrophoresis (2D-DIGE) as well as newer quantitative methods as isotopic chemical labeling (e.g. iTRAQ, dimethyl labeling (DML)), multiple reaction monitoring (MRM) and label-free quantification (Fig. 2).

Figure 2. Overview of selected methods for quantitative proteomics. Metabolic labeling techniques including SILAC and SILAM were not used to study neurodegenerative diseases in human patients. DML – dimethyl labeling, iTRAQ – isobaric tags for relative and absolute quantification, ICAT – isotope-coded affinity tags, SILAC – stable isotope labeling with amino acids in cell culture, SILAM – stable isotope labeling of mammals, MRM/SRM – multiple/selected reaction monitoring, 2-DE – two-dimensional gel electrophoresis, AQUA – absolute quantification. Picture was modified after Craft et al [59].

Quantitative gel electrophoresis methods

2-DE and 2D-DIGE are classical and robust methods to compare protein expression levels between normal and disease states[60,49,61,62]. As described before, protein spots are detected and can be compared between different 2D-gels by 2D-imaging analysis software. The alignment of different gels should be carefully considered to ensure accuracy of quantification. To overcome the problem of gel-to-gel differences and therefore incorrect protein quantification, in 2D-DIGE approaches proteins are labeled by fluorescent cyanine dyes which allow the combined analysis of several samples on a single 2D-gel [62]. Finally, protein spots of interest are excised, enzymatically digested and analyzed by LC-MS/MS or MALDI-TOF-MS. In neuroproteomics, 2-DE and 2D-DIGE are commonly used to identify differentially regulated proteins in neurodegenerative diseases like AD, PD, ALS and MSc (see below).

Chemical labeling methods

Coupled to significant improvements of mass spectrometry instrumentation, quantitative MS-based methods like chemical labeling techniques with stable isotopes evolved to enable large scale and in-depth protein studies. One way of protein and peptide labeling includes the usage of chemical isobaric tags (e.g. iTRAQ, ICAT, TMT). iTRAQ (Isobaric Tag for Relative and Absolute Quantification) allow the unique possibility to multiplex up to 4 (4-plex) or 8 (8-plex) samples together in one LC-MS/MS experiment to increase the accuracy of protein quantification [63]. Initially, proteins are enzymatically digested to peptides and then labeled with isobaric tags at the free amino-termini and epsilon-amino groups of lysine residues. The isobaric tags contain a reactive group for peptide binding, a balancer group, a cleavage site

specific for CID and a reporter ion. After labeling with different tags, which all have the identical mass, samples are mixed and analyzed together in one LC-MS/MS run. Quantification occurs in MS/MS mode after fragmentation due to the detection of cleaved reporter ion masses (reporter ion mass: 114.1, 115.1, 116.1 and 117.1 in 4-plex iTRAQ).

Next to isobaric tags, peptides can also be chemically labeled with isotopomeric dimethyl labels for quantitative purposes (dimethyl labeling)[64]. After enzymatic digestion of proteins, primary amines of peptides react with formaldehyde to form dimethyl isotopomers with a mass addition of 28, 32 and 36 Da. Therefore, up to 3 samples can be multiplexed and measured together in one LC-MS/MS run (3-plex). In MS mode, the peptide triplets with a mass difference of 4 Da are then used for relative quantification between the 3 samples.

Chemical labeling of peptides by tags or dimethyl labels occurs at a late stage of sample preparation and could be therefore a reason for technical variations between samples affecting the accuracy of quantification. Nevertheless, both chemical labeling techniques enable global proteome quantification of mostly all types of sample/tissue including human, e.g. post-mortem brain tissue and CSF of patients with neurodegenerative diseases. Recently, global proteome studies were performed in our group using DML to analyze patients with AD [9] and ALS [65].

Label-free quantification

In comparison to labeling techniques for quantitative MS-based proteomics, non-label methods have the benefit to utilize protein quantification without any kind stable isotope labeling. Therefore, label-free quantification is not laborious and can be applied to all biological samples. Two label-free techniques are frequently used: quantification based on peptide-ion intensity, which compare ion abundances of a peptide between LC-MS/MS runs, and spectral counting based on the numbers of MS/MS spectra per peptide [66,67]. Both methods rely on the correlation that the ion abundance as well as the number of fragmentation spectra are linearly proportional to protein abundance in a single LC-MS/MS run.

Label-free quantification is suitable for proteomic studies using sub-cellular fractionation or chromatographic protein/peptide separation but it requires high resolution MS instrumentation and reproducibility across multiple samples to ensure high accuracy of quantification. For neuroproteomics studies, label-free quantification has been recently used to analyze changed protein expression e.g. in brain tissue of AD patients [68] and spinal cord tissue of ALS patients [69].

Furthermore, newly developed methods for label-free quantification as SWATH [70] and MS^E [71,72] could gain in importance for neuroproteomics analysis due to high technical reproducibility and accuracy. Both techniques rely on data-independent acquisition of ion spectra. In SWATH experiments, all fragment ions are detected by cycling through specified precursor isolation windows, whereas all precursor and fragment ions are monitored at different collision energy levels in MS^E mode. Due to high throughput protein identification and quantification as well as targeted data analysis strategy both methods might be established for clinical applications in the future.

Multiple-reaction monitoring

Multiple-reaction monitoring or selected reaction monitoring (SRM) is a MS-based, targeted protein measurement to detect relative and absolute peptide levels [73]. MRM is most widely performed on triple quadrupole mass spectrometer (QQQ), using the first quadrupole (Q1) for the selection of parent ions, the second quadrupole (Q2) for fragmentation and the third quadrupole (Q3) to scan and filter fragment ions.

In contrast to global protein profiling methods as described before, MRM allows the measurement of specific target peptides with high selectivity as well as with high sensitivity to detect low abundant proteins in complex samples. In addition, absolute protein quantification (AQUA) can also be performed by MRM using stable isotope containing tryptic or synthetic peptides.

In recent neuroproteomics studies, MRM was used to analyze the expression levels of targeted proteins in AD and MSc patients. Furthermore, MRM is a promising technique to complement Enzyme-linked Immunosorbent Assay (ELISA) assays as a diagnostic tool in clinical practice.

4. MS-based proteomics discoveries in neurodegenerative diseases

The neurodegenerative diseases (i.e. Alzheimer's disease, Parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis) are a large group of disorders characterized by irreversible loss of neurons of brain and spinal cord tissue. The progressive neurodegeneration in turn leads to shared disease symptoms e.g. destroyed memory, impaired learning functions, communication difficulties, as well as changes in personality. The neuropathology of these diseases is linked to the aggregation and accumulation of misfolded proteins that leads to the

formation of intra- and extra-cellular aggregates, such as senile plaques in AD, Lewy bodies in PD and hyaline inclusions in ALS.

Typically, the clinical diagnosis of such diseases cannot be made until the disease has progressed to the point that dementia or motoric dysfunctions arise. Although significant improvement in clinical diagnosis and care for patients with these diseases has been made, still there is currently no curative treatment for any of these disorders. Thus, the need for identification, development and validation of biomarkers for diagnosing different forms of dementia that meet the criteria of precision, specificity and repeatability is increasingly important. Additionally, it is extremely important to find reliable biomarkers that can distinguish neurodegenerative disorders to begin an early treatment.

In the following section, we will focus on biomarker discovery for the four most common CNS disorders: AD, PD, ALS and MSc using MS-based proteomic methods in order to summarize the recent advances in this rapidly developing field. Therefore, we will cover only a small part of proteomic research done in the field of biomarker discovery.

The overview of recently published neuroproteomic studies differentiating between AD, PD, ALS, MSc and control samples, including the source of CNS samples, applied MS-based techniques and the biomarker candidates discovered are listed in Table 2.

Alzheimer's disease

AD is the most common form of neurodegenerative disease, accounting for 50-60 % of all diagnosed cases [74]. Although the exact etiology of AD remains to be defined, histopathologically AD is characterized by deposition of the aggregated β -amyloid ($A\beta$) peptide in the form of extracellular senile plaques and hyperphosphorylated tau protein in the form of intracellular neurofibrillary tangles causing massive neuronal and synaptic degeneration. Several neuropathological processes have been proposed to connect with the onset and progression of Alzheimer's disease. Primarily, the "amyloid cascade hypothesis" posits that accumulation of $A\beta$, cleaved from the amyloid precursor protein (APP) by β -secretase and γ -secretase [75]. Impaired mitochondrial functions, oxidative stress and inflammatory processes have also been suggested to play a role in AD pathogenesis [76]. Currently, combined analysis of 42 amino acid form of $A\beta$ ($A\beta_{42}$), total tau (tTau) and hyperphosphorylated tau (pTau) in CSF are employed AD diagnosis[77]. CSF of AD patients shows decreased values of $A\beta_{42}$ and increased values of tTau or pTau.

During the last years MS-based proteomic technologies have been applied quite intensively to study AD-related changes in protein levels of CSF and plasma samples as well as post mortem brain tissues. Classic proteomics platform, two-dimensional gel electrophoresis (2-DE) in combination with mass spectrometry or tandem mass spectrometry was shown to be very effective for analysis of CNS samples. Most of proteomic studies on neurodegenerative disorders have been performed using 2-DE. Recently, using 2-DE and LC MS/MS, Henkel et al. [78] identified 20 potential AD plasma biomarkers, ten of these proteins were either involved in the pathophysiology of AD or the A β -peptide processing pathway. The 2-DE study [79] revealed altered levels of 47 novel candidate protein biomarkers in AD as compared to control CSF samples. Alteration of four proteins (neuronal cell adhesion molecule (NCAM), chitinase 3-like 1, chromogranin A, carnosinase I) was confirmed by ELISA measurements. The same approach was applied by Yin et al. [80], they identified a large number of significantly regulated proteins in AD CSF samples. Additionally they showed that the increased level of neural cell adhesion molecule 1 in the CSF of AD patients compared with normal controls is higher than that of PD patients, indicating that NCAM1 is a possible marker for distinguishing AD from PD. In another 2-DE study[81], increased levels of clusterin and apolipoprotein J proteins were found in plasma of the AD patient samples compared to controls. Song et al. [82] detected a panel of 18 signal proteins as markers of AD and mild cognitive impairment using 2-DE and LC MS/MS approach.

Over the past years, the alternative gel-free MS-based proteomics approaches have been widely used for identification and quantification of possible CNS protein biomarkers that fall outside the range of 2-DE. Gel-free shotgun approach can be combined with quantitative proteomic technique, e.g. stable isotope labeling or label free method, and can be easily used for large-scale proteomic experiments. In our recent study [9], by employing a stable isotope DML MS approach we identified 827 brain proteins, 69 of which were significantly changed in AD compared non-neurological control brains. A quantitative proteomic strategy based on the cloud-point extraction method for the separation and enrichment of hydrophobic MPs in combination with DML MS analysis was also employed by our group [17] to quantitatively identify protein expression changes in AD temporal neocortex samples. 62 proteins were found to be differentially regulated including three novel hydrophobic transmembrane proteins: down-regulated sarcoplasmic/endoplasmic reticulum calcium ATPase2 and up-regulated orphan sodium- and chloride-dependent neurotransmitter transporter NTT4 and C9orf5.

Label free quantitative proteomics was used to investigate the protein expression in the brain cortex [68,83] and substantia nigra [84] of AD patients, leading to the identification of 197, 13 and 19 differentially expressed proteins, respectively.

The A β isoform pattern in CSF and brain tissue samples has been the subject of extensive target proteomic studies aimed to distinguish AD patients from non-demented subjects. Pannee et al. [85] have applied MRM-MS technique to quantify a β 42, a β 40 and a β 38 in human CSF. Apolipoprotein E has also been measured in serum using this approach, showing a significant decrease of its level in serum of AD patients [86]. Choi et al. [87] reported on a nanoLC-MRM/MS analytical assays for the measurements of the A β 1-40, A β 1-42, retinol-binding protein and cystatin C in human CSF and validated MS findings using ELISA.

Recently, immunoaffinity enrichment technique, e.g. immunoprecipitation, followed by high resolution MS, was used to analyze the levels of two species APP α and APP β in CSF from AD and non-demented controls [88]. Portelius et al. [89] have demonstrated the ability to apply immunoprecipitation in combination with MALDI-TOF MS or LC-MS/MS to determine the A β isoform pattern in the cerebellum, cortex and hippocampus of AD subjects. Combination of immunoprecipitation and LC-MS/MS approach was used by Thorsell et al. [90] for the determination of neurogranin in the CSF samples. The results reveal a significant increase in the neurogranin levels in the CSF from AD patients and a trend towards increasing levels in the MCI group.

Parkinson's disease

Parkinson's disease is the second most common progressive degenerative CNS after AD. It is characterized by motor function impairment caused by loss of the dopamine (DA)-containing neurons in the brainstem and the presence of intracellular protein inclusions in the substantia nigra, known as Lewy bodies. It is suggested that the abnormal accumulation of proteins, particularly heavily ubiquitinated alpha-synuclein (α -syn), may play role in the pathogenesis of PD [91]. Diagnosis of typical cases of PD is mainly based on symptoms as well as on clinical tests e.g. computed tomography and magnetic resonance imaging of brain scans.

2-DE followed by LC-MS/MS is a very common approach in analysis of differentially expressed proteins in Parkinson's disease. Recently, Alberio et al. [92] verified a list of potential PD biomarkers, proposed by different authors and visible in 2-DE plasma maps of 90 subjects and developed a panel of 9 PD-related plasma proteins (haptoglobin, transthyretin, apolipoprotein A-1, serum amyloid P component, apolipoprotein E, complement factor H,

fibrinogen γ , thrombin, complement C3) using 2-DE MS/MS approach. The elevated levels of transthyretin and haptoglobin were also found in PD serum and CSF samples using 2-DE in combination with LC-MS/MS [93,94] or MALDI-TOF MS [95]. Zhang et al. [96] employed a 2-DE MS/MS coupled with iTRAQ labeling to quantitatively identify the differentially expressed proteins in PD serum samples. 26 proteins were found to be differentially expressed including up-regulated sero-transferrin and clusterin and down-regulated complement component 4B, apolipoprotein A-I, α -2-antiplasmin and coagulation factor V. Two-dimensional difference gel electrophoresis (2D DIGE) technique, in combination with MALDI-TOF MS analysis was recently applied to CSF samples from PD patients and controls, detecting 8 proteins that were expressed at significant levels between these two groups [97]. Additionally, by using Western blotting (WB) analysis authors showed that the expression of tetranectin was reduced in the CSF from PD patients and elevated in AD, while the expression of apoA-I was changed only in the CSF from PD patients. Using 2-DE-LC-MS/MS approach Guo et al. [98] identified 14 differentially expressed proteins (including isoforms of the same proteins in some cases) in the CSF of PD patients and demonstrated for the first time that the levels of autotoxin and SOD1 are up-regulated in CSF of PD dementia patients.

Targeted proteomics has also been using in the study of PD. Using a immunoprecipitation method combined with LC-MS/MS approach Öhrfelt et al. [99] were able to detect and identify known and novel isoforms of alpha-synuclein in PD brain tissue homogenates, including N-terminally acetylated full-length α -syn and two N-terminally acetylated truncated forms of α -syn. Lehnert et al. [100] detected 16 proteins altered in CSF of PD patients using iTRAQ labeling method. Validation of these candidates was then carried out by MRM method, confirming however that only tyrosine-kinase non-receptor-type 13 and netrin G1 were significantly changed in PD and control subjects.

Hong et al. [101] have recently published the biggest proteomic study on CSF levels of alpha-synuclein and observed reduced levels of α -syn in more than 100 PD patients compared to both 50 AD patients and 132 controls after controlling for blood contamination. Additionally, they found DJ-1 as another potential protein candidate which level was significantly decreased in PD cases versus controls or AD patients.

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis is an incurable and the most common type of adult motor neuron disease that leads to progressive paralysis and death within a few years of onset [102]. Its pathology is characterized by irreversible degeneration of the upper and lower motor neurons in cord spinal cord, brainstem, and motor cortex, which causes progressive muscle weakness and muscle atrophy, while leaving the intellect usually completely undamaged throughout the course of the disease. However, the mechanism of ALS onset in humans is unknown and no effective treatment is available at present. The diagnosis of ALS is currently based on clinical and electrodiagnostic evidence since there is no clinical biological marker exists for early in vitro-based diagnostic test for ALS during life. Due to the extremely quick and irreversible neurodegenerative progress in ALS, it became of particular interest in biomarker discovery.

Possible candidates for biomarkers studied so far, are mostly proteins detected in cerebrospinal fluid. Mendonça et al detected by 2-DE and MALDI-TOF MS elevated levels of Parkin-like protein in CSF of ALS patients. Parkin-like protein is an ubiquitin E3 ligase, and mutations in the parkin gene are the most common cause of familial PD. Additionally, authors identified increased levels of several zinc and iron binding proteins in ALS CSF samples. These metals are essential for many metabolic processes and are related to neurodegenerative disease [103]. Elevated levels of alpha-1-antitrypsin have also been presented in CSF samples in two different proteomic studies using MALDI-TOF MS approach [104,105]. Furthermore, Brettschneider et al. [105] have found increased levels of heat shock protein1, fetuin-A precursor, transferrin, nebulin-related anchoring protein and transthyretin in ALS CSF samples. Interestingly, that SELDI-TOF-MS analysis performed by Ryberg et al. [106] has shown the reduced levels of native transthyretin and increased levels of cysteinyl-glycine-transthyretin, a modified form generated by oxidative damage.

Proteomic approaches have been applied to study protein expression changes in postmortem tissues such as the spinal cord from patients diagnosed with ALS. Recently, Hanrieder et al. [8] reported for the first time the MALDI imaging-based protein profiling and relative quantification of post-mortem spinal cord samples obtained from ALS patients and controls. They found that two protein species including a C-terminally truncated form of ubiquitin were found decreased in ALS spinal cord samples compared with controls and validated these findings by Western blot and immunohistochemistry experiments. Zhou et al. [69] employed a label-free quantitative MS approach to find differentially expressed proteins in ALS spinal cord tissue, detecting 14 proteins that were dramatically altered between ALS and control

cases. The protein galectin-3 emerged as a lead biomarker candidate on the basis of its differential expression as assessed by immunoblot and immunocytochemistry. Further analysis of galectin-3 in CSF samples showed that ALS patients had approximately twice as much galectin-3 as normal and disease controls.

However, since characteristic clinical features for ALS are usually appeared in the muscles and because spinal cord tissues sampling is very invasive for the living patient, muscle tissue of ALS patients is very valuable material to perform global surveys on changes in protein expression with clinical characteristics of ALS. In our recent study [65], a shotgun proteomics and quantitative DML were employed to analyze the global changes in human skeletal muscle proteome of ALS *vs* healthy cases for the first time. A panel of 11 ALS-related muscle proteins (medium-chain specific acyl-CoA dehydrogenase, glycogen debranching enzyme, myomesin-1, cytochrome c oxidase subunit 6C, phosphoglycerate mutase 1, four and a half LIM domains protein 1, myosin light chain 3, creatine kinase S-type, protein-arginine deiminase type-2, Kelch repeat and BTB domain-containing protein 10, transitional endoplasmic reticulum ATPase) could be identified using DML LC-MS/MS approach.

Multiple sclerosis

Multiple sclerosis is the most common cause of chronic disabling disorder of CNS and is generally regarded as an autoimmune disease to myelin components that occurs in young adults. MSc is characterized clinically by a variable remitting relapsing and relapsing course and pathologically by the chronic inflammation in the CNS causing axonal damage, progressive accumulation of focal plaques of demyelination in the white matter and neurologic disability [107]. MSc can be divided into two main types: a relapsing type and a progressive type. Both MSc types starts with an episode of so-called clinically isolated syndrome (CIS) [108]. The etiology of the disease is still unknown and currently there is no available diagnostic test to predict disease course and prognosis in individual patients. Currently, magnetic resonance imaging and evidence of dissemination of the lesions in space and time are used for the diagnosis of MSc [109].

In recent years a number of papers appeared describing proteomic analysis of CSF or brain tissue of MSc patients. 2D-E and 2D-DIGE has been widely used in many of the studies in biomarker discovery for MSc. In order to elucidate proteome signatures, Amin et al. [110] used 2D-DIGE MS/MS to compare the protein expression profile in serum of different

clinical subtypes of MSc patients and healthy controls. They found 46 differentially regulated proteins, three highly expressed proteins (transthyretin, zinc alpha-2-glycoprotein and immunoglobulin kappa light chain) were selected for further evaluation by independent immunoassays. Ottervald et al. [111] reported the analysis of CSF samples of patients with different subtype of MSc using two-dimensional gel electrophoresis and LC-MALDI-MS/MS approach. They selected ten proteins candidates for further confirmation by microbead-based multiplex immunoassay. One of the interesting validated candidates found by Ottervald et al. was significantly up-regulated proteins fetuin-A. Previously it was shown that fetuin-A is markedly elevated in demyelinated lesions and in gray matter within MSc brain tissue and CSF fetuin-A can be used as biomarker of disease activity in MSc [112]. In a recently published study of Liu et al. [113] two different relatively accurate quantitative technologies, 2D-DIGE and UPLC/Q-TOF MS, were used to analyze the differential proteomic expression in MSc CSF samples. 2D-DIGE MALDI-TOF MS analysis resulted in 39 differentially expressed proteins, whereas UPLC/Q-TOF MS approach showed 68 differentially expressed proteins between the MSc and the control groups with 23 regulated proteins common in the two methods. The following ELISA measurement of the level of cystatin C, which was one of the proteins identified by the combination of the two technologies, confirmed the results of the two quantitative proteomic approaches. Using the 2-D DIGE technique followed by MALDI-TOF MS identification, Tumani et al. [114] for the first time compared CSF proteome in CIS patients with and without progression to MSc. They identified nice differentially expressed proteins that could discover the risk of CIS patients to convert to clinically definite MSc. The differential expression of one of the promising candidate proteins (Fetuin-A) was confirmed using ELISA analysis. Qin et al. [115] reported a decrease of vitamin D-binding protein in CSF samples of MSc patients compared to controls using 2D-E combined with MALDI-TOF MS and validated the MS findings by means of an ELISA. Surprisingly, that Ottervald et al. [111] found an increased CSF level of vitamin-D-binding protein in MSc versus control subjects.

Schutzer et al. [10] employed a label-free quantitative MS approach to identify proteins in the CSF of first-attack MSc patients compared to established relapsing remitting (RR) MSc and controls. They found that the CSF proteome of these patients is distinguishable from established RR-MSc, particularly by gray matter components (axon, neuron, and synapse), and that gray matter rather than myelin is more proximally involved in the initiation of MSc.

Targeted proteomic technology such, e.g. SRM-MS has been also used for absolute quantification and verification of potential biomarkers in MSc. Recently, Kroksveen et al. [116] performed the largest SRM study of differentially abundant proteins in MSc. Authors identified and verified five significantly regulated proteins (α -1-antichymotrypsin, contactin-1, apolipoprotein D, clusterin, kallikrein-6) that are associated with the conversion from CIS-MSc to clinically definite MSc type.

Füvesi et al. [7] have applied a differential shotgun proteomic approach based on iTRAQ labeling and nanoLC-MALDI TOF/TOF MS to identify potential protein biomarkers in CSF related to rapid progression in MSc. Seven proteins including Ig kappa and gamma-1 chain C region, complement C4-A, fibrinogen beta chain, serum amyloid A protein, neural cell adhesion molecule 1 and beta-2-glycoprotein 1 were found to be more abundant in both fulminant MSc samples but not in the elapsing-remitting MSc sample compared to the control. Stoop et al. [117] reported the results of quantitative CSF protein and peptide profiling of MSc patients using MALDI-FT-ICR MS. Proteins with higher expression in MSc than in non-inflammatory controls include proteins such as chromogranin A, clusterin and complement C3. Salvisberg et al. [118] demonstrated for the first time that modifications of the tear proteome can reflect biological abnormalities associated with MSc. Among the differential proteins, α -1 antichymotrypsin was found to be significantly increased using iTRAQ labeling method with further confirmation by Western blot and ELISA.

Conclusion

Mass spectrometry-based proteomics methodologies hold a great potential for many applications in basic neuroscience research and in clinical neuroproteomics. Due to its inherent high sensitivity, specificity and speed mass spectrometry has matured into a promising and powerful tool for defining proteomes, and, particularly, for the discovery of potential biomarker candidates. This review provides coverage of recently established methodology and MS-based quantitative protocols for clinical neuroproteomics research. The rapid progress that has been made in this field and intensive efforts have been put to identify promising potential biomarker candidates which will be of significant clinical value in the early diagnosis and prognosis of neurodegenerative diseases. Proteomics-based approaches

are also being used in clinical trials and protein-protein interaction neuroproteomics studies. Despite of some limitations, proteomics represents an important choice of research in biomarker development and clinical interventions. At present a large number of promising findings are validated within different laboratories around the world and the outcome has a very high likelihood of rendering in-depth molecular understanding of the physiological and pathological changes found in patients with neurodegeneration. However, in spite of immense advances in this field the search for an “ideal biomarker or biomarker panel” still continues. To maximize the clinical value of found candidate biomarkers from proteomic profiling results the targeted proteins need to be thoroughly validated in combination with clinical data, imaging technologies and quantitative detection assays.

Table 1. Most commonly employed protein extraction and purification techniques

Extraction		Protein purification
<i>Mechanical disruption</i>	<i>Main components of lysis buffers</i>	<i>Enrichment/purification techniques</i>
<ul style="list-style-type: none"> • Blender • Ultrasonic bath • Ultrasonic probe • French press • Glass bead agitator • Motor and pestle 	<p><u>Detergents:</u></p> <p><i>i. Ionic:</i> e.g. SDS, deoxycholate, CTAB</p> <p><i>ii. Bile acid salts:</i> e.g. Deoxycholic acid</p> <p><i>iii. Non-ionic:</i> e.g. Triton X-110, Triton X-114, octyl glucoside, Tween 20</p> <p><i>iv. Zwitterionic:</i> e.g. CHAPS, CHAPSO</p> <p><u>Chaotropes:</u> e.g. urea, thiourea, guanidinium salts</p> <p><u>Organic solvents:</u> e.g. methanol, acetonitrile, chloroform</p> <p><u>Organic acids:</u> e.g. formic acid, TFA</p> <p><u>Salts:</u> e.g. NaCl</p>	<ul style="list-style-type: none"> • Dialysis • Ultrafiltration • Precipitation • Gel filtration • Solid phase extraction

Table 2. An overview of recently published neuroproteomic studies differentiating between AD, PD, ALS, MSc and control samples, including the source of CNS samples, separation technologies, applied mass spectrometry-based techniques, main results and validated differential abundant proteins between diseased cases and controls by independent technology.

Source	Quantitative method	Proteins/peptides identified, quantified/alterd proteins	Validation by independent technique	Year	REF
<i>Alzheimer's disease</i>					
Brain tissue (neocortex)	DML, LC MS/MS	69 differentially regulated proteins.	-	2014	[9]
Brain tissue (neocortex)	DML, LC MS/MS, WB	62 differentially regulated proteins.	Sarcoplasmic/endoplasmic reticulum calcium ATPase2, lysosome-associated membrane glycoprotein 1, 14-3-3 protein gamma and alpha-enolase	2014	[17]
Serum	SRM MS	Significant decrease of serum ApoE levels in AD patients (45) versus control (43).	Apolipoprotein E (microsphere-based fluorescence-activated cell sorting (FACS))	2014	[86]
Brain tissue (cortex)	MALDI-TOF MS	36 differentially regulated synaptic proteins	-	2013	[119]
CSF	Immunoaffinity enrichment, LC-MS/MS	2-fold increase of tau.	Tau (immunoassay)	2014	[120]
CSF	MRM	12 differentially regulated proteins.	A β 1-40, A β 1-42, retinol-binding protein and	2013	[87]

			cystatin C (ELISA)		
CSF	Immunoprecipitation, LC MS/MS	Significant increases in soluble amyloid precursor protein α and β levels.	-	2013	[88]
CSF	SRM MS	Significant decrease in level of $\alpha\beta 42$, $\alpha\beta 40$ and $\alpha\beta 38$.	-	2013	[85]
Brain tissue (cortex)	1-DE, LC MS/MS,	25 differentially regulated proteins.	IRSp53 and internexin (WB)	2013	[121]
Plasma	2-DE, LC MS/MS	20 differentially regulated proteins.	-	2012	[78]
Brain tissue (cortex)	LC MS/MS	197 differentially regulated proteins.	PKC-gamma, NUMBL (WB)	2012	[68]
Brain tissue (substantia nigra)	Nano-UPLC MS/MS	19 differentially regulated proteins.	Heat shock 70 kDa protein 2 (WB)	2012	[84]
Brain tissue (cortex)	LC MS/MS	13 differentially regulated proteins.	Ubiquitin carboxy-terminal hydrolase 1 and syntaxin-binding protein 1 (WB)	2012	[83]
Brain tissue (cortex, hippocampus and cerebellum)	Immunoprecipitation, MALDI-TOF MS	Significant decrease in level of $\alpha\beta 4-42$ and $\alpha\beta 1-40$ and $\alpha\beta 1-42$ isoforms in AD brains.	-	2011	[89]
CSF	2-DE, LC MS/MS	47 novel candidate protein biomarkers.	Neuronal cell adhesion molecule, chitinase 3-like 1, chromogranin A, carnosinase I (ELISA)	2011	[79]
CSF	Immunoprecipitation, LC MS/MS	Elevated neurogranin levels in AD and MCI patients.	Neurogranin (immunoblotting)	2010	[90]
Plasma	2-DE, LC MS/MS	Significant increases in clusterin/apolipoprotein J	Clusterin (WB)	2010	[81]

		level in AD patients versus controls.			
Plasma	2-DE, LC MS/MS	18 differentially regulated proteins	-	2009	[82]
CSF	2-DE, LC MS/MS	Significant changes in levels of contactin-1, contactin-2, carnosine dipeptidase 1, 120 kDa isoform precursor of neural cell adhesion molecule 1, α -dystroglycan, secreted protein acidic and rich in cysteine-like protein 1 precursor, isoform 2 of calyntenin 1, neuronal pentraxin receptor.	Neural cell adhesion molecule 1, α -dystroglycan and neuronal pentraxin receptor (WB)	2009	[80]
<i>Parkinson's disease</i>					
Plasma	2-DE, LC MS/MS	9 different proteins (haptoglobin, transthyretin, apolipoprotein A-1, serum amyloid P component, apolipoprotein E, complement factor H, fibrinogen γ , thrombin, complement C3) were identified as a potential diagnostic pattern.	-	2013	[92]
CSF	2-DE, MALDI-TOF MS	21 differentially regulated proteins.	Superoxide dismutase and tetranectin (WB)	2013	[122]
CSF	iTRAQ, MRM	16 differentially regulated proteins in PD.	Tyrosine-kinase non-receptor-type 13 and netrin G (MRM)	2012	[100]

CSF	2-DE, LC-MS	6 proteins: fibrinogen, transthyretin, apolipoprotein E, clusterin, apolipoprotein A-1, and glutathione-S-transferase-Pi, were found to be different.	-	2012	[93]
Serum	2-DE, LC MS/MS, iTRAQ	26 proteins were differentially expressed. Significant increases of sero-transferrin and clusterin levels and decrease of complement component 4B, apolipoprotein A-I, α -2-antiplasmin and coagulation factor V levels in PD patients versus controls.	Apolipoprotein A-I, sero-transferrin and α -2-antiplasmin (WB)	2012	[96]
Brain tissues (superior parietal gyrus)	Immunoprecipitation, LC MS/MS	Novel isoforms of alpha-synuclein.	N-terminally acetylated full-length α -syn and two N-terminally acetylated truncated forms of α -syn (WB)	2012	[99]
Plasma	2-DE MS, ELISA	2 proteins: IgG kappa L and human serum amyloid P component were found differentially expressed.	Serum amyloid P component (ELISA)	2011	[123]
Serum	MALDI-TOF-MS combined with magnetic bead-based weak cation exchange	5 differentially regulated proteins.	-	2011	[124]
CSF	2D DIGE, MALDI-TOF MS	8 differentially regulated proteins by MS analysis.	Tetranectin and apolipoprotein A (WB)	2011	[97]
CSF	2-DE, MALDI-TOF	Significant increases in	Transthyretin and	2010	[95]

	MS	transthyretin and haptoglobin levels in PD patents	haptoglobin (WB)		
Serum	2-DE, LC MS/MS	15 differentially regulated proteins by MS analysis.	KIAA0325 protein, myosin heavy chain IIx/d and transthyretin (WB)	2010	[94]
CSF	2-DE, LC MS/MS	8 differentially regulated proteins.	Tetranectin and apolipoprotein A-I (WB)	2010	[125]
CSF	LC MS/MS	2 differentially regulated proteins: DJ-1 and alpha-synuclein.	DJ-1 and alpha-synuclein (ELISA)	2010	[101]
CSF	2-DE, LC MS/MS	14 differentially regulated proteins.	-	2009	[98]
CSF	2-DE, MALDI-TOF MS	6 differentially regulated proteins (serum albumin precursor, serum albumin chain-A, mutant globin, proline rich repeat 14, hemoglobin β fragment, and serum transferrin N-terminal lobe).	Hemoglobin β fragment was attenuated and serum transferrin N-terminal lobe (WB)	2009	[126]
<i>Amyotrophic lateral sclerosis</i>					
Muscle	DML, LC MS/MS	11 proteins (medium-chain specific acyl-CoA dehydrogenase, glycogen debranching enzyme, myomesin-1, cytochrome c oxidase subunit 6C, phosphoglycerate mutase 1, four and a half LIM domains protein 1,	-	2014	[127]

		myosin light chain 3, creatine kinase S-type, protein-arginine deiminase type-2, Kelch repeat and BTB domain-containing protein 10, transitional endoplasmic reticulum ATPase) were significantly altered in ALS vs control muscles			
Spinal cord tissue	MALDI-imaging mass spectrometry (IMS)	Significant reduction of two protein species in ALS spinal cord.	Neuronal nuclear antigen and ubiquitin (WB)	2013	[8]
CSF	2-DE, MALDI-TOF MS	Up-regulation of Parkin-like and several iron and zinc binding proteins.	-	2012	[128]
CSF	Chromatographically functionalized paramagnetic beads, MALDI-TOF-MS, MRM	5 significantly regulated proteins.	Secreted phosphoprotein 1, alpha-1-antitrypsin, nerve growth factor inducible precursor protein, plasminogen, cathepsin D, cystatin C (ELISA)	2012	[104]
CSF	2-D-DIGE, MALDI-TOF MS	Significant increases in the level of 6 proteins (heat shock protein1, alpha-1 antitrypsin, fetuin-A precursor, transferrin, transthyretin (TTR), nebulin-related anchoring protein).	Fetuin-A and transthyretin (ELISA)	2010	[105]
CSF	SELDI-TOF-MS	Significant increases in the level of C-reactive protein	C-reactive protein (ELISA)	2010	[106]

		and decrease of level of transthyretin and cystatin C.			
Spinal cord tissue, CSF	LC MS/MS	14 differentially regulated proteins.	Galectin-3 (WB)	2010	[69]
<i>Multiple sclerosis</i>					
Serum	2-D-DIGE, MALDI-TOF MS	46 differentially regulated proteins by MS analysis.	Kappa light chain and zinc alpha-2-glycoprotein (WB)	2014	[110]
Tear	iTRAQ	Among 185 identified tear proteins, alpha-1 antichymotrypsin was the only one to be significantly increased.	Alpha-1 antichymotrypsin (WB, ELISA)	2014	[118]
CSF	iTRAQ	Five proteins significant differentially regulated (alpha-1-antichymotrypsin, contactin-1, apolipoprotein D, clusterin, kallikrein-6) were significantly differentially abundant in several of the group comparisons (CIS-MSc and controls and/or CIS and controls).	Alpha-1-antichymotrypsin, contactin-1, apolipoprotein D, clusterin, kallikrein-6 (SRM)	2013	[116]
Peripheral blood mononuclear cells	2-DE, MALDI-TOF MS	5 differentially regulated proteins	-	2013	[129]
Brain tissue (substantia nigra)	Nano-UPLC MS/MS	17 differentially regulated proteins.	-	2012	[84]
CSF	iTRAQ, nanoLC-MALDI TOF/TOF MS	7 proteins (Ig kappa and gamma-1 chain C region,	-	2012	[7]

		complement C4-A, fibrinogen beta chain, serum amyloid A protein, neural cell adhesion molecule 1 and beta-2-glycoprotein 1) were more abundant in both fulminant MSc samples but not in the elapsing-remitting MSc sample compared to the control.			
CSF	LC MS/MS	32 differentially regulated proteins.	-	2012	[130]
Serum	MALDI-TOF MS	Significant increases in the level of nucleolin protein fragment.	Nucleolin (WB)	2012	[131]
CSF	2-DE, LC-MALDI-MS/MS, Microbead-based multiplex immunoassay	Significant increases in the level of 4 proteins (alpha-1 antichymotrypsin, contactin 1, fetuin A, vitamin D binding protein) and significant decrease in the level of 6 proteins (alpha-1 macroglobulin, fibulin 1, angiotensinogen, neural cell adhesion molecule 1, neuronal cell adhesion molecule precursor, superoxide dismutase 1, alpha-2 macroglobulin).	-	2010	[111]
CSF	2D-DIGE, MALDI-TOF MS, UPLC/Q-TOF MS	23 regulated proteins common in the two approaches. Down-regulation	Cystatin C (ELISA)	2009	[113]

		of cystatin C in MSc was confirmed by ELISA.			
CSF	2-D-DIGE, MALDI-TOF MS	Serin peptidase inhibitor found to be upregulated, 8 proteins (alpha-1-B-glycoprotein, fetuin-A, apolipoprotein A4, haptoglobin, zinc alpha-2-glycoprotein, retinol-binding protein, superoxid dismutase 1, transferrin) were down-regulated.	Fetuin-A (ELISA)	2009	[114]
CSF	MALDI-FT-ICR MS	Significant increases in the level of 3 proteins (chromogranin A, clusterin, and complement C3).	-	2009	[117]
CSF	2-D-DIGE, MALDI-TOF MS	13 proteins showed more than 1.5-fold difference in expression levels between ALS and control groups.	Vitamin D-binding protein (ELISA)	2009	[115]

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Conflict**of****interest**

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