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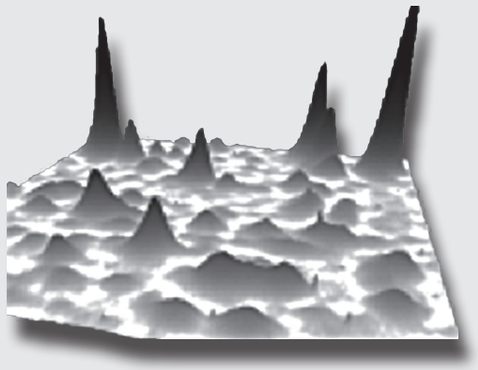

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PROTEOMICS IN NEUROLOGICAL DISEASE

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Stockholm 2009

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To Jenny

ABSTRACT

Neurodegenerative and neuroinflammatory diseases are conditions affecting the central nervous system that in the end have dramatic impacts on the affected individuals and their families. Today, large efforts are made to understand the disease origin and progression. This thesis focuses on Multiple Sclerosis (MS), which is the most common neurological disease among young adults. The diagnosis of MS is based on a series of clinical and neuroimaging criteria, and at present no reliable prognostic tools are available.

We have aimed at developing a solid technical platform for investigation of potential biomarkers in MS. New purification methods to remove abundant proteins from the cerebrospinal fluid and tissue from individuals with MS and controls were developed. The samples were further analyzed using proteomic techniques, two-dimensional gel electrophoresis, mass spectrometry and bioinformatics.

We demonstrated that proteins in MS plaques, adjacent tissue and non-affected brain tissue were differentially expressed. Cerebrospinal fluid samples of individuals affected with MS encompassing its different disease phases and control individuals were analyzed. A reference disease, Post-Polio Syndrome (PPS), was included and regarded as a non-inflammatory condition. Prediction models were constructed and univariate analysis of the protein expression was performed. The results revealed that there was a large heterogeneity in protein profiles between the MS subgroups. In PPS individuals, a protein profile based on three proteins could predict the disease with a high sensitivity and specificity. Interestingly, in contrast to the prevailing assumption, the identification of these proteins indicated that there is an ongoing neuroinflammation and neurodegeneration in PPS.

To further evaluate the MS results we developed a multiplex quantitative immunoassay based on the expression pattern of ten proteins. A new cohort comprising individuals affected with MS and control individuals was assembled, and the expression of the proteins was analyzed. Classification models based on the biomarker panel could identify 70% of the relapsing-remitting MS and 80% of the secondary progressive MS individuals and controls correctly. Thus, protein expression profiles differ between the different forms of MS.

In conclusion, we have developed a proteomic platform that has enabled the discovery of potential biochemical biomarkers of diagnostic and prognostic value in MS and PPS. Further analysis of the protein expression patterns has also added biological information, which may prove useful for the understanding of etiology and disease course.

LIST OF PUBLICATIONS

I. Extraction and proteomic analysis of proteins from normal and multiple sclerosis postmortem brain. Jia Newcombe, Bodil Eriksson, Jan Ottervald, Yang Yang, Bo Franzén. *Journal of Chromatography B,* 2005, **815** 191–202

II. Protein profiling of plaques in Multiple Sclerosis. Jan Ottervald, Bo Franzén, Bodil Eriksson, Jia Newcombe.
Manuscript

III. MULTIPLE SCLEROSIS: Identification and clinical evaluation of novel CSF biomarkers. Jan Ottervald, Bo Franzén, Kerstin Nilsson, Lars I. Andersson, Mohsen Khademi, Bodil Eriksson, Sven Kjellström, György Marko-Varga, Ákos Végvári, Robert A. Harris, Thomas Laurell, Tasso Miliotis, Darius Matusevicius, Hugh Salter, Mats Ferm, Tomas Olsson.
Under revision, *Journal of Proteomics*

IV. Identification of novel candidate protein biomarkers for the post-polio syndrome — Implications for diagnosis, neurodegeneration and neuroinflammation. Henrik Gonzales*, Jan Ottervald*, Kerstin C. Nilsson, Niclas Sjögren, Tasso Miliotis, Helena Von Bahr, Mohsen Khademi, Bodil Eriksson, Sven Kjellström, Ákos Végvári, Robert Harris, György Marko-Varga, Kristian Borg, Johan Nilsson, Thomas Laurell, Tomas Olsson*, Bo Franzén*. *Journal of Proteomics*, 2009, **71**, 670–681
(* These authors contributed equally.)

CONTENTS

LIST OF ABBREVIATIONS

2DE	Two dimensional gel electrophoresis
A1AC	Alpha-1 anti-chymotrypsin
A2MG	Alpha-1 macroglobulin
APP	Amyloid precursor protein
BBB	Blood-brain barrier
CHCA	Alpha-cyano-4-hydroxycynnamic acid
CIS	Clinical isolated syndrome
CK-B	Creatine kinase, B-type
CNS	Central nervous system
CSF	Cerebrospinal fluid
DIGE	Fluorescence Difference Gel Electrophoresis
DRP-2	Dihydropirimidinase-related protein 2
EAE	Experimental Autoimmune Encephalomyelitis
EDSS	Expanded Disability Status Scale
ESI	Electrospray ionization
ELISA	Enzyme-linked immunosorbent assays
GoA	Guanine nucleotide-binding protein G – alpha unit
GUAD	Guanine deaminase
GFAP	Glial fibrillary acidic protein
HC	Healthy Control
ICAM-1	Intercellular adhesion molecule 1
IFNγ	Interferon gamma
Ig G	Imunoglobulin G
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-16	Interleukin 16
IPGs	Immobilized pH gradient strip
LC-MALDI TOF	Liquid chromatography MALDI TOF
MALDI TOF	Matrix-assisted laser desorption/ionization time-of-flight
MBP	Myelin basic protein
MOG	Myelin oligodendrocyte glycoprotein
m/z	Mass to charge
NSE	Neuron specific enolase
OND	Other Neurological disease
PCA	Principal component analysis
PLP	Proteolipid protein
PLS	Partial Least Square
PMF	Peptide mass fingerprint
PPM	Parts per million
PPMS	Primary Progressive Multiple Sclerosis
PPS	Post-Polio Syndrome
PTMs	Post-translational modifications

QTOF	Quadruploe Time Of Flight
RRMS	Relapsing Remitting Multiple Sclerosis
SPMS	Secondary Progressive Multiple Sclerosis
TNFα	Tumor necrosis factor alpha
VCAM-1	Vascular cell adhesion molecule 1
ZO-2	Zonula occludens protein 2

1 GENERAL BACKGROUND

1.1 THE CENTRAL NERVOUS SYSTEM

The main task of the central nervous (CNS) is to register and coordinate the body's movements and functions, and to enable cognition, memory and learning. The tissue contains neurons, glial cells, astrocytes, oligodendrocytes, microglia and blood vessels. The functional unit is the neuron, which is composed of the cell body from which dendrites branch, and the axon, which is surrounded by myelin sheaths that are provided by oligodendrocytes to support and protect the neuron. The myelin sheaths also facilitate the transmission of nerve signals along the neuron. The signals are finally conducted to other neurons via synapses through the release of neurotransmitting peptides and/or proteins (Fig. 1).

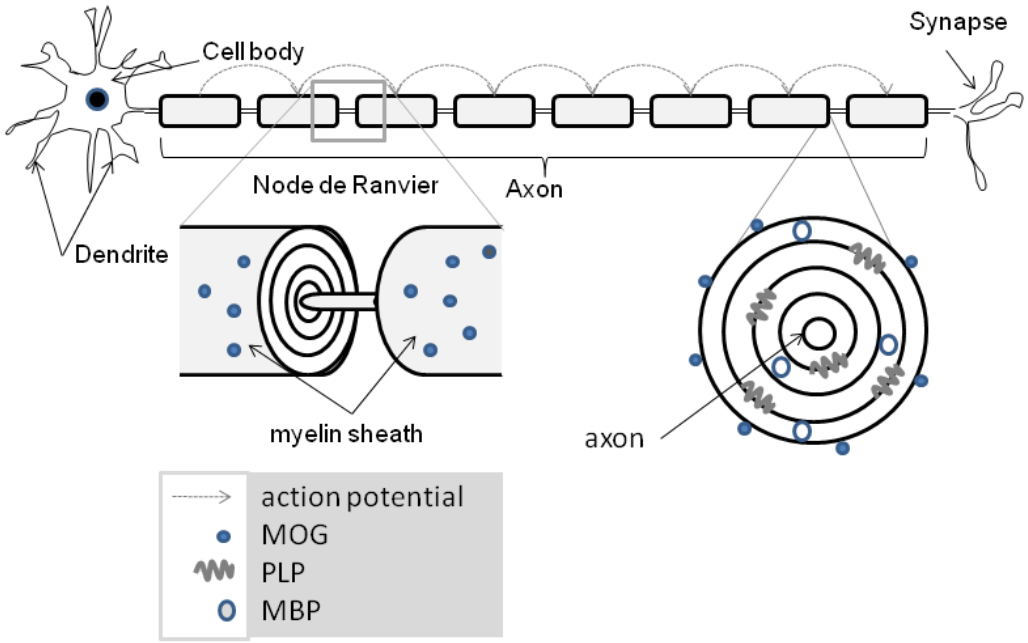

Fig.1 Schematic image of the components and cellular structure of a neuron.
Proteins of interest with regard to autoimmune reactions are illustrated. Myelin oligodendrocyte glycoprotein (MOG) is located on the plasma membrane surface, proteolipid protein (PLP) in the membrane and myelin basic protein (MBP) in the cytoplasm of the myelin sheaths.

The blood brain barrier (BBB) protects and shields the CNS by regulating the content of the cerebrospinal fluid (CSF) that surrounds the brain and spinal cord (Fig.2). Due to the dynamic environment in which the brain operates, it is of great importance that the BBB is properly preserved and ensures that the right components are delivered to the brain to maintain homeostasis. The major part of the CSF is produced by ependymal cells in the choroid plexus, and in total ~500 ml CSF is produced during 24h [1]. However, the maximum rate of production, ~40ml/h at midnight, decreases to ~5ml/h at noon [2], implying intra-individual differences in protein or peptide concentrations during the day. Other cells in the CSN are astrocytes, which fulfil a nourishing and supportive function and microglia that are involved in immunological processes in the brain.

1.2 DISEASES AFFECTING THE CENTRAL NERVOUS SYSTEM

Great efforts are taken to develop new technologies in order to gain a better understanding of the biology underlying the neuroinflammatory and neurodegenerative diseases. Diseases within this category encompass Alzheimer's disease (AD), Parkinson Disease (PD), Huntingtons disease (HD) and Multiple Sclerosis (MS) [3-6].

Inflammation is pivotal in MS, but also considered in primary neurodegenerative diseases such as AD and PD [7-9]. In PD, increased serum levels of interleukin 2 (IL-2) and tumor necrosis factor alpha (TNFα) have been observed, indicating ongoing inflammation [10, 11]. Moreover, increased levels of interleukin 6 (IL-6) and TNFα have been observed in brain tissue and CSF from PD patients [12]. In AD and PD, increased levels of interleukin 1 (IL-1) and IL-6 have been determined in CSF [13]. The connection between degeneration and inflammation has been investigated in several studies. In AD, immune-reactive complex and activated microglia have been observed [14], and in MS evidence of inflammation has been observed by macrophage, B- and T-cell infiltrates in the acute and relapsing forms, but also in secondary progressive and primary progressive forms [15]. In many neurological disorders the homeostasis is changed, resulting in a persistent altered balance between the different cell types present within the CNS [7, 8, 16].

1.3 MULTIPLE SCLEROSIS

The disease is the most common neurological disease among young adults, with a mean age of onset at 30 years of age [17]. The estimated number of individuals affected with MS is >1,000,000 worldwide [18]. The disease prevalence is 5-7/100000 individuals, and of these two-thirds are females [19]. Different disease mechanisms have been proposed throughout the years [20-23]. Today MS is regarded as a progressive, autoimmune disease leading to demyelination and axonal damage [24]. This process includes recruitment of leucocytes via increased production of cytokines by activated cells [25]. Previous studies suggested that TNFα could reflect the relapses and IL-6 the immune activity in MS individuals [26]. There is infiltration of T-cells into the CNS, and subsequently infiltrating macrophages attack the myelin sheaths surrounding the axons. This phenomenon can be visualized using magnet resonance imaging (MRI) [27], through which characteristic focal plaques in the white matter of the CNS can be observed. In addition, differential expression of TNFα and interferon gamma (IFNγ) can be detected also when the disease is clinically silent in between relapses [28], (Fig. 2).

To gain deeper biological understanding of the disease, animal models of MS in the form of Experimental Autoimmune Encephalomyelitis (EAE) and cell models have been employed [29-31]. One important characteristic in MS is that the BBB)ruptures, allowing proteins to diffuse and lymphocytes to migrate into the CSF. These cells together with those already present in the CNS can be activated by the newly encountered proteins [27]. In a study in EAE mice it was shown that $CD4^+$ Th17 cells induced production of the chemokines CXCL1 and CXCL2. By blocking the CXCL receptor, inhibition of the breakdown of BBB was observed, and leukocyte recruitment into CNS was inhibited [32]. Immunological attack by activated T-cells of specific proteins on the myelin sheaths has been observed, and myelin basic protein (MBP) was first regarded as an autoantigen [33-35]. Moreover, myelin oligodendrocyte glycoprotein (MOG) [36, 37] and proteolipid protein (PLP) may also represent autoantigens in MS [38, 39] (Fig.1). Interestingly, when peripheral blood mononuclear cells from MS individuals and healthy individuals were stimulated with MOG peptides an increased number of reacting cells secreting IFNγ, was observed in MS but not in healthy controls (HC) [40].

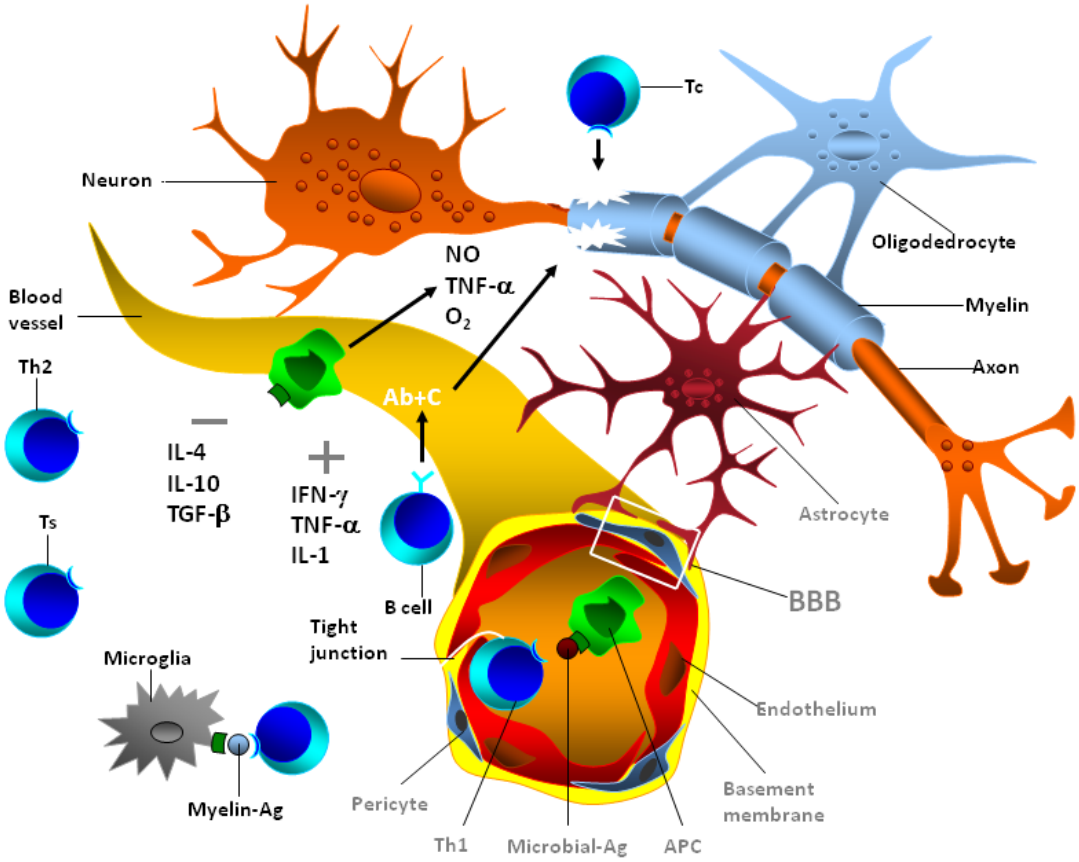

Fig. 2 Some of the cells and proteins that may be involved in the immunological process during Multiple Sclerosis (kindly provided by Dr. M. Khademi)

MBP has been investigated from many different angles, and an early finding was that it is expressed in different isoforms, including phosphorylation and partial deamination [41], in MS. Other studies have shown that different levels of citrullination (conversion of a positively charged arginine residues to an uncharged citrulline) occur, and that citrullinated MBP-specific T-cells can give rise to an enhanced recall response in MS individuals [42, 43]. Post-translational modifications (PTMs) seemed to be of great importance in MS in a study investigating the levels of phosphorylated arginine 107 in MBP. The level of phosphorylation observed in normal samples was reduced or absent in individuals affected by MS [44]. Another PTM, oxidation of MOG induced by malondialdehyde, has been reported to increase its immunoreactivity, with EAE mice developing a more severe disease [45] (Fig. 1).

Current diagnosis relies on clinical features with evidence of lesions differing in time and space as described in [46]. Today the criteria include options to use MRI to visualise lesions [47], and CSF biochemical measurements that include assessment of the oligoclonal band(s) of IgG.

The IgG-index = (CSF/S IgG ratio)/(CSF/S albumin ratio) and the barrier index = CSF/serum albumin quotients (Quotent Alb = CSF albumin/serum albumin). Importantly, the disease bouts must be separated in time and space for the definite diagnosis of clinically defined MS [46]. Pathological findings have suggested the division of the disease into four different subgroups based on differences in cell composition and in the degree of myelination in the plaques [48]. Due to the complexity of the disease, a number of different proteins or substances have been proposed as biomarkers that reflect the biological activity related to the disease (Table 1).

Biomarker category	**Potential biomarker**
Immune system activation	IL-1,IL-2, IL-6, TNFα CCR5, CXCR3 Anti-MBP, Anti-MOG Complement factor 3 Complement factor 4 Oligoclonal bands
Demyelination	MBP
Oxidative stress	Nitric oxide
Neuronal/axonal damage	Neurofilament Tau NSE
Remyelination	Glial fibrillary acidic protein (GFAP) Neural cell adhesion molecule
Gliosis	S-100 GFAP

Table 1. A summary of potential biomarkers during MS, reflecting different biological activities.

The disease course usually starts with a first attack termed the clinical isolated syndrome (CIS) [49]. With a relapse the patient is diagnosed as relapsing-remitting MS (RRMS). The disease can then continue with further relapses or be stable for several years. However, 60-80% of the individuals with time develop secondary progressive MS (SPMS). SPMS is characterized by a gradually increased disability of the individual [50]. Primary progressive MS (PPMS) is a less frequent type that constitutes ~10% of all cases. This variant displays a gradual worsening from onset.

There are treatments that reduce the relapse rate in RRMS individuals. Interferon beta 1-α and interferon beta-1β were initially used due to their anti-viral effects, but they also have immunomodulatory effects [51]. The annual rates of reduction of relapses were ~30-35% following treatment with interferon beta-1α [52, 53]. Glatiramer acetate is a mixture of synthetic polypeptides composed of four amino acids, and the mechanism of action is believed to be either an inhibition of the binding between presented MBP peptides and the T-cell receptor of pathogenic T cells [54] or a T2 cytokine bias. In a clinical study, the rate of relapses was reduced by 25% following treatment with glatiramer acetate [55].

Another drug with highly selective action on a specific target is natalizumab, which has beneficial effects in RRMS individuals. Natalizumab is an antibody that specifically binds to α4β1-integrin and inhibits the binding of T-cells to vascular cell adhesion molecule 1 (VCAM-1) at the endothelium, thereby preventing the entrance of T-cells into the CNS. The rate of relapses following treatment with natalizumab was decreased by 68%, and 92% fewer lesions were detected by MRI [56]. In another study, reduced cell counts in CSF and increased levels of TNF and IFN-γ mRNA in mononuclear cells from peripheral blood were observed. Furthermore, natalizumab treatment improved the quality of life in individuals with severe disease [57].

1.4 POST-POLIO SYNDROME

Survivors of poliomyelitis may develop increased or new symptoms decades after the acute infection, a condition known as *post-polio syndrome* (PPS). The condition affects 20-60% of previous polio patients. The underlying pathogenesis is not fully understood, and accurate diagnosis is not feasible. Approximately twenty million persons worldwide are affected to some degree. Thus, PPS is one of the most prevalent motor neuron diseases [58]. Survivors of acute paralytic disease later in life develop symptoms such as increased weakness of the musculoskeletal system, fatigue, pain, gait disturbances, breathing difficulties as well as swallowing problems [59]. Consequently, it may be difficult to differentiate symptoms of an active PPS disease from those of normal aging. The current diagnosis is based on thorough clinical examinations in order to eliminate other possible causes, and thus identification of diagnostic biomarkers is highly desirable. Treatment options for PPS are limited. However, patients treated with high-dose intravenous immunoglobulins experienced improvement in a clinical study [60].

1.5 BIOMARKERS

The use of biomarkers has been helpful over time within medicine, for example high blood pressure correlates to the development of cardiovascular diseases [61]. To define biomarkers Floyd and colleges have proposed the following:

A **Biomarker** or biological marker is *"a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic response to a therapeutic intervention* [62]".

A **Clinical end-point** is *"a characteristic or variable that reflects how a patient feels, functions or survives* [62]".

A **Surrogate end-point** is *"a biomarker intended to substitute for a clinical end-point. A surrogate end-point is expected to predict clinical benefit (or lack of benefit or harm) based on epidemiologic, therapeutic, pathophysiologic or other scientific evidence* [62]" (Fig. 3).

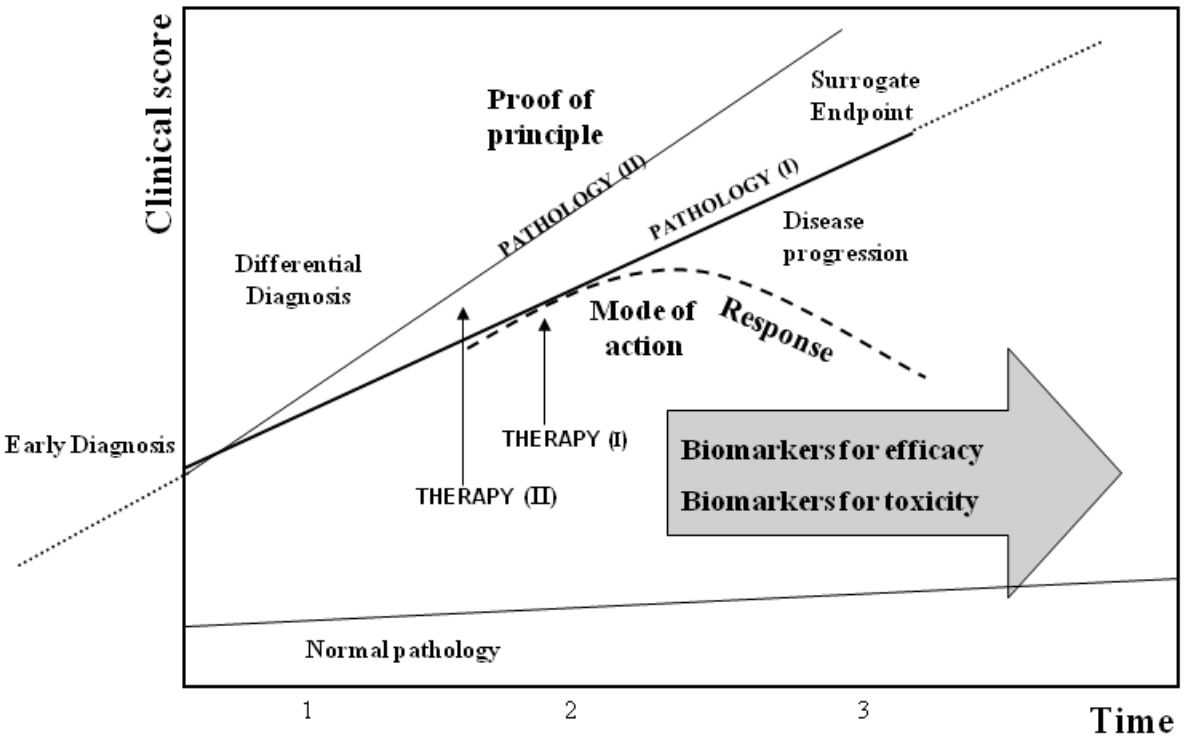

Fig. 3. Strategic planning and implement of the study and the framework of potential biomarkers can reveal tremendous amounts of biological information.

Thus, if used correctly, biomarkers can potentially enhance the development of pharmaceutical innovations both by reducing time and lowering the expenses in clinical trials. A more comprehensive discussion about biomarker end their use is found in [63].

1.6 BIOCHEMICAL BIOMARKERS IN MULTIPLE SCLEROSIS

In MS many different hypothesis-driven biomarkers have been proposed to represent different stages of the disease course, including inflammation, axonal damage and repair mechanisms, reviewed by Bielekova and Martin [63]. Large efforts have been made to understand the individual events occurring in brain tissues during a clinical manifestation [64].

During inflammation, pro-inflammatory cytokines and adhesion molecules are differentially expressed in lesions in MS individuals compared to non-inflammatory and normal CNS tissues, TNFα and interleukin 4 (IL-4) exhibiting higher expression [65]. Examining intercellular adhesion molecules that are necessary for the infiltration and activation of invading leucocytes into the CNS, it was shown that soluble intercellular adhesion molecule 1(ICAM-1) and TNFα receptor were expressed at higher levels in serum and CSF of MS individuals compared to controls [66]. During the inflammatory events it has been shown that production of the pro-inflammatory interleukin 16 (IL-16) is decreased in active lesions [67].

To detect an active demyelination process in MS individuals, MBP levels were used as a measurement for active demyelination [68]. However, the clinical disability and CSF levels of MBP obtained did not correlate [69]. With respect to cytokine profiles, interleukin-1 (IL-1) has been detected together with TNF-α in active disease [70], but compared to inflammatory neurological diseases there were no differences in expression levels [71].

Previous studies have indicated that some proteins can be useful in detecting neurodegeneration in CIS individuals. Measurement of the concentrations of tau, phospho-tau, S100B, amyloid beta and neuron specific enolase (NSE) in CSF and serum are informative in this respect. Interestingly, the NSE concentration in CSF and serum was decreased in CIS individuals. With regard to the other biomarkers, no changes were obtained in the concentrations between groups. Moreover, no correlation between Expanded Disability Status Scale (EDSS) and the concentration of investigated proteins was observed [72].

Neurofilaments have been detected in CSF in MS individual and are suggested as a marker for axonal damage disease activity [73]. Nitric oxide has also been proposed as a marker for disease activity in MS with elevated levels during relapse [74] (Fig. 2).

Unbiased biomarker discovery has been performed in individuals with MS to some extent using proteomics techniques [75-79]. However, the sample sizes when using CSF or post-mortem samples can be a limitation, and if pooled material is used this can be a disadvantage [80]. Moreover, it is of importance to choose individuals with no prior medical treatment. Due to the disease complexity, a number of different proteins or substances have been proposed as biomarkers to reflect the biologically active stage of MS ongoing in the individual (Table 1).

2 CURRENT INVESTIGATION

2.1 AIM

- One of the aims of this thesis was to develop and establish a new platform comprising affinity and purification methods with the final goal of achieving higher sensitivity and to increase the specificity using proteomics technologies.
- A second goal was to investigate samples from individuals affected by MS at a protein level and to identify potential biomarkers that could be used for diagnostic and/or prognostic purposes, or that could be used to monitor treatment effects.
- A third goal was to identify differentially expressed proteins in order to increase the understanding of the underlying disease mechanisms.

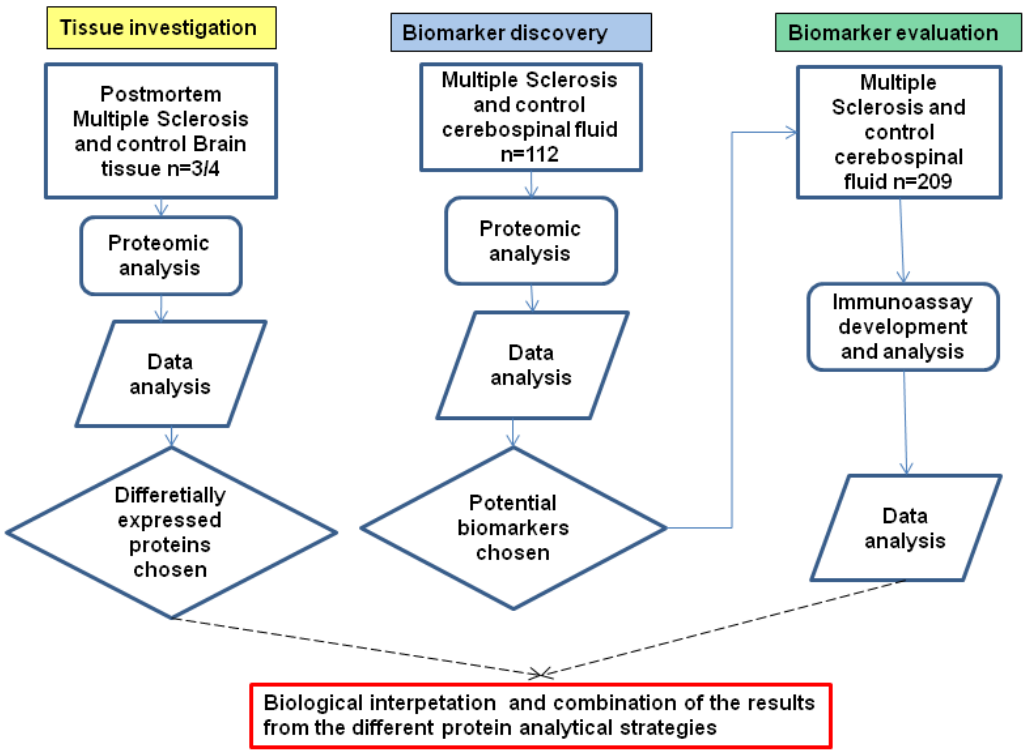

Fig. 4 Study outline with decision points for this work.

2.2 COMMENTS ON MATERIALS AND METHODOLOGIES

2.2.1 Patient data collection

All study enrolments followed the recommendations of the declaration of Helsinki and the studies were approved by the Ethics committee of the Karolinska Institute. Oral and written information was given to the patients and confirmed consent in writing was received before inclusion.

Study I and study II : Normal control and MS post-mortem brain tissue samples were obtained from the NeuroResource tissue bank, London, UK. The study was approved by the local ethics committee. Brain samples were screened with oil red-O and haematoxylin for histological evaluation before dissection. Each MS tissue block was dissected into separate samples of plaque and the macroscopically normal-appearing white matter immediately adjacent to each lesion. Normal control or MS tissues were finely chopped to facilitate solubilization for fractionation, and then separated into either duplicate or triplicate homogenous 30–50 mg samples depending on amount of tissue available, prior to rapid freezing in tubes for homogenization.

Study III: Samples were collected at Karolinska University Hospital Stockholm, Sweden, during investigation of individuals with possible MS using diagnostic criteria as described in [46]. As controls we selected samples from persons with other neurological diseases without having oligoclonal IgG bands in their CSF. These individuals had a variety of other neurological signs and symptoms including trigeminal neuralgia, unspecified sensory symptoms, visual disturbance, neurasthenia and headache. Routine blood tests, CSF analysis and brain MRI did not reveal any sign of inflammatory disease in this cohort of controls. The patients in the treatment study received a monoclonal antibody, natalizumab (anti-VLA-4; Tysabri, Biogen Idec, Cambrige, MA, USA) at a dose of 300 mg administrated by i.v infusion as routine treatment every fourth week.

Study IV: Consecutive PPS individuals with a history of acute poliomyelitis and new problems such as increased muscle weakness, muscle fatigue and pain in muscle groups who were earlier affected by poliomyelitis using diagnostic criteria as described in [81] were included. Control patient CSF samples were derived from either non-diseased volunteers, HC or persons with neurological signs or symptoms for other reasons than PPS, other neurological disease (OND). These patients were regarded as being non-inflammatory and the lumbar puncture had been performed as a part of routine clinical analysis of these patients. All samples were assessed for erythrocyte contamination before analysis. The CSF of these patients displayed no signs of inflammation in the form of pleocytosis, increased IgG index or oligoclonal bands.

A comparison with samples from persons with SPMS was added. SPMS samples were age-matched to the PPS group and can be considered to be an inflammatory disease reference material. CSF from an independent population of persons with PPS and OND were used to validate Kallikrein 6 expression levels. CSF was obtained from 37 individuals with PPS and 30 individuals diagnosed as having other neurological diseases. Since tissue samples from MS lesions are unfeasible to obtain, the CSF represents the closest available biological sample reflecting the underlying pathology of diseases influencing the brain.

In studies I and II, which are based on post-mortem tissue from MS-affected individuals and controls, the samples were difficult to obtain due to the complexity of accessing high quality post-mortem brain tissue. However, since it was possible to produce biological replicates we consider the study design as being solid. For studies III and IV, we developed an affinity purification system to remove the most abundant proteins in CSF i.e. albumin and immunoglobulin G (IgG). In studies III & IV the individuals had not yet received treatment at the time of sampling in order to obtain samples with relevant protein changes. However, in the evaluation part (pilot study, paper III) individuals receiving routine treatment were included in order to detect potential treatment effects in RRMS individuals. Studies III & IV were performed in a randomized style and all samples were processed in parallel (for an overview of the studies, see Fig. 4).

2.2.2 Proteomics

Proteomics can be defend as *'The identification, characterization and quantification of all proteins involved in a particular pathway, organelle, cell, tissue, organ or organism that can be studied in concert to provide accurate and comprehensive data about that system'*[80]. Classical proteomics is usually described as two-dimensional gel electrophoresis (2DE) followed by protein expression profiling [82, 83] and protein identification with mass spectrometry [84].

Today, modern protein analysis and characterization is increasing and developing with high speed. Applying pathway analysis of complex samples, regulation of many different biological systems can be revealed. By using the properties that proteins encompass, size and charge, it is also possible to separate complex protein and peptide mixtures and to identify the components using mass spectrometry.

One major advantage of 2DE is that quantitative information can be obtained for a large number of proteins in parallel, including many different post-translational modifications (PTM's). If detailed

characterizations of differentially expressed proteins are successful, we may identify new pathways and create new hypothesis regarding the disease or cellular system undergoing study. The steps include image and data analysis with estimation of the protein levels obtained from the gel followed by identification and characterization assisted by mass spectrometry. The approach using 2DE yields substantial information about the sample. The limitation is that it only provides information about proteins in the chosen window depending on the choice of immobilized pH gradient strip (IPGs) and gel composition, in our studies pI 4-7 and 15-200 kDa (Fig. 5). That decision will then eliminate potential candidates such as cytokines and chemokines due to the fact that they are small in size <15kDa and present in low concentrations. By using newly developed techniques, it is now possible to compare two different samples/individuals in the same gel, and to add a third reference sample to make normalization and thereby decrease the gel variation from ~20% to below ~5% [85].

Today, the technical direction moves towards gel-free quantitative mass spectrometry-based applications [86-88]. However, as reviewed by Kubota *et al,* a combination of gels and mass spectrometry can be advantageous when dealing with large data points and post-translational modifications [89]. Different methodologies from 2DE to advanced mass spectrometry-based techniques have been applied on many diseases. In the neurodegenerative field different techniques have contributed to the understanding of the biology behind diseases, excellently reviewed by Claudle and colleagues [90].

Unbiased proteomics approaches have previously identified potential candidate biomarkers in different neurological disorders [76, 77, 91-93]. It should be noted that the work is both time- and labour-intensive, and the outcome often produces large lists of potential biomarkers for which further follow-up demands large efforts. However, the potential markers can also add new biological information by pure serendipity that may provide new insights into disease mechanisms. Indeed, initial unbiased proteomics studies have identified the protein 14-3-3 as a disease marker which following validation has been used as a diagnostic tool in Creutzfeldt-Jakob disease [94].

2.2.3 Two-dimensional gel electrophoresis

It is possible to use the properties in size and isoelectric point (pI) for separation of proteins using 2DE. The technique has developed from O´Farrel, and further refinement has been made by introduction of IPGs [82, 83, 95-97] (Fig. 5).

Usually the technology can reveal up to 2000 different protein variants through separation in two dimensions, the first based on isoelectric properties and the second on size. Analysis and detection of proteins in a complex mixture is enabled with good separation and resolution (Fig. 4). Different protein staining techniques have been employed during the years, from Coomasie Blue to silver staining, where the cost and sensitivity is low for the former, but conversely higher in both respects for the latter (down to 0.1 ng/protein spot) [98, 99].

However, development of new staining techniques (Sypro Ruby, ProQ-dimond) has increased the sensitivity and dynamic range of the protein analysis and facilitates subsequent identification and characterization by mass spectrometry [98, 100, 101]. By the development of fluorescence difference gel electrophoresis (DIGE), the introduction of multiple fluorescent dyes for labeling of protein samples prior to the 2DE separation have increased the sensitivity (saturation labeling) and reproducibility (inclusion of an internal standard) [85].

In order to analyze the protein expression on the gels different analytical softwares have been developed [102, 103], and different variance problems have been discovered [104]. However, these questions have been addressed and resolved by development of new imaging software systems [105-107].

In summary, from a technological point of view, if we would have the possibility to conduct these studies today, the choice of method had most likely been 2DE-based techniques again, using DIGE instead of Sypro Ruby. In this way the variation might be decreased from ~20% to ~5% and the total amount of protein loaded onto the gels would be ~40 μg/gel. This would give the possibility to run IPG with different intervals, 3-6, 4-7 and 6-10, which would yield approximately 4,500 protein spots to analyze.

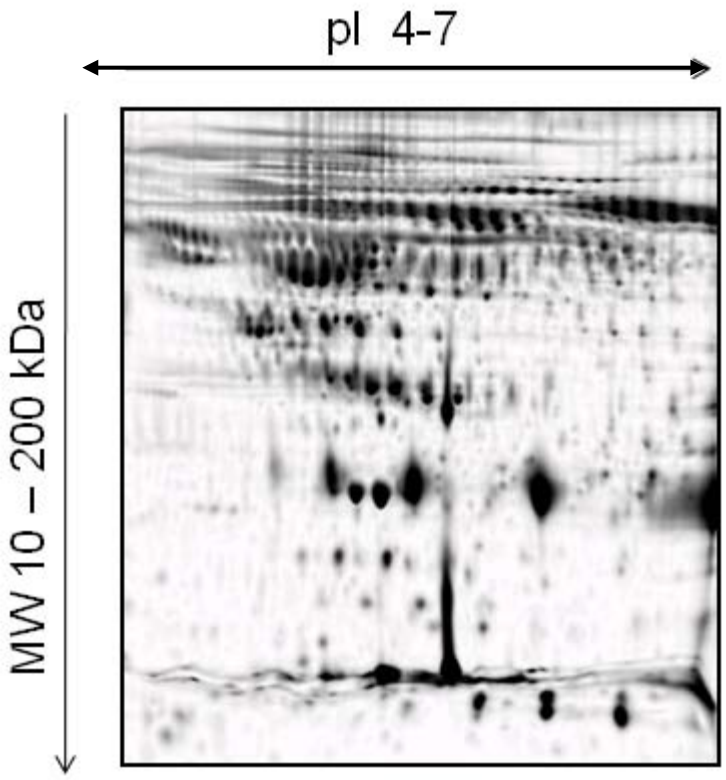

Fig. 5 A representative 2DE gel from study III.

2.2.4 Mass spectrometry

The technology has the analytical key to the understanding of the biological information of the samples by reveling the sequence and/or amino acid information and, thus to be able to identify the proteins of interest. The technique provides accurate mass measurements due to the mass-to-charge *(m/z)* ratio of proteins, peptides and peptide fragments. The peptide fragments can give further information of amino acid sequence and possible modifications such as phosphorylations and oxidations or other PTMs. Mass spectrometers are usually built comprising three components, ion source, mass analyzer and detector (Fig. 6).

Using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) the sample is mixed with a matrix compound in excess and thereafter subjected to a sample plate where the matrix and sample co-crystallize. The principle underlying MALDI-TOF is that the co-crystallized sample/matrix is irradiated in a vacuum chamber with laser pulses, and it is thereafter subjected to ionization and desorption

processes. The ions are accumulated in the extraction region, and after a time delay the ions are accelerated by an electric field towards a gird at ground potential. Thereafter the ions are allowed to

drift into the flight tube until they hit the detector. The velocity of the ions is based on their mass, a higher mass having a slower velocity and a lower mass having a higher velocity. In general, the obtained spectrum contains between 500 to 3000 laser shots depending on the concentration of the peptides in the sample. When analyzing peptides the machine is operated in reflectron mode to increase the resolution, and to analyze proteins the linear mode is preferable (Fig. 6). A limitation with MALDI TOF and peptide mass fingerprinting (PMF) is when analyzing complex samples, or when the digestion produces to low amount of peptides. Therefore it is best to use in combination with 2DE, whereby the proteins are usually separated.

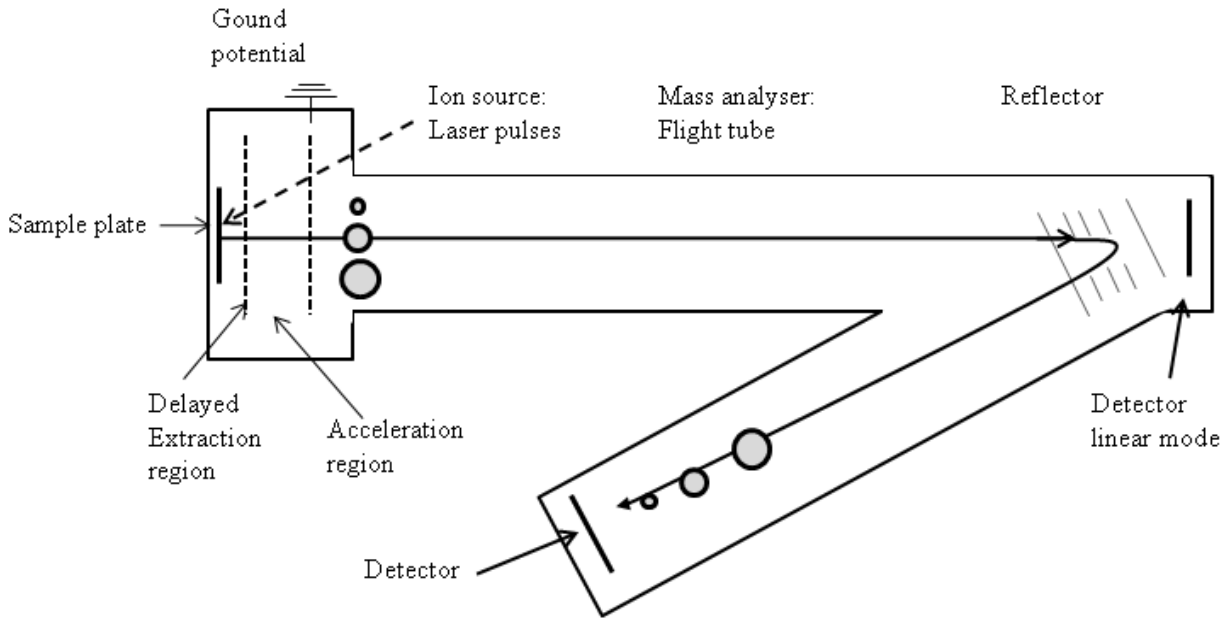

Fig. 6. Schematic illustration of a MALDI-TOF mass spectrometer with the possibility to operate in linear or reflectron mode. Ions that are separated in the flight tube will arrive to the detector at different times.

The development of mass spectrometry has generated new models with higher resolution and sensitivity, allowing fragmentation of MALDI-generated precursor ions, thereby providing information about the amino acid sequence. Two instruments have been developed to produce fragmentation of peptides, quadrupole time-of-flight (QTOF) which consist of a MALDI ion source connected to an analytical quadrupole [108] and MALDI TOF/TOF that has two time-of-flight analyses [109]. In the collision cell the precursor ion dissociates, mainly due to the rupture of the peptide bonds, into product ions that are measured by their *m/z*. The obtained spectrum is unique for the individual

peptides analyzed. Depending on the size of the analyte (peptide or protein), specific matrices are chosen: to analyze smaller peptides alpha-cyano-4-hydroxycynnamic acid (CHCA) is usually preferred, and for intact proteins sinapinic acid is used [84, 110].

Another ionization technique used is electrospray ionization (ESI) that generates charged molecules at atmospheric pressure [111]. The sample solution is sprayed from a thin capillary, producing a fine spray, and a strong electric field is applied. The ionization process occurs during evaporation of the solvent induced by gas or heat. ESI produces a continuous beam of ions, in contrast to MALDI TOF.

Development of ESI has yielded low-flow electrospray, nano ESI, in which an extremely thin needle is used to produce the beam [112]. This produces smaller droplets, and the advantage is that very small sample amount is required. By using external pumps, combinations of different separation columns can be used, and thereby the resolution is increased. One method to separate the proteins and peptides is reversed phase liquid chromatography (RP-LC) which takes advantage of hydrophobic interactions and allows purification by removing salt and other water soluble impurities. The sample is loaded onto a column packed with solid phase adsorbents carrying hydrophobic groups (C4, C8, C18) that bind the peptides, followed by washing and elution with an organic solvent, e.g. acetonitrile.

In order to achieve a high quality peptide prior to PMF or fragmentation analysis, the protein needs to be enzymatically digested and purified. The standard procedure is to digest the protein with trypsin, cleaving proteins C-terminally of the positively charged amino acids arginine (R) and lysine (K) and thereby providing a large number of peptides available for analysis. There are alternative endoproteases available for protein cleavage; Lys-C (cleaves after K) and Arg-G (cleaves after R) and chymotrypsin that cleaves after phenylalanine, tyrosine, tryptophan, leucine and methionine, which can be utilized when necessary.

The detected *m/z* peptides and amino acid sequence can be further searched in databases e.g. SwissProt and TREMBL, and be matched to theoretically expected enzyme digests for each protein sequence that exist in the database [113, 114].

In studies I & II, proteins were digested with trypsin and the samples were further purified using C-18 Zip-Tip before application of MALDI TOF and PMF for identification of interesting proteins.

In study III & IV proteins were digested with trypsin, and the samples were further purified using a Micro-tech Workstation as described in [115] and/or Liquid chromatography-matrix-assisted laser desorption/ionization mass spectrometry (LC-MALDI TOF) reversed-phase chromatography. Protein identification was undertaken using database searches of MS/MS data obtained from a MALDI-TOF/TOF instrument. In all studies we used Mascot (Matrix Sciences, UK) as the search engine.

2.2.5 Multiplex immunoassay

The multiplex immunoassay was developed and the samples were analyzed at Rules Based Medicine (Austin, Texas, USA http://www.rulesbasedmedicine.com/). The assays were based on two different enzyme-linked immunosorbent assays (ELISA) in sandwich format. The principle behind sandwich ELISA is that a microtiter plate is coated with antibodies, and thereafter the sample is added into the wells and incubated for a time interval. Detection is performed with a secondary antibody, tracer, with a coupled enzyme or fluorofor. In study III we used a biotinylated detection antibody, and detection was facilitated by adding streptavidin-phycoerythrin.

2.2.6 Data analysis

Studies I & II: All statistical tools used in study I and II were provided by the PDQuest™ software. The first statistical analysis was a Student's t-test (p=0.05 significance level) between replicate groups consisting of multiple closely related gels from the same sample. In one analysis, plaque samples were in one replicate group and white matter samples adjacent to these lesions were in another replicate group, both originating from the same tissue block. The Student's t-test revealed the number of spots differing significantly between the replicate groups. These differences were compared again to determine how many of the significantly differing spots were present in two pairs of replicate groups using a Boolean test to combine information from different groups of data. Data was exported to other systems for principal component analysis (PCA) using the SIMCAP 8.1 program (Umetri AB, Sweden) in combination with the Spotfire 6.2 program (Spotfire AB, Sweden). Spotfire was only used for visualization of data.

Study III: In order to identify a minimal number of variables with maximal ability to discriminate between MS and controls, Partial Least Squares (PLS) [116] modelling was applied to the data. Two-group classification (prediction) models were built for CIS, RR remitting, RR relapsing and SPMS samples versus the control group, as well as a combined MS model (RR remitting, RR

relapsing and SPMS) versus the control group. Furthermore, two additional patient groups were formed based on clinical parameters, one low neurodegeneration (low ND) group, and one high neurodegeneration group (high ND). The criteria for inclusion in the low ND group were clinical diagnosis CIS and RR, low MRI and expanded disability status scale (EDSS), see [46], below or equal to 3. This approach resulted in identification of eight individuals. The criteria for inclusion in the high ND group were clinical diagnosis SP and EDSS score above or equal to 3.5, resulting in 11 patients. A PLS model was built to identify variables that separated these two groups. This model was denoted the 'ND' model.

To optimize model parameters and to select important variables, a procedure that shares resemblance with both cross-validation and bootstrap was devised. The samples were randomly divided into one training set (2/3 of the samples) and one test set (the remaining 1/3). A model was built on the training set, a variable importance measure. The so-called variable importance in the projection (VIP) was calculated for all variables and the test set was predicted on the model. Note that the variable ranking and selection was only performed on the training set, as inclusion of the test set in the variable selection can result in substantial overestimation of the predictive ability of the model. New models were built on successively reduced numbers of variables as ranked by the VIP parameter. This process was repeated for 100 random partitions of the samples into training and test sets for models with a varying number of PLS components (1-10). The prediction rate was calculated as an average of the percentage correctly classified samples across the 100 iterations. When using PLS, a cut-off threshold needs to be set in order to establish which class a certain sample belongs to. Cut-off optimization was also performed through the cross-validation/bootstrapping procedure as described below for the minimum number of variables achieving an acceptable prediction performance in order to obtain balanced prediction rates between the two classes in the model [117].

For selection of the most important variables for each comparison, an overall VIP rank was formed using the whole set of samples, and taking the maximum VIP for two different datasets based on two different approaches to handle missing values. To filter out low expressed proteins, the following filter criteria were applied to the VIP list: to be classed as increased, the mean level in the disease group should be above 100 parts per million (PPM), with a fold change above 1, and the spot should be present in at least 60% of patients in the disease group. The criteria for a decrease was analogous, with the spot present in at least 60% of control patients, the mean level in control above 100 PPM and fold change below –1.

The data analysis described above was used to select ten biomarker candidates for further evaluation. To investigate whether any of the ten selected proteins were differentially expressed between MS and controls, two different types of analysis were conducted using new datasets. In one analysis a linear model with disease group and sex as fixed effects was applied individually to each protein variable, using SAS version 9.1.3. Pairwise comparisons for each MS subgroup versus the control group were carried out as contrasts within the model to estimate the relative difference in means between the two groups. In addition to a point estimate of the relative difference in means, two-sided 95% confidence intervals were estimated and associated two-sided p-values were calculated. The level for statistical significance was set to 0.05. No multiplicity adjustments were performed on the p-values.

In the other analysis, classification (prediction) PLS models using all ten proteins were constructed using the PLS package of R [118]. Two separate models were constructed, one with RRMS *vs.* controls, and one with SPMS *vs.* controls. Models were constructed using 6 components, and by setting the status of controls to 0 and the MS groups to 1 prior to training. Predicted values following LOO (leave-one-out) cross-validation were classed as being either above or below a given threshold.

The performance of the models was calculated for a range of threshold values (0-1.0, increment 0.1). Performance was measured as Sensitivity (True Postive / (True Positive + False Negative) and Specificity (True Negative / False Positive + True Negative) at that range of threshold values. The results were plotted as a Receiver Operating Characteristic (ROC) curve.

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For the comparison of protein levels, post- *vs.* pre-natalizumab treatment, patient data was modelled in SAS v9.3 using a mixed effects model with patient as random effect and time (post- or pre-) as fixed effect. This model takes into account that observations from the same patients are correlated, but assumes that observations from different patients are independent. Six and 12 months duration data were modelled separately. The model was applied to each protein individually. Within the model, relative estimates of the mean post versus pre treatment difference were calculated, along with a two-sided 95% confidence interval and associated two-sided p-value. The level for statistical significance was set to 0.05. No adjustments to the p-values for testing many variables were performed.

Study IV: Aiming at of identifying a reduced number of protein spots of predictive value, we applied multivariate predictive modeling to the data. The modeling was conducted in two steps. First, a training set consisting of 36 OND/HC and nine PPS was used to select the protein spots with the highest predictivity and to optimize model parameters. To optimize model parameters and to select important protein spots we applied a cross validation procedure to the training set data, coupled with a variable (corresponding to a protein spot) selection procedure. Briefly, in the cross-validation procedure, the training set was internally divided into a training and a test set randomly, and this was repeated a number of times. The prediction rate was recorded as an average of the test set predictions over all cross-validation rounds. Note that the variable (protein spot) selection was only performed on the training set, as inclusion of the test set in the variable selection can result in substantial overestimation of the predictive ability of the model. In the next step, the predictivity of the model and the associated selected protein spots was evaluated using an independent test set consisting of six PPS and seven OND/HC samples. We used the PLS method to build predictive models of the data.

2.3 RESULTS AND DISCUSSION

The aims of study I were to establish a fractionation procedure for reducing the levels of the most abundant proteins (GFAP intermediate filament and tubulin) in normal and pathological CNS tissues, and to optimize electrophoresis conditions in order to facilitate proteomic analysis of changes in disease-related protein expression in CNS.

Two important considerations in the development of the CNS protein fractionation used in this study were the prevention of proteolysis during preparation of samples for electrophoresis, and the removal of CNS lipids. Inhibition of proteolytic enzymes is important as there are high levels of these enzymes in active MS lesions, and therefore a protease inhibitor cocktail was included in the buffer. Lipids were removed effectively by several steps of centrifugation. This was necessary because high levels of lipids are present in brain and spinal cord white matter, and even traces of lipids can cause streaking of proteins in gels [119]. Increasing the size of the IPG strip from 18 cm to 24 cm improved the resolution on the gels, but the time required to run the gels did not influence the results. In summary, the fractionation and proteomic methodologies developed in this study can be utilized for the identification and quantification of disease-related proteins in neurological or psychiatric disorders.

In study II we used the method developed during study I and analyzed MS plaques, adjacent tissue and normal brain tissue. We identified proteins that had interesting expression profiles according to the statistical analysis. Seven of the proteins that were differentially expressed in the different tissues are reported in Table 2. Digitized information was analyzed using PDQUEST software (Bio-Rad) and 2-D Gaussian fitting was used to detect and quantify partially overlapping spots as well as isolated spots. In accordance with our results, it was recently reported that lamin B is expressed in MS plaques [120]. Moreover, in another study based on MS plaques the majority of our seven identified proteins were reported, further supporting our findings [76].

Table 2. Differentially expressed proteins in MS lesions.

		Acute MS lesion		**Chronic MS lesion**	
Protein	*Normal tissue*	*Adjacent tissue*	*Plaque lesion*	*Adjacent tissue*	*Plaque lesion*
Creatine kinase, B-type (CK-B)	+++	+	+	++	++
Dihydropirimidinase -related protein 2 (DRP-2)	++	+++	++	+	++
Guanine nucleotide-binding protein G – alpha unit (GoA)	+	++	++	++	+
Guanine deaminase (GUAD)	+	++(+)	++(+)	++	+
Guanine deaminase (variant, GUAD)	+	++	++	++	+
Lamin B (fragment)	+	++	++	+	+
Zonula occludens protein 2 (ZO-2)	++	++	+	+	+

Expression levels: Low = +, Intermediate = ++ and High = +++ in the different tissues.

We also identified 12 different variants of the protein 14-3-3 and its isoforms τ, β, γ and ε (Fig.7). The expression pattern of the proteins could separate the individuals when performing hierarchical clustering. 14-3-3 and its different isoforms have previously been detected in CSF from CIS individuals indicating early neurological events [121] (Fig. 7). DRP-2 has been found to be modified by oxidation in AD brains [122, 123], and this may be a sign of oxidative stress. Our study exhibited high expression of DRP-2 in adjacent tissue of acute MS plaque, but the potential level of oxidation was not investigated.

Using the fractionation method from study I, we could detect and quantify proteins that had previously been below the detection limit using 2DE. However, a disadvantage is that 2DE based proteins studied have a limited size and pI (10-200kDa, and 4-7). Nevertheless, we detected post-

translational modifications using 2DE, observing two isoforms of GoA and GUAD that were differentially expressed. It is unlikely that this result could have been detected using a non-gel-based technology.

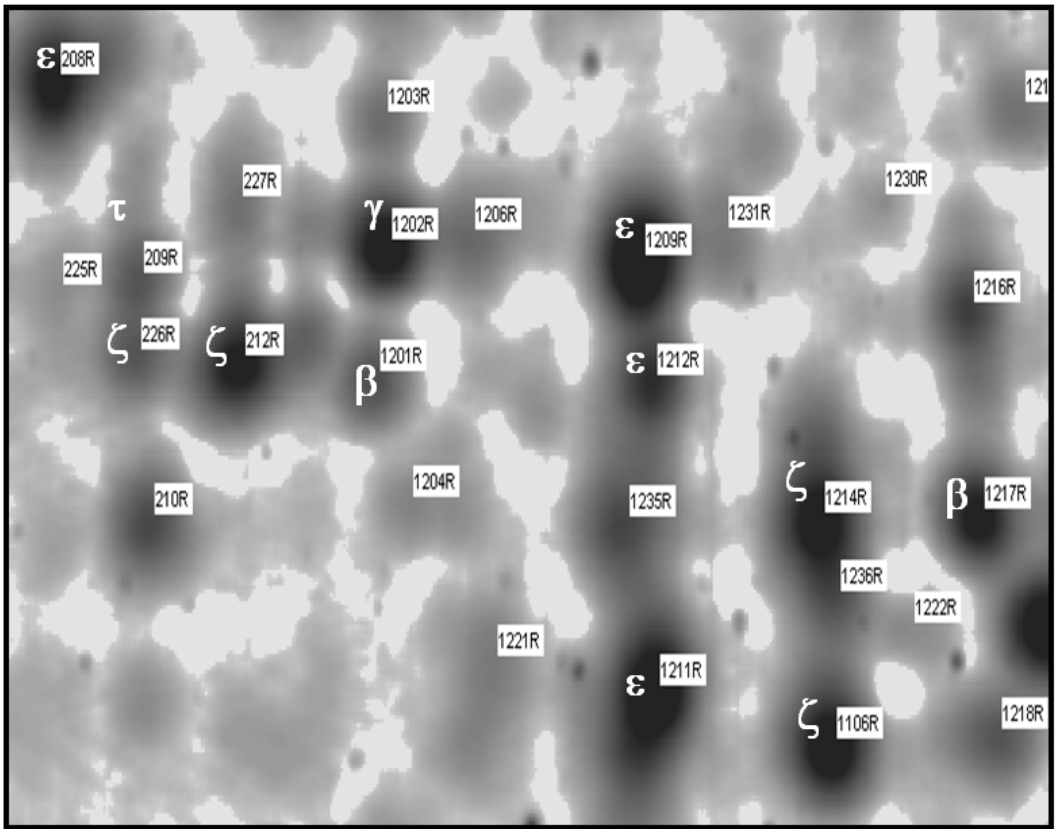

Fig. 7 Zooming in the location of the gel containing the different isoforms of the 14-3-3 protein which were identified and characterized using MALDI-TOF analysis.

Importantly, a biomarker can be obtained from different sources, mRNA or proteins. In the case of gene expression, approximately 28,000 genes are recognised HGNC Home Page (www.genenamnes.org). However, mRNA can be subjected to different processing including alternative splicing, and therefore the amount of protein products produced can change [124]. At the protein level modifications can occur, e.g. phosphoryation [125], glycosylation [126] and activation by cleavage [127], enabling protein-protein interactions and signal transduction. The number of possible protein isoforms exceeds >1,000,000, and thus characterization of the potential biomarker may be necessary. All proteins isoforms may be expressed in tissues, but the local homeostasis will affect their expression and levels.

In study III we analysed affinity-purified CSF from individuals affected by MS (including disease subgroups), controls and a reference disease PPS in a proteomics-based biomarker discovery study. We used an affinity column to remove albumin and IgG from the samples in order to increase the sensitivity, as ~80% of the protein content in CSF is albumin and IgG. This procedure enabled detection of proteins at lower concentrations. By selective removal of these proteins there is also a possibility that interesting information is lost since albumin and IgG are carries of other proteins and peptides. However, one good argument for the purification is that we were able to study many proteins in CSF that have never have been investigated before. Moreover, we knew exactly which protein window we are looking into.

To achieve the maximum level of information in each sample ~600 μg of protein was loaded onto each gel. The subsequent data analysis based on a prediction model and hierarchical clustering yielded 50 proteins that could be used for identification of MS individuals (Fig. 8). The top 50 proteins in each subgroup were further subjected to mass spectrometry, and of these only ~20% were positively identified due to low concentrations or bad recovery from the gels.

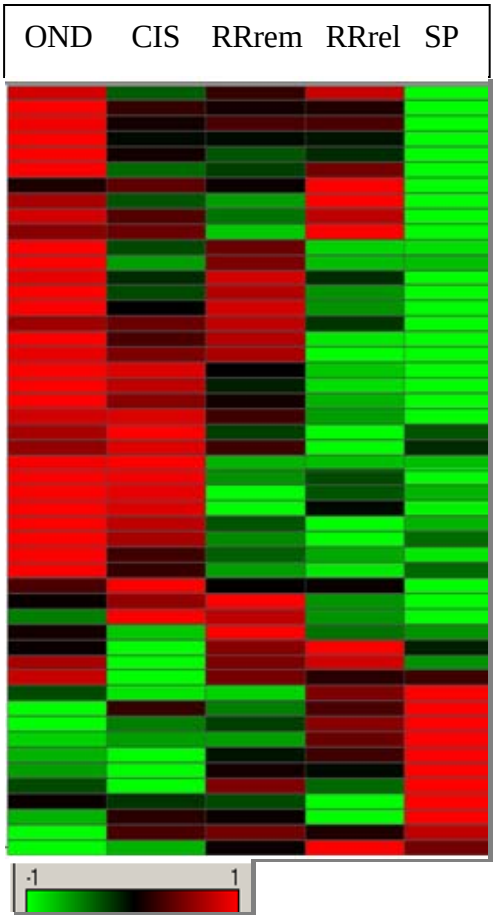

Fig. 8 Hierarchical clustering of the top 50 proteins obtained from the proteomic biomarker study

Colour-code: red represents increased and green decreased protein expression.

This information together with that from the gel analysis, e.g trains of spots usually representing isoforms of the same protein, yielded ten candidate biomarkers that were selected for further evaluation by quantitative immunoassay using an independent cohort of MS and control individual samples. The candidates were chosen to make the protein profile more similar to that of the secondary progressive condition in MS in order to be able to detect changes in the progression of the disease.

To further explore potential connections between the differentially regulated proteins in the CIS and SPMS groups within the 2DE study, we used the Ingenuity pathway system (http://www.ingenuity.com). It became evident that there were links to amyloid precursor protein (APP) processing, which has previously been shown to be involved in AD [128]. The observation indicates that there is an ongoing neurodegenerative process both in the early stage (CIS group, Fig. 9) and the later stage (SPMS, Fig. 10) during MS progression.

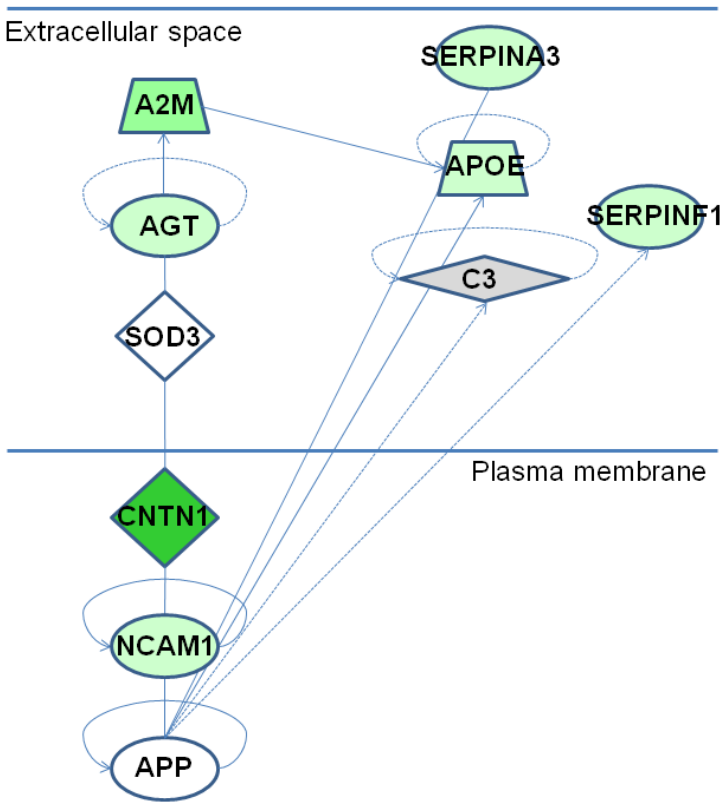

Fig. 9. Differentially regulated proteins from the CIS group and their connection to APP.
Green represents decreased levels and gray conflicting results. Abbreviations; AGT, angiotensinogen; A2M, alpha 2 macroglobulin; APOE, apolipoprotein E; APP, Amyloid precursor protein; C3, complement 3; CNTN1, contactin 1; SEPINF1, pigment epithelium derived factor; SERPINA3, alpha 1 antichymotrypsin;, SOD3, superoxide dismutase 3.

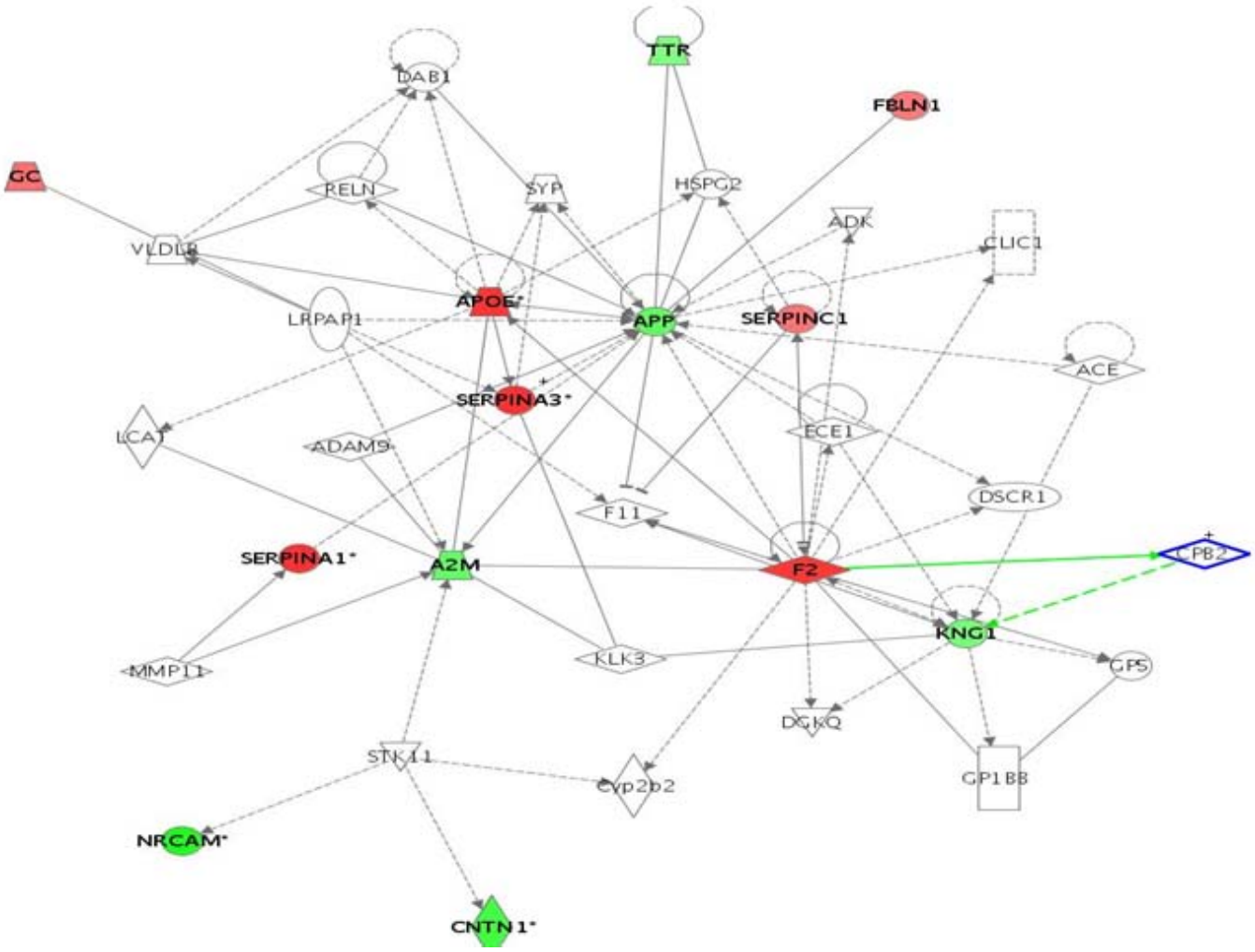

Fig. 10. Differentially regulated proteins from the SPMS group and their connection to APP.

Green represents decreased levels and red increased levels. Abbreviations for the regulated proteins; APP, Amyloid precursor protein; APOE, apolipoprotein E; A2M, alpha 2 macroglobulin; CNTN1, contactin 1; F2, complement factor B; FBLN1, fibulin 1; GC, vitamin D-binding protein; KNG1, kininogen 1; NRCAM, Neuronal cell adhesion molecule; SERPINA1, alpha-1 antitrypsin; SERPINA3, alpha 1 antichymotrypsin; SERPINC1, antithrombin; TTR, transthyretin.

As proteomics is a labour-intensive technique which is not a standard procedure in clinical laboratories, we decided to translate our findings into a multiplex immunoassay platform. During the biomarker discovery phase of study III, we selected 10 potential biomarkers for further evaluation using a multiplex immunoassay. One consequence of shifting platform from proteomics to immunoassay is that the protein expression pattern may change. The immunoassay is based on antibodies recognizing a specific part of the protein, thus information regarding PTMs is not included as in 2DE. On the other hand, the sensitivity of the immunoassay is usually very high, enabling detection of proteins at low concentrations. Despite the change of analytical platform the pattern obtained gave interesting results.

It is worth noting that the protein apolipoprotein E was not included in the analysis, although it has been regarded as a risk factor for MS. However, in our study we detected and identified ~20 different isoforms of ApoE in the CSF, and five of these exhibited MS-related expression profiles. This implies that further characterization of the proteins needs to be performed to determine which should be further evaluated and validated.

The immunoassay showed significant increases in the CSF levels of alpha-1 anti-chymotrypsin (A1AC), alpha-1 macroglobulin (A2MG) and fibulin 1 in RRMS individuals as compared to control subjects. In SPMS four additional proteins; contactin 1, fetuin A, vitamin D binding protein (VDBP) and angiotensinogen (ANGT) were increased as compared to control subjects. In particular, ANGT was increased 3-fold in SPMS, indicating its potential as a biomarker of disease progression in MS. In PPMS, A1AC and A2MG exhibited significantly higher CSF levels than controls, with a trend of increase for ANGT. In a previous study it was demonstrated that ANGT was produced and secreted by astrocytes and that the protein was involved in the maintenance of BBB by cleavage of ANGT to angiotensin II [129]. Moreover, there was a decreased number of immunopositive ANGT perivascular astrocytes in MS lesions. The 3-fold increase of ANGT in SPMS may imply that the activities of renin and angiotensin converting enzyme (ACE) are changed in MS. Increased levels of ACE but decreased levels of angiotensin II have been reported in RRMS individuals [130]. Taken together, this indicates that a disturbance in the renin/angiotensin system occurs in MS.

Further evaluation was conducted in a pilot study of CSF from RRMS individuals before and after treatment with natalizumab. The mean protein levels for the 9 patients treated for 6 months appear generally to be decreased following treatment. For the groups of patients that were treated for 12 months there were no significant differences between post- and pretreatment. However, the treatment study did not include placebo control individuals, and the interpretation is consequently difficult since treatment effects cannot be separated from time effects in a post- versus pre-treatment comparison. Moreover, the baseline mean levels for the patient group treated for 6 months were more similar to the RRMS mean levels, and the baseline mean levels for the patient group treated for 12 months were generally lower, being more similar in range to the control group, which also complicates the interpretation.

In summary, classification models based on the biomarker panel could identify 70% of the RRMS and 80% of the SPMS and control individuals correctly. However, the panel of biomarkers needs to be further validated in independent patient cohorts. The panel may also be extended with other proteins that are interesting from different neurodegenerative and neuroinflammatory perspectives, and selected as biomarker candidates.

In study IV we demonstrate a protein profile that has the potential to be used as a diagnostic tool for PPS. The proteins identified in this study are known to be involved in different pathways associated with tissue damage and apoptosis. These data together with previous observations of an ongoing inflammation with cytokine production provide evidence for the hypothesis that PPS may be caused by an active inflammation and a neurodegenerative process. Thus, there is potential for development of different treatments for this clinical condition.

3 CONCLUSIONS

The aim of this thesis was to develop purification techniques to gain higher specificity and sensitivity using proteomics technologies, and to apply these on MS samples in order to identify biochemical biomarkers and increase the understanding of the biology behind the disease. Conclusions can be summarized as follows:

Employing the purification methods, we could detect and quantify proteins that have not been described previously in the diseases using 2DE.

Using 2DE-based proteomics we were able to detect protease activity and post-translational modifications in MS tissue and individuals affected with PPS. Our observations support the hypothesis that there is an ongoing active neuroinflammation and neurodegeneration in PPS, which has been partly controversial until recently.

The approach to use unbiased 2DE-based proteomics to discover a protein pattern consisting of 10 proteins as potential biomarkers for MS was succsesful, and the prediction model classified the individuals into the different MS subgroups. For PPS, the prediction model classified the individuals with high precision. Identification of additional proteins from the MS subgroups also generated biological information.

Further evaluation of the potential MS biomarkers was performed using a quantitative multiplex immunoassay. Analyzed data obtained from the assay revealed different protein expression patterns of RRMS and SPMS. The predictions of the two MS groups were classified with high accuracy. The natalizumab-treated RRMS individuals in the pilot study also exhibited interesting protein profiles.

4 FUTURE PERSPECTIVES

There is a continued need for understanding disease development, progression and treatment effects in MS. Validated biomarkers would be useful in complex diseases like MS in order to monitor disease progression and effects of new treatment strategies. Investigation of different compartments of the human body; plasma, CSF and tissues, followed by system biology investigations will be important to reveal connected pathways.

The combined experience from the studies we have conducted is that there is a further need to develop tools to understand the complexity of human biology. The technical development of different technologies, e.g. quantitative mass spectrometry and powerful data mining tools makes the future encouraging.

To use unbiased biomarker discovery studies and subsequently transfer the results in order to specifically analyze different diseases, mass spectrometry-based multiple reaction monitoring (MRM) can be employed.

There is a need to create collaboration networks in order to be able to process the enormous amounts of data evolving form the scientific community and to interpret them correctly. Increased collaboration between the clinical and the scientific community to promote the use of advanced analysis instruments in clinical practice is desired.

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