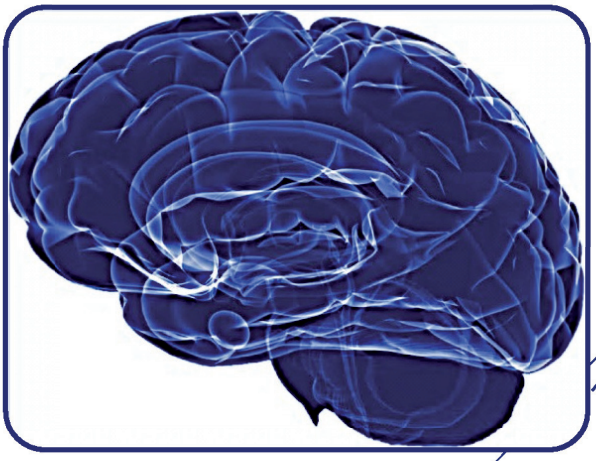
IDENTIFICATION OF POTENTIAL BIOMARKERS IN MULTIPLE SCLEROSIS



Vaibhav Singh

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To my parents

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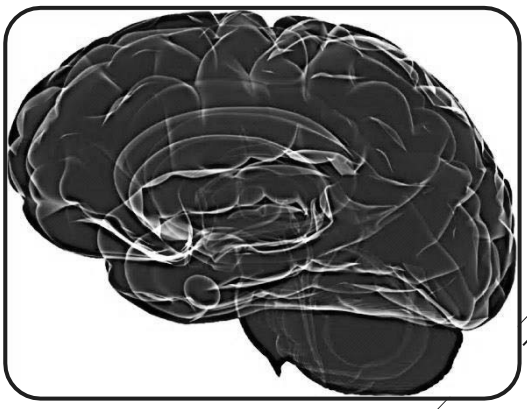
Introduction



Chapter 1/1.1

Proteomics technologies for biomarker discovery in multiple sclerosis

Vaibhav Singh, Rogier Q. Hintzen, Theo M. Luider, Marcel P. Stoop.

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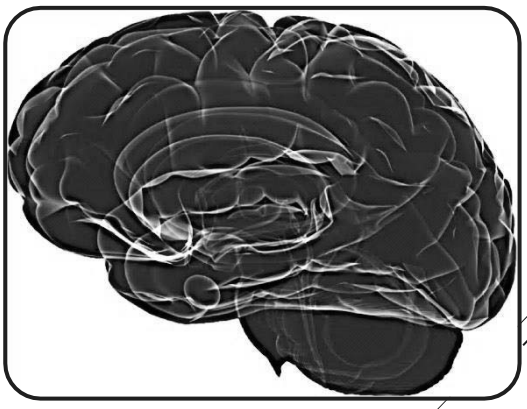

ABSTRACT

Multiple sclerosis is a disabling inflammatory and neurodegenerative disorder that predominantly affects young adults. There is a great need for biomarkers, which could elucidate pathology as well as provide prognosis of disease progression and therapy response in Multiple sclerosis. Rapidly evolving, technology driven applications such as mass spectrometry based proteomics are currently being developed for this purpose. In this review, we will outline the current status of the field and detail a number of the bottlenecks as well as future prospects of this type of biomarker research.

1. INTRODUCTION

Multiple sclerosis is an inflammatory and neurodegenerative disease of the central nervous system (CNS), with a complex and incompletely understood aetiology. The disease appears to evolve/occur in individuals that are genetically susceptible to the disease, but only those who are exposed to certain unknown environmental triggers develop the disease. It is believed that the disease process has an early phase with increased migration of auto-reactive lymphocytes across the blood-brain barrier (BBB) into the CNS (1). One of the major adverse effects that can be observed in multiple sclerosis patients is damage to the myelin sheath that enwraps axons, and is physiologically needed to increase the speed of signal transmission. This process culminates in the hallmark of the disease; the formation of the sclerotic lesions from which the disease gets its name. Other cells affected besides the myelin-forming oligodendrocytes are neurons with their axons. One of the main issues in multiple sclerosis is the heterogeneity of the disease, as observed by the different patterns of plaque formation in patients (2) and the variability of clinical symptoms in patients. The majority of patients (80-85%) have the relapsing remitting subtype of the disease, in which relapses are followed by periods of remission. Around two thirds of these patients eventually enter a secondary, progressive phase of the disease. In 10-20% of multiple sclerosis patients the disease is progressive from onset (3).

In recent years new techniques have become available for analysis of biological samples in search of potential biomarkers for diseases such as cancer, Alzheimer's disease and also multiple sclerosis. These biomarkers can be useful in a number of different ways, like for example prognosis, monitoring of disease progression and early detection of diseases, which is of specific importance in multiple sclerosis as early detection and subsequent early treatment retards disease development (4). Additional purposes include detection of new possible therapy targets, monitoring of response to certain therapies as well as increasing our understanding of disease pathology (5). In this review we will discuss available mass spectrometry-based proteomics technologies, sample selection considerations and future prospects for multiple sclerosis research.

2. BIOLOGICAL SAMPLES

2.1. Blood

Blood is a promising body fluid for biomarker discovery because of its easy collection and the presence of brain specific proteins and peptides produced in the CNS. These may reflect pathological changes occurring within the brain and spinal cord (6). In blood

TNF-α and CCL2 have been suggested to be indicators of inflammatory responses in primary progressive multiple sclerosis (7), whereas kallikreins has been linked to the secondary progressive stage of multiple sclerosis (8). Blood is a rich source of disease related proteins due to its large dynamic range (> 1015) (9). Since it is located distantly from CNS, the detection of disease related proteins may be complicated by dilution and fast clearance of proteins by the liver and kidneys. Additionally metabolic changes may occur and there is also the possibility that highly abundant proteins like albumin in blood may mask detection of low abundant proteins, therefore making detection difficult (10, 11). In addition, proteases may be effective during the travel from the CNS to the blood stream that may truncate proteins of interest and even more make the exercise to find reliable and clinical relevant markers a challenge.

2.2. Urine

Urine is another interesting body fluid that attracts attention because of its non-invasive collection method. Past studies have reported neopterin and nitric oxide metabolites as marker of disease activity in multiple sclerosis (12). The possibility of detecting interesting protein biomarkers in urine is limited by metabolic variations observed in urine, relatively low protein content overwhelmed by a few high abundant proteins (for example albumin and tamm-horsefall protein (13)), high salt concentration and presence of relatively high levels of low molecular proteins. Based on these difficulties Thongboonkerd and co-workers concluded that urine may not the best choice especially for proteomics based biomarker research for CNS disorders (14). However the proteolytic cleavage into low molecular weight products can also be related to protease activity that could be partly disease specific (15).

2.3. Cerebrospinal fluid

While blood and urine are easily obtainable, the key endorsement of cerebrospinal fluid (CSF) as a body fluid for biomarker detection of CNS disorders is its direct connection and close proximity to the CNS. However, its collection is obviously more invasive and elaborate than the collection of blood.

As a consequence of fluid transports in the CNS, most of CNS-disease related proteins are likely to diffuse into the CSF. Therefore CSF is able to reflect directly the ongoing pathological conditions in the brain (16). This fluid is partly produced in choroid plexus of the brain at a rate of 500 ml/day and very rich in brain specific proteins. Normally, the concentration of protein in CSF (0.2-0.5g/l) is far lower than in serum (60-80g/l) (17), mainly because of the vastly lower concentrations of highly abundant blood proteins that could mask detection of low abundant proteins by sophisticated techniques. Together this makes CSF an interesting body fluid for biomarker research for CNS disorders.

2.4. CNS tissue

Post-mortem brain tissue, and in particular brain lesions, allow for the possibility to detect changes caused by the pathology of multiple sclerosis at possible sites of the disease activity. These lesions are a combination of demyelination and inflammation occurring in different parts of CNS. Histological patterns of active demyelinating lesions vary from patient to patient (18). Lesion tissue obtained post-mortem from multiple sclerosis patients has been shown to have differences in RNA expression compared to normal appearing white matter (19). Consequently, if differences exist on a transcriptomics level, then effector molecules like proteins and metabolites might also be differentially abundant at the site of disease activity. Active multiple sclerosis lesions could be of great interest to approach disease in a targeted manner using an efficient technique called laser capture microdissection. This technique does not alter or damage the morphology and chemistry of the tissue and the surrounding cells. Using this type of method Han et al. have reported increased protein C inhibitor protein and tissue factors in chronic active multiple sclerosis lesions (20). Availability of this kind of material is limited, so it is difficult to obtain sufficient numbers of samples to perform meaningful statistics on detected biomarkers. Therefore biobanking of brain tissue material is, along a good description of the obtained material, essential. The Netherlands Brain Bank (www.nin.knaw.nl) is a nice example of brain tissue banking. Additionally, tissue is an interesting biological option for validation of biomarkers detected in other types of biological samples (Figure 1).

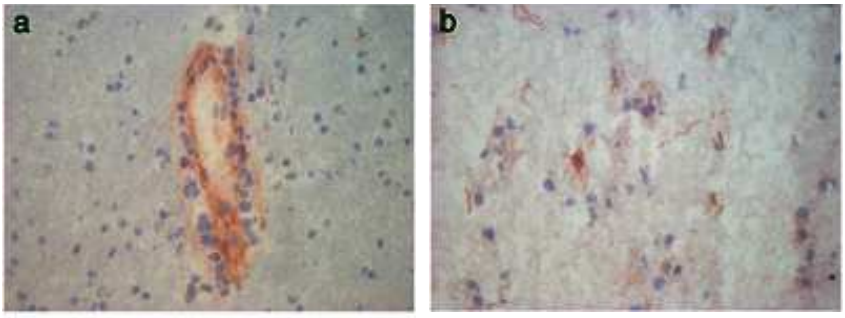

Figure 1. Staining of brain tissue for the protein clusterin. In several studies on CSF, clusterin has been shown to be elevated in multiple sclerosis patients. Histochemical staining of human normal white matter brain sections for clusterin of active (a) and inactive (b) lesions shows an increased abundance of clusterin in active lesions, validating the implication of clusterin in the disease (Picture taken from the dissertation of Marcel Stoop, "Cerebrospinal Fluid Proteomics of Multiple Sclerosis Patients").

2.5. Animal models

Animal models may be a good alternative and a more experimentally accessible road to investigate tissue for multiple sclerosis. However, the translation of results observed

in the animal model to the human situation can be quite difficult. Experimental autoimmune encephalomyelitis (EAE) mimics the CNS inflammation observed in multiple sclerosis to a certain extent. EAE can be induced in a number of different animals, such as marmoset monkeys and rodents. Rodent EAE is a well-studied model which has helped scientists to understand underlying biology of CNS inflammation, and has shown the importance in testing of therapeutic approaches to multiple sclerosis immunology and pathology in animal models (21). However, therapeutic success and detection of biomarkers in EAE cannot be directly translated to humans because of the phylogenetic distance between rodents and humans. In contrast, a non-human primate EAE model such as the marmoset monkey, which is phylogenetically closer to humans, has more similarities at molecular and functional level with our nervous system and immune response (22, 23), and could potentially be more efficient and beneficial in pre-clinical trials and investigation (24).

3. PRE-ANALYTICAL FACTORS

The proteome can be affected by several pre-analytical factors prior to measurement, resulting in inaccuracies in the data. These factors include sample collection and sample storage. Sample collection according to a strict, standardized protocol is essential for preventing unnatural variation in the proteome of the samples (25). Adherence to such a protocol would make future proteomics studies and the exchange of samples between laboratories, for potential biomarker validation studies, easier. The importance of sample storage conditions for biomarker research for multiple sclerosis is perhaps best illustrated by the identification of an N-terminal cleavage product of cystatin C in CSF of multiple sclerosis patients by Surface-Enhanced Laser Desorption/Ionization (SELDI) mass spectrometry as a potential biomarker (26), which was later shown to be an artefact caused by sample storage at −20°C instead of −80°C (27). This example illustrates both the necessity of storing both case and control samples under the same conditions as well as the limits of −20°C storage of biological samples. Additional issues arise when the samples are subjected to multiple freeze/thaw cycles or are not centrifuged correctly (28), so essentially biological samples should be aliquoted and centrifuged before storage and all samples should be stored at −80°C.

Another factor that should be controlled rigorously when performing CSF proteomics experiments is blood contamination. Protein concentrations in blood are 400-600 fold higher in blood than in CSF, so any contamination of CSF samples by blood, due to traumatic CSF tap, will invariably skew the protein abundances in CSF tremendously, leading to erroneous measurements (29). Essentially, any CSF sample in which blood-specific proteins such as haemoglobin or apolipoprotein B-100 can be

identified by mass spectrometry should be excluded from CSF proteomics studies (30). Individual sample selection is also a topic that should be considered carefully before commencing a proteomics experiment. Although overall proteome differences due to gender and age do not appear to be extensive, several proteins are exceptions to this observation. Thus, these proteins should be evaluated with great care, in the context of age and gender of the selected samples, if these prove to be significantly differentially abundant between case and control groups (31).

4. "OMICS" STRATEGIES

The complexity that surrounds the aetiology and pathology of multiple sclerosis suggests that effects of disease processes will not just be present at a single biological level. Indeed, even a genetic component, potentially precluding biological effects, as evidenced in genetic isolates, has been shown to be present in multiple sclerosis (32). Thus, all "omics" disciplines (genomics, proteomics, metabolomics and all variants thereof) are of potential interest in multiple sclerosis research. Specific genetic variations influence susceptibility for the disease (33) but environmental factors are also of great influence in multiple sclerosis disease evolution (34). Transcriptomics, located one step downstream from genomics, uses microarrays to analyze RNA expression profiles to provide valuable insights on mechanisms of damage, repair and regeneration (35). With these techniques post-mortem brain tissue can be compared on RNA expression levels to detect gene expression differences between acute, chronic active and chronic inactive lesions (36). Even closer to the active disease processes than DNA and RNA are proteins and metabolites. These molecules are probably more directly involved in the acute pathological issues in multiple sclerosis and therefore interesting as biomarkers. Metabolomics biomarker studies are, to date, limited in number, due to technical difficulties regarding metabolite stability, metabolite identification and the potential introduction of sample variation due to collection and preparation (37). Proteomics, the study of the function as well as the primary structure of proteins, allows for the large scale study of protein profiles in complex biological samples, typically in an unbiased fashion (38). Currently, it is anticipated that a future merger of proteomics with cell biology will yield a better understanding of regulation of cellular processes, both in healthy and diseased states (39).

5. PROTEOMICS-BASED BIOMARKER RESEARCH

Mass spectrometry (MS) is the core technique in proteomics. Hence, the evolution of mass spectrometry techniques towards high resolution and more high through-put machinery has been essential for the advancement of the field. Current MS instruments such as Fourier transform ion cyclotron resonance, Orbitrap and quadrupole time-of-flight mass spectrometers have greatly enhanced the quality of proteomics data. Flexibility on either side of the mass spectrometer, in the form of different modes for ionization, sample separation and data analysis, enables the adaptation of differ-

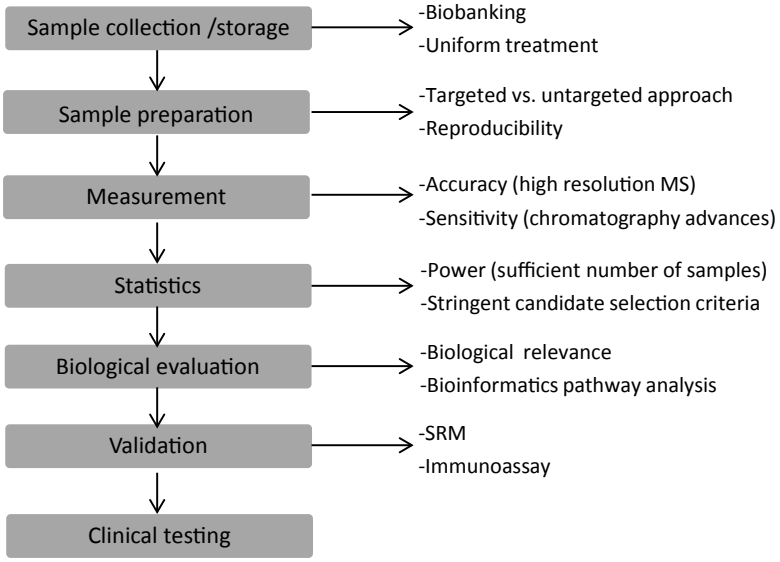

Figure 2. Flow chart for an extended proteomics experiment. The process starts with the collection of well annotated samples and continues on through the bioinformatics stage. Validation of biomarkers and the relation of biomarkers to biological and pathological relevance are especially important as these factors determine the value of the reported biomarker. For all stages of the experiment some key considerations are highlighted. The last stage, clinical testing, is often problematic, as it is difficult to trans- late biomarkers to clinical practice.
Definition: Selected reaction monitoring (SRM) is defined as a targeted MS technique for the detection and accurate quantification of a predetermined set of proteins in a complex background. In a SRM-MS assay, one or two signature proteotypic peptides which are unique to the protein of interest are selected to represent the protein. Proteotypic peptides are the peptides that uniquely identify each protein and are consistently observed when a sample mixture is examined by a (tandem) mass spectrometer. In an SRM experiment, a predefined precursor ion and one of its fragments are selected by the two mass filters of a triple quadrupole mass spectrometer and monitored over time for precise quantification. This is an instrument which has a capability to selectively isolate precursor ions corresponding to the m/z of the signature peptides and to selectively monitor peptide-specific fragment ions. SRM assays do not require the generation of high affinity antibodies, and yet have lower limit of detection to pg/ml protein concentrations.

ent experimental designs based on the mass spectrometry core of the analysis. After sample selection a typical proteomics experiment consists of four distinct stages: sample pre-treatment, protein or peptide separation, protein of peptide identification, and a bioinformatics-supported data analysis stage (Figure 2).

5.1. Sample pre-treatment

Sample pre-treatment and the separation of peptides or proteins in a sample are interchangeable components of a proteomics experiment. In some approaches one precedes the other while in other strategies it is the other way around. Decisions on sample preparation include the choice to either digest the proteins enzymatically or not to digest and whether to analyze the naturally occurring peptides and proteins. Whereas both approaches have merits, the majority of approaches contain an enzymatic digestion of proteins, typically by trypsin.

A second available choice is the option to utilize stable isotope labels to quantify differences or to pursue label-free approaches. Potential tags that can be included to quantify differences between cases and controls are isobaric tags for relative abundance and quantitation (ITRAQ), isotope coded affinity tags (ICAT) and 18O labelling. Using these techniques quantification of peptides can be achieved based on the intensity ratio of heavy and light forms of the labelled peptides. Label-free quantitation either employs spectral counts or peak areas for quantitative measures. Whereas the stable isotope labels complicate the sample pre-treatment procedures, they do not necessarily result in better quantitative data (40).

5.2. Protein or peptide separation

Protein or peptide separation is not always essential when performing proteomics experiments on biological samples. Studies without separation strategies have yielded a number of potential CSF markers for differences between multiple sclerosis and controls (41) as well as significant differential protein abundances between different multiple sclerosis disease types (42). However, given the complexity of biological samples, fractionation of the contents does allow for a more in-depth analysis of the proteome. Proteins in a sample can be separated by a number of different methods, such as 2-dimensional gel electrophoresis (2-DGE), liquid chromatography (LC) or "protein chips" (43). 2-DGE separates proteins according to charge and size in two discrete steps, but this technique is limited due to the relatively high labour intensity of the technique and troubled by co-migration issues. The online application of nanoscale LC techniques offers an increased potential to detect low abundant proteins, but like 2-DGE this technology is also relatively time consuming (44). Additionally, quantitative analysis using online coupled LC and MS is less straightforward as 2-DGE, but the

reproducibility and the ease of comparing relatively large numbers of samples is more developed in LC based techniques.

Protein chip arrays, used in combination with SELDI mass spectrometry, fractionate and enrich subpopulations of proteins from complex mixtures on the chip surface. Although SELDI has been applied to the analysis of potential biomarkers, there remain reproducibility, sensitivity, and protein sequencing concerns regarding this technique (30).

5.3. Protein identification

One of the key elements in proteomics is protein identification. This is typically done by one of two methods: database searching and de novo sequencing. Database searching employs algorithms to match the experimental fragmentation spectra to known amino acid sequences in a database, which subsequently gives a score related to a statistical probability of a correct identification. De novo sequencing, i.e. sequencing without the assistance of a linear database aims to identify peptides which are not present in a database (45). By using the experimental tandem mass spectrometry data a sequence can be proposed by using the information of fragments of the peptides observed. A combination of database searching and de novo sequencing is a powerful method to increase the number of identified peptides in a proteomics setting (46), which is one of the key goals to proteomics in the near future as, currently, many potentially biologically interesting peptide peaks in this type of research remain unidentified.

5.4. Bioinformatics-supported data analysis

Data analysis has long been one of the bottlenecks of proteomics research (47). Only very recently software applications that allow for the analysis of large numbers of complex liquid chromatography mass spectrometry (LC-MS) runs have become available. These advances will allow for more in-depth analysis of biological samples due to the separation of the components of the sample which will allow for detection, and comparison between sample groups, of lower abundant proteins (48).

6. RECENT MULTIPLE SCLEROSIS CSF PROTEOMICS DEVELOPMENTS

Besides proteomics analysis of CSF peptides (49) and proteins (50-59) that differentiate between multiple sclerosis patients and controls, recently some other potential biomarker avenues have been explored. One of the key events in multiple sclerosis disease development is the patients' conversion from a clinically isolated syndrome of demyelination to clinically definite multiple sclerosis. Two recent studies have analyzed CSF samples with proteomics techniques to investigate this conversion and

identified a number of proteins, like for example chitinase 3-like protein 1 and fetuin-A, whose abundances were altered in patients that converted to clinically definite multiple sclerosis (60, 61). These findings could be very intriguing stepping stones in the investigations of multiple sclerosis disease evolution. Secondly, another recent study has reported on the effects of Natalizumab treatment in a cohort of multiple sclerosis patients. A small number of proteins were found to be decreased after six months of treatment, but after twelve months of treatment this effect could no longer be observed (62). Finally, an interesting approach was taken by Obermeier et al., who matched transcriptome immunoglobulin data to CSF immunoglobulin proteome data to identify specific immunoglobulin sequences relating to multiple sclerosis pathology, which could, due to the known involvement of immunoglobulins in inflammatory disorders, be a very promising avenue for biomarker research for multiple sclerosis (63).

7. BOTTLENECKS

Although the proteomics field is rapidly developing and new advances in technology and analysis software are reported in quick succession, several bottlenecks for the application of proteomics to clinical applications remain. Until recently one of the major bottlenecks was the lack of advanced analysis software options for large datasets of LC-MS experiments. Especially retention time alignment has long remained a difficult issue, as retention times can vary between runs. For meaningful comparison between the samples these small differences in retention time need to be corrected, and this is typically done by advanced software options, which have become available in recent years (64). However, this was not the only bottleneck for proteomics applications in biomarker research. Additional, still remaining, issues are the techniques' bias towards high abundant proteins, the lack of validation of found biomarkers, the perception of limited reproducibility of mass spectrometry-based proteomics research and the lack of clear, well-defined bioinformatics analysis options to relate the mass spectrometry data to the underlying biology. The proteomics field is rapidly developing and new advances in technology and analysis software are reported in quick succession, several bottlenecks for the application of proteomics to clinical applications remain. Until recently one of the major bottlenecks was the lack of advanced analysis software options for large datasets of LC-MS experiments. Especially retention time alignment has long remained a difficult issue, as retention times can vary between runs. For meaningful comparison between the samples these small differences in retention time need to be corrected, and this is typically done by advanced software options, which have become available in recent years. However, this was not the only bottleneck for proteomics applications in biomarker research. Additional, still remaining, issues are the techniques'

bias towards high abundant proteins, the lack of validation of found biomarkers, the perception of limited reproducibility of mass spectrometry-based proteomics research and the lack of clear, well-defined bioinformatics analysis options to relate the mass spectrometry data to the underlying biology.

7.1. Protein enrichment or depletion

The large dynamic range of proteins in biological samples is one of the major obstacles in clinical proteomics. As high throughput sample fractionation techniques are limited in capacity, additional steps are often required to detect and quantify the lower abundant proteins (65). Two main options exist to enable the detection of these proteins; depletion of high abundant proteins and specific enrichment of a group of target proteins.

Depletion of high abundant proteins increases the dynamic range of the measurement for the remaining proteins (66). Commercial products have been developed for depletion of up to 20 high abundant proteins from biological samples with high reproducibility. However, one of the main problems with depletion techniques is the specificity of the depletion procedure. Several high abundant proteins, like albumin for example, are transport proteins to which many other proteins can attach. By removing albumin from the complex mixture, the proteins that albumin binds to, are co-depleted, which affects the remaining, depleted sample with regards to its content (67). So, while the removal of the high abundant proteins is reproducible, the side effects of this procedure may be quite variable.

One of the alternatives to protein depletion is targeted enrichment, which is a straightforward fractionation strategy for the detection of low abundant proteins (68). Using targeted enrichment strategies specific sub-proteomes can be isolated from complex samples which greatly enhances the capacity for the detection of low abundant proteins. Affinity to azide resins and immobilized metal ions can be exploited to enrich for post translational modifications, such as glycosylation (69) and phosphorylation (70), respectively. Post translational modifications are known to play a major role in pathophysiological processes, and consequently specific enrichment of these modifications is of significant interest for elucidation of disease pathology. There are a large number of affinity capture approaches, as any epitope or functional group one can raise an antibody to, or can design a chemical coupling to, like active sites of enzymes, can be captured (71).

Due to the influential inflammatory component of multiple sclerosis affinity enrichment of immunoglobulins would be an interesting avenue to explore. In lung carcinoma it has recently been shown that patients have different amino acid sequences in the complementarity determining regions of IgG compared to controls (46). This would be of considerable interest for multiple sclerosis, because immunoglobulins have

been widely reported to be involved, and multiple sclerosis-specific complementarity determining regions could be very intriguing biomarkers. However, all these targeted enrichment strategies are limited by their lack of high throughput and by the fact that researchers need to already have defined a target proteome before analysis commences and consequently can only study a part of the total proteome in a given experiment.

7.2. Biomarker validation

One of the key components of a biomarker study is the validation phase. Unfortunately this part of the biomarker pipeline has also long been one of the bottlenecks of biomarker research (72). Immunoassays and antibodies allow for analysis of small numbers of proteins, but the lack of commercially available reagents, combined with high costs, lengthy production and purification times, have rendered the large majority of biomarker candidates untestable. MS-based quantification methods, SRM (Figure 3) in particular, are interesting alternatives to immunoassays due to their independence on antibodies and the possibility of rapid and cost-efficient development (73, 74). This targeted MS technique is not burdened by Bonferroni correction requiring multiple testing issues, which are potentially problematic in other MS methods. Additionally, in contrast to immunoassays, SRM techniques offer good multiplexing capabilities, enabling researchers to simultaneously quantify numerous peptides in parallel. SRM quantification uses stable isotope labelled peptides as internal standards, which must be synthesized for all candidate markers to ensure the best possible results. One pos-

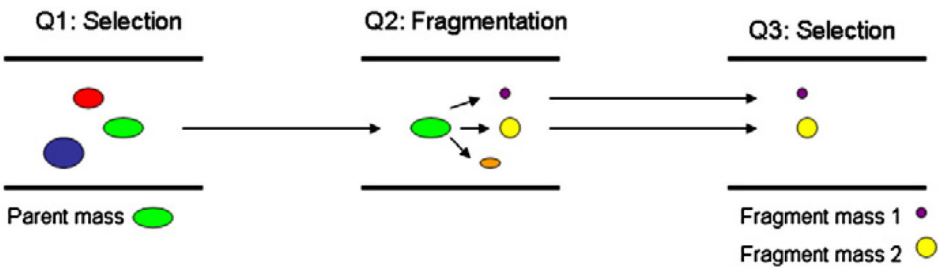

Figure 3. Schematic of a SRM measurement. In SRM measurements complex (digested) biological samples can be quantitatively measured by using the quadrupoles (Q1, Q2 and Q3). In the first quadrupole the target (the parent mass) is selected. Only molecules of that mass are allowed to enter the second quadrupole, in which this molecule is fragmented in specific parts. Then in the third quadrupole one of the fragments is selected, and only this fragment is permitted to reach the detector where it is measured. The sensitivity of the measurements can be increased by fractionation of the biological samples before entry into the first quadrupole, for example by liquid chromatography gradient. By programming the machinery for multiple parent masses, these SRM measurements can be multiplexed and a relatively large number of targets can be quantified in a single experiment, provided that for all targets an internal standard is present in the sample. For accurate quantitative measurements multiple transition states (multiple fragment masses) are preferred, as this increases the accuracy of the measurements significantly.

sible drawback of SRM for peptide quantification is the limited concentration range in which reliable quantification in complex, unfractionated biological samples can be performed. Sample fractionation, followed by SRM quantification is possible with low CV's in biological samples, but this substantially decreases the high sample throughput, which is one of the main strengths of SRM protein quantification (75). However, the high reproducibility across laboratories and instrument platforms as reported by Addona et al. is a very strong selling point for SRM protein quantification (76) that should foster greater acceptance by the clinical community of this type of technology for validation of candidate biomarkers. And while it seems unlikely that immunoassays will be replaced by MS-based protein quantification in the near future, even though both methods are similar with regards to sensitivity, SRM-based approaches, coupled with expected developments in MS instrumentation with regards to specificity and detection limits, might facilitate the alleviation of one of the big existing bottlenecks in biomarker studies.

7.3. Reproducibility

To understand accurate protein dynamics, a highly reproducible proteomics analytical method is required (77). As proteomics studies often include extensive sample pre-treatment procedures, this required reproducibility is not merely limited to the mass spectrometry method but extends to every single part of the process from sample collection to data analysis. Currently published proteomics biomarker studies for multiple sclerosis show only limited overlap between them, but this is not necessarily due to a lack of reproducibility, but rather due to differences in approach and clinical starting material. Different sample pre-treatment methods and different mass spectrometry tools are of great influence to the end results in these types of studies, which must be accounted for when assessing these data. The fact that different mass spectrometry techniques have confirmed protein differences of patients compared to controls on multiple occasions is quite promising and to be expected based on the current assessment of proteomics reproducibility in the scientific community (78-80). In fact, the sample pre-treatment and clinical parameters of the sample appear to be of greater influence on reproducibility than the mass spectrometry methods. Therefore, it appears that two factors are essential for enhanced reproducibility of biomarker studies. The first is adherence of researchers to a consensus on sampling, storage and biobanking of biological samples (25), as this would limit variations in samples and reproducibility issues prior to commencement of the proteomics study. This would also allow easier exchange of samples between institutes to increase sample sizes in proteomics studies, which would allow for more advanced statistical analysis, and consequently better defined results. Secondly, a standard has recently been proposed for reporting on proteomics studies (81). Reporting proteomics studies according to the recommendations

proposed by Mischak et al. would increase the quality standards and scientific validity of these reports and could power significant progress in clinical medicine.

7.4. Bioinformatics

Processing and management of acquired mass spectrometry data is one of the major bottlenecks in proteomics studies. Due to the fact that data is generated in large quantities, extraction of the meaningful knowledge from the results presents especially large challenges (82). After resolving one of the major bottlenecks in the application of biocomputing to proteomics data by development of a standardized universal output format for mass spectrometry data (83), two major issues remain. The first issue is the use of a large number of different databases, which includes publicly available well maintained libraries as well as home-made derivates consisting of sub-populations of these libraries, for peptide and protein identification by proteomics researchers. Although the plethora of available databases perhaps increases the chance of identification of peptides from MS/MS spectra, chances of reproduction of the results by other laboratories are decreased and results become complex and confusing due to the lack of insight in the contents of these databases. A major step forward in this area would be the systematic use of an up-to-date, well maintained database accessible by all researchers and laboratories. The second, more complex issue is the development of standard approaches to discern relevant biological networks and pathways from proteomics data. This is an essential step for the application of proteomics techniques on biological issues and should be given high priority, as these developments are still lagging behind.

8. FUTURE PERSPECTIVES

Mass spectrometry-based proteomics is a research field that has depended largely on developments in instrumentation during its recent period of rapid growth. Only very recently developments in analysis software, sample pre-treatment applications and biological sample availability have begun to catch up. The increasing interest of life sciences in proteomics still stands to gain significantly from improving those specific areas further.

Aberrant cleavage of proteins combined with the biological activity of many peptides in body fluids in normal and diseased states, makes analysis of endogenous peptides and small molecules an interesting alternative to trypsin-digested proteome. Analysis of endogenous peptides, a research field labelled peptidomics (84), could be an interesting addition to tryptically digested proteins for detection of biomarkers, which has already been shown to be applicable to multiple sclerosis CSF studies (49).

Proteomics is only one of the "omics" disciplines, and while it is a key part of Systems Biology due the effector role that proteins play in biological processes and the fact that proteins are very rich in information, the other "omics" are also of great value for biomarker studies. Integration of data from multiple disciplines, like for example fusion of metabolomics and proteomics data as shown by Blanchet et al. in an EAE model for multiple sclerosis (85), is a new approach to combine a larger part of Systems Biology in a single analysis. Additionally, mRNA expression profiles and protein expression profiles contribute to a more refined description of biological systems due to the fact that they appear to be largely complementary (86). So combining the different "omics" fields in experiments from the same biological system would seem to increase our understanding of complex biological processes.

Adherence to the recommendations and protocols for sampling, sample storage and reporting on proteomics biomarker studies (25, 81) should also be very beneficial for the advancement of proteomics in clinical settings. The standardized sampling and storage will allow for collaboration between research groups without variation in the results due to differences in sample handling prior to the mass spectrometry measurements. The main advantage here will be the possibility to create larger sample sets which would allow for more advanced analysis and consequently more reliable results. Additionally the reporting standards for proteomics studies will be greatly beneficial for the reproducibility of the results and the ability of outsiders to compare between proteomics studies. In particular the characterization of the samples, which would also allow for better matching between cases and controls (87), and the details on the bioinformatics analysis are of great import in this area (82).

Finally, the introduction of new instrumentation and instrument application remains a strong force in the advancement of proteomics for biomarker research. Recently mass cytometry, a combination of mass spectrometry and flow cytometry, was introduced as an elegant option for the analysis of target analytes in single cells (88). Using this technique, mass spectrometry can be elevated from a level in which a group of cells, isolated by laser capture microdissection for example, are examined, to a level where mass spectrometry examines every cell as an independent "test tube" (89). Isolation and mass spectrometry analysis of T lymphocytes at the initiation of immune responses using mass cytometry would of great interest for multiple sclerosis, as lymphocyte migration to the central nervous system is one of the key events in the disease.

9. CONCLUDING REMARKS

Biomarkers for disease diagnosis, monitoring of disease progression, monitoring of the effects of treatment and disease prognosis are urgently needed for complex disorders

such as multiple sclerosis. Despite the fact that mass spectrometry based techniques are limited by a variety of factors, like for example the large dynamic range of proteins in biological samples, new developments are rapidly emerging to take proteomics to the next level. By standardizing critical elements of proteomics studies in the area of sample collection, sample storage, sample documentation and data analysis, combined with the constantly growing progress in the field, proteomics can potentially evolve to a level of genuine clinical proteomics in the near future.

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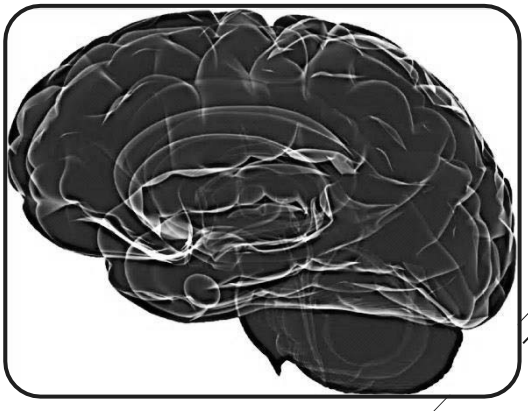
Chapter 1/1.2

General aspects of multiple sclerosis



Multiple sclerosis (MScl) is a chronic, autoimmune, inflammatory neurological disease of the central nervous system (CNS) (1). MScl attacks the myelinated axons in the CNS, and destroys the myelin and the axons to different degrees (2, 3). It has a lifetime prevalence of 1 per 1000 individuals in the western world (4). It is twice as common in women as in men, and people of Northern European descent happen to be at the highest threat for MScl (1, 2, 5). The MScl is diagnosed on the basis of clinical findings and supporting evidence from additional tests, such as magnetic resonance imaging (MRI) of the brain and testing for oligoclonal bands in cerebrospinal fluid (CSF). MScl usually presents in adults of 20 to 45 years age. Sometimes, MScl presents in childhood or late middle age (5). The cause of MScl is not known, but it seems to involve a combination of genetic susceptibility and a nongenetic trigger, for example a virus, change in metabolism, environmental factors, which all together result in a self-sustaining autoimmune disorder that leads to recurrent immune attacks on the CNS (5). No single diagnostic test exists for MScl (1). Diagnosis is based on the evidence of (a) at least two different lesions (plaques or scars) in the white matter of the CNS; (b) At least two different episodes during the disease course; and (c) chronic inflammation of the CNS, which is determined by analysis of the CSF. This is in the absence of an alternative explanation for the clinical signs or symptoms. A general diagnosis of MScl is allowed in the presence of one or more of these criteria, which may be refined according to the subsequent course of the disease.

TYPES OF MScl

The MScl patients can be categorized into four major groups based on the course of the disease (1) (Figure 1):

(A) Relapsing remitting (RR) MScl

This is the most common form. It affects about 85% of MScl patients. Most people with this type of MScl first experience symptoms in their early 20s. It is noticeable by flare-ups (relapses) of symptoms followed by periods of remission, when symptoms improve or disappear. The patterns of nerves affected, severity of attacks, degree of recovery, and time between relapses varies from person to person. Eventually, most people having RR MScl will enter a secondary progressive phase of MScl.

(B) Secondary progressive MScl

This type of MScl may develop in some patients with RR MScl. The disease course continues to worsen with or without periods of remission or leveling off of symptom severity.

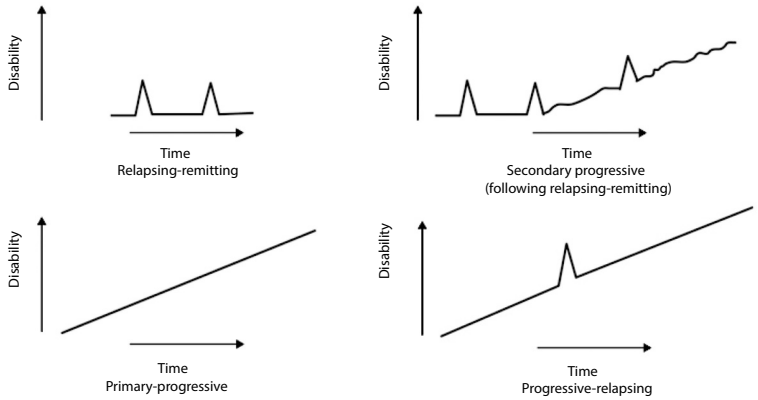


Figure 1. MScl Classification. Graphical representation of four types of disease courses. All the graphs display level of disability over time.

(C) Primary progressive (PP) MScl

It affects approximately 10-20% of MScl patients. In PP MScl, symptoms continually worsen from the time of diagnosis. There are no well-defined attacks, and there is a little or no recovery. There are several aspects of PP MScl that differentiates it from other types of MScl, such as 1) at the time of diagnosis people with PP MScl are usually older than an average age of 40; 2) This type of MScl usually leads to disability earlier than RR MScl; 3) Roughly equal numbers of men and women develop PP MScl. Whereas in other types of MScl, women outnumber men by three to one.

(D) Progressive-relapsing MScl

This is a rare form of MScl. It affects less than 5% of patients. From the start it is progressive with intermittent flare-ups of worsening symptoms along the way.

Variation in clinical course reflects the complexity in MScl pathophysiology. Categorization of MScl is important to help with the prediction of disease severity and response to the treatment.

CHILDHOOD-ONSET OF MScl

MScl that onset before the age of 18 is defined as childhood MScl. Nearly, 3-5% of all individuals with MScl experience disease onset before the age of 16 (6, 7). It is increasingly recognized and represents 2-5% of the total MScl cases (7). This disorder was previously known as early onset MScl. Once diagnosed, almost all children are considered to have RR MScl, with most symptoms of MScl similar to those seen in adults. Children experience 2-3 times more frequent relapses than adults with early

onset (8). Generally, children with MScl have a higher relapse rate and reach permanent disability at a younger age than adult-onset MScl. In children, progression of motor disability as measured by an expanded disability status scale (EDSS) takes longer than in adults (9). Secondly, childhood onset MScl patients have a higher T2 lesion (part of conventional MRI technology) burden than adult MScl patients, which supports the conclusion that MScl in children is more inflammatory (10). Diagnosing of MScl in children is more challenging than in adults due to the frequency of other childhood disorders with similar symptoms and characteristics. The main challenge is to exclude other CNS disorders that could mimic MScl, and to distinguish between MScl and various temporary demyelinating syndromes that can occur in children. Some of these patients are reclassified as MScl based on the nature of the clinical events, laboratory findings, and subsequent MRI changes. While the risk of developing MScl following an episode of acute disseminated encephalomyelitis (ADEM) in childhood is <10%, the risk following an episode of clinically isolated syndrome (CIS) has been shown to be between 26% to 62% in several recent studies using the international study group criteria (11, 12).

Early and correct diagnosis of childhood onset MScl is essential for immediate treatment to diminish ongoing neuro-inflammation and irreversible neurodegeneration (13) and still remains an understudied topic.

MScl PREGNANCY (A NATURAL DISEASE MODIFIER)

MScl affects women in their pregnancy years. In this respect, the first large prospective study of natural history in pregnant women with MScl was PRIMS (pregnancy in multiple sclerosis) study (14). This study clarified the possible influence of pregnancy and delivery on the clinical course of MScl (14, 15). The study showed reduction in the relapse rate during pregnancy, as compared to the year before pregnancy, especially marked in the third trimester, and a significant increase in the relapse rate in the first trimester after postpartum (Figure 2).

Several other studies also confirmed the same (16). It is interesting to note that the decrease in relapse rate during pregnancy is more pronounced than the effect obtained with any disease modifying treatment (15). It seems that pregnancy has a protective effect on the disease activity, which is also reproduced in the experimental autoimmune encephalomyelitis (EAE) model in guinea pig, rats and rabbits (17, 18). During pregnancy high placental and maternal production of sex hormones is known and these are the most intensive biological features (Figure 3). It is also shown that oestrogen and progesterone can suppress EAE (19). Pregnancy is also characterized by major immunological changes that recedes with delivery. In this regard, a shift from cell mediated

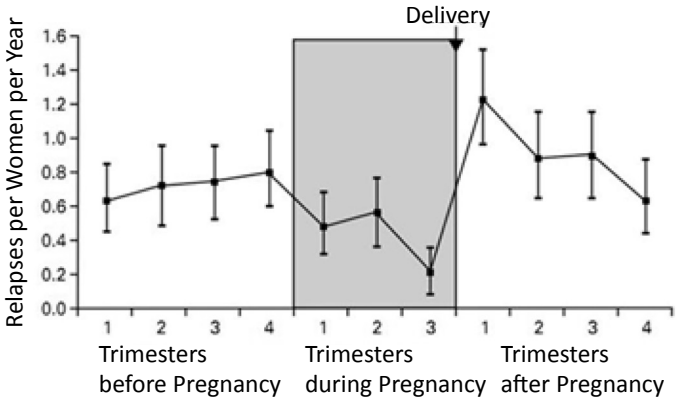


Figure 2. Figure shows the rate of relapses per women per year for each three month period before, during and after pregnancy. Reproduced with permission; Brain (2004); 127:1353-1360.

Thy1 responses to enhanced humoral immunity and a Th2 profile is also noted during pregnancy (20) (Figure 3). The fetal placental unit secretes cytokines, such as IL10, that down regulates the production of maternal factors mediating cellular immunity. On the contrary, delivery might be associated with reversal of this cytokine balance-similar, in some respects, to the process of graft rejection (20). Further, there are systemic changes that have a direct impact on the activity of the disease process in MScl (21).

A better understanding of the biological mechanisms underlying these pregnancies related changes in disease activity could usefully illuminate ideas on the pathogenesis and may even suggest new treatment strategies. For this purpose body fluids such as urine can be studied as a marker of disease activity (22).

DISEASE-MODIFYING THERAPIES (NATALIZUMAB)

Currently, no Food and Drug Administration Agency (FDA) approved treatment exists for MScl patients. Disease-modifying agents, shortens the duration of acute exacerbations, decreases their frequency, and gives symptomatic relief (5) thus maintaining function and improving the quality of life (24). There are eight FDA-approved therapeutic agents, which can decrease the disease activity and progression in RR MScl patients. Natalizumab (Tysabri) is one of these therapeutic agents.

In MScl patients, the formation of inflammatory lesions may contain lymphocytes (T cells, B cells and NK cells) and monocytes, that gain access to the brain parenchyma from the circulation by first adhering to vascular endothelial cells (25, 26).

Natalizumab is an effective drug for the treatment of RR MScl. It is a recombinant humanized immunoglobulin (IgG4) monoclonal antibody that is produced from mam-

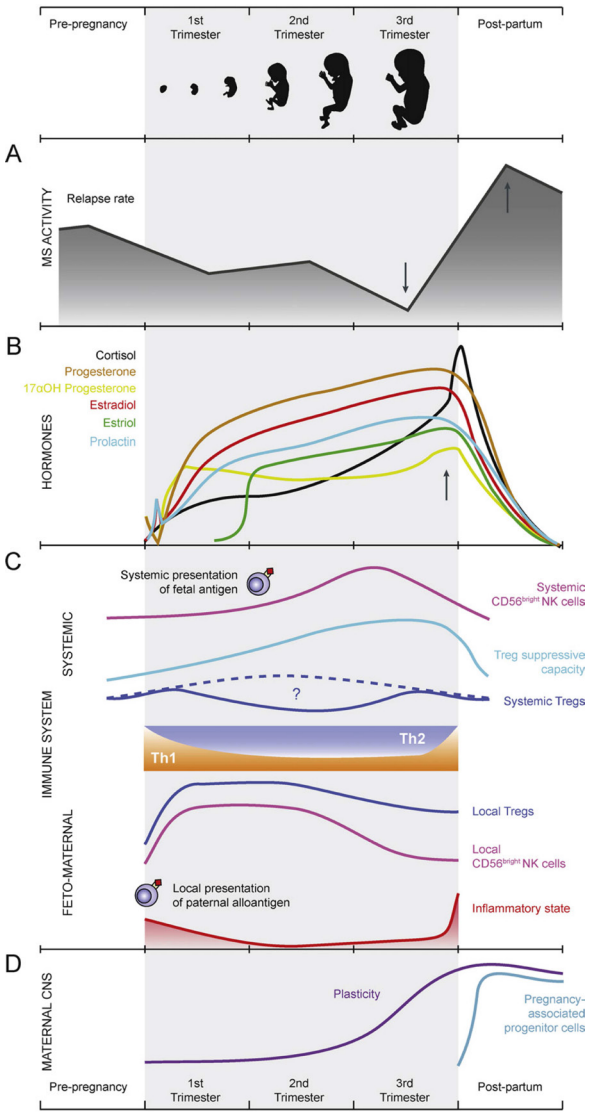


Figure 3. Pregnancy and MScl. (A) MScl relapse rate decreases during the third trimester (arrow) and increases in postpartum period (arrow).
(B) Immunomodulatory properties of raised steroid hormones might be a cause for beneficial effects of pregnancy. They reach a peak during the third trimester of pregnancy (arrow) therefore overlap with the strongest decrease in relapse rate.
(C) Immunological processes occur both locally at the feto-maternal interface and to a smaller extent systemically. Although a systemic Th2-like shift is evident starting in the second trimester and lasting until pre-parturition.
(D) The advantageous effects of pregnancy in MScl may not be restricted to variation of the maternal immune system, but could in part also reflect effects on the maternal CNS, either by increasing resilience to immune-mediated attacks or by stimulating endogenous repair mechanisms. (Reproduced with permission, J Reprod Immunol. (2013); 97:140-6, see reference (23).

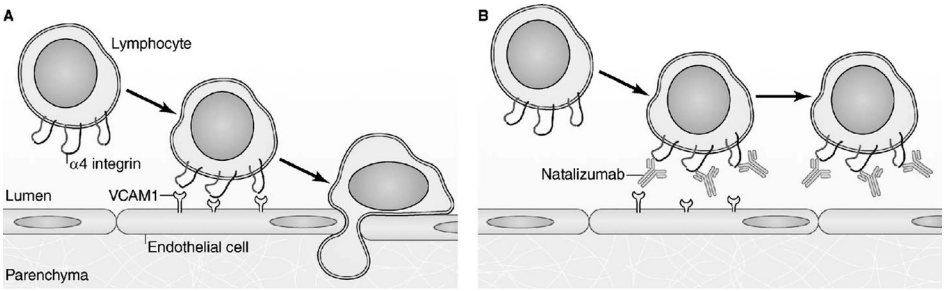


Figure 4. Figure shows blockade of lymphocytic diapedesis by Natalizumab in MScl.
(A) Alpha 4 integrin binds to vascular cell adhesion molecule 1 (VCAM1) on inflamed brain endothelium. This interaction provides lymphocytes entree to the CNS.
(B) Natalizumab blocks binding of lymphocytes to VCAM on inflamed brain endothelium, therefore preventing lymphocyte access into the CNS. (Reproduced with permission; J. Cell Biol. (2012) 199: 413–416)

malian cells. It is the first in a class of agents named selective adhesion molecule inhibitors. Natalizumab binds to the 4 subunit of α4β1 (CD49d; VLA4) and α4β 7 integrins expressed on the surface of leukocytes (except neutrophils), and it inhibits the α4 mediated adhesion of leukocytes to their counter receptors (2) (Figure 4). In MScl patients, Natalizumab treatment is associated with a reduced migratory capacity of immune cells and a prolonged decrease in lymphocyte counts in the CSF (27, 28). Natalizumab markedly reduces the attack rate in patients with MScl and significantly improves all measures of severity (29). Like the beta interferon and glatiramer acetate, Natalizumab's precise mechanism of action in MScl patients has not been completely defined. Treatment with Natalizumab might have different side effects that require monitoring. Adverse effects must be recognized and identified.

With respect to the above, the effect of Natalizumab in MScl patients before and after treatment should be evaluated. Therapy biomarkers can state whether it is successful and functional or not.

NEUROBIOLOGY

Neurons are cells that transmit information to other nerve cells, muscle, or gland cells. They have a cell body, an axon, and dendrites. Small cellular processes called dendrites receive the input. A longer process called an axon, or nerve fiber, carries the information away from the cell in the form of bioelectric signals, often called impulses. It allows the neuron to communicate with other neurons and with cells outside the nervous system. Neurons and their axonal and dendritic processes embedded in a glial network provide additional structure and function. The brain contains at least ten times

more glia (also known as glial cells or, neuroglia) than neurons. Glia contributes to the cellular architecture of CNS. It is the non-neuronal cells that maintain homeostasis, form myelin, and provide support and protection for neurons in the brain and peripheral nervous system. They are of four types: 1) Astrocytes (star-shaped cells), these are commonly found between neurons and blood vessels. They provide architecture for neurons and define anatomical boundaries and also act as a source of growth factors and cytokines; 2) Oligodendrocytes, these resemble astrocytes but are smaller and have fewer processes. They form in rows along myelinated axons, and they synthesize and maintain the myelin sheath; 3) Microglia, are small cells and have fewer processes than other types of neuroglia, they are scattered throughout the CNS, where they help support neurons and phagocytize bacterial cells and cellular debris; 4) Ependyma, form the inner lining of the central canal that extends downward through the spinal cord, these cells also cover the specialized capillaries called choroid plexuses associated with the ventricles of the brain. Here they help to regulate the composition of CSF.

MScl PATHOLOGICAL FEATURES

MScl pathology is characterized by inflammation, demyelination (with limited remyelination), axonal injury and astrocytic scar formation. Although MScl is classically considered as white matter disease (31, 32), the recent methodological approach also point to strong contribution of cortical gray matter damage. The pathology of grey matter lesions varies from white matter lesions. The cortical lesions (at autopsy) are less inflammatory, with less macrophage, lymphocyte infiltration and decreased complement activation (33) as compared to the white matter lesions. However, cortical inflammation can be an important feature also in early MScl stages (32). A detailed evaluation of the white matter pathology shows four different lesion patterns (34), all of them harboring macrophages, T cells and some B cells.

MScl IMMUNOLOGY

B cells and autoantibodies in the pathology of MScl

MScl is characterized by increased permeability of blood-brain barrier (BBB); this allows immune cells such as macrophages, T and B cells, to infiltrate into the CNS. According to the traditional view of MScl pathophysiology, CD4+ T helper cells react with myelin components and trigger an inflammatory cascade in the CNS that results in demyelination and axonal loss. However, there are several studies that suggest the involvement of B cells and autoantibodies in the pathology of this disease (35-38).

A clarification for this shift of focus (39) towards B cells in the pathogenesis of MScl comprises: (a) An intrathecal immunoglobulin (Ig) synthesis with the oligoclonal Ig band in the CSF; (b) The clonal expansion of B cells in the CSF. Clonal expansion of B cell populations in the lesion is indicated by the antibody spectrum characterized fluid or in the tissue itself. Some data suggest that the process is driven by specific antigens itself. Relatively less is known about the antigen specificity of B lymphocytes and plasma cells in MScl lesions;

(c) In the meninges of some patients with a progressive disease course, presence of follicle-like aggregates is observed. Presence of B cells, plasma cells and excess Ig both in brain lesion and CSF of patients with MScl also provides a basis for treatments directed against B cells. Four possible mechanisms for B cells associated MScl pathophysiology are described such as: (a) antigen presentation, (b) antibody production, (c) cytokine regulation and (d) the formation of new lymphoid structure in the meninges. B cells express a number of surface receptors at different phases of their development, for example CD19, CD20, CD40, CD22 and BAFF-R. In MScl, certain stages of B cells maturation may be crucial for determining the pathogenicity activity of B cells. B cells have pro-inflammatory as well as anti-inflammatory functions. Previous studies, conducting clinical trials with rituximab and ocrelizumab, monoclonal antibodies which deplete cells of the CD20 positive B cell lineage, show that the pro-inflammatory features of B cells dominate in most of the MScl patients. Still, as those antibodies spare long-lived plasma cells (which lack CD20) and therefore should hardly remove the amount of circulating Ig, their effect on autoantibodies is more complex and suggests that the depletion of B cells is beneficial in a manner independent of Ig (40).

Immunoglobulin G (IgG)

IgG play a role in MScl courses in at least a subset of patients. This is suggested by some evidences, for instance: (a) Oligoclonal antibody response generated by a limited repertoire of activated B cells, a diagnostic finding in the CSF (36). The increased level of IgG in the CSF represents an intrathecal antibody synthesis, which is related with oligoclonal banding and it also indicates that an immunological process is active in the CNS. Oligoclonal bands are shown to found in >95% of patients with clinically definite disease (41); (b) Therapeutic success of plasma exchange in a proportion of MScl patients (42); (c) The demonstration of demyelinating and axopathic IgG in MScl patient serum (43), and (d) The Ig deposition and complement activation in a subset of MScl patients (34).

In B-cell immune responses, IgG play several important roles, first by inserting on the surface membrane, they act as a receptor, bind to specific antigens and transmits activation signals that start the immune response. Furthermore, in secreted soluble form they function as antibodies, binding and assigning antigen for destruction by

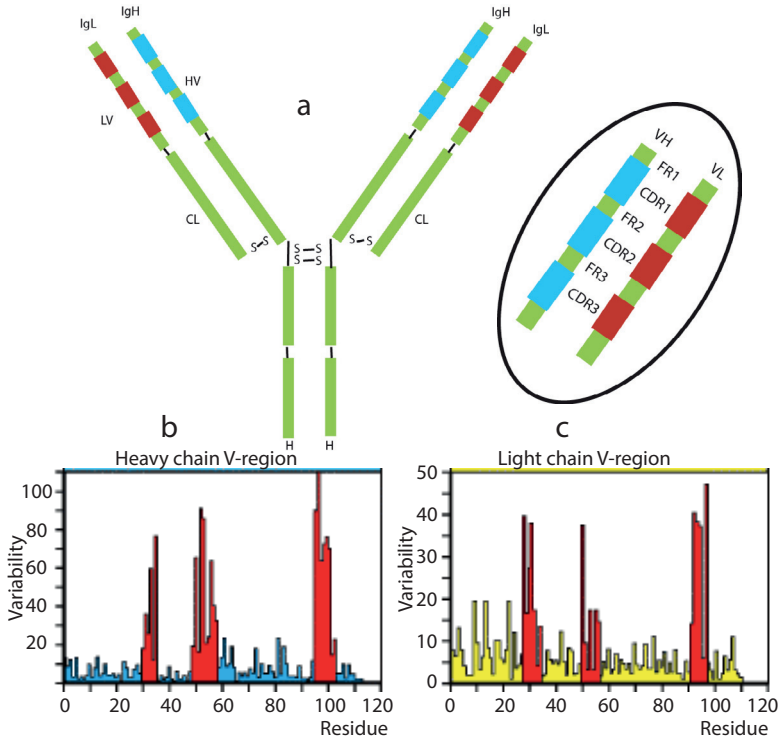


Figure 5. The IgG molecule (a) comprises two of each type of light (IgL) and heavy (IgH) chain. These are covalently joined by disulphide bonds (shown as S–S) between cysteine residues. The variable domains (V) of the heavy chain (VH) and the light chain (VL) contribute to the binding site of the antibody molecule. The variable regions of the IgH and IgL chains, contains framework regions (FR1, FR2 and FR3) and three hypervariable or complementarity-determining regions (CDRs; CDR1, CDR2 and CDR3). CDRs determine the recognition and binding site to the antigen, in the three-dimensional structure. Figure (b and c) shows variability in V regions of the IgH and IgL chains. The degree of variability, at each different position is, graphically shown for the entire V region of IgH (b) and IgL chain (c). The three arms of hypervaribility CDR1, 2 and 3 residue position numbers are shown on the X axis.

macrophages or proteases (via complement), thus exerting effector functions of the immune response.

In both situations, Igs use the variable (V) segment. The Ig V region and corresponding antigenic epitope fit together no less tightly than the key and its lock. The V region of the Ig is formed in the course of B cell differentiation through a complex series of diverse events. The step in naïve B cell development includes recombination of germline genes, addition of non-germline (N) encoded elements, and somatic mutation to improve further the fit (affinity) of Ig after an encounter with the actual antigen.

Typical monomeric Ig is made up of two light and two heavy chains interconnected by disulphide bonds. Binding sites, each formed by the V regions of adjacent light and

heavy chains. The Ig V regions are thus the structural basis for antibody diversity. Ig V regions are composed of framework segments with genetically conserved sequences and interspersed hypervariable region (sequences specific for each individual specific Ig). They combine to form the molecular surface of an antigen-binding site. Structural genes for the Ig V region fall into three sets: variable (V), diversity (D) and joining segments clustered on the chromosome as linearly arranged gene segments. Each group contains a large number of individual genes and human heavy chain has hundreds of V, dozens of D and several J genes (44) (Figure 5).

NEED FOR MScl BIOMARKERS IN BODY FLUIDS

MScl has pathophysiological complexities; it is a heterogeneous disease with respect to clinical symptoms, neuroradiological findings and treatment responses. MScl is normally diagnosed only after repeated bouts, that is, only after some time following disease onset. A golden standard for the diagnosis of MScl is inadequate. However, the diagnosis of MScl relies on a combination of clinical features with imaging and CSF abnormalities (oligoclonal Ig bands). These later changes can be counted among immunological biomarkers, but they are not specific for MScl. In terms of disease management, early diagnosis and appropriate and timely therapeutic intervention are crucial factors in establishing favorable outcomes. Thus, there is an urgent requirement for easily accessible biomarkers. Mass spectrometry-based proteomics approach has developed as a powerful method that has the potential to accelerate MScl biomarker discovery in body fluids.

OUTLINE OF THE THESIS

This thesis focuses on five topics in MScl: 1) two major subtypes, 2) early-onset, 3) involvement of pathogenic IgG (auto) antibodies (humoral immune response,) 4) effect of disease modifying therapy (Natalizumab), and 5) pregnancy.

The goal of the study on two different MScl subgroups (RR and PP MScl), was to differentiate and to identify bioclinical markers by comparing CSF proteins. For childhood-onset MScl study, our aim was to improve diagnosis. The main goal in this study was to find CSF protein markers expressed during first event of CNS demyelination that can help to distinguish children with MScl from children with monophasic ADS. The aim of our study on humoral immune response in CSF was to perform the clonal characterization of the CSF IgG antibody repertoire and to find common characteristics of the antigen binding sites exclusively among RR MScl patients. Further, we monitored

the effects of Natalizumab drug treatment on RR MScl. This was to study potential CSF markers that are relevant for CNS pathology and identify the protein markers for effects of the therapy. The main objective of our study on pregnancy in MScl was to identify biological mechanism of the decreased activity of disease during pregnancy. To this we performed a longitudinal study in RR MScl patients at 28-30 weeks during pregnancy and at 4-8 weeks after delivery. To quantify and identify disease associated proteins different types of high resolution mass spectrometer was applied on samples from MScl patients. Overall, the thesis here emphasized the identification of biomarkers related to MScl and to improve further understanding of the disease.

This thesis can be divided into six parts:

Chapter 1.1 outlines the current research scenario of the MScl and a detailed number of bottlenecks as well as a future prospect of mass spectrometry based proteomics biomarker research.

Chapter 1.2 describes the general characteristics of MScl.

In **Chapter 2** we report the CSF proteomic profile of two different MScl types, PP and RR MScl. In this chapter, we set out to identify the proteins that differentiate between the two disease types using quantitative MALDI-FT-MS mass spectrometry.

Chapter 3 deals with the childhood onset MScl and monophasic ADS. In this chapter we describe the results of a nano scale liquid chromatography coupled online to high resolution mass spectrometer (LC-MS) based trypsin profiling study for quantification and identification of proteins in CSF samples of the children with MScl.

In **Chapter 4** we have investigated clonality of CSF IgG using LC-MS. The aim of this work is to detect specific proteomic profiles of CDR (in CSF IgG) present in MScl patients but absent in controls.

In **Chapter 5** we describe the results of a LC-MS tryptic profiling study on RR MScl patient's CSF before and after one year of Natalizumab treatment. The differentially expressed proteins were confirmed by SRM and ELISA measurements.

In **Chapter 6** we describe the result of urinary proteome analysis of pregnant women (during third trimester and at first postpartum period) with MScl using LC-MS. We describes the identification of protein that are related to pregnancy and proteins that change significantly different in MScl compared to normal pregnancy.

In **Chapter 7** the finding of the studies are summarized and discussed with the indication for future directions.

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Chapter 2

Proteomics comparison of cerebrospinal fluid of relapsing remitting and primary progressive multiple sclerosis

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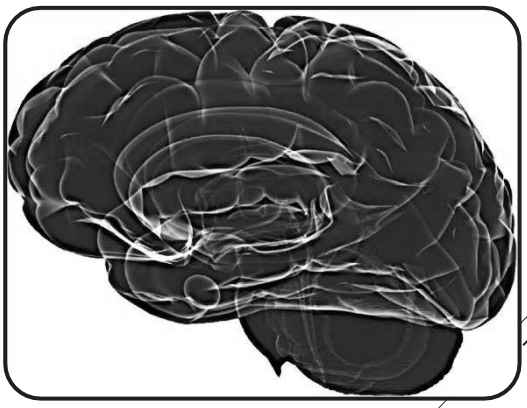

ABSTRACT

Based on clinical representation of disease symptoms multiple sclerosis (MScl) patients can be divided into two major subtypes; relapsing remitting (RR) MScl (85-90%) and primary progressive (PP) MScl (10-15%). Proteomics analysis of cerebrospinal fluid (CSF) has detected a number of proteins that were elevated in MScl patients. Here we specifically aimed to differentiate between the PP and RR subtypes of MScl by comparing CSF proteins. CSF samples (n=31) were handled according to the same protocol for quantitative mass spectrometry measurements we reported previously. In the comparison of PP MScl versus RR MScl we observed a number of differentially abundant proteins, such as protein jagged-1 and vitamin D-binding protein. Protein jagged-1 was over three times less abundant in PP MScl compared to RR MScl. Vitamin D-binding protein was only detected in the RR MScl samples. These two proteins were validated by independent techniques (western blot and ELISA) as differentially abundant in the comparison between both MScl types. The main finding of this comparative study is the observation that the proteome profiles of CSF in PP and RR MScl patients overlap to a large extent. Still, a number of differences could be observed. Protein jagged-1 is a ligand for multiple Notch receptors and involved in the mediation of Notch signaling. It is suggested in literature that the Notch pathway is involved in the remyelination of MScl lesions. Aberration of normal homeostasis of Vitamin D, of which approximately 90% is bound to vitamin D-binding protein, has been widely implicated in MScl for some years now. Vitamin D directly and indirectly regulates the differentiation, activation of CD4+ T-lymphocytes and can prevent the development of autoimmune processes, and so it may be involved in neuroprotective elements in MScl.

INTRODUCTION

Multiple sclerosis (MScl) can be divided into two major subtypes based on clinical representation of disease symptoms in the patients (1). Between 85-90% of patients can be classified as having the relapsing remitting (RR) MScl subtype, in which disease relapses are followed by periods of remission, and 10-15% of all MScl patients are diagnosed with the primary progressive (PP) subtype (2). Even within a single large Dutch MScl pedigree of 26 patients with similar genetic background, the percentage of patients with a PP phenotype remained 15% (3).

By definition, in PPMScl progression is characterized by a progressive course without relapses or remissions from the onset of the disease (4). PP patients tend to have lower inflammatory lesional activity, for which no immunological or genetic explanation has been identified yet. The scarce comparative neuropathological studies show a large overlap in lesional pathology, but indicate less inflammatory activity for PP, with still substantial axonal damage (5). The general picture is that relapse onset and PP forms share substantial characteristics. In other words, it has remained a challenge to identify the biological parameters that determine a PP disease course.

Although proteomics analysis of active multiple sclerosis lesions may be a straightforward approach to study the processes involved in MScl pathways (6), this is very difficult to perform in living individuals. In most cases the pathology of the disease can only be investigated in post-mortem material, which quite frequently represents the end-stage of the disease. The study of CSF taken during disease appears a good alternative. CSF is in close contact with the CNS parenchyma and collects the products of the inflammatory and neurodegenerative processes of MScl activity.

Proteomics analysis of CSF has detected a number of proteins that were elevated in MScl patients (7, 8). Additionally, differentially abundant proteins identified by proteomics, such as apolipoprotein A1 (9) and chromogranin A (10) were validated by other techniques. Other studies added additional data on elevated immunoglobulin expression in MScl CSF, as well as increased levels of apolipoprotein E (11, 12). Yet in all currently reported proteomics CSF studies of MScl patients either only a single subtype of MScl patients or a combined group of all subtypes of MScl was studied, whilst the differences between the subtypes of MScl remained unexplored. Because RR MScl and PP MScl are very different in terms of disease course and disease progression, this also has therapeutic consequences. Hence, there are probably also differences on a biological and pathological level, which could, if determined, be very useful for elucidation of the biology and pathology of both disease types.

In the current study we specifically aimed to differentiate between the MScl patients and healthy controls and between both subtypes of MScl by comparing CSF proteins and peptides. Subsequently, the identified biomarker proteins and peptides were

discussed in relation to the different pathological processes observed in RR MScl and PP MScl.

METHODS

Ethics statement

The Medical Ethical Committee, de commissie medisch ethische vraagstukken, of the Erasmus University Medical Centre in Rotterdam, The Netherlands, approved the study protocol and patients gave written consent. The approval numbers for this study are 200.721/2001/75 and 2006/188.

Patient selection

The CSF samples of MScl patients, divided into two groups, RR MScl (13) and PP MScl (14), were collected from untreated patients undergoing routine diagnostic procedures by an experienced neurologist (RQH), and matched for presence or absence of oligoclonal bands. The healthy control CSF samples were taken from patients receiving spinal anesthesia prior to non-neurological minor surgical interventions, such as knee and hip replacements, groin rupture and Achilles tendon rupture. All samples were handled in exactly the same way after sampling, using a procedure that has been previously reported (12). In brief, immediately after sampling, the CSF samples were centrifuged to discard cells and cellular elements and the total protein concentration and albumin concentrations were determined. The number of oligoclonal bands and the intrathecal cell count were also reported. The remaining volume of the samples was aliquoted and stored at −80°C, where they remained until sample preparation for this study. No extra freeze-thaw cycles were allowed.

Sample preparation, measurement and analysis

The CSF samples were handled according to the same protocol for quantitative MALDI-FT-ICR MS measurements we reported previously (12), which consists of a blinded experiment in which the samples were digested by trypsin and subsequently measured by MALDI-FT-ICR (APEX IV Qe 9.6 Tesla MALDI-FT-ICR mass spectrometer (Bruker Daltonics, USA)). After calibration by means of omnipresent albumin peaks, an analysis matrix is generated. A univariate analysis, in which a p-value was determined for every peak position, was used for statistical analysis, in which two groups were compared at a time, for a total of three individual comparisons between the groups. The differentially abundant peaks ($p < 0.01$) in the comparisons were considered for identification purposes. The fold increase of every identified peptide with $p < 0.01$ in the comparisons was determined to confirm differential abundance between groups.

Assessment of the statistical background, by means of permutation of a series of 50 scramblings of the samples for each comparison, was used to define a cut-off number for the determination of statistically significant differences between groups. In this permutation procedure all samples are randomly assigned a new group number, scrambling the sample group compositions, prior to performing the univariate analysis to determine the p-values for each peak position. By this method the number of peaks that is assigned a p-value below 0.01 by chance is determined. Iterative repetition of this procedure allows for a statistically relevant mean with standard deviation that could be taken as a realistic background value for this not normally distributed data.

The differentially abundant peptides were identified by nano-LC-ESI-Orbitrap MS. These measurements were carried out on a Ultimate 3000 nano LC system (Dionex, Germering, Germany) online coupled to a hybrid linear ion trap / Orbitrap MS (LTQ Orbitrap XL; Thermo Fisher Scientific, Germany). Five µL digest were loaded on to a C18 trap column (C18 PepMap, 300µm ID x 5mm, 5µm particle size, 100 Å pore size; Dionex, The Netherlands) and desalted for 10 minutes using a flow rate of 20 µL /min 0.1% TFA. Then the trap column was switched online with the analytical column (PepMap C18, 75 µm ID x 150 mm, 3 µm particle and 100 Å pore size; Dionex, The Netherlands) and peptides were eluted with following binary gradient: 0% - 25% solvent B in 120 min and 25% - 50% solvent B in further 60 minutes, where solvent A consist of 2% acetonitrile and 0.1% formic in water and solvent B consists of 80% acetonitrile and 0.08% formic acid in water. Column flow rate was set to 300 nL/min. For MS detection a data dependent acquisition method was used: high resolution survey scan from 400 – 1800 Th. was performed in the Orbitrap (value of target of automatic gain control AGC 106, resolution 30,000 at 400 m/z; lock mass was set to 445.120025 u (protonated (Si(CH3)2O)6) (15)). Based on this survey scan the 5 most intensive ions were consecutively isolated (AGC target set to 104 ions) and fragmented by collision-activated dissociation (CAD) applying 35% normalized collision energy in the linear ion trap. After precursors were selected for MS/MS, they were excluded for further MS/MS spectra for 3 minutes. The MS/MS identifications were obtained using in the Bioworks 3.2 (peak picking by Extract_msn, default settings) software package (Thermo Fisher Scientific, Germany), and its' SEQUEST feature, using minimum XC scores of 1.8, 2.2 and 3.75 for reliable identification of single, double and triple charged ions respectively in the UniProt-database (version 56.0, human taxonomy (20069 entries)). Carboxymethylation of Cysteine (+57.021 u) as fixed and oxidation of Methionine (+15.996 u) as variable modifications and tryptic cleavage were considered. The number of allowed missed cleavages was 2, the mass tolerance for precursor ions was 10 ppm and for fragment ions 0.5 Da. The cut-off for mass differences with the theoretical mass of the identified peptides was set at 2 ppm.

Contamination of CSF by serum of plasma is a possible issue in CSF peptide profiling, because if one or more of the samples is contaminated by serum or plasma, the comparison of CSF peptide profiles is inevitably skewed by the higher total protein concentrations in serum or plasma (16). To prevent inclusion of contaminated CSF samples in this study, the CSF samples were checked for specific blood contamination. If a hemoglobin peptide could be identified by nanoLC-ESI-Orbitrap (C18 column) with sufficiently high confidence score or if the mass peak 1274.7255 (part of hemoglobin gamma) has a signal to noise of 4 or higher in MALDI-FT-ICR measurements, the sample was discarded from further analysis due to plasma/serum contamination. Another blood specific protein, apolipoprotein B100 was checked in the same way as possible blood contamination.

Immuno-assays for validation of differentially abundant proteins

For two proteins, for which we found differentially abundant peptides, we performed validation experiments. This was done by commercially available ELISA (for vitamin D-binding protein) and by western blot (for protein jagged-1), using a validation cohort of patients consisting of 10 RR MScl and 10 PP MScl samples. The samples of the validation cohort were demographically and clinically comparable to the original cohort of patients (average EDSS RR MScl group (standard deviation in brackets): 2.5 (0.8), average EDSS PP MScl group 2.8 (1.0), and the disease duration, presence/absence of oligoclonal IgG bands and male female ratio were all similar to the original cohort (no p-values below 0.05) using a t-test to compare the groups). Additionally we also measured the original samples using this ELISA and western blot. For the first protein, vitamin D-binding protein, we performed a commercially available ELISA (Immundiagnostik, Germany) according to the manufacturer's specifications. For the second protein that was differentially abundant between the both MScl types, jagged-1, we performed a two-step western blot using goat anti-jagged1 antibodies (primary antibody) and anti-goat antibodies (secondary antibody) (Sigma Aldrich, United States). Protein transfer was checked by Ponceau staining. Quantitative assessment of the gel bands after photoluminescence was performed using Image J (freely available at www.rsb.info.nih.gov/ij).

RESULTS

Clinical information

In total 34 CSF samples were used for mass spectrometry analysis, while twenty of these samples and twenty additional samples were used for validation experiments. All samples analyzed by mass spectrometry were tested as being negative for serum/

Table 1. CSF sample information: The concentrations, age, Expanded Disability Status Scale (EDSS) score and disease duration values are averages with standard deviation in brackets. None of the variables in these tables differed significantly between the groups (all t-tests showed p-values higher than 0.05).

	PP MScl	RR MScl	Controls
Number of samples	10	11	10
Protein concentration (g/L)	0.398 (0.118)	0.391 (0.135)	0.386 (0.110)
Albumin concentration (g/L)	0.254 (0.104)	0.228 (0.082)	0.205 (0.090)
Age	48.1 (9.0)	43.9 (14.1)	51.1 (13.7)
EDSS	3.2 (0.8)	2.8 (0.9)	-
Disease duration (years)	3.4 (1.3)	2.6 (1.5)	-
Male/Female ratio (% females in group)	6 / 4 (40%)	6 / 5 (45%)	8 / 2 (20%)

plasma contamination by MALDI-FT-ICR measurements. However, in three samples we were able to identify hemoglobin peptides with sufficiently high XC scores for confident identification using nano LC-ESI-Orbitrap measurements. These three samples, 2 PP MScl and 1 RR MScl, were subsequently excluded from further analysis. The analysis matrix, which was used to profile the differences in peptide profile between the three groups, consisted therefore of 31 CSF samples (Table 1).

Peak detection and data analysis

After the MALDI-FT-ICR spectra were loaded into PeptrixTM software package, they were each tagged with a group number (1, 2 and 3 for PP MScl, RR MScl and controls respectively). Calibrating using five omnipresent albumin peaks was followed by generation of an analysis matrix with the intensity of all peaks of every sample recorded for all detected peaks. Using the Wilcoxon-Mann-Whitney test to compare the groups pair-wise, the comparison between both MScl types resulted in 15 peak masses with p-values below 0.01. By scrambling the sample groups the number of background peaks was determined at 17, so the number of differentially abundant peptide peaks in the comparison of the two MScl types is around the level of the number of background peaks, indicating that the difference between these two groups appears to be nonexistent or at least at background level. However, the proteins that were identified with low p-values in this comparison were of substantial interest in a MScl context.

A total of 43 peptide peaks with a p-value below 0.01 were observed for the comparison of PP MScl versus the controls. The comparison of RR MScl versus the controls had 41 peak masses with p-values lower than 0.01. Seventeen of the peak masses with $p < 0.01$ were present in both comparisons.

Identification

Identification of the differentially abundant peptides was performed by measuring all samples using the nanoLC-ESI-Orbitrap. Due to the prefractionating by nanoLC far more peak masses and identifications are generated by ESI-Orbitrap than there are peak masses in the analysis matrix generated by quantitative MALDI-FT-ICR. Although many of the identified peptides do not correspond to peak masses in the analysis matrix, we were able to identify a number of differentially abundant peptides for all three comparisons (full list, including charge states and sequence coverage. Of the 43 differentially abundant peptide masses that were observed using MALDI-FT-ICR mass spectrometry in the comparison of PP MScl versus the controls we were able to identify 29 peptides. These peptides included several peptides of Ig gamma-1 and Ig kappa. Another differentially abundant peptide was identified as a part apolipoprotein D, which has previously been shown to be elevated intrathecally in MScl patients (17). In the comparison of RR MScl versus controls we were able to identify 24 of the 41 differentially abundant peptide masses, which, as was the case with the comparison of PP MScl versus the controls, also included several peptides of Ig gamma-1 and Ig kappa as well as apolipoprotein D. In fact, of the 24 peptides identified in this comparison, 14 were also identified in the comparison of PP MScl versus the controls, indicating that the differences of the two MScl types compared to the controls are remarkably similar. Additional proteins with low p-values in the comparisons of both MScl types with the controls include StAR-related lipid transfer protein 4 (2.551 fold increase in PP MScl compared to controls and 2.837 fold increase in RR MScl compared to controls), LON peptidase N-terminal domain and RING finger protein (2.847 fold increase in PP MScl and 2.212 fold increase in RR MScl), and ryanodine receptor 1 (2.129 fold increase in PP MScl and 1.877 fold increase in RR MScl).

PP MScl versus RR MScl

The comparison of PP MScl versus RR MScl showed a limited number of differentially abundant peptide peaks. Of these peaks 7 were identified, the most notable being protein jagged-1 (Table 2). This particular protein was over three times less abundant in PP MScl compared to RR MScl. Another interesting differentially abundant protein is vitamin D-binding protein, which was not detected by mass spectrometry in the PP MScl samples but was detected in the RR MScl samples. Other proteins, such as serine/threonine kinase NLK and sodium leak channel non-selective protein were more abundant in PP MScl than in RR MScl, although for the latter protein the difference was small (1.18 fold increase).

Because our main interest was focused on the differences between the two MScl types we selected two differentially abundant proteins from that comparison for validation purposes using 10 PP MScl and 10 RR MScl samples measured by mass

Table 2. Differentially abundant peptides and proteins in the comparison of PP MScl versus RR MScl. M* denotes oxidation of methionine residue.

Acc. number	Protein	# of pept.	p-value	Peptide	Abund. in PP MScl	Fold change	Incidence in PP MScl (%)	Incidence in RR MScl (%)
P02774	Vitamin D-binding protein	1	0.0092	ELPEHTVKLCDNLSTKNSK	↓	-	0	55
P61769	Beta-2-microglobulin	1	0.0014	VEHSDLSFSK	↑	-	70	0
P78504	Protein jagged-1	1	0.0087	TCMEGWM*GPECNRAICR	↓	3.188	30	63
Q8IZF0	Sodium leak channel non-selective protein	1	0.0069	GKSLETLTQDHSNTVRYR	↑	1.180	80	18
Q8NEB9	Phosphatidylinositol 3-kinase catalytic subunit type 3	1	0.0015	SALM*PAQLFFK	↓	-	0	73
Q9NXT0	Zinc finger protein568	1	0.0071	DQGGHSGERPYECGEYR	↓	1.786	80	82
Q9UBE8	Serine/threonine kinase NLK	1	0.0041	YHTCM*CKCCFSTSTGR	↑	-	60	0

Table 3. The results of the validation experiments in the original sample set. By ELISA measurement vitamin D binding protein is more abundant in RR MScl than in PP MScl in the original sample set (p = 0.017), based on the average (+/− standard deviation) concentrations in CSF. Protein jagged-1 (western blot) is more abundant in RR MScl than in PP MScl in the original sample set (p = 0.019), based on the averages (+/− standard deviation) in photoluminescence readout.

Original sample set	**PP MScl**		**RR MScl**		
	Average	**Standard deviation**	**Average**	**Standard deviation**	**p-value (t-test)**
Vitamin Dbinding protein concentration in pg/ml - (ELISA)	13716	5881	19594	3938	0.017
Protein jagged-1 photoluminescence readout (western blot)	8304	3553	16640	9563	0.019

spectrometry and an additional 10 PP MScl and 10 RR MScl samples from independent patients. Validation by ELISA showed that the concentration of vitamin D-binding protein was significantly lower (t-test, $p < 0.05$) in the PP MScl group compared to the RR MScl group in the samples also measured by MALDI-FT-ICR MS (Table 3). A similar result was observed in the new, clinically and demographically comparable validation samples (Table 4). After western blotting, quantitative assessment of the gel bands showed that protein jagged-1 was indeed less abundant in PP MScl than in RR MScl. A t-test on the photoluminescence readout values of the original sample set showed a p-value below 0.05 when comparing the RR and PP MScl samples (Table 3). The same comparison in the validation samples also showed a p-value below 0.05), indicating a significant differential abundance of jagged-1 in the two MScl types (Table 4). In order to place the identified proteins in a biological context, they were uploaded to the Ingenuity Pathways Analysis service (Ingenuity Systems) for to network analysis. Six of the seven differentially abundant proteins were placed in a network relating to neurological disease (Figure 1).

Table 4. The results of the validation experiments in the validation sample set. By ELISA measurement vitamin D binding protein is more abundant in RR MScl than in PP MScl in the demographically and clinically comparable validation sample set (p = 0.032), based on the average (+/− standard deviation) concentrations in CSF. Protein jagged-1 (western blot) is more abundant in RR MScl than in PP MScl in the validation sample set (p = 0.041), based on the averages (+/− standard deviation) in photoluminescence readout.

Validation sample set	**PP MScl**		**RR MScl**		
	Average	**Standard deviation**	**Average**	**Standard deviation**	**p-value (t-test)**
Vitamin D-binding protein concentration in pg/ml ELISA)	15411	6186	23125	8458	0.032
Protein jagged-1 photoluminescence readout (western blot)	9462	3867	19868	14393	0.041

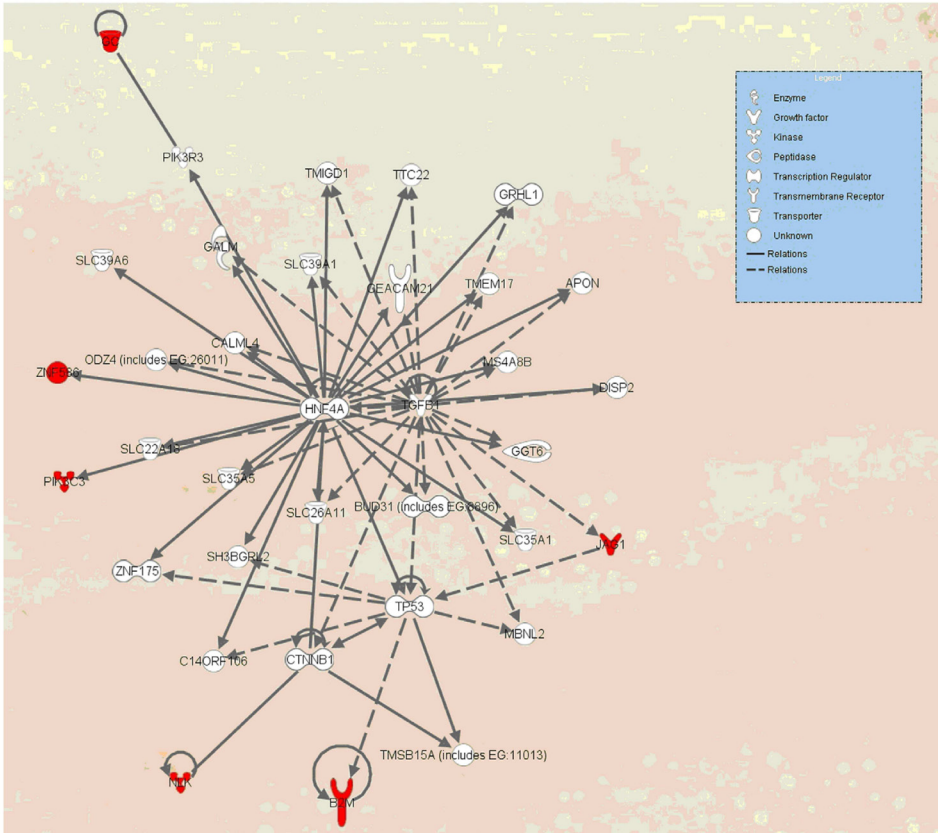

Figure 1. Six of the seven differentially abundant proteins (in red) identified in the comparison of the two MScl disease types (PP and RR) fit into a network related to neurological disease.

DISCUSSION

The main finding of this comparative study is the observation that the proteome profiles of CSF in PP vs RR MScl patients overlap to a large extent. This is in line with the lack of clear-cut differences between the two major clinical MScl sub-groups, at genetic, immunological and neuropathological levels. Interestingly, our approach using sensitive state-of the art mass spectrometry techniques, led to the identification of a few distinct CSF proteins, some of them with biological functions that appear of direct interest for MScl pathology. Two of these proteins were validated by other techniques as well as in a validation sample set. The lack of statistical power, due to the low number of biological samples available for this study, led to a less than ideal statistical analysis, meaning that the reported p-values may not be directly associated to disease pathology. However, the reported unvalidated proteins are of significant biological interest,

and among these are several immunoglobulin proteins, which have been previously reported to be elevated in CSF of MScl patients (18, 19).

The number of peaks with low p-values in the comparison of the two MScl types is lower than the number of background peaks, so this is a strong indication that, even though there may be peptides and proteins that are differentially abundant in the comparison, overall the difference between the two disease types appears to be undetectable by means of the univariate analysis. One of the peptides of protein jagged-1 is over three times less abundant in PP MScl and also is observed with a lower incidence in this group (Table 2). Protein jagged-1 is a ligand for multiple Notch receptors and involved in the mediation of Notch signaling, which influences neuronal function and development (20). The Notch signaling pathway has long been known to influence cell fate in the developing nervous system. Jagged-1 has been found to be highly expressed in hypertrophic astrocytes within and around active MScl plaques lacking remyelination, while, in contrast, there was negligible jagged-1 expression in remyelinated lesions suggesting involvement of the Notch pathway in remyelination in MScl (21). Later, linkage equilibrium screening implicated a number of genes, including the jagged-1 gene, as susceptibility genes for MScl in a large contingent of Europeans (22). It has also been suggested that jagged-1 has therapeutic potential in the treatment of CD8+ T cell mediated diseases, due to its ability to deliver indirect negative signals into CD8+ T cells in vivo (23). Additionally, animal models have shown that elevated expression of Notch and jagged-1 expression does not appear to be a limiting factor in remyelination, but the animal model study reports that there were no quantitative differences in Notch1 expressing cells in slow and rapidly remyelinating lesions, indicating that Notch–Jagged signaling is not a rate-limiting determinant of remyelination in rodent models of demyelination (24). Additionally immunohistochemistry experiments have shown that constituents of the Notch pathway are expressed in remyelination in an animal model of T-cell- and antibody-mediated CNS demyelination (25). However, network studies based on the quantitative expression levels of 20 genes in over one hundred individuals identify jagged-1 as a new therapeutic target whose differential behavior in the MScl network was not modified by immunomodulatory therapy, illustrating how network analysis can predict therapeutic targets for immune intervention and identifying the immunomodulatory properties of jagged-1, making it a new therapeutic target for MScl and other autoimmune diseases (26).

The identified peptide of vitamin D-binding protein is not observed in any of the PP MScl samples, but small peaks of this peptide are detected in 6/11 RR MScl samples. Impaired vitamin D homeostasis has been widely implicated in MScl for some years now (27-29). This vitamin directly and indirectly regulates the differentiation, activation of CD4+ T-lymphocytes and can prevent the development of autoimmune processes (30, 31), and so it may be involved MScl. Considering that the geographic

incidence of MScl indicates an increase in MScl with a decrease in sunlight exposure, that vitamin D is produced in the skin by solar or UV irradiation and that high serum levels of 25-hydroxyvitamin D have been reported to correlate with a reduced risk of MScl, a protective role of vitamin D has been suggested (32). More than 99% of 25-hydroxyvitamin D, the principle circulating metabolite of vitamin D is bound to proteins, of which approximately 90% is bound to vitamin D-binding protein (33, 34). Recently, a CSF proteomics study showed that serum levels of vitamin D-binding protein were decreased significantly in RR MScl patients compared to other neurological disorders (35). While our results do not indicate a differential abundance difference between the MScl subtypes and the controls, the two disease types did vary significantly in CSF levels of vitamin D-binding protein, with RR MScl showing a higher abundance. Since a neuroprotective function has been suggested for vitamin D, it may be that in PP MScl this neuroprotective pathway is at least partially deficient, resulting in a significantly more disabling disease manifestation. However, it should not be forgotten that vitamin D-binding protein has pleiotropic functions, beyond vitamin D metabolism. It can significantly enhance the chemotactic response to complement fragment C5a (36), and there are substantial stimulatory effects on macrophages (37). In light of the increasingly recognized role of innate immunity in the progressive phase of MS pathogenesis (38), vitamin D-binding protein appears an interesting candidate mediator.

The identification of a peptide of beta-2-microglobulin as differentially abundant in the comparison between both MScl types is somewhat misleading. Another seven peptides of this protein were identified among the peak masses in the analysis matrix that had high p-values in the comparison of PP and RR MScl, so it is very likely that the low p-value of the peptide of beta-2-microglobulin reflects high abundant protein variations, suggesting this low p-value is most likely a false positive. In contrast, the other proteins that are differentially abundant in this comparison were either identified by the single peptide listed in Table 2 or by multiple peptides that had low p-values.

The identification of peptides of albumin and serotransferrin stands out in the tables of the differentially abundant peptides and proteins. While the identifications are essentially correct it must be noted that only 2.4% of the identified albumin peptides and 4.8% of the identified serotransferrin peptides had p-values below 0.01, which means that the values for the peptides of these two particular proteins are most likely due to other reasons than large abundance differences in these two proteins. In comparison, most of the other differentially abundant proteins that we identified were found by a small number of peptides with a low p-value or a single peptide with a low p-value. For these proteins we did not observe any other peptides with non-significant p-values, with the exception of bromodomain adjacent to zinc finger domain protein 1A and Rho GTPase-activating protein 18-like. For both of these proteins another peptide was identified with high p-value, indicating these proteins were likely not differentially

abundant. The peptides with low p-values of these proteins were also characterized by a low fold increase, making them less interesting for independent immunoassay follow-up.

Because of the healthy state of the CSF control group, no intrathecal inflammatory response was to be expected in this group. Therefore the very clear difference in immunoglobulin abundance in the comparison with the both MScl types can be explained. In the two comparisons of both MScl types with the control group, several proteins are present in both comparisons, for example Ig gamma-1 chain C region and apolipoprotein D. Apolipoproteins have been previously implicated in MScl. Proteomics studies have shown apolipoprotein E abundances to be elevated in CSF of MScl patients compared to controls (9, 12). In the central nervous system apolipoprotein D is a lipocalin that is mainly expressed in glia, but also in neurons. This protein has been repeatedly implicated in MScl, and it has been shown that it has a neuroprotective effect in a number of neurodegenerative diseases by controlling the level of peroxidated lipids, which coincides with glial activation in mouse models of encephalitis (39). A previous proteomics study showed increased levels of apolipoprotein D in patients with a clinically isolated syndrome of demyelination, indicating that abundance levels of this protein are highest in MScl patients at the time of their first exacerbation (10, 17). Another potentially interesting protein found to be differentially elevated in the comparisons of both MScl types with the controls is ryanodine receptor 1. This receptor is involved in the maintenance of the calcium-equilibrium in brain tissue. The release of toxic levels of positively charged calcium ions may, due to the deleterious effects of excitotoxicity, represent a key mechanism of axonal degeneration in disorders such as MScl (40).

In conclusion, the CSF peptide profile of the control samples differed from both MScl types, with, not unexpectedly, proteins related to immune response showing the highest fold increase in abundance in the MScl types compared to the controls. Even though the CSF peptide profiles measured by MALDI-FT-ICR of PP MScl and RR MScl were quite similar, still a few differences could be observed, most notably regarding the molecules confirmed by immunoassay, protein jagged-1 and vitamin D-binding protein.

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Chapter 3

Gray matter related proteins are associated with childhood multiple sclerosis

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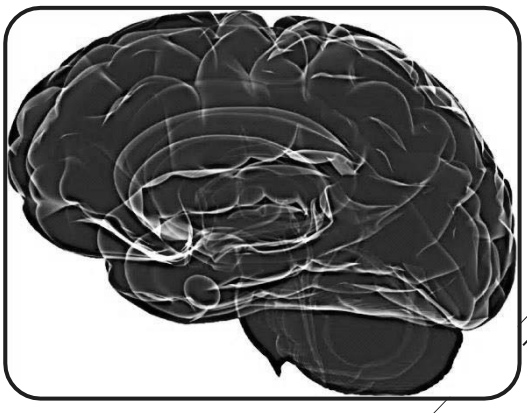

ABSTRACT

Objective: Identification of CSF biomarkers for multiple sclerosis (MScl) in children with an initial acquired CNS demyelinating syndrome (ADS).

Methods: CSF was collected from a cohort of 39 children with initial ADS, of them 18 were diagnosed with MScl and 21 had a monophasic disease course. Proteomic analysis of trypsinised CSF (20 µl) was performed by nano liquid chromatography Orbitrap mass spectrometry. Univariate statistical analysis was used to identify differentially abundant proteins between childhood-onset MScl and monophasic ADS.

Results: A total of 2260 peptides corresponding with 318 proteins were identified in the total set of samples. Of these 2260 peptides, 88 were identified as most distinctive between MScl and ADS. 53 peptides, corresponding to 14 proteins, had higher abundance in children with MScl as compared to monophasic ADS. Twelve out of these 14 proteins were linked to neuronal functions and structures such as synapses, axons and CNS proteases (example: neurofascin, carboxypeptidase E, brevican core protein, and contactin-2). The other two were functionally related to immune function. The 35 peptides identified with decreased abundance in children with MScl corresponded to seven proteins. Six of them linked to innate immune function (example: haptoglobin, haptoglobin-related protein, c4b-binding protein alpha chain and monocyte differentiation antigen CD14) and one to cellular adhesion (protein diaphanous homolog 1).

Conclusion: At first onset of ADS, CSF of children diagnosed with MScl showed increased abundance of CNS grey matter related proteins, whereas CSF of children with a monophasic disease course showed increased abundance of innate immunity related proteins.

INTRODUCTION

A few percent of all multiple sclerosis (MScl) patients experience their first event in childhood (1, 2). In children, such a first acquired demyelinating syndrome (ADS) can present with a spectrum of clinical features including optic neuritis (ON), transverse myelitis (TM), acute disseminated encephalomyelitis (ADEM), neuromyelitis optica (NMO), and other clinically monofocal or polyfocal symptoms (3, 4). In most children with monophasic ADS, the disease course remains monophasic. However, a significant part of these children will subsequently be diagnosed with MScl (5, 6). In this group, the time between the initial monophasic ADS, and the second MScl defining attack was typically <2 years (5).

Current diagnosis of MScl uses the combination of clinical features, cerebrospinal fluid (CSF) findings such as positive oligoclonal bands (OCB), and magnetic resonance imaging (MRI) criteria for dissemination in time and space (1, 5, 7). These factor are insufficient to predict the disease course at first event. The availability of a biomarker that helps to differentiate between children with monophasic ADS, and those subsequently diagnosed with MScl is needed. Moreover, identification of CSF proteins that are associated with childhood onset MScl can provide further insight into the disease pathophysiology. So far, only one study was published that compared CSF in MScl (n=8) patients with monophasic ADS (n=11) in children. Zooming in on neuronal peptides and non-myelin proteins in CSF, this study suggested for disturbed axoglial biology during early MScl (8) event. Involvement of central nervous system (CNS) gray matter components (which relates to neuronal cell bodies, dendrites, axons, oligodendrocytes and synapses) has also been shown in early MScl initiation in one other study that was performed in adults (>18 yrs.) clinically isolated syndrome (CIS) and established MScl patients(9).

In the present study, we have investigated CSF, a body fluid that reflects ongoing CNS pathology (10) in a fully unbiased manner. Samples were analyzed by high resolution, and sensitive nano-liquid chromatography Orbitrap mass spectrometry (LC-MS) (11). Our aim was to find CSF protein markers expressed during first event of CNS demyelination that can help to distinguish children with monophasic ADS (n=21) from children with MScl (n=18).

MATERIALS AND METHODS

Patients

Children (<18 years old), who were diagnosed with either monophasic ADS or MScl were identified by the Dutch Study Group for Pediatric MScl, which includes 13 major

pediatric neurology centers in The Netherlands as described earlier(12). Diagnosis (12) of pediatric MScl patients was made in case they had a second demyelinating attack of the CNS and/or MRI evidence of a new lesion at least one month after onset (1). Initial phenotypes of participants were defined by clinical history and physical examination. This study included 41 participants; 22 children diagnosed with monophasic ADS, and 19 children diagnosed with clinically defined MScl. After collection, all CSF samples included in this study were immediately centrifuged at 3000g to remove cell debris, subsequently coded, frozen and stored at −80°C until analysis. An aliquot was subsequently used for routine CSF diagnostics. The remaining volume of the samples was aliquoted and stored at −80°C. The study was approved by the Clinical Research Ethics Board of the Erasmus University. Written informed consent was obtained from family of patients.

Sample preparation and LC-MS measurements

CSF samples (20 µL) were digested using an in-solution trypsin digestion protocol followed as previously described (13). Samples prepared were analyzed by LC-MS/MS using an Ultimate 3000 nano RSLC system (Thermo Fischer Scientific, Germering, Germany) online coupled to a hybrid linear ion trap / Orbitrap mass spectrometer (LTQ Orbitrap XL; Thermo Fisher Scientific, Bremen, Germany). Five microliter of each tryptic digest were loaded onto a C18 trap column (C18 PepMap, 300 µm ID x 5 mm, 5 µm particle size, 100 Å pore size; Thermo Fisher Scientific, The Netherlands) and desalted for 10 minutes using 0.1% TFA in water at a flow rate of 20 µL/min. The trap column was switched online with the 50 cm long analytical column (PepMap C18, 75 µm ID × 500 mm, 2 µm particle and 100 Å pore size; Dionex, Amsterdam, The Netherlands) and peptides were eluted with the following binary gradient: 3% - 25% eluent B for 120 min and 25% - 50% eluent B for a further 60 min, where eluent A consisted of 0.1% formic acid in ultrapure water and eluent B consisted of 80% acetonitrile and 0.08% formic acid in water. The column flow rate was set to 250 nL/min (oven temperature: 40°C). For electro-spray ionization (ESI), metal-coated nano ESI emitters (New Objective, Woburn, MA, USA) were used and a spray voltage of 1.5 kV was applied. For MS/MS analysis a data dependent acquisition method was used: a high resolution survey scan from 400 – 1800 m/z was performed in the Orbitrap (automatic gain control (AGC) 106, resolution 30,000 at 400 m/z; lock mass set to 445.120025 m/z(14). Based on this survey scan the 5 most intense ions were consecutively isolated (AGC target set to 104 ions) and fragmented by collision-activated dissociation (CAD) applying 35% normalized collision energy in the linear ion trap. Precursor masses within a tolerance range of +/− 5 ppm that were selected once for MS/MS were excluded for MS/MS fragmentation for 3 minutes (dynamic exclusion).

Protein identification and quantification

MS/MS spectra were extracted and converted into mgf files using extract_msn (part of Xcalibur version 2.0.7, Thermo Fisher Scientific Inc. Amsterdam, The Netherlands). Sequencing of the fragmentation spectra was conducted by a Mascot MS/MS database search (version 2.3.01, Matrix Science Inc., London, UK) against the human subset of the uniprot database (20,253 Homo sapiens entries; version: June 1st 2013). The following search parameters were applied: a maximum of two missed cleavages, tryptic cleavage, oxidation as a variable modification of methionine (+ 15.995 u) and carbamidomethylation as a fixed modification of cysteine (+ 57.021 u). Further peptide mass tolerance was set to 10 ppm and fragment mass tolerance to 0.5 u. To estimate the false discovery rate (FDR), a decoy database search was conducted. Next, peptide and protein probabilities were determined by using Scaffold (version 3.6.3, Proteome Software Inc., Portland, OR) and finally the identification result was filtered by a protein and peptide threshold probabilities of minimal 95% and a protein ought to be identified by at least 2 peptides. Peptide FDR was 0.2% and protein FDR was 3.4% (corresponds with 95% probability). LC-MS data were analyzed using the Progenesis LC-MS software package (11) (version 3.6, Nonlinear Dynamics Ltd, Newcastle - upon - Tyne, United Kingdom). Individual runs were aligned with each other in this software by at least 200 vectors using the automated alignment option to compensate for variations of retention times. Subsequently, peak picking and integration of peak areas were performed and peaks (features) with +2 and +8 and at least 2 isotopes were selected. Identification results (as described before) were imported and assigned to the corresponding peaks (2260 peaks identified). Subsequently, the result data were exported in excel format and further normalized abundance (computed by Progenesis) used for further statistics.

Statistical analysis

The Wilcoxon test (unpaired, two tailed) was used to analyze the differences in the abundance of peptides between MScl, and monophasic ADS with $p<0.01$ being considered statistically significant. Relative quantitative differences of peptide abundances between MScl and monophasic ADS samples were calculated as log2 ratio between median abundances of both groups. A set of significantly distinct peptides and proteins was determined by applying following criteria: (a) peptides that had at least 1.5 fold difference in expression at a p-value < 0.01; (b) protein identified by at least 2 peptides, and (c) at least 40% differentially expressed peptides ($p<0.01$) per protein, whereby peptides of a given protein were required to have representation in the same direction (increased or decreased abundance in MScl). In the above dataset we also excluded those proteins that had only 1 peptide differentially identified out of total two.

To verify these findings and to determine the statistical background level, we performed a permutation analysis on the entire dataset (2260 peptides) between

MScl (n=18) and monophasic ADS (n=21) sample groups included with the above mentioned criteria. Whereby, we determined the statistical background level on a set of significantly distinct peptides. The random permutation test, on the dataset with randomized sample group assignment, was repeated 1000 times through which, the resulting thresholds were saved.

For all calculations and graphics, we used the R software package (R version 3.0.2, http://www.R-project.org) (15). For other calculations and graphics, SPSS 15.0 (SPSS, Chicago, IL, USA) and Microsoft Excel 2010 were used.

RESULTS

Patient characteristics

We have analyzed 18 MScl and 21 monophasic ADS patients. One MScl and one monophasic ADS patients were excluded because of <200 required alignment vectors found during Progenesis LC-MS analysis. From 18 patients diagnosed with MScl, 5 had ON, 2 had TM, 3 had clinical monofocal, and 8 had clinical polyfocal symptoms as their first symptoms of onset. From the remaining 21 patients with monophasic ADS, 2 had experienced ON, 2 had TM, 8 had clinical polyfocal and 9 had ADEM. No significant differences were observed between the two groups in terms of gender, CSF levels of total protein, albumin, albumin CSF/serum quotient, leukocyte and IgG concentration. The mean age at onset of children diagnosed with MScl (14.17 ± 1.5) was found to be significantly higher in comparison to monophasic ADS (6.89 ± 4.9), reflecting the epidemiology of these phenotypes. In addition, CSF elevated IgG index and positive oligoclonal bands (OCB) were more frequently present in children with MScl ($p \leq 0.01$). Patient characteristics are given in table 1.

Identification of proteins that discriminates MScl from monophasic ADS

We have detected 50,119 peptide precursors from Progenesis label-free analysis LC-MS experiment from all trypsin digested protein in CSF samples. A Mascot database search in the human subset of the Uniprot database resulted into 2260 unique peptides that corresponded with 318 proteins. The total protein concentrations of digested peptide samples quantified (integrated UV area at 214 nm) during LC–MS measurements did not show any significant difference ($p = 0.54$) between CSF samples of children with MScl and monophasic ADS group. To check technical variability, at regular interval we measured 12 reference samples (pooled CSF samples from all patients). Here, also the total protein concentrations of digested peptide samples quantified (integrated UV area at 214 nm) during LC – MS measurements between Reference group 1 (n=6) and 2 (n=6) did not show any difference (Reference group 1 (n=6) =382.94 ± 153.28;

Table 1. Clinical characteristics and routine CSF findings of children with multiple sclerosis (at disease onset) and the monophasic acquired demyelinating syndrome. Data are presented as the mean ± SD or median and range of patients. In case of missing data, the number of patients with available data is indicated between parentheses.

	Monophasic ADS (n=21)			MScl (n=18)			p-value[2)]
	Mean ± SD	Median	Range	Mean ± SD	Median	Range	Monophasic ADS *vs* MS
Age at onset, years	6.89 ± 4.91 (n = 21)	5.81	1.14 - 17.11	14.17 ± 1.50 (n = 18)	14.29	11.14 - 16.21	p<0.01
Sex, % females	71.4	N/A	N/A	61.1	N/A	N/A	N.S.
Protein g/l	0.36 ± 0.21 (n = 21)	0.3	0.18 - 1.15	0.33 ± 0.14 (n = 17)	0.32	0.19 - 0.75	N.S.
Albumin g/l	0.23 ± 0.20 (n = 11)	0.16	0.11 - 0.79	0.18 ± 0.07 (n = 14)	0.17	0.08 - 0.36	N.S.
Leukocytes x10/6	37.12 ± 34.91 (n = 20)	28.5	1 - 118	20.98 ± 25.68 (n=17)	13	1.0-87	N.S.
IgG g/l	0.05 ± 0.07 (n = 11)	0.03	0.01 - 0.27	0.05 ± 0.03 (n = 13)	0.05	0.03 - 0.12	N.S.
IgG index	0.61 ± 0.10 (n = 12)	0.58	0.5 - 0.79	1.36 ± 0.72 (n = 17)	1	0.67 - 3	p<0.01
Elevated IgG index[1)], n (%)	2 (16.7)	N/A	N/A	16 (94.1)	N/A	N/A	p<0.01
Positive OCB, n (%)	2 (12.5) (n = 16)	N/A	N/A	14 (87.5) (n = 16)	N/A	N/A	p<0.01
Relapsing disease, n (%)	0 (0)	N/A	N/A	18 (100)	N/A	N/A	p<0.01

[1)] IgG index considered elevated 0.68 or higher (6) [2)] p value was calculated by Mann-Whitney test, and p<0.01 was considered significant.
Abbreviations used: ADS, monophasic acquired demyelinating syndrome; **d**, Days; **MScl**, multiple sclerosis; **N/A**, not applicable; **N.S.**, not significant; **OCB**, oligoclonal bands.

versus Reference group 2 (n=6), 429.34± 661.90, p=0.3). In addition, a number of MS/MS fragmentation spectra also did not show any significant difference between samples of children with MScl and monophasic ADS. In particular, the measured MS/MS fragmentation spectra for MScl, and monophasic ADS samples were 16796 ± SD 1795 and 16971 ± 1153 (p=0.72) respectively. Moreover, the database identified MS/MS spectra for MScl and monophasic ADS samples were 1607 ± 280 and 1626 ± 256 (p=0.83) respectively.

Comparing abundance of identified peptides (n=2260) from CSF samples of children with MScl and monophasic ADS patients and using the stringent criteria as described in statistical analysis, we found a total of 88 differentially abundant peptides. Of these 88 peptides, 53 were significantly increased (table 2) and 35 decreased in CSF samples of

Table 2. Identification of proteins differentially expressed in CSF of pediatric multiple sclerosis patients (n=18) as compared to monophasic acquired demyelinating syndrome (n=21). The table shows 21 proteins, from which 14 were identified with increased and 7 with decreased abundance in MScl. The table includes the direction of difference in MScl, name of the protein, p-value and fold expression difference. All proteins given in the table were identified with at least two unique peptides, differentially abundant peptides ($p<0.01$) had at least 1.5 fold difference in expression (median) between groups; For the same protein, 40 percent of identified peptides were differentially expressed with the expression in the same direction (i.e. either higher or lower abundant in MScl) of the same protein.

Trend in MScl[a)]	Description	Sig./total[b)]	Fold change Average (Min-Max)[c]	p-value[d)] Average (Min-Max)
Increased abundance in MScl	Amyloid-like protein 2	2/2	3.8 (2 - 5.5)	0.006 (0.003 - 0.008)
	Neurofascin	3/3	2.5 (2.1 - 3)	0.002 (0.001 - 0.002)
	Carboxypeptidase E	2/3	2.4 (2.1 - 2.6)	0.0004 (0.0002 - 0.0005)
	Neuronal growth regulator 1	2/3	2.3 (2.1 - 2.5)	0.0008 (0.0008 - 0.0009)
	Contactin-2	4/9	2.3 (1.5 - 3.2)	0.002 (0.0005 - 0.003)
	Amyloid beta A4 protein	6/11	2.2 (1.8 - 2.6)	0.002 (0.00003 - 0.008)
	Brevican core protein	5/7	2.1 (1.7 - 2.6)	0.002 (0.0001 - 0.005)
	Disintegrin and metalloproteinase domain-containing protein 22	2/2	1.9 (1.7 - 2.1)	0.0004 (0.0001 - 0.0006)
	Tyrosine-protein phosphatase non-receptor type substrate 1	3/4	1.9 (1.5 - 2.1)	0.003 (0.001 - 0.006)
	Dickkopf-related protein 3	6/11	1.8 (1.6 - 2)	0.002 (0.00004 - 0.006)
	Neuronal cell adhesion molecule	9/18	1.8 (1.6 - 2.1)	0.004 (0.001 - 0.009)
	Ig kappa chain V-III region POM	2/2	1.8 (1.7 - 1.8)	0.002 (0.0009 - 0.003)
	Ig gamma-1 chain C region	5/11	1.7 (1.5 - 1.8)	0.0016 (0.0002 - 0.005)
	Kallikrein-6	4/7	1.7 (1.5 - 1.9)	0.001 (0.0006 - 0.004)
Decreased abundance in MScl	Apolipoprotein B-100	7/17	661 (3.5 - 3930)	0.0026 (0.00002 - 0.01)
	C4b-binding protein alpha chain	2/4	8.1 (2.9 - 13.4)	0.008 (0.007 - 0.008)
	Haptoglobin	18/29	3.7 (2 - 12.1)	0.002 (0.00004 - 0.01)
	Haptoglobin-related protein	2/4	3.3 (3.1 - 3.5)	0.0002 (0.00001 - 0.0005)
	Leucine-rich alpha-2-glycoprotein	2/3	2.6 (2.3 - 3)	0.008 (0.007 - 0.009)
	Monocyte differentiation antigen CD14	3/3	1.9 (1.8 - 2)	0.006 (0.004 - 0.009)
	Protein diaphanous homolog 1	2/2	1.8 (1.7 - 1.9)	0.005 (0.001 - 0.009)

a) Trend, Protein abundance is either significantly increased or decreased in MScl as compared to monophasic ADS.

b) Sig. Number of differentially identified peptides $p<0.01$ / Total, is the total number of identified peptides for same protein.

c) Fold change, i.e. fold expression difference calculated based on median abundance from 18 MScl and 21 ADS patients, Shown is average of fold difference for all peptides of the same protein. Minimum and maximum range for the same is indicated.

d) p-value was calculated by Wilcoxon test. Given in the table is the average p-value and range for all differentially abundant peptides of the same protein.

children with MScl (table 2)as compared with monophasic ADS. Peptides with increased abundance (n=53) in the MScl group corresponded with 14 proteins and peptides with decreased abundance (n=35) corresponded with 7 proteins. An inventory of these 21 proteins is given in table 2. Moreover, fold expression difference between MScl and monophasic ADS groups groups and statistical significance are plotted simultaneously for our entire data set (n=2260 peptides) as a Volcano plot (figure 1). This permutation analysis resulted in 20 ± 41 (median 9) false positive peptide markers, which indicated that our observations are not due to chance alone, because more than 90% of the permutations yielded 0-4 significant hits i.e. in 900. Only 4 times out of 1000 more than 88 hits with low p-value were detected (FDR: 0.4%). Whereas, contrasting to this mere background chance, from the actual data set (true hits) 88 peptides were identified. Therefore, comparison of permutated data with the real data indicated that the occurrence of differentially abundant peptides related to MScl or monophasic ADS was highly significant (p<0.001).

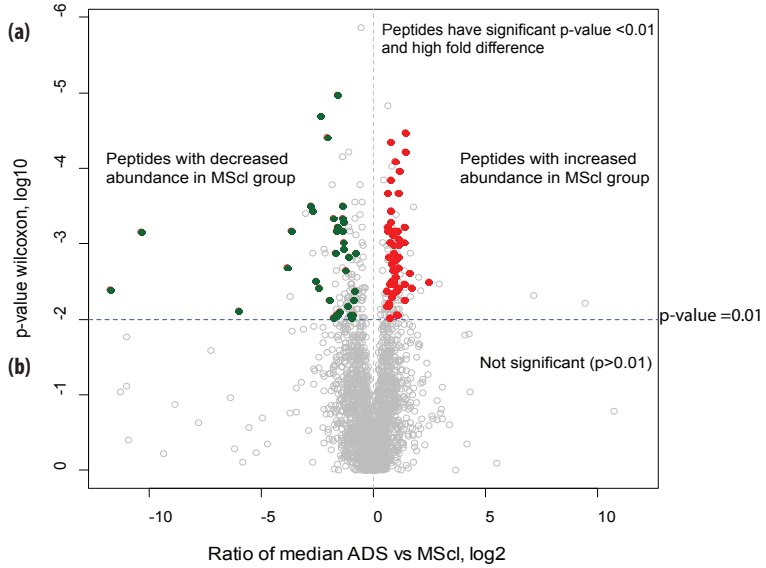

Figure 1. Volcano plot: p-value versus fold change. Peptides (n=2260) showing distribution of fold change and statistical significance. In plot each point represents a peptide, and shows the ratio between MScl (n=18) and monophasic ADS samples (n=21) plotted against the level of statistical significance. Y-axis shows p-values, obtained (plotted at log10) from a Wilcoxon test performed between abundances of peptides. X axis shows ratio of median between MScl and monophasic ADS (plotted on log 2).
(a) Above the dashed horizontal line, red points (n=53 peptides) were found with increased in abundance (right side of the vertical line), green points (n=35 peptides) decreased in abundance (at left side of the vertical dashed line) in the MScl group (compared to monophasic ADS).
(b) Peptides shown in gray colour, below the dashed horizontal line did not pass stringent statistical criteria for identification of candidate peptide.

Identified proteins with increased abundance (n=14) in MScl (table 2) were: amyloid-like protein 2 (2/2, 2 significant peptides for a total of 2 peptides), neurofascin (3/3), carboxypeptidase E (2/3), neuronal growth regulator 1 (2/3), contactin-2 (4/9), amyloid beta A4 (6/11), brevican core protein (5/7), disintegrin and metalloproteinase domain-containing protein 22 (2/2), tyrosine-protein phosphatase non-receptor type substrate 1 (3/4), dickkopf-related protein 3 (6/11), neuronal cell adhesion molecule (9/18), Ig kappa chain V-III region (2/2), Ig gamma-1 chain C region (5/11) and kallikrein-6 (4/7). Proteins identified with decreased abundance in MScl (n=7) were: apolipoprotein B-100 (7/17), c4b-binding protein alpha chain (2/4), haptoglobin (18/29), haptoglobin-related protein (2/4), leucine-rich alpha-2-glycoprotein (2/3), monocyte differentiation antigen CD14 (3/3), and protein diaphanous homolog 1 (2/2). The function of these 14 proteins and overlap with previous studies are summarized in table 3. Among the 14 proteins with increased abundance in the MScl, 12 were associated with CNS structure and functions (86%), especially to the grey matter (table 3), compared to 17% of the total identified proteins related to CNS structure and functions. Seven proteins identified with decreased abundance in the MScl (relative to monophasic ADS group) were components of the innate immune system and inflammation (table 3B).

Proteins identified in the current study did not exhibit any myelin related proteins, for instance; myelin oligodendrocyte glycoprotein and myelin basic protein.

We examined for the influence of age of onset on the 88 candidate peptides abundances for children with MScl and monophasic ADS by correlation analysis. We found a mean coefficient of determination (± SD) for MScl 0.04 ± 0.06, and for monophasic ADS 0.05 ± 0.05. Thus, by correlation analysis no significant correlation was found between age and peptide abundance for children with MScl and monophasic ADS for all 88 peptides.

DISCUSSION

In the current study, we have used LC-MS proteomic approach to search for differences in CSF proteome between children with MScl and monophasic ADS in children. Benefit of this Orbitrap technique is the possibility to identify relatively vast amounts of different peptides and at the same assess their abundances, in a small sample volume. We observed a striking difference between the two groups, using stringent statistical criteria. We searched for the known functions of the 88 peptides corresponding to 21 distinctive proteins (14 proteins increased and 7 decreased in abundance in MScl), making use of available/biological databases (www.geneontology.org and www.nextprot.org) and the literature. Among the 14 proteins with increased abundance in MScl, 12 were associated with CNS structure and functions, especially to the gray matter. Three

Table 3. Table summarizes function of differentially abundant markers and overlap with previous studies. Most of the proteins were assigned as either neuronal or immune related molecules based on previous reports and database searches. First column shows name of the protein and Uniprot accession number (given in the bracket, it denotes protein ID). Second column shows function of proteins. Third column shows comparison with a previous study on children MScl (Dhaunchak and colleagues) using ADS-MScl (n=8, mean age 12 yrs.) *vs.* monophasic ADS (n=11, age 10 yrs.). The fourth column shows overlap with the work of Schutzer and colleagues, they used CIS-MScl n=9, age 18-42 yrs. and established MScl & controls (n=6, 31-54 yrs.)

3A. Function of proteins identified with increased abundance in MScl

Differential proteins (Accession number)	Functions and expressions	Dhaunchak et al. (8)	Schutzer et al. (9)
Amyloid-like protein 2 (Q06481)	Relative of amyloid precursor protein family, memory processes(36), concentrated in synapses(37), regulates neuronal stem cell differentiation during cortical development(38)	×	** Variant
Neurofascin (O94856)	Cell adhesion, synapse formation(39), located at CNS paranodal domain and expressed by oligodendrocytes, target for autoantibody-mediated axonal injury in MScl (21)	No sig. diff.	×
Contactin-2 (Q02246)	Axon connection, majorly expressed on CNS juxta-paranodal domain	No sig. diff.	***
Amyloid beta A4 protein (P05067)	Component of amyloid plaques (Alzheimer brains), neurite growth, neuronal adhesion and axonogenesis	×	×
Brevican core protein (Q96GW7)	Major proteoglycan in perisynaptic extracellular matrix of brain(40), inhibit neurite outgrowth from cerebellar granule neurons(24)	No sig. diff.	Decreased in first-attack CIS-MScl relative to established RR-MScl and controls
Carboxypeptidase E (P16870)	Found in brain and throughout neuroendocrine system, synthesis of most neuropeptide(41)	Same trend (4 sig./21 total, p=0.004)	×
Neuronal growth regulator 1 (Q7Z3B1)	Trans-neural growth-promoting factor, promotes outgrowth and expressed on reactive astrocytes after entorhinal cortex lesion (in mice)(42)	No sig. diff.	×
Tyrosine-protein phosphatase non-receptor type substrate 1 (P78324)	Supports adhesion of cerebellar neurons, neurite outgrowth and glial cell attachment(29)	×	×
Neuronal cell adhesion molecule (Q92823)	Paranodal region of CNS, axo-glial contact(43)	No sig. diff.	**
Disintegrin and metalloproteinase domain-containing protein 22 (Q9P0K1)	Expressed in the juxta-paranodal complex (43).	Not same trend (2 sig./10; p=0.01, 0.02)	×

3A. Function of proteins identified with increased abundance in MScl (continued)

Differential proteins (Accession number)	Functions and expressions	Dhaunchak et al. (8)	Schutzer et al. (9)
Dickkopf-related protein 3 (Q9UBP4)	Highly expressed in the brain and spinal cord, influences synapse formation(9),	×	**
Kallikrein-6 (Q92876)	Brain-related serine protease, elevated in active MScl, regulate early CNS demyelination in a viral (mouse) model of MScl (27).	×	**
Ig gamma-1 chain C region (P01857)	Immune response, elevated in adult MScl and CIS as compared to non-inflammatory neurological diseases(16)	×	×
Ig kappa chain V-III region POM (P01624)	Immune response, elevated in adult MScl as compared to non-inflammatory and inflammatory neurological diseases(16)	×	×

3 B. Function of proteins identified with decreased abundance in MScl

Differential proteins	Functions	Dhaunchak et al (8)	Schutzer et al. (9)
Apolipoprotein B-100 (P04114)	Innate immune related, not produced in CNS(44), observed in CSF and can cross a dysfunctional blood-CSF barrier(44)	×	×
C4b-binding protein alpha chain (P04003)	Innate immune defense, involved in complement activity(45)	×	×
Haptoglobin (P00738)	Innate immune defense(30)	×	×
Haptoglobin-related protein (P00739)	Innate immune defense(46)	×	×
Leucine-rich alpha-2-glycoprotein (P02750)	Induced by inflammation, cell adhesion, high expression in the deep cerebral cortex(26), novel marker of granulocytic differentiation (30))	×	×
Monocyte differentiation antigen CD14 (P08571)	Main modulator of innate immune system(32)	×	×
Protein diaphanous homolog 1 (O60610)	Coordinates cellular dynamics by regulating microfilament and microtubule function(34), role in cell–matrix adhesions, variant related to innate immune function (35)	×	×

Abbreviations and definitions: ×, Protein was not identified; ****,** Increased expression in first-attack CIS MScl group compared to established RR-MScl and controls; ** Variant, Variant of same protein was found with increased expression in first-attack CIS MScl group compared to established RR-MScl and controls; ***, Protein was found with increased expression in first attack CIS-MScl relative to RR-MScl but decreased in first attack CIS-MScl vs. controls; ****, Decreased expression in first-attack CIS-MScl vs. established RR-MScl and controls; **No sig. diff.**, Protein was identified in pediatric CSF samples but there was no statistical difference in abundance was found between MScl and monophasic ADS group comparison; **Same trend**, when protein abundance was compared between two group (for ex MScl and monophasic ADS), expression (either increased or decreased in MScl) of identified protein showed overlap with our study; **Not same trend**, When abundance of protein was compared they did not show expression in the same direction (either increased or decreased expression in MScl) relative to our study.

of these 12 proteins were CNS related proteases, and other two proteins were related to immune functions. The functions of these 14 proteins are summarized in table 3 A. Seven proteins identified with decreased abundance in MScl relative to monophasic ADS were components of the innate immune system and inflammation (see table 3 B). Thus, in the MScl group, there was a clear over-representation of neuronal and axoglial proteins (table 3 A). Whereas in the monophasic ADS group, proteins associated with innate immunity were over-represented (table 3 B).

Recently, two research group demonstrated the identification of axoglial and grey matter proteins using mass spectrometry in CSF of MScl patients (8, 9). Similar to our study, Dhaunchak and his colleges compared CSF of 8 pediatric MScl patients with 11 monophasic ADS (8). The overlap of some proteins in the MScl group is noticeable (e.g. carboxypeptidase E), despite clear differences in sample handling such as depletion of abundant proteins with possible carrier function for other proteins, and exclusion of proteins with less than 5 kDa weight.

Our results show also overlap with molecules identified in the CSF of adult acute onset MScl cases, Schutzer and colleagues performed the mass spectrometry analysis in the CSF on CIS cases versus established relapsing remitting MScl (RR-MScl) and controls(9). They showed proteins that distinguished these CIS patients from both established RR-MScl and controls. For example, they showed significant increase of kallikrein 6, dickkopf-3 in first attack MScl in comparison to established RR-MScl and Controls. They also showed a significant increase in contactin-2 (neuronal membrane protein) in first-attack MScl patients relative to established RR-MScl whereas decrease in CIS compared to controls (9) (table 3 B).

Our findings stipulate the neurodegenerative arm of MScl neuropathology, also in children, and already present at the earliest stage of clinical disease. In analogy with the findings of others (8, 9) and ourselves in previous studies (16-19) we also observed a striking lack of myelin proteins in these clear-cut cases of acute demyelination. We assume this may not directly imply absence of such free proteins in this type of pathology, but rather it may reflect the specific physicochemical properties of the hydrophobic myelin components, and perhaps different pathways of elimination from the CSF, e.g. via draining macrophages (20). In any case we have to be cautious in using the dominant presence of gray matter over white matter proteins as suggestive proof that neurodegeneration is a primary event in MScl, and would precede demyelination.

The presence of CNS grey matter may simply represent damage by inflammation, and the molecules identified may provide leads to a better understanding of this presumed inflammation induced neurodegeneration. It should be stressed that not all differential proteins were over-represented in MScl. Some were under-represented, pointing at more complex mechanisms such as a perturbation in the physiology of the axoglial apparatus (8).

A disturbing factor in our study could be the fact that, due to the skewed occurrence of monophasic disease at younger age, both groups were not matched according to age. We doubt however whether this has influenced the results, as we did not see an age effect in any of the two groups (MScl and monophasic ADS) on abundance of the 21 identified proteins.

From the 21 proteins identified here, 14 showed increased abundance in MScl, and their known functional roles are summarized in table 3 A. Two proteins were associated with the amyloid beta A4 protein family. Amyloid-like protein 2 is concentrated at neuronal synapses and has a role in memory processes. Amyloid beta A4 protein is associated with neurite growth, neuronal adhesion and axonogenesis.

Four (of 21) proteins: contactin-2, neurofascin, neuronal growth regulator 1 and brevican core protein were shown to be located at the paranodal, juxta-paranodal region of the CNS of myelinated axons in the CNS. Myelinated axon can be divided into axon initial segments, nodes, paranodes, juxtaparanodes (begins at the innermost axo-glia junction of paranode) and internodes. Contactin-2 is axonal glycoprotein which is shown at juxta-paranode domain of myelinated axons (9). Neurofascin plays a role in the assembly of nodes of Ranvier in the CNS (21). Two isoforms of neurofascin are shown to interact with contactin-associated protein and contactin-1 to form paranodal junction that attaches the myelin loop to the axon; and helps to separate voltage gated sodium channel at node and potassium channel at juxtparanode region (22). Disruption of neurofascin localization shows early changes preceding demyelination and remyelination in MScl (23). Interestingly, contactin-2 and neurofascin have are autoimmune targets in MScl (22). Neuronal growth regulator 1 is shown to be located at paranodal region of CNS and play a role in axo-glia contact at the node of Ranvier. Identification of contactin-2 and neurofascin in CSF of children with MScl is consistent with a previous study (8) however, they did not find significant difference in CSF of children with MScl and monophasic ADS (table 2 and 3). Among the 4, brevican core protein is known as CNS specific proteoglycan at the surface of neuroglial sheaths, where it is enriched in perisynaptic extracellular matrix (24).

Among the proteins with increased abundance in MScl (of 14), the proteases/peptidases protein named as, carboxypeptidase E plays a role in synthesis of most neuropeptides (25). Next, disintegrin and metalloproteinase domain-containing protein 22 are highly expressed in brain and localized at juxta-paranode. This molecule presumed to be work as a major neuronal receptor (26). An other brain related protease was kallikrein-6, which is a secreted serine protease, and it is described to regulate early CNS demyelination in a viral model (expression in the brain and spinal cord of mice) of MScl (27). Additionally CSF kallikrein-6 elevated level is reported in MScl (adults) as compared to neurological controls (28) (table 3A).

Among other proteins with increased abundance in MScl (of 14), tyrosine-protein phosphatase non-receptor type substrate 1, is implicated in the neurite outgrowth and glia-cell attachment and shown to support adhesions of cerebellar neurons (29). Next the highest expression of dickkopf related protein 3 is reported in the brain and spinal cord (table 3A). Thus the majority of proteins with increased abundance in our MScl group (compared to monophasic ADS) were neuronal related with the exception of two immune function related proteins Ig gamma-1 chain C region and Ig kappa chain V-III region POM (table 3 A).

Our study reports seven proteins with decreased abundance in pediatric onset-MScl as compared to the monophasic ADS (table 2) group. Six of these seven proteins have previously been reported as specific component of innate immune functions (table 3 B). Haptoglobin an acute phase protein (30) was identified as most distinctive one (18/29, 18 significant peptides for a total of 29 peptides) from those which were elevated in monophasic ADS group (compared to children with MScl). Another study in adults showed increased haptoglobin concentration in NMO comparison to adult MScl patients (31). C4b-binding protein alpha chain which is a crucial component of complement cascade inhibits function of complement component. Interestingly, we also found monocyte differentiation antigen CD14, which is shown to be mainly expressed on cells of monocytic lineage (macrophages and monocytes) (32). Higher CD14 levels might be linked with increased levels of cytokines triggering inflammatory processes in ADS children. Monocytic cells secrets soluble sCD14 (activation product of activated monocytes) so this may affect the enormous macrophase activation during acute monophasic ADS (33).

Among seven identified proteins protein diaphanous homolog is a member of the formin family (34), it is expressed in brain and, their variants are shown to be required for innate immune response to gram-negative bacterial Infection (35).

Overall in MScl group we found a significant over-representation of proteins associated with changes in CNS gray matter, axons, synaptic regulation, node of Ranvier and brain protease (table 3 A). Several of these proteins are part the axoglial apparatus and may relate to disturbances in axo-glia interaction (8) (table 3 A). Two of them (contactin-2 and neurofascin) have been identified as possible axo-glial auto-antigens in MScl (22).

The overlap of proteins observed in previous studies (8,9) (table 3 A) as part of the axo-glia apparatus, and grey matter provides validation for these proteins. Further insight into the role of these proteins in early-onset MScl can be useful for disease process understanding and might be useful as a future tool to differentiate MScl from monophasic ADS in children. These pathologically relevant proteins (mostly CNS grey matter related), elevated in CSF of early-onset MScl in children might be involved in early disease mechanisms. Further insight into the role of these proteins can be useful

for disease process understanding. Moreover, such proteins might be useful as a future tool to differentiate children MScl from children with monophasic ADS. In addition, knowing the start of MScl could immediate an earlier treatment with disease modifying therapies. However, the current research is designated as discovery phase study, which serves as a base for the follow-up on verification and validation phase studies which can provide in clinically valuable biomarkers. In future, it would be interesting to further validate our findings with an independent technology more importantly in an independent sample group.

In conclusion our data indicate that monophasic ADS can be differentiated from MScl in children primarily by CNS grey matter proteins and immune related proteins. Our findings point to perturbed axoglial physiology as a hallmark of the earliest events of MScl pathogenesis.

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Chapter 4

Cerebrospinal-fluid-derived Immunoglobulin G of different multiple sclerosis patients shares mutated sequences in complementarity determining regions

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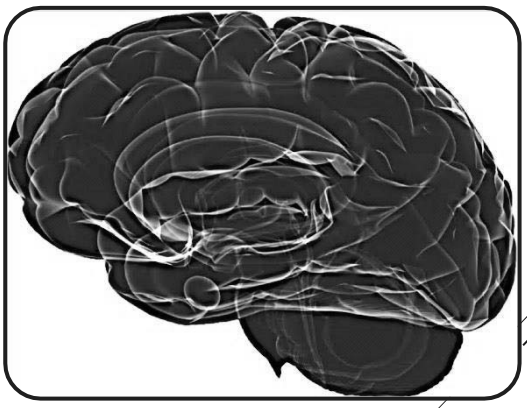

ABSTRACT

B lymphocytes play a pivotal role in multiple sclerosis pathology, possibly via both antibody dependent and independent pathways. Intrathecal immunoglobulin G production in multiple sclerosis is produced by clonally expanded B cell populations. Recent studies indicate that the complementarity determining regions of immunoglobulins specific for certain antigens are frequently shared between different individuals. In this study, our main objective was to identify specific proteomic profiles of mutated complementarity determining regions of immunoglobulin G present in multiple sclerosis patients but absent in healthy controls. To achieve the above objective, from cerebrospinal fluid of 29 multiple sclerosis patients and 30 healthy controls we purified immunoglobulin G and separated the corresponding heavy and light chains by SDS - PAGE. Subsequently, bands were excised, trypsinized and measured with high - resolution mass spectrometry. We sequenced 841 heavy and 771 light chain variable region peptides. We observed 24 heavy and 26 light chain complementarity determining regions that were solely present in a number of multiple sclerosis individuals. Using stringent criteria for the identification of common peptides, we found five complementarity determining regions shared in three or more patients and not in controls. Interestingly, one complementarity determining region with a single mutation was found to be in common in six patients. Additionally, one other patient carrying a similar complementarity determining region with another mutation was observed. In addition, we found a skew in kappa to lambda ratio and in the usage of certain variable heavy region which was previously being observed at the transcriptome level. At the protein level, cerebrospinal fluid immunoglobulin G share common characteristics in the antigen binding region between different multiple sclerosis patients. The indication of a shared fingerprint may indicate common antigens for B cell activation.

INTRODUCTION

Autoimmune mechanisms play a central role in the pathogenesis of multiple sclerosis (MScl). Recent trials indicate that B - lymphocyte depletion therapy can substantially reduce disease activity in relapsing remitting (RR) MScl patients (1). Clinical amelioration after depletion seems to precede reduction in autoantibody levels, possibly because this treatment rapidly affects the antigen-presenting cell functions of B cells (2). This finding has boosted interest in studies on the pathogenic role of auto - reactive B - cells. Despite the success of inhibiting antibody - independent functions of B cells, arguments remain for an additional chronic pathogenic role for auto - antibodies within the central nervous system (CNS). This includes: a) the presence of antibodies in cerebrospinal fluid (CSF) and brain tissue (3); b) depositions of antibody within areas of demyelination along with local complement activation (3); c) myelin oligodendrocyte glycoprotein (MOG) specific antibodies in some subpopulations of MScl patients (4, 5). Additionally, KIR 4.1 was recently identified as a target of autoantibody response in subgroup of person with MScl (6).

It has been shown that the distribution of genes used to generate antibodies in B cells from CSF and lesions of MScl patients are skewed from naturally expected distributions. Several groups described clonal B cell populations within the CNS, sometimes even skewed to certain families of variable heavy (VH) regions (7, 8). No common motifs have yet been found to be shared between different MScl patients. This would be in line with classical immunological insight that suggests that it is extremely rare to find common sequences in the immunoglobulin G (Ig) variable regions amongst different individuals. However, this view has recently been challenged (9-11). Both after vaccination and in paraneoplastic syndromes like anti - Hu, strikingly identical shared complementary determining region (CDR) motifs between patients were observed (12). Of note is also a surprising study in which it was observed that malignant chronic lymphocytic leukemia B cells in different patients all recognized a single fungal antigen and showed shared use of CDR3 sequences between different individuals (13).

A novel approach to study Ig gene usage in biofluids of MScl is the use of proteomic sequencing. Obermeier and colleagues described overlap between Ig B cell (CSF) transcriptomes and proteomes in four individual MScl cases, without inter - individual overlapping sequences (14). However, this elegant proof of principle study was limited to four MScl patients and there was no comparison between patients and controls. The possibility to sequence CSF Ig at the protein level (10, 14, 15) may bring along some advantages. The genetic approaches used so far share the benefit that complete sequences can be identified at a single cell level, still Ig derived from such clones do not necessarily represent the actual Ig repertoire found in CSF. Furthermore, where genomic studies are restricted to CSF cells, humoral CSF studies also include Ig proteins from

other anatomical brain areas, such as parenchyma, meninges and Virchow Robin spaces. Finding common characteristics of the antigen binding sites of Ig between patients may provide leads into the question whether common antigenic stimuli are responsible for recruitment of intrathecal B cells in MScl. We previously described a new approach by using advanced nano - scale liquid chromatography coupled online to a high resolution mass spectrometer (LC - MS) (10, 16, 17), a reliable and powerful method for sensitive detection of CDR peptides (18). Moreover it can also be used to compare CDR peptide profiles between a relatively large number of patients and controls.

Our main question was if we could detect specific proteomic profiles of CDR present in MScl patients, but absent in controls. Here, we report a number of common CDR sequences in Ig of a group of MScl patients that were not observed in healthy controls. In addition we showed disturbed kappa (κ)/lambda (λ) chain ratios in CSF Ig of MScl patients and VH family usage in patients compared to controls.

MATERIALS AND METHODS

Clinical Samples: MScl patients and Non - Neurological controls

CSF samples were collected from untreated MScl patients, which were selected by an experienced neurologist (RQH) and were followed prospectively by the Rotterdam Multiple Sclerosis Center ErasMS at the Department of Neurology at the Erasmus Medical Center (Rotterdam, the Netherlands). The procedure for CSF sample collection was as described previously (19). All MScl patients had defined relapsing remitting MScl or a clinically isolated syndrome according to the 2005 McDonald criteria for MScl (20). The control individuals were free of any neurological disorders. They underwent minor, non - neurologically indicated, surgeries. CSF was taken prior to the administration of sedatives as part of the anaesthesia procedure. Therefore this group is further referred to as healthy controls. Immediately after collection, the CSF samples were centrifuged (10 minute at 3000 rpm) to discard cells and cellular elements, and the supernatant was aliquoted and stored at −80°C, until further use for this study. Blood contaminated CSF samples were excluded based on the presence of erythrocytes detected by microscopic examination immediately after sampling. One aliquot of a sample was used for routine CSF diagnostics in the Clinical Chemistry department of Erasmus MC. This diagnostic procedure for the MScl patients included quantification of total protein, albumin, assessment of the number of Oligoclonal bands (OBs), and Ig index. Moreover, these samples taken from another aliquot were previously studied to determine intra - individual variations in CSF protein abundances (Stoop et al., 2010). This study was approved by the institutional ethical committee of the Erasmus MC and written informed consent was obtained from all participants.

Ig Quantification Assay

An ELISA assay using 96 well plates (Immuno 96 Micro Well TM Solid Plate, Thermo Fisher, Bremen, Germany) was used to determine Ig concentrations in CSF samples. In this assay affiniPure F(ab')2 Fragment Goat Anti-Human IgG (H+L) (Jackson ImmunoResearch laboratories, Suffolk, United Kingdom) at a concentration of 1.3 mg / ml, was used to coat the wells as a capture antibody. As a detection antibody, horse radish peroxidase conjugated polyclonal secondary antibody, antihuman Fc Goat (Anti human IgG HRP Sigma-Aldrich, Saint Louis, MO) was used. Samples were incubated with antibodies for five minutes and gently shaken (400rpm) at 4°C on a thermo-cycler shaker (Eppendorf). 3,3',5,5',- tetramethylbenzidine (TMB, Sigma-Aldrich, Saint Louis, MO) (100µL per well) was used as a substrate for horseradish peroxidase that developed a soluble blue reaction product. Reaction was stopped with 100µL 1M hydrochloric acid. Ig concentration was determined photometrically by absorbance at 450nm and quantified using an eight point's calibration curve ranging from 1.0×10^{-5} to 0.5 µg/µl.

Ig Purification

Ig was purified from CSF samples by Melon Gel IgG Spin Purification kit (Pierce, Rockford, IL) according to the manufacturers' protocol for serum with slight modifications. All CSF samples were diluted at a ratio of one to three with purification buffer. We used 100 µL CSF from patients and 200 µl from controls based on the Ig concentration assay (two times more CSF volume for controls were used in comparison to the patients to normalize for Ig concentration). Subsequently, the diluted CSF was added to a spin column containing Melon Gel resin. After 15 minutes of incubation, the spin column was centrifuged at 5000 g and the flow through was collected that contained the purified Ig. Ig concentrations were determined in the flow through fractions after purification (with above described Ig ELISA). Equal amount of Ig across all patients and controls were then taken and subsequently lyophilized (Sublimator 400, Zirbus Technology, Tiel, the Netherlands) for six hours. The lyophilized Ig fractions were then stored at −20°C for one day before proceeding to separation by SDS - PAGE.

For SDS - PAGE separation loading buffer was added to each lyophilized sample and heated at 90°C for 10 minutes. Purified Ig antibodies were resolved into heavy (IgH) and light chain (IgL) by reducing one - dimensional SDS - PAGE using Bio - Rad Mini - Protean electrophoresis system gels (10% polyacrylamide gels of 0.75 mm thickness). The gels were stained with Novex® Colloidal Blue Staining (Invitrogen, Carlsbad, CA) according to the manufacturers' instructions. Overnight destaining was performed for visualization of IgH and IgL chain protein bands and subsequently gels were scanned.

In - Gel Trypsin Digestion

We excised gel bands manually in a laminar flow cabinet as a preventive measure to minimize environmental keratin and other contaminating protein - like materials. Protein bands were cut into plugs and transferred into Eppendorf tubes. We performed reduction by dithiothreitol and alkylation with iodoacetamide. Subsequently in-gel digestion was performed overnight at 37°C and further peptide extraction procedures were performed (18). After peptide extraction, samples were dried for three hours in a vacuum centrifuge (SPD 1010, Thermo Savant, Holbrook, NY), and afterwards stored at −80°C until LC - MS measurements.

Chromatography Separation and Mass spectrometric Measurement

Before LC - MS measurements, the dried peptide samples were dissolved in 40 µL of an aqueous solution of 0.1% TFA and sonified. The samples were measured with a nano LC system (Ultimate 3000, Thermo Fisher Scientific, Amsterdam, the Netherlands) coupled on - line to a hybrid linear ion trap/Orbitrap mass spectrometer (LTQ-Orbitrap-XL, Thermo Fisher Scientific, Bremen, Germany).

Samples were loaded onto a trap column (PepMap C18, 300 µm ID x 5 mm length, 5 µm particle size, 100 Å pore size; Thermo Fisher Scientific) and washed and desalted for 10 minutes using 0.1% TFA (in water) as loading solvent. Then the trap column was switched online with the analytical column (PepMap C18, 75 µm ID x 250 mm, 3 µm particle and 100 Å pore size; Thermo Fisher Scientific) and peptides were eluted with the following binary gradient: starting with 100 % solvent A, then from 0 % to 25 % solvent B in 60 min and from 25 % to 50% solvent B in further 30 min, where solvent A consisted of 2% acetonitrile and 0.1% formic acid in HPLC grade water, and solvent B consisted of 80 % acetonitrile and 0.08% formic acid in HPLC grade water. All LC solvents were purchased from Biosolve, Valkenswaard, the Netherlands. Column flow rate was set to 300 nL/min, and eluting peptides were measured first by a UV detector (at a wavelength of 214 nm in a 3 nL nano flow cell; Thermo Fisher Scientific) and consecutively introduced into the MS. For electro - spray ionization (ESI), metal - coated nano ESI emitters (New Objective, Woburn, MA, USA) were used and a spray voltage of 1.5 kV was applied. For MS detection, a data - dependent acquisition method was used: high-resolution survey scan from 400–1800 Th. was detected in the Orbitrap (target of automatic gain control=106, resolution=30,000 at 400 m/z, lock mass set to 445.120025 Th. (protonated [Si (CH3)2O]6 (21)).

On the basis of this full scan the five most intensive ions were consecutively isolated (AGC target set to 104 ions) and fragmented by collision activated dissociation (CAD, applying 35 % normalized collision energy), and detected in the ion trap. Precursor masses within a tolerance range of +/− 5 ppm that were selected once for MS/MS were excluded for MS/MS fragmentation for three minutes or until the precursor intensity fell below a

S/N of 1.5 for more than 5 scans. Samples were prepared and measured in a randomized order. Internal quality control sample was measured once in every five measurements. Before each run, a blank run was performed to monitor background of the system.

DATA ANALYSIS AND PEPTIDE IDENTIFICATION

Peptide identification by Mass and Fragmentation

Acquired LC - MS profiles for the separate purified IgH chain and the IgL chain datasets were analyzed separately using the Progenesis LC - MS software package (version 2.6, Nonlinear Dynamics Ltd, Newcastle - upon - Tyne, United Kingdom). In this software package individual runs were aligned with each other to compensate for variations of retention times (samples which could not be aligned by at least 200 vectors using the automated alignment option were excluded for further analysis as recommended by the manufacturer). Before peak - selection, integration of the area of the peaks was performed. The resulting peaks could then be associated with the amino acid sequence information if corresponding fragmentation spectra were available. From raw data files, MS/MS spectra were extracted and converted into mgf files using extract_msn (part of Xcalibur version 2.0.7, Thermo Fisher Scientific Inc.). Sequencing of the fragmentation spectra was conducted by a Mascot MS/MS database search (version 2.3.01, Matrix Science Inc., London, UK) against the human subset of the NCBInr sequence database (National Center for Biotechnology Information database for non - redundant sequences; version 15th August 2010, Homo sapiens taxonomy; 232854 sequences). The following settings were used for the database search: a maximum of two missed cleavages, tryptic cleavage, oxidation as a variable modification of methionine (+ 15.995 u) and carbamidomethylation as a fixed modification of cysteine (+ 57.021 u). Further peptide mass tolerance was set to 10 ppm and fragment mass tolerance to 0.5 Da. For peptide identification minimum ion score of 25 was required. The resulting peptide identifications were filtered using Scaffold (version 3.2.0, Proteome Software Inc., Portland, OR). Peptide false discovery rates (FDRs) were calculated by Scaffold on the basis of FDR= (false positive)/(false positive + true positives)). On average FDRs was determined always lower then 0.1%. Filter criteria for the generated identification result table were set to greater than 95 % peptide probability (and greater than 95 % protein probability. At this stage all non - Ig proteins (examples: albumin, transferrin, keratin etc.) were filtered out on basis of their protein names to focus solely on the Ig proteins. Subsequently, the identified peptides and proteins were imported into the Progenesis software package and linked to their corresponding peptide peak. The Progenesis analysis matrix contained mass, charge, intensity, abundance and MS/MS fragmentation spectra of detected peptides.

The abundance listed for all peaks can be defined as background or signal. To remove background from real peak signals we used the following procedure: First around 30 randomly chosen peaks in all samples with low intensity region were reviewed in regards of their isotopic pattern. Peaks with more than two isotopes were classified as valid peaks, and peaks with no isotope or just one or two isotopes were classified as background. We performed manual checks on all peptides of interest to confirm background level and the detection of exact location and overlap of peaks among samples in the above mentioned Progenesis software package. The specific aim of this work was to find CDR peptide fragments which were present in common in MScl patients and not in the control group. Therefore we filtered the datasets for candidates that were found in at least three MScl patients and were absent in control patients.

Manual Confirmation of Peptide Identifications

For further confirmation of identification of peptides as depicted above, we assessed the MS / MS fragmentation spectra. If an identical peptide was shared among patients, for each individual sample one should expect similar spectral patterns (in terms of mass fragmentation and retention time window) to be present in different samples. Additionally, we evaluated the isotopic patterns in the mass spectrometry spectra in terms of number and appearance of isotopes. Using the method described above, those marker peptides that did not pass our filter were not qualified as marker.

CDRs Identification (assigning location in Ig structure)

All identified amino acid sequence were aligned to a variable (V), Diverse (D), Joining (J), constant (C) – region Ig germ line sequences of human (Homo sapiens) derived from the IMGT database (ImMunoGeneTics information system® http://www.imgt.org; Montpellier, France (22). As described previously (16, 17, 23), we used the BLASTp search algorithm (BLASTp algorithm; NCBI Blast version 2.2.22) to align the identified peptide sequences to the corresponding Ig.

All peptide alignments with bit scores greater than 12.5 were selected for further analysis. Peptides aligned to the variable domain germ line were further submitted to the IMGT/domain gap alignment tool (http://www.imgt.org), which positions the peptide to the germ line sequence in the IMGT unique numbering residue system. The alignment with the most homologous germ line allele was provided by the IMGT tool and included in the data if the identity score was at least 70%. By this approach we were able to locate a peptide to CDRs or frame work region. A CDR peptide was defined as a peptide containing minimum three amino acids in the CDR part, irrespective of framework length. Moreover, the methodology overview is described in the form of flow chart (figure 1).

Statistical Analysis

We determined a set of CDR peptides that were exclusively present in at least three MScl samples but not in any control samples (true result). To determine the probability that this finding is not due to chance alone, we performed permutation tests. We permutated (randomized) the sample group assignment (patient or control group) and rerun the determination of exclusively present CDR peptides 5000 times (false hits). Relative frequency of occurrence of exclusive CDR peptides in a randomized sample set was calculated by dividing the false hits exceeding the true result by the numbers of randomization trials. For these computations we used the statistics package R (R version 2.15.2, www.r-project.org). Other statistical analysis of data and graphical presentations were performed using GraphPad Prism (GraphPad Software version 5.00, www.graphpad.com) or by Excel 2010 function.

RESULTS

In total 59 samples were included for analysis of the IgH comparison set (n = 29 MScl versus n = 30 controls). IgL comparison set included 55 samples (n = 29 MScl versus n = 26 controls). Four IgH samples (2 MScl and 2 controls) and six IgL samples (1 MScl and 5 controls) were excluded after label - free analysis because of weak alignment and UV information.

Patient characteristics

All patient characteristics are shown in table 1. Significant differences in the gender and age (Mann - Whitney test, $p < 0.0001$) were observed between MScl patients and controls. All other parameters did not show any differences (Mann - Whitney test, $p > 0.05$) between the groups. MScl patients have slightly increased Ig concentrations in CSF. Therefore we normalized CSF Ig concentration. After normalization, UV quantified area (peptide abundance) obtained during LC - MS measurements did not show any significant difference (Mann - Whitney test, $p = 0.80$ IgH and $p = 0.18$ IgL) in concentration of digested peptides between groups.

VH and VK family distribution in MScl patients and Controls

The peptide spectral count (MS / MS identification based) information (Scaffold based) acquired at the individual level (method figure 1) was used to analyze Ig VH family distribution between MScl patients and controls. An alignment match score of 70 % was used as a cut off value and a mean score of 90 % ± 8.5 % was observed. The mean sequence length was 10 ± 2.1 amino acids (mean ± SD). Peptide counts were normalized at the individual patient level to the total number of peptides found for the given

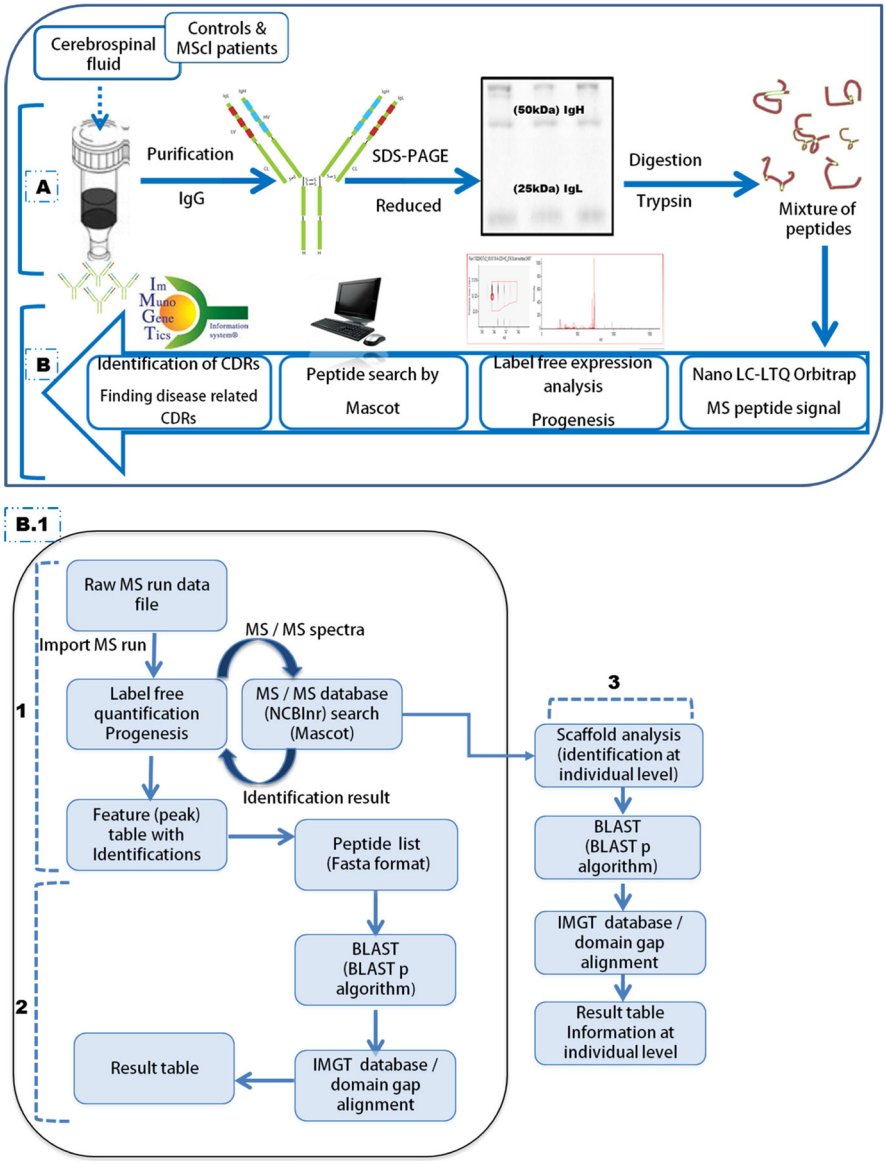

Figure 1. Schematic illustration of the proteomics based methodology used to assign shared CDR exclusive presence in CSF Ig of MScl patients.

(A) Ig was purified from CSF of MScl patients (100 µL) and non - neurological controls (200 µl) based on the Ig concentration assay. Purification was performed by Melon gel IgG Spin Purification kit. Purified Ig was separated into IgH and IgL chain by reducing one - dimensional SDS - PAGE gels. (B) After In - Gel trypsin digestion of excised IgH and IgL bands, the mixture of peptides was measured by Nano LC - LTQ Orbitrap MS. Mass spectra were analyzed by Progenesis software and peptide search was performed by Mascot. Identified peptides were used for CDR identification using IMGT database and BLASTp search algorithm to find MScl specific CDRs.

(B.1) Detail of part B: (1) Nano LC - LTQ Orbitrap MS generated, LC - MS profiles (raw MS run data) for the IgH and IgL datasets. They were analyzed separately using Progenesis LC - MS software package (label free quantification). Sequencing of the MS / MS spectra was executed by a Mascot MS / MS database search against the human subset of the NCBInr sequence database. Afterwards, the identified peptides were imported into the Progenesis and linked to their corresponding peak. The peak abundance (UV area under curve) information was used for CDRs presence or absence analysis. (2) Identified peptides were extracted and converted into FASTA format. They were aligned to a V, D, J and C element of Ig (Homo sapiens) germ line sequences derived from the IMGT database. We used the BLASTp search algorithm to align the identified peptide sequences to the corresponding Ig fragments. Next, they were submitted to the IMGT / domain gap alignment tool. This analysis provided alignment details of CSF Ig peptides in comparison to Ig germ line that included: gene name, homology match score, mutation/mismatch and start and end position. (3) Peptide identification details were uploaded in Scaffold program. The Scaffold file contains a peptide identification view report based on the spectral counts (at MS / MS level). Peptide counts were obtained at the individual level for each sample and were exported to a spreadsheet containing detailed information about the protein and peptide hits. On basis of the resulting combined peptide set, alignment summary in comparison to the germ line were assigned at the individual level (using a BLASTp algorithm and IMGT database). This information was used for VH and VK family distribution analysis.
Abbreviations used: BLAST, basic local alignment search tool; BLASTp, basic local alignment search tool for protein; IMGT, ImMunoGeneTics information system; Feature (or Peak), object of defined mass with an identified charge state, retention time and isotopes characterized by the analysis software.

Table 1. Clinical characteristics of MScl patients and non-neurological controls. Clinical characteristics are presented as median (25th and 75th percentiles). The differences were identified by Mann-Whitney test. Statistical Significance considered at p <0.05.

	IgH			IgL		
	Control (N=30)	**MScl (N=29)**	**p value**	**Control (N=26)**	**MScl (N=29)**	**p value**
Age (Yrs) at sampling	52 (39 - 68)	31 (26-41)	<0.0001	53 (41-67)	33 (27-42)	<0.0001
Gender	13 ♂ 17 ♀	2 ♂ 27 ♀	<0.0001	13 ♂ 13 ♀	2 ♂ 27 ♀	<0.0001
Total protein (g/l)	0.39 (0.33-0.50)	0.37 (0.260.46)	0.07	0.39 (0.33-0.51)	0.36 (0.26-.45)	0.05
Albumin (g/l)[a)]	0.19 (0.17-0.35)	0.19 (0.140.24)	0.61	0.18 (0.10-0.36)	0.19 (0.15-0.24)	0.87
Abnormal CSF Ig index[b)] (OBs)[c)]	N/A	21 (23)	N/A	N/A	21 (23)	N/A
Time from CIS to CSF sampling[d)]	N/A	5 (2- 38)	N/A	N/A	5 (2-40)	N/A

Abbreviations used: IgH: Heavy chain IgL; Light chain: N / A; Not applicable; OBs: Oligoclonal bands; ♀: Female; ♂: Male
a) Albumin concentration, (g/ l) is reported for n = 11 IgH and n = 8 for IgL. b) Ig index, (CSF / serum Ig): (CSF / serum albumin), was denoted as increased if greater than 0.67. c) OBs, Presence of CSF - specific OBs. d) Time from CIS to CSF sampling, Time from first attack to CSF sampling in months and defined as first neurological episode caused by inflammation of demyelination.

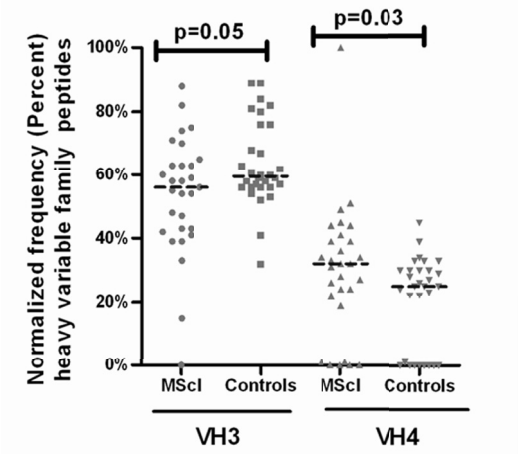

Figure 2. The family usage of VH3 and VH4 peptides in MScl in comparison to controls. The family usage of IgH repertoire expressed in CSF of MScl and controls is shown. Horizontal lines represent median value. Peptide counts were normalized at the individual patient level to the total number of peptides in given family. Frequencies (percent) of peptides are plotted at the Y axis. Usage of peptides assigned to each family was compared between both groups. Slight over representation of VH4 family (Mann-Whitney test, p = 0.03) was observed in MScl patient group (N = 29) in comparison to the control group (N = 30).

IgH chain family. Usage of peptides assigned to each family was compared between both groups. Log transformed data was not normally distributed (by D' Agostino and Pearson omnibus normality test), therefore a non -parametric statistical test was used to determine statistical significance using raw data. In comparison to controls, VH 4 was found to be slightly but statistically significant (p = 0.03) and trend towards increased VH 3 in MScl was observed (p = 0.05) (Figure 2). No significant differences were found for the other six VH (p > 0.05) families. Family usage of the VK chains between MScl patients and controls was also performed in the similar way like VH. We found peptide alignment match mean score 86 % ± 11 %. The mean amino acid sequence length was 10 ± 2.6 (mean ± SD). Analysis did not show significant difference in between groups.

Disturbed κ/λ ratio in MScl patients

The κ/λ ratio was analyzed between controls (n = 26) and MScl patients (n = 29). The ratio of κ and λ light chains was determined from the abundance data for representative peptides from the LC - MS dataset (Progenesis). The peptides used were C region peptides (n = 10) having an alignment match score of 100 % ± 0 % (mean ± SD) for κ (n = 6) and 97 % ± 3 % (n = 4) for λ peptides. The κ to λ mean ratio in control was 2.52 ± 1.71 and in MScl 4.06 ± 2.76. A two tailed t - test was applied since on log scale transformed data normal distribution was found by means of normality testing. The ratio showed significant elevation in MScl group (p = 0.03) (Figure 3).

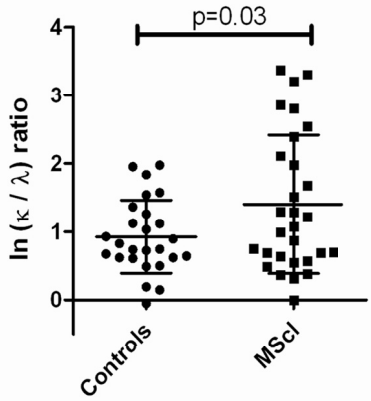

Figure 3. The κ to λ ratio in CSF Ig of MScl patients (n = 29) and controls (n = 26). The κ to λ ratio in CSF Ig of MScl and controls is expressed on a logarithmic (ln) scale and shown as means with SD. Ratios showed elevation in MScl group (unpaired t - test, p = 0.03).

Identical CDR peptides identification in several MScl patients

As a result of nano – LC - LTQ Orbitrap MS measurements of tryptically digested Ig bands, we found 54,057 MS peaks totally in the IgH chain and 56,664 in the IgL chain comparison set by Progenesis. The database (NCBInr) analysis by Mascot identified 1086 peptides for IgH samples. Similarly (by NCBInr) we identified 920 peptides for IgL samples. Next, the BLASTp algorithm aligned our peptide (experimental) sequences to a database (IMGT) of germline sequences that were present in naive B-cells (as described in method). The best matching germline allele was selected from the database. Comparison of the peptide with the best matching germline allele also revealed which amino acids were most likely mutated during rearrangement and affinity maturation. In this way, from the 1086 IgH peptides, 809 sequences were assigned to the V region, 32 sequences to the J region and 99 sequences to the C region. Similarly (by IMGT), from the 920 IgL peptides, 722 peptides were assigned to the V region, 49 sequences to the J region and 99 to the C region. Next, within the IgH variable domain, we could assign 41 peptides to the CDR1, 128 to the CDR2 and 171 to the CDR3. Within the IgL variable domain, we could assign 78 peptides to the CDR1, 233 to CDR2 and 51 to CDR3. Moreover, a summary of identification is shown as a flowchart. Many of the IgH and IgL peptides in our dataset were unassigned. Possible reason for this could be rearranged CDR3 sequences having too little similarity to the germline sequence to allow for alignment. We were interested in peptides that were observed only in the MScl group. Therefore, based on the presence or absence of these peptides in the MScl group we found 24 IgH and 26 IgL peptides in the MScl group that could only be identified in the MScl group and not in controls. In contrast as expected peptides derived from the Ig C region were equally distributed in both patients and controls (data not shown).

We searched for those CDR related peptides in the MScl group that shared among multiple MScl individuals. Next, we zoomed into the CDR regions to assess for peptides that were shared in at least three MScl patients and absent in the controls. We found nine IgH peptides and six IgL peptides. At lower threshold (i.e. shared in at least two MScl) we observed 14 IgH peptides and 13 IgL peptides (data not shown).The elucidated results here emphasized the fact that CDR peptides were from MScl patients, and were not from three or more controls. As a confirmation step, a second round of assessment was performed by means of 2-D view analysis in the Progenesis software package. To check whether the fragmentation spectra of a peptide were similar in all patients in whom it was identified, we critically scrutinized and assessed the fragmentation spectra of peptides. At this point, we found five peptides that were proven to be shared between three or more MScl patients. The exact characteristics are shown in table 2.

To determine the probability, that exclusive presence of CDR in MScl is not due to chance alone we permutated the entire dataset repeatedly 5,000 times for CDR marker category. Analysis showed that CDR exclusive presence in MScl is not due to chance in IgH ($P = 0.0005$) and also in IgL ($P = 0.0007$). Five peptides were found to be specific to three or more patients in the MScl group. Interestingly, one of the mutated CDR2 commonly used was seen in seven different MScl patients. IMGT Alignment analysis showed that this CDR peptide had different mutations (T, E, and N) or insertion (F) at the same spot. The QDGSE**T**YYVDSVK (amino acid given in bold, mismatch/mutation from Ig germline) peptide was quantified in six MScl patients and not in 30 controls (Figure 4). Further, four out of the six MScl patients were identified by MS/MS (and not in controls), giving additional support for proper sequence identification (Figure 4). Sequence alignment with the human germ line sequences (derived from IMGT database) showed homology to the IGHV 3-7 and threonine (T) amino was found mutated/mismatched from the lysine (K) of the germ line. A similar peptide, QDGSE**E**YYVDSVK was identified in three MScl patients. Alignment showed the mutation of a glutamic acid (E) at the same spot. In addition to the peptide described above, two CDR resembling mutations QDGSE***TF***YVDSVK (bold italic, insertion) and QDGSE**N**YYVDSVK were also exclusively observed solely in one MScl patient. Next, the IDWDDDKYYSTSLK peptide was quantified exclusively in four MScl patients. For two of these MScl patients fragmentation spectra were obtained that showed identical peptide MS/MS, supporting robustness of the peptide identification. Alignment analysis showed homology to the IGHV 2-70*01 (CDR2) for these peptides, another identical CDR, IDWDDDKYY**T**TSLK with mutation was observed solely in one MScl patient. Next, the YNSAPLTFGGGTK peptide was identified exclusively in three patients. Homology search showed alignment to the CDR3 of gene IGKV 1-27 *01 and IGKJ 4 *01. Finally, the LLI**H**GAS**N**R peptide was identified solely in three MScl patients. Alignment showed homology to the IGKV 3-20 *02 CDR2 and histidine (H) and asparagine (N) were found mutated from the tyrosine (Y) and serine (S) compared to the germ line.

Table 2. Exclusive presence of common CDR motifs in CSF Ig of MScl patients. Table shows CDR peptide precursor mass, charge, confidence score for the sequence based on the MS/MS spectra, amino acid sequence, location details in Ig structure, expression in controls and patients, gene information and match score with Ig germ line.

m/z	z	Confidence	CDRs[a)] (Peptide by MS/MS)	CDR [b)]	Mutation	Expression		Germ line[d)]	Gene name[e)] (Allele name)	Homology (%)	Reference
						MScl[c)]	Control				
745.838	2	64	QDGSE**T**YYVDSVK[a')]	CDR 2	Yes	6/29	0/30	QDGSEKYYVDSVK	IGHV 3–7 (IGHV 3–7*02)	92.3	(14, 33, 34)
759.833	2	65	QDGSE**E**YYVDSVK[a')]	CDR 2	Yes	3/29	0/30	QDGSEKYYVDSVK	IGHV 3–7 (IGHV 3-7*02)	92.3	(14, 33, 34)
583.605	3	43	IDWDDDKYYSTSLK[a'')]	CDR 2	No	4/29	0/30	IDWDDDKYYSTSLK	IGHV 2–70 (IGHV 2-70*01)	100	(34, 35)
490.786	2	47	LLI**H**GAS**N**R	CDR 2	Yes	3/29	0/26	LLIYGASSR	IGKV 3–20 (IGKV 3-20*02)	77.8	-
656.830	2	52	YNSAPLTFGGGTK	CDR 3	No	3/29	0/26	YNSAPLTFGGGTK	IGKV 1-27 & IGKJ4 (IGKV 1-27*01 & IGKJ4*0)	100	-

Abbreviations used: CDRs, complementarity determining region (CDR1, CDR2, CDR3); MS/MS, tandem mass spectrometry; z, charge, **a)** Amino acid sequence of IgH and IgL CDR obtained based on the MS/MS spectra. CDR is underlined; the mutation in amino acid from the germline is shown in bold. **a'** and **a''** denote sequences resembling sequences with a different mutation, present exclusively in one patient **b)** The position of sequence in Ig structure is indicated. **c)** The number of MScl patients in which these shared CDR motifs were found (shared in patients/total number of patients). **d)** Sequence translated from the Ig germline and derived from IMGT database. **e)** The most homologous IgH and IgL germ line gene.

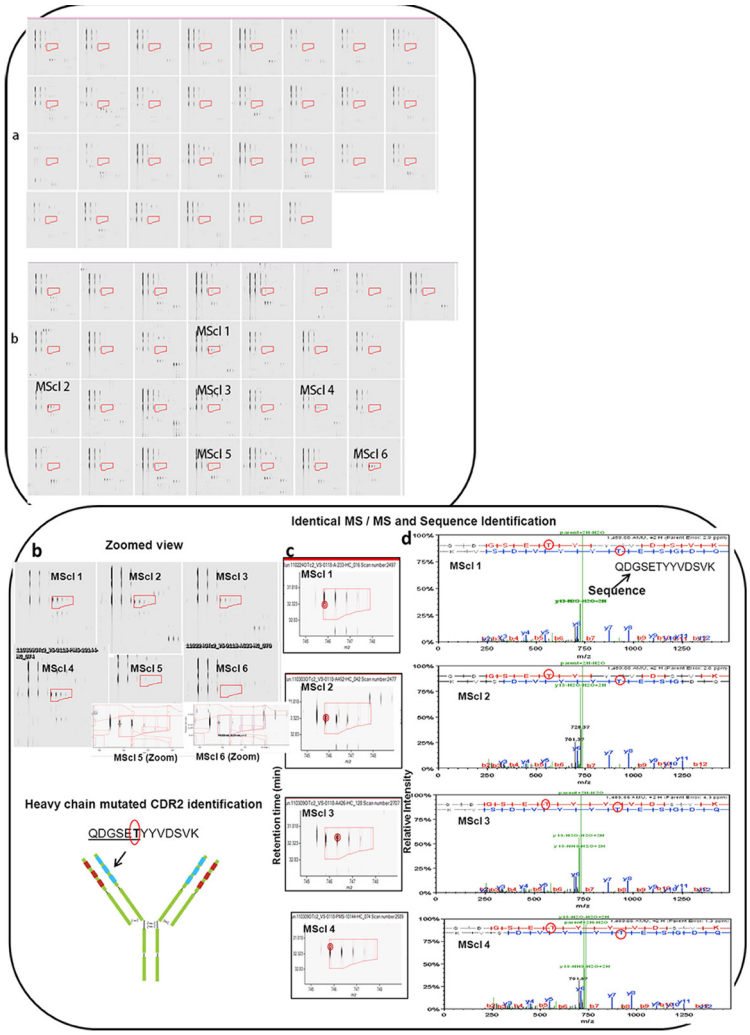

Figure 4. QDGSETYYVDSVK mutated CDR2 identification in six MScl patients and not in 30 controls. Peptides were analyzed by Progenesis LC-MS and comparison between MScl and control was performed. After alignment of the chromatograms in the Progenesis software, 2D profiles were compared to find expression profile between both groups. (a) Precursor mass of the peptide not found in any of the 30 controls. (b) Precursor mass of the peptide identified in six out of 29 MScl patients. The region covered in a) and b) by red box/isotope boundaries indicates area of interest and shows precursor mass isotopic pattern, peptide abundance was calculated as the sum of the peak areas within these isotope boundaries. (c) Example of identical MS/MS fragmentation spectra in different MScl samples. Red circle (at left) in the figure indicates location of retention time and mass (m/z) where MS / MS scan was triggered. Almost identical MS/MS fragmentation spectra were observed in different patients. (d) A MS/MS spectra containing the m/z values and abundances of peptide ion products. Longer series of contiguous y and b ions shows a higher probability of correct identification. The sequence proposes a mutated CDR2 peptide. Red circle shows mutated (T) amino acid from germ line that was found during alignment using IMGT database. A CDR peptide was defined as a peptide containing a minimum of three amino acids in the CDR part irrespective of framework length.

DISCUSSION

The main observation of this study was the shared use of identical mutated sequences in CDR of purified Ig from CSF of different MScl patients. In contrast, in a set of 30 healthy controls no overlap in the tryptic peptide sequences of CDR regions existed. To the best of our knowledge, this is the first study that uses a proteomics approach to analyse and compare the sequences of purified Ig from CSF of a significant large set of MScl patients and controls. Most studies until now have investigated clonality at the transcriptome level (8, 27-29). In these previous studies no common sequences between distinct individuals had been reported. These studies used FACS based cell sorting method or CSF cell isolation and ended up with limited number of CD19+ B cells repertoire or/and CD138+ plasma cells (8, 28). Further they used cloning at nucleic acids level by means of PCR based technologies. Apart from the larger number of patients investigated, here we have targeted the CSF Ig at the protein level and did not investigate CSF B cells.

Although, all MScl CSF samples tested here had signs of elevated intrathecal Ig production we cannot claim that the public sequences shared here between distinct individuals are responsible for the OBs seen in immunoelectric focussing used in routine hospital chemistry. It should be realised that the sophisticated MS technique applied here makes it possible to detect peptide sequences at concentrations far below the threshold for routine immunoelectric focussing (18).

The current paradigm in Immunology is that antigen specificity of B cells is determined via random mechanisms, and therefore one would expect different sequences of the antigen binding CDR between different individuals. This has recently been challenged by a number of observations. First, Scheid et al. cloned 576 new Human immunodeficiency virus (HIV) antibodies from four unrelated individuals and found that despite extensive hypermutation, these antibodies shared consensus sequences in both framework and CDR V - regions of IgH chains (9). In another study in Sjögren's syndrome, secreted human Ro52 antibody from different patients were found to share public V region sequences in unrelated patients (30). Finally, our group observed 28 common Ig - derived sequences in the paraneoplastic anti Hu - syndrome that were specific for auto-antigen and were found exclusively in samples from a single autoantibody defined clinical neurological entities (12).

Our study in MScl patients appears to follow the same paradigm challenging pattern. A considerable set of 24 IgH and 26 IgL CDR peptides were exclusively present in MScl patients. We found nine peptides in the IgH set and six peptides to be shared among at least three MScl patients. These numbers were somewhat higher with a lower threshold for sharing Ig in at least two MScl patients. At lower threshold (shared in two MScl patients), we observed 14 CDR peptides in IgH and 13 in IgL exclusively in MScl

patients. This indicates that under (auto) antigenic pressure there may be common selection mechanisms for the production of intrathecal Ig production by B lymphocytes. The suggestion of shared sequences within different MScl individuals is reminiscent to what has been reported on recruited T lymphocytes in this disease (31).

Although the reason remains unproven it would not be farfetched to speculate that some CDR sequences may better survive the clonal selection process than others, perhaps due to a stronger binding of the 3 D structure of the CDR to the antigen. Better insight in common selection mechanisms for (auto) antibodies in MScl and identification of inter-individually shared specific CDR sequences might even deliver markers for subgroup identification.

Apart from focusing on the CDR regions, we also investigated possible use of common VH and lgL chain families. VH repertoires can be divided into seven families based on sequence similarity (22). Previous studies at the genomic level indicated a skewed use of VH4 in MScl (8, 32). Although it is hard to draw firm conclusions here, it was striking to see over-representation of peptide sequences ($p = 0.03$) of the VH4 family in MScl patients compared to controls. In addition, we observed a trend towards increased VH3 family usage in MScl ($p = 0.05$). No significant difference was observed for the use of VK chain between both groups.

An additional observation here has been the increased κ/λ ratios in the MScl group. This observation is in agreement with previous studies (33) using conventional assays for IgL detection.

Compared to the other studies, a limitation of our study was that we do not know the antigen specificity of the V-regions identified here. In fact we do not show complete Ig sequences, due to the fact that trypsin digestion is needed for this approach. In light of the shared sequences observed in known antigen-specific antibodies in HIV infection, anti-Hu paraneoplastic disease and Ro52 autoimmunity it would be of future relevance to investigate possible use of public V - region sequences in purified specific antibodies against MScl candidate antigens such as anti MOG, anti neurofascin and anti KIR 4.1 (6, 34, 35).

In the available previous studies no attempts were shown to match the identified sequences with those available in public data bases such as BLAST (NCBI). We here performed such a cross-check and were surprised to notice that similar sequences were found in other studies investigating B cells from CSF of MScl patients.

Although the used technique makes it impossible to show the sequence of complete Ig proteins, it is striking that these peptides have also been identified in other MScl studies. For example our study showed a common CDR usage in seven different patients. This QDGSEKYYVDSVK peptide which is part of the CDR2 belongs to the IGHV 3-7 germ line. We found different amino acid mutations (T, E or N) and insertion (F) at the same spot (K) of when compared with the germ line. QDGSE**T**YYVDSVK and QDGSE***TF***YVDSVK

sequences were published in the database from the study assessing intrathecal CSF B cells sequences on two MScl patients using RT - PCR approach (24). The peptide QDGSE***T F***YVDSVK was also shown by a different study that sequenced CSF OBs in four MScl patients using proteomics approach (14). Furthermore gene IGHV 3-7 associated with same has also been shown in previous studies performed on CSF B–cells of MScl patients (14, 24, 25). Next, gene IGHV2-70 related to IDWDDDKYYSTSLK and IDWDDDKYY**T**TSLK has also been described in different previous studies performed on CSF B cells of MScl patients (25, 26). The peptide IDWDDDKYYSTSLK has no mutation, while it is plausible that this peptide may be found in a larger healthy population, we presume that this peptide is enriched in the MScl population. Certain V-genes may be preferentially selected in the antibody response against certain antigens (Repertoire bias) (7). Thus, such peptides, especially as a part of a larger panel of peptides, help in the identification of patient populations even if they are not unique to that population. Next, peptide YNSAPLTFGGGTK does not contain amino acids introduced by somatic hypermutation but it was generated by V(D)J - recombination. Nevertheless it is unique because of its particular deletion of two nucleotides of the V-region in conjunction with one nucleotide of the J-region plus the lack of any additional N-nucleotides. In all, three of the CDRs observed in three or more MScl patients have been linked to MScl in the past.

In MScl patient's the phenomenon of repertoire bias (7, 8, 32, 36) has been described, whereby specific genes from the germline repertoire are favoured in the panel of antibodies that is produced during the immune process. These pressures drive antibodies in convergent directions. Indeed one might expect many CDR3 sequences as they are important for the specificity of an antibody. However, two aspects may favour CDR 1/2 instead. First, CDR3 are generally highly mutated on the border of V, D and J germline alleles. As such they are difficult to identify by homology to the germline sequences, and may remain unidentified. Second, it may be that the highly mutated CDR3 are in fact mostly unique to an individual, and that motifs shared between individuals are instead found in the moderately mutated CDR1/2 regions.

To conclude, this proteomic study shows for the first time CDR peptides shared between individual MScl patients and not in controls. There was a striking overlap with a few CDR peptides identified in other studies that assessed B cell clonality in MS CSF at the nucleic acid levels. Whether such common B cell responses are indeed driven by auto-antigens remains to be determined. It will be of interest to study common V-region use in known autoreactive Ig's that appear to play a role in MScl (6).

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Chapter 5

Effects of Natalizumab treatment on the cerebrospinal fluid proteome of multiple sclerosis patients

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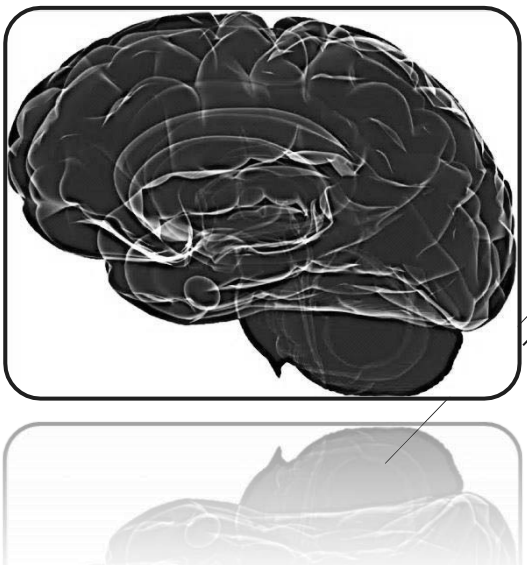
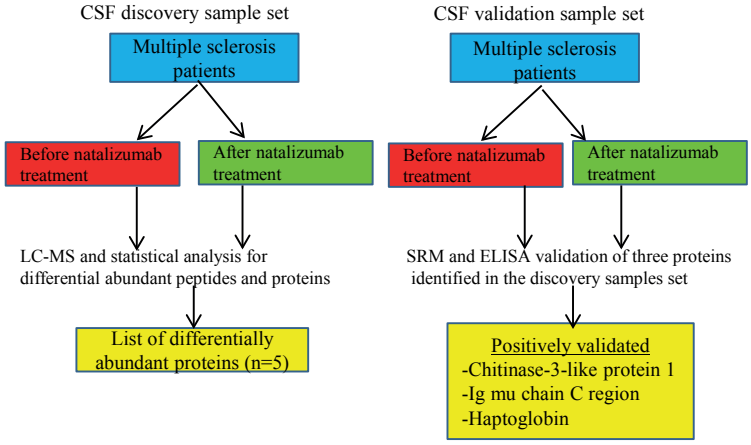


ABSTRACT

Natalizumab is a very effective, relatively new drug for the treatment of relapsing remitting multiple sclerosis. Inflammatory and neurodegenerative processes in the central nervous system are presumed to cause adverse effects during the course of this disease. To monitor the effects of Natalizumab treatment on the cerebrospinal fluid (CSF) proteome of patients, CSF samples were taken from patients before commencing treatment as well as after one year of treatment. Profiling proteomics experiments using electrospray Orbitrap mass spectrometry and pair wise comparison of patients before and after one year of Natalizumab treatment revealed a number of candidate biomarkers that were significantly differentially abundant between the before- and after treatment groups. Three proteins were subsequently validated using selected reaction monitoring (SRM) in a new, independent sample set. All three proteins, Ig mu chain C region and haptoglobin, both known inflammation-related proteins, as well as chitinase-3-like protein 1, were confirmed by SRM to be significantly lower abundant in CSF of multiple sclerosis patients after one year of Natalizumab treatment. The findings for chitinase-3-like protein 1, a presumed biomarker for more rapid progression from a first clinically isolated syndrome to clinically definite multiple sclerosis, was further confirmed by ELISA measurements.



INTRODUCTION

Multiple sclerosis (MScl) is a common cause of neurological disability in young adults. The cause of the severe neurological disability is the interruption of myelinated tracts in the central nervous system (CNS) (1). Most pathogenesis models for the disease support the existence of two connected processes that occur in patients with MScl; inflammation and neurodegeneration (2). These processes are not uniformly represented across patient populations but can predominate selectively in individual patients, contributing to the heterogeneity of phenotypic expression of the disease (3). The majority (approximately 85%) of MScl patients have the relapsing remitting (RR) MScl subtype of the disease (4). RR MScl is characterized by relapses with increased disease activity, which occur at irregular intervals, followed by periods of remission. It is believed that the disease process starts with increased migration of auto-reactive lymphocytes across the blood-brain barrier (5). Treatment for RR MScl is focused on the reduction of the number of relapses. These therapeutic agents, such as interferon beta and glatiramer acetate, are moderately effective and reduce the annualized relapse rate by about a third (6, 7). The monoclonal antibody Natalizumab (brand name: Tysabri) is an effective agent for relapsing remitting multiple sclerosis patients shown by a 68% reduction of relapse rate plus a strong effect on MRI lesion parameters (8).

The therapeutic effect of Natalizumab is mediated by suppression of leukocyte migration into the central nervous system by blocking the Very-Late Activation Antigen 4 ligand on immune cells(9). As a consequence, a reduction of inflammation and inflammatory mediators might be observable in the central nervous system of Natalizumab treated patients. There are indications that also neurodegeneration-related proteins can be influenced by Natalizumab treatment (10).

We anticipated that the strong effects of Natalizumab on MS disease activity plus the possibility to study CSF samples before and after treatment provides a unique model to study potential CSF markers that are relevant for CNS pathology, and may eventually even serve as markers for effects of therapy. Therefore we here employed state-of-the-art quantitative proteomics techniques to differentiate between cerebrospinal fluid (CSF) samples of multiple sclerosis patients before and after treatment with Natalizumab.

METHODS

CSF samples were taken from relapsing remitting MScl patients before Natalizumab treatment commenced at the University Medical Center Eppendorf (Hamburg, Germany). The post-treatment samples were taken at the same hospital one year after the

Table 1. Details of all patients and related samples included in this study (discovery and validation sample sets). The values for age, disease duration, EDSS score (Expanded Disability Status Scale) and protein concentrations are averages (with standard deviation in brackets). In both sample sets the change in EDSS, as well as the protein concentration in the discovery sample set, from before treatment to after treatment is not significant (p>0.01).

	Discovery set samples (Germany)	Validation set samples (Sweden)
Gender distribution (M/F)	4/13	5/15
Age (years)	37.1 (8.1)	41.4 (9.8)
Disease duration (years)	8.4 (5.8)	11.2 (6.6)
EDSS before treatment	3.7 (1.3)	4.3 (1.9)
EDSS after treatment	3.6 (1.4)	4.2 (1.9)
Protein concentration before treatment (mg/L)	381.7 (75.1)	-
Protein concentration after treatment (mg/L)	318.8 (87.3)	-

start of Tysabri treatment. The Tysabri treatment consisted of a year-long treatment of monthly Natalizumab infusion (300 mg intravenously, infused over approximately one hour, every four weeks). All patients gave their consent for the repeated CSF sampling. Immediately after sampling, the CSF samples were centrifuged for 10 min at 3000 rpm to discard cells and cellular elements. The samples were subsequently used for routine CSF diagnostics, which included quantification of total protein concentration (table 1). The remaining volume of the samples was aliquoted and stored at −80°C, where they remained until sample preparation for this study. The sample details are listed in table 1 (discovery sample set column).

From each CSF sample, 20 µL was added to 20 µL of 0.2% Rapigest (Waters, Milford, MA) in 50 mM ammonium bicarbonate buffer. After 30 min incubation periods with 1,4-dithiothreitol (60°C) and, subsequently, iodoacetamide (37°C), 4 µL of 0.1 µg/µL gold-grade trypsin (Promega, Madison, WI)/3 mM Tris-HCl (pH 8.0) was added to each sample. The samples were incubated overnight at 37°C. To adjust the pH of the digest to pH < 2, trifluoroacetic acid (TFA) was added to the mixture prior to the final incubation step at 37°C for a duration of 45 minutes to stop the enzymatic digestion reaction.

Mass spectrometry measurements were carried out on a Ultimate 3000 nano LC system (Dionex, Germering, Germany) online coupled to a hybrid linear ion trap/Orbitrap MS (LTQ Orbitrap XL; Thermo Fisher Scientific, Germany). Five µL digest (i.e. 2 µL CSF) were loaded on to a C18 trap column (C18 PepMap, 300 µm ID ×5 mm, 5 µm particle size, 100 Å pore size; Dionex, The Netherlands) and desalted for 10 minutes using a flow rate of 20 µL/min 0.1% TFA. Then the trap column was switched online with the analytical column (PepMap C18, 75 µm ID ×150 mm, 3 µm particle and 100 Å pore size; Dionex, The Netherlands) and peptides were eluted with following binary gradient: 0%–25% solvent B in 120 min and 25%–50% solvent B in further 60 minutes, where

solvent A consist of 2% acetonitrile and 0.1% formic in water and solvent B consists of 80% acetonitrile and 0.08% formic acid in water.

Column flow rate was set to 300 nL/min. For MS detection a data dependent acquisition method was used: high resolution survey scan from 400–1800 Th. was performed in the Orbitrap (value of target of automatic gain control AGC 106, resolution 30,000 at 400 m/z; lock mass was set to 445.120025 u (protonated (Si(CH3)2O)6(11)). Based on this survey scan the 5 most intensive ions were consecutively isolated (AGC target set to 104 ions) and fragmented by collision-activated dissociation (CAD) applying 35% normalized collision energy in the linear ion trap. After precursors were selected for MS/MS, they were excluded for further MS/MS spectra for 3 minutes.

The raw data was pre-processed using the Progenesis LC-MS software package (version 2.6, Nonlinear Dynamics, Newcastle-upon-Tyne, United Kingdom). Peptides were identified and assigned to proteins by exporting features, for which MS/MS spectra were recorded, using the Bioworks software package (version 3.2; Thermo Fisher Scientific, Germany; peak picking by Extract_msn, default settings). The resulting .mgf file was submitted to Mascot (version 2, Matrix Science, London, United Kingdom) for identification to interrogate the UniProt-database (release 2010_04; taxonomy: Homo sapiens, containing 77399 sequences). Only ions with charge states between +2 and +7 were considered and only proteins with at least two unique peptides (Mascot ions sore > 25, (i.e. a peptide probability cut off value of 0.01)) assigned to them were accepted as true identifications. Modifications: carbamidomethylation of cysteine was set as fixed and oxidation of methionine as variable modification, allowing a maximum of 2 missed cleavages. Mass tolerance for precursor ions was set to 10 ppm and for fragment ions at 0.5 Da. The Mascot search results were imported back into the Progenesis software to link the identified peptides to the detected abundances of these peptides. The peptide abundances were normalized to the total ion current to compensate for experimental variations using an algorithm available in the analysis software. Subsequently the data were exported in Excel format.

The abundances of all identified peptides were compared between the groups of samples (before and after treatment with Natalizumab) by performing a paired t-test on all individual peptides. Proteins of which 50% or more of the peptides had a low p-value in this t-test ($p<0.01$) were deemed to be significantly differentially abundant between the two groups.

Validation by Selected Reaction Monitoring

To validate the found differentially abundant proteins a validation sample set of CSF samples of Natalizumab treated relapsing remitting multiple sclerosis patients was collected at the Karolinska Institute in Stockholm, Sweden. The CSF samples, which were

taken from 20 patients both before and after treatment were stored at −80°C before shipping on dry ice to the analysis laboratory (Table 1). Subsequently these samples were enzymatically digested following the exact same protocol as used for the original samples. The digested CSF samples were spiked (at 20 fmol/µL, corrected for peptide purity (99%)) with known concentrations of stable isotope-labelled peptide standards corresponding to sequences 100-112 (NVPLPVIAELPPK) and 113-120 (VSVFVPPR) of Ig mu chain C region (P01871), sequences 119-131 (TEGDGVYTLNNEK) and 162-170 (ILGGHLDAK) of haptoglobin (P00738), and sequences 322-335 (GNQWVGYDDQESVK) and 370-377 (FPLTNAIK) of chitinase-3-like protein 1 (P36222) for quantification by selected reaction monitoring (SRM).

Peptides in spiked CSF digests were separated by reversed-phase chromatography on an Ultimate 3000 nano LC system (Dionex). Spiked CSF digest (1 µL) was loaded onto a C18 trap column (PepMap C18, 300 µm ID by 5 mm length, 5 µm particle size and 100 Å pore size; Dionex) and washed for 5 min at a flow rate of 20 µL/min with 0.1% TFA in H2O. Next, the trap column was switched in line with the analytical column (PepMap C18, 75 µm ID by 150 mm length, 3 µm particle size and 100 Å pore size; Dionex). Peptides were eluted at a flow rate of 300 nL/min with the following gradient: 0%–45% solvent B in 30 min, where solvent A is H2O/acetonitrile (ACN) 98%/2% (vol/vol), 0.1% formic acid (FA) and solvent B is H2O/ACN 20%/80% (vol/vol), 0.1% FA. Separation of the peptides was monitored with a UV detector (absorption at 214 nm).

SRM analysis was performed on a 4000 QTRAP (AB Sciex, Concord, Canada) in the positive ion mode. Three transitions were attempted for quantification for all peptides (NVPLPVIAELPPK, transitions y3, y9, and y11; VSVFVPPR, transitions y3, y4 and y5; ILGGHLDAK, transitions b6, b7 and y7; GNQWVGYDDQESVK, transitions y7, y8 and y9; and FPLTNAIK, transitions y5, y6 and y7). No transitions could be quantified for the TEGDGVYTLNNEK peptide of haptoglobin, as this peptide could not be detected on the ESI-QqQ. Hence haptoglobin was quantified on the values obtained for the ILGGHLDAK peptide only. All transitions of the FPLTNAIK peptide of chitinase-3-like protein 1 proved to be lacking sensitivity for quantification, so these were subsequently excluded from the quantitative analysis. The y7 transition of NVPLPVIAELPPK of chitinase-3-like protein 1 was excluded from the calculations due to interference, hence this peptide was quantified by two transitions, whereas the remaining three quantified peptides were quantified by three transitions. The linearity of the SRM assay was determined in the range of 100 amol/µL to 100 fmol/µL, and peak heights proved to be linearly well correlated to spiked peptide concentrations ($R2 > 0.9947$ for all 11 quantified transitions. The instrument was optimized for collision energy en declustering potential per individual peptide, and the cycle time was set to 0.9850 seconds for a total of 2132 cycles per sample. Limits of detection and quantification were not determined, however, all quantified transitions had acceptable S/N ratios ($S/N > 4$). We

performed data analysis using the SRM data analysis program Skyline (version 1.1). Peak areas were checked for interference with other peaks in the biological samples and if necessary adjusted to eliminate interference, prior to exportation of the heavy/light peptide ratio values. Concentrations of the analyte peptides were determined based on the ratio between the peak area of the analyte peptide to the peak area of the spiked isotope-labelled internal peptide standard, which was added at a known concentration, using the average value calculated per peptide based on all quantifiable transitions. Hence the concentration of the GNQWVGYDDQESVK peptide is based on two transitions, whereas the concentrations of the NVPLPVIAELPPK, VSVFVPPR and ILGGHLDAK peptides are based on three transitions.

Validation of Chitinase-3-like protein 1 by ELISA

To further confirm the differential abundance of chitinase-3-like protein 1 between untreated and Natalizumab treated multiple sclerosis patients, an ELISA for this protein was purchased, and performed according to the manufacturers (R&D Systems, Abingdon, United Kingdom) instructions.

RESULTS

Using ESI-Orbitrap mass spectrometry a total of 3289 peptides were identified during the measurements of the "before treatment" and "after treatment" CSF samples of the seventeen relapsing remitting multiple sclerosis patients in the discovery sample set. These peptides relate to 578 unique proteins, of which 301 were identified by 2 or more peptides. Using a paired t-test to compare the CSF samples from before treatment to the samples from after treatment, 122 peptides had a p-value below 0.01 (statistical background determined by permutation: 33+/− 21). Five proteins identified by two or more peptides were identified as having at least 50% of the peptides with p-values below 0.01 in the comparison between the samples before and after treatment. These proteins, namely haptoglobin, Ig mu chain C region, Ig mu heavy chain disease protein, serine protease inhibitor and scavenger receptor cysteine-rich type 1 protein M130, are listed in Table 2. Additionally, chitinase-3-like protein 1, a potential CSF marker for conversion of clinically isolated syndrome of demyelination (CIS) to clinically definite MScl (12), was identified by eleven unique peptides. One of these peptides had a significant p-value in the statistical analysis, and an additional five peptides showed low, nearly significant p-values (between 0.016 and 0.071). All peptides of chitinase-3-like protein 1 were decreased in the Orbitrap measurements of the CSF samples after treatment. Hence, although not statistically significant according to the predetermined criteria, this protein did show a trend towards statistical significance.

Table 2. The fourteen peptides identified as differentially abundant between the "before treatment" and "after treatment" groups.

Primary accession number	Protein	Number of peptides with p<0.01 / total number of peptides	Higher abundant in group
P00738	Haptoglobin	15/26	Before treatment
P01871	Ig mu chain C region	7/12	Before treatment
P04220	Ig mu heavy chain disease protein	1/2	Before treatment
P05154	Serine protease inhibitor	1/2	Before treatment
Q86VB7	Scavenger receptor cysteine-rich type 1 protein M130	1/2	Before treatment

Because this protein has been previously implicated as a conversion factor for CIS to MScl, we decided to include chitinase-3-like protein 1 in the validation stage of the study. The majority of proteins related to the inflammatory response, including previously reported potential markers for multiple sclerosis complement C3, complement C4 and clusterin(13), were lower abundant after Natalizumab treatment, although this did not reach statistical significance, with the exception of haptoglobin and the immunoglobulin proteins listed in table 2. Previously reported markers of neurodegenerative processes, chromogranin A and contactin-1(14), were not significantly changed after treatment, although both proteins were higher abundant after treatment. None of the four proteins reported to be decreased in abundance after six months of Natalizumab treatment by Ottervald et al.(10) was statistically significantly changed after six months of Natalizumab treatment. Alpha-1 antichymotrypsin was slightly less abundant after treatment and the other three proteins, contactin-1, neuronal cell adhesion molecule and neural cell adhesion molecule 1, were slightly more abundant after treatment. Three proteins, haptoglobin, Ig my chain C region and chitinase-3-like protein 1, were selected for validation using SRM. The other three proteins listed in table 2 were not selected for validation because they were identified by two peptides,

Table 3. SRM results for the three proteins selected for validation. The listed concentrations for the proteins are averages (with standard deviations in brackets). The p-values indicate that all three proteins (chitinase-3-like protein 1, Ig mu chain C region and haptoglobin) are significantly lower in concentration in CSF of multiple sclerosis patients after Natalizumab treatment.

Protein	Peptide	CSF concentration before Natalizumab treatment (mg/L)	CSF concentration after Natalizumab treatment (mg/L)	p-value
Chitinase-3-like protein 1	GNQWVGYDDQESVK	0.153 (0.101)	0.100 (0.065)	0.038
Ig mu chain C region	NVPLPVIAELPPK	1.061 (1.142)	0.538 (0.522)	0.024
Ig mu chain C region	VSVFVPPK	0.179 (0.175)	0.072 (0.080)	0.026
Haptoglobin	IGGHLDAK	0.803 (0.610)	0.433 (0.339)	0.002

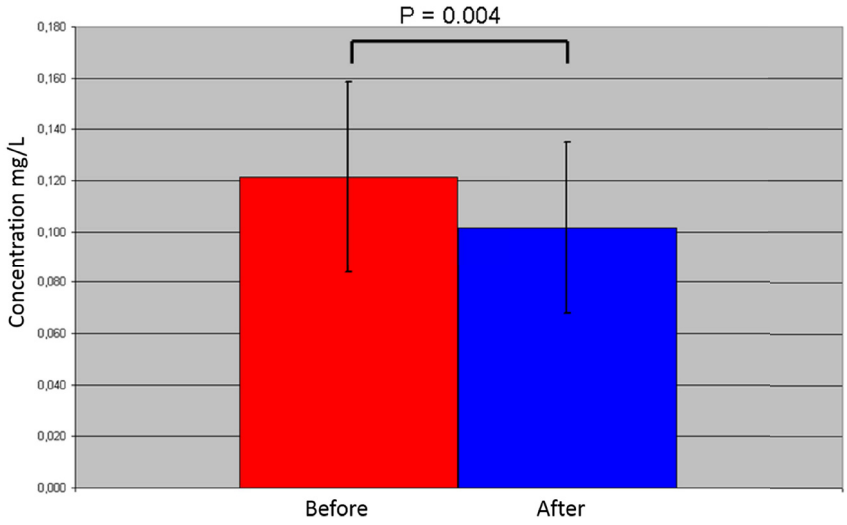


Figure 1. Concentrations of chitinase 3-like protein 1 in CSF of MScl patients before (red) and after (blue) treatment with Natalizumab as determined by ELISA. The concentration of this protein in CSF is significantly lower after treatment (p = 0.004).

and only one of these was significantly different between the sample groups. This validation procedure showed that the peptides of these proteins were significantly lower in concentration in CSF of MScl patients after treatment with Natalizumab in a new, independent sample set (p = 0.038 for chitinase-3-like protein 1, p = 0.024 and p = 0.026 for Ig mu chain C region, and p = 0.002 for haptoglobin, Table 3), confirming the results obtained by the ESI-Orbitrap. The concentrations of these peptides (and related proteins) in individual patients were decreased in CSF after treatment with Natalizumab in nearly all patients. Additionally, an ELISA for chitinase-3-like protein 1 was performed for further confirmation, which showed a significant decrease in abundance of this protein after treatment (p =0.004) in CSF of MScl patients in the validation sample set (Figure 1).

DISCUSSION

In this study, we analyzed the CSF proteome of MScl patients treated with Natalizumab to assess the effects of the drug on the proteins in the CSF by comparing paired samples from patients before treatment and twelve months after treatment. Overall the changes in the CSF proteome after twelve months treatment with Natalizumab were limited, but we were able to make a number of interesting observations. The applied label free LC-MS strategy of this study allowed for the semi-quantitative analysis of

over 300 proteins, which, although surely do not entail the entire CSF proteome, allows analysis of the CSF proteome beyond merely the high abundant proteins. The three-hour chromatography gradient is capable of separating the digested peptides of the CSF proteome sufficiently to enable an in-depth analysis of the CSF proteome, although fractionation or depletion of the samples would potentially allow for even a more in-depth analysis.

The observation that the proteins related to inflammation are less abundant in CSF after Natalizumab treatment is in line with the proposed mechanism of action of the drug (15). Natalizumab, a humanized monoclonal IgG4κ antibody, selectively binds to the α4-integrin component of adhesion molecules found on lymphocytes and monocytes. This binding inhibits interaction with cells expressing the ligand of VLA-4, vascular cell adhesion molecule-1 (VCAM-1), consequently blocking adherence of activated leukocytes to endothelial cells, which is a required step of the extravasation into inflamed tissue (16). Hence inflammation is inhibited in MScl patients due to the fact that Natalizumab prevents the migration of autoreactive leukocytes out of blood vessels into target organs, such as the CNS, by blocking the adhesion molecules of the α4-integrin component of adhesion molecules on leukocytes.

Also in line with the proposed mechanism of Natalizumab is the lack of significantly differentially abundant neurodegenerative proteins after treatment. As the drug mechanism firmly points towards an effect on the inflammatory part of the disease processes, an effect on neurodegeneration was not directly expected to be observed here. Although a previous study shows that axonal damage in MScl is reduced by Natalizumab treatment, it is anticipated that a highly effective anti-inflammatory treatment reduces axonal loss, as inflammatory activity is associated with axonal damage (17). Consequently, these neurodegenerative effects are likely secondary to inflammatory processes. In contrast to the study by Ottervald and co-workers, in the presented study CSF samples from 6 months after the start of Natalizumab treatment were not included (10). The neurodegeneration-related proteins, alpha-1 antichymotrypsin, contactin-1, neuronal cell adhesion molecule and neural cell adhesion molecule 1, which Ottervald et al. described as significantly less abundant after six months of treatment, but not after twelve months treatment, were not significantly differentially abundant after twelve months of Natalizumab treatment in our study. Since no CSF samples were taken after six months of Natalizumab treatment and analysed in this study, as well as the fact that the experimental procedures differed substantially between the studies, it is difficult to compare them. Three of the four proteins reported as lower abundant by Ottervald and co-workers (10) were even slightly higher abundant after treatment, although these values did not reach significant levels. Hence, no proteins that had previously been related to neurodegenerative effects in MScl could be observed in CSF to be affected by Natalizumab treatment in this study.

A number of the differentially abundant proteins found to be less abundant after Natalizumab treatment (Table 2) is related to the inflammatory response, which is in the pathogenetic hallmark of MScl. Consequently, the observation that inflammatory response-related proteins with a known higher abundance in CSF of MScl patients such as immunoglobulins and haptoglobin (18) are downregulated after treatment with a drug that blocks the entry of inflammatory cells into the CNS and the subsequent inflammatory effects stands to reason. All proteins identified as significantly higher abundant after treatment were only identified by a single peptide, which is less reliable than identification by multiple peptides. Two of the differentially abundant proteins, haptoglobin and Ig mu chain C region (both lower abundant after treatment), were subsequently validated by a different, quantitative mass spectrometry method in an independent data set. The validation experiment confirmed the Orbitrap profiling results for both proteins even though one of the peptides of haptoglobin could not be quantified for technical reasons.

Both IgM and haptoglobin are related to the inflammatory response and have been previously implicated in multiple sclerosis (19, 20). The proposed mechanism of action of Natalizumab includes inhibition of leukocyte infiltration into the CNS, therewith dampening the local inflammatory reaction. In light of this the lower abundance of Ig mu chain C region and haptoglobin in CSF of multiple sclerosis patients after Natalizumab treatment may not be surprising. Still, it is of note that a previous study has linked intrathecal IgM production with MS disease activity (19).

The third and final protein that was quantified in the SRM validation experiment, chitinase 3-like protein 1, was not significantly differentially abundant according to the stringent criteria set for the Orbitrap profiling experiments ($p < 0.01$ for 50% of the peptides of a protein). However the fact that a clear trend towards statistical significance could be observed, coupled with a report that chitinase 3-like protein 1 is a potential marker for conversion of CIS to clinically definite MScl (12), prompted the inclusion of this protein in the validation experiment. Both the validation by SRM ($p = 0.038$) as well as by ELISA ($p = 0.004$) confirmed the Orbitrap profiling experiment. The difference in p-values between the validation (statistically significant) and profiling (not statistically significant) experiments is most likely due to the selectivity of the validation method, as accurate quantification is more reliable using targeted methods such as SRM and ELISA, compared to untargeted methods like full scan label free Orbitrap measurements. The values measured for the differentially abundant proteins in the individual patients were, in the vast majority of the cases, lower abundant after Natalizumab treatment. Two patients were exceptions to this observation, but for which we could not identify an obvious clarification. Nevertheless, the observed trend in the remaining individuals is a clear illustration of the differential abundance of the proteins in these Natalizumab treated multiple sclerosis patients.

Some caution is needed about the interpretation of our data, as this study (because of medical ethical reasons) did not include a control group of patients followed over time without receiving Natalizumab treatment. The possibility of a placebo effect on biomarkers or other unknown factors theoretically remains. Despite this note of caution, the treatment associated differences in this study group were striking, the statistical thresholds for relevance were relatively high and the findings have a biological rationale.

Chitinase 3-like protein 1, which is also known as YKL40, is a chitin-binding, but chitinase activity-lacking member of the glycoside hydrolase 18 chitinase family (21). One of the main cell types that secretes this protein are macrophages, linking the protein to the inflammatory response. Increased abundance of this protein in all likelihood is not specific for conversion of CIS to MScl or response to Natalizumab treatment in MScl patients, but rather a marker for inflammatory activity in the affected compartment, which is supported by the observation of increased levels in serum of several types of cancers and diseases such as rheumatoid arthritis (22-24). Additionally, the role of the chitinase family in the pathogenesis of MScl is further exemplified by the observation of increased plasma levels of chitotriodase in MScl patients compared to controls (25, 26).

In conclusion, in this hypothesis-free proteomic approach we observed that Natalizumab treatment of MScl patients resulted in a decrease in the levels of inflammation-related proteins in the CSF, which is in line with the proposed mode of action of natalizumab. CSF proteins related to neurodegeneration were not affected by Natalizumab treatment; although statistical power may have been insufficient to detect this most interesting was a decrease in the CSF levels of chitinase 3-like protein 1 after twelve months treatment with Natalizumab. The relevance of this marker for MScl activity has recently been indicated because this molecule is a potential marker for conversion of CIS to clinically definite MScl.

ACKNOWLEDGEMENTS

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Chapter 6

Proteomics urine analysis of pregnant women suffering from multiple sclerosis

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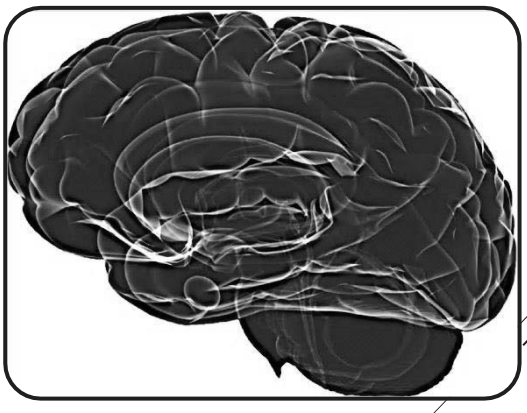
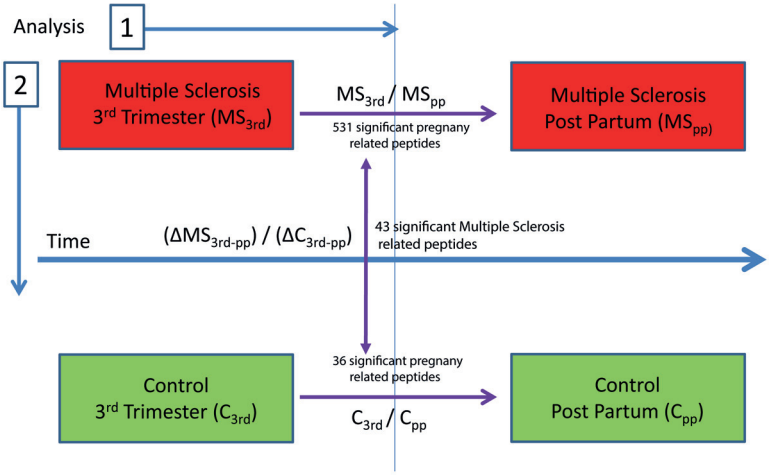


ABSTRACT

Multiple sclerosis (MScl) disease frequently remits during the third trimester of pregnancy but exacerbates in the first postpartum period. In this context, we investigated protein identification, its abundance and its change related to these two periods in urine. Using mass spectrometry (LTQ Orbitrap), we identified 1699 tryptic peptides (relates to 402 proteins) in urine from 31 MScl and 8 control at these two periods. Pregnancy related peptideswere significantly elevated (p<0.01) in MScl patients as compared with controls (Analysis 1; 531 peptides in MScl and 36 peptides in controls higher abundance in the third trimester compared to postpartum). When comparing the longitudinal differences (Analysis 2), we identified 43 (related to 35 proteins) MScl associated peptides (p<0.01) with increased or decreased difference ratio in MScl compared with controls. The most discriminating peptides identified were trefoil factor 3 and lysosomal associated membrane protein-2. Both proteins have a role in the innate immune system. Three peptides with a significant decreased ratio were plasma glutamate carboxypeptidase, Ig mu chain C region, osteoclast associated immune like receptor. Our results indicate that the protein expression pattern in urine of MScl patients contain information about a remote CNS and brain disease processes.



Conclusion: Pregnancy related proteins in urine are elevated in Multiple Sclerosis

INTRODUCTION

Multiple sclerosis (MScl) is a chronic demyelinating disorder of the central nervous system (CNS) (1). During pregnancy, it is described that disease activity is at the lowest level. Compared to a period of one year before pregnancy, a 70% decline in MScl disease activity is noted during the third trimester of pregnancy (2, 3). This decline is followed by an increased annualized relapse rate in disease activity at first postpartum period most clearly in the first three months after delivery, which even goes up to ~70% above the pre-pregnancy rate (4, 5). Most of our knowledge regarding pregnancy-related remission in MScl disease activity is derived from conventional studies that focused on individual components, such as estrogen (6-10). Others have focused on anti-inflammatory thymus cell antigen 1 (Thy1) to thymus cell antigen 2 (Thy2) shifts in peripheral blood (11) and T-cell subset fluctuations during pregnancy (12). In the above context, recently, Gilli and colleagues (13) studied pregnancy-related remission in MScl disease activity on RNA microarray platform using immune cell population of blood/peripheral blood mononuclear cells (PBMCs) and noticed a decrease in inflammation related genes during MScl pregnancy. Brynedal et al. (14) also showed that the disease bouts in MScl primarily depend on events outside of the CNS, which highlights the importance to study changes in urine and other bio-fluids. It can be assumed that the biological mechanisms of this declined disease activity in pregnant women with MScl will involve in altering the protein level in urine. The biological mechanisms or this decreased activity of disease during pregnancy has not been investigated yet neither at protein nor at the DNA/RNA level. Therefore, large-scale identification of proteins and comparison at protein levels in tissues and body fluids compared to genomics data of PBMCs is still needed to get information to understand these phenomena. Our main aim was to identify MScl specific proteins that are potentially related to the changed frequency of relapses and remissions during pregnancy (5), in order to find proteins that are related to the protective effect that pregnancy has on the central nervous system in MScl. These pathologically relevant proteins might be potential treatment targets for MScl or merely be of interest with regards to their function or location in the biological pathways involved in MScl, pregnancy or the disease modifying effects of pregnancy in MScl. We used high resolution nano-scale liquid chromatography online coupled to a high resolution mass spectrometer (LC-MS) to investigate if MScl associated peptides exist in urine. We used urine, longitudinally obtained during pregnancy. In the current study, we quantified and identified the urinary proteome during third trimester (28-30 weeks) of pregnancy, and at the first postpartum period (within 4-8 weeks after delivery) in pregnant women with and without MScl. We emphasized and discussed the tryptic peptides and proteins that are related to pregnancy and proteins that change significantly different in MScl compared to normal pregnancy.

EXPERIMENTAL SECTION

Patients

The described study was approved by the Clinical Research Ethics Board of Erasmus University Medical Center, and all patients provided written informed consent. The exclusion criteria for both patients and controls were recurrent abortion, hypertension, diabetes mellitus or, other systemic diseases. Women with full term pregnancy were included in the study. Normal pregnant controls (n=8) were recruited from the outpatient clinic of Obstetrics, Erasmus MC. The study included 31 MScl patients during their pregnancy at third trimester (28–30 weeks), and the first postpartum (4–8 weeks) visit. MScl patients and controls were matched for age. Patient data collected were: maternal age, disease duration, gestational age at delivery, pre-eclampsia, birth weight, breast feeding, data related to relapse rate before pregnancy, during the third trimesters of pregnancy, and in three months after delivery. The study was designed in such a way that the absence of clinical infection was determined by medical history and questionnaires. MScl patients did not receive any disease modifying therapies during the course of the study. MScl patients and controls had no pregnancy related disorders and no urinary tract infections. The time of sample collection was remained consistent (between 10 am and noon) throughout the study. After collection, urine samples were centrifuged (3000 g) to remove cell debris, and the supernatant was stored at −80°C for subsequent proteomics analysis. Urine creatinine concentrations were determined by routine analysis of the Clinical Chemistry department of the Erasmus MC.

Urine protein purification and in-solution trypsin digestion

Protein was purified as previously described (15), with the exception of a molecular weight cut-off (MWCO) filter, which was not used in the present study. Briefly, samples were taken from the –80°C freezer and thawed at room temperature (RT). Urine (1.2 ml) was mixed with 0.6 ml of 3 M urea, 15 mM NH_4OH, 0.03% SDS and kept at RT for 30 min. Next, 1.5 ml of the mixture was desalting using PD-10 columns (GE Healthcare, Uppsala, Sweden). Columns were equilibrated with 25 ml 0.01% NH_4OH, and subsequently samples were loaded on to these columns. A volume of 2 ml of equilibration buffer was applied to the column, the flow-through was collected and lyophilized (Sublimator 400, Zirbus Technology, Tiel, The Netherlands), and stored at −20°C. Next, these samples were subjected to trypsin digestion. Lyophilized samples were re-suspended in 35 µL LC water. To 25 µL of each sample 25 µL 0.2% Rapigest (Waters Corporation, Milford, MA) 100 mM ammonium bicarbonate was added. The reduction was performed at 5 mM DTT for 30 min heating at 60°C. Thereafter samples were cooled down to RT (approximately 25 min), and subsequently alkylated with at 15 mM for 30 min in darkness and at RT. Afterwards, 500 ng of trypsin (Promega, Madison, WI, USA) was

added (yielding an estimated protease to protein ratio of 1:20). The samples were then incubated overnight at 37°C. The digestion was stopped by adding 5% TFA to obtain a final concentration of 0.5% TFA (pH<2) followed by incubation at 37°C (at 450 rpm) for 30 min. Prior LC-MS analysis, 1 µL of each sample were loaded onto a nano LC system (Thermo Fisher Scientific, Germering, Germany), peptides were eluted by a short 15 min gradient (3.2% to 40% acetonitrile), and abundance of eluting peptides was measured by UV absorbance at 214 nm. Total UV absorbance of all eluting peptides was used to normalize overall abundance differences between various samples and adjust injection volumes for subsequent LC-MS analysis.

LC-MS measurements and database search

Tryptic digested samples were measured on a nano-LC system (Thermo Fisher Scientific, Germering, Germany) coupled in-line to a hybrid linear ion trap/Orbitrap mass spectrometer (LTQ-Orbitrap-XL Thermo Fisher Scientific, Bremen, Germany). Samples were loaded on to a trap column (PepMap C18, 300 mm ID 5 mm length, 5 mm particle size, 100 Å pore size; Thermo Fisher Scientific), washed and desalted for 10 min, using 0.1 % trifluoroacetic acid (TFA) (in water) as loading solvent. Next, the trap column was switched in-line with the analytical column (PepMap C18, 75 mm ID x 500 mm, 2 µm particle and 100 Å pore size (Thermo Fisher Scientific). Peptides were eluted with the following binary gradient (starting with 3% solvent B, then from 3% to 25% solvent B in 60 min and from 25% to 50% solvent B in 30 min, where solvent A consisted of 0.1% formic acid (restituted with HPLC grade water), and solvent B consisted of 80% acetonitrile and 0.08% formic acid (restituted with HPLC grade water). The column flow rate was set to 250 nL/min, and eluting peptides were measured first by a UV detector at a wavelength of 214 nm in a 3 nL nano flow cell (Thermo Fisher Scientific), and subsequently introduced into the MS. All LC solvents were purchased at Biosolve, Valkenswaard, the Netherlands.

For electro-spray ionization nano ESI emitters (New Objective, Woburn, MA, USA) were used and a spray voltage of 1.5 kV was applied. For MS detection, we used a data-dependent acquisition method: high resolution survey scan from 400–1800 Th. was detected in the Orbitrap (target of automatic gain control = 1 E7, resolution = 30,000 at 400 m/z, lock mass was set to 445.120025 Th (protonated (Si (CH3)2O)) 6). On the basis of this full scan the five most intensive ions were consecutively isolated (AGC target set to 10,000 ions), and fragmented by collisional activated dissociation (applying 35% normalized collision energy), and detected in the ion trap. Precursor masses within a tolerance range of ±5 ppm that were selected once for MS/MS were excluded for MS/MS fragmentation for the next 3 min, or until the precursor intensity drop below an S/N of 1.5 for more than five scans. Orbitrap full scan spectra and ion trap MS/MS fragmentation spectra were acquired partially simultaneously. All urine

samples were prepared and measured in random order. In order to monitor the background of the LC-MS system each run was preceded by a blank run. Also, to check the system performance at optimum, a pooled urine sample (10 µl from each) was used as an internal quality control, and was measured once in every five measurements for assessing the quality of the measurements in time (where the quality control check was based on a check for number of identifications and retention time shift with regards to potential retention time alignment).

MS/MS spectra (Orbitrap) from the raw data files of each sample were converted into mgf files using Extract-MSN (part of Xcalibur version 2.2, Thermo Fisher Scientific Inc.), and used to perform database searches using Mascot (version 2.3.01; Matrix Science Inc., London, UK), against the human subset of the Uniprot KB/Swissprot database (version 2011_10, human taxonomy, 20,257 entries). Therefore, following settings were used carbamidomethylation of cysteine (+57.021 u) as fixed and oxidation of methionine (+15.996 u) as a variable modification, tryptic enzyme specificity was allowed a maximal of 2 miss-cleavages. The mass tolerance for precursor ions was 10 ppm, and the mass tolerance for fragment ions was 0.5 Da. Database search results were further processed by Scaffold (version 3.6.3, Proteome Software Inc., Portland, OR) to merge the individual search results, compute protein grouping and calculate peptide and protein probabilities. The resulting peptide identifications were filtered using scaffold. Peptide false discovery rate (FDRs), related to peptide identification, was calculated by Scaffold on the basis of FDR = (false positive) / (false positive + true positives)). The average protein FDR was determined always lower than 0.16 % for proteins and 1 % for peptides. The FDR was determined by a decoy database.

Progenesis Label-free quantification

The LC-MS measured data files were imported into Progenesis LC-MS software package (Version 3.1; Nonlinear Dynamics Ltd, Newcastle-upon-Tyne, UK). Samples were aligned to a reference run (selected objectively by the software) for their retention time using an automated LC-MS algorithm option. Peptide abundances (abundance is defined as area under the peak) were calculated as the sum of the peak areas within the isotope boundaries obtained from the mass spectrometry scan. Only peaks with a charge state ranging from +2 to +8, with two or more isotopes were included for further analyses. LC-MS quantified peptides were further normalized to the total ion current to compensate for experimental variations utilizing an algorithm available in the Progenesis analysis software. Database search results subsequently imported into Progenesis, and assigned to their corresponding peptide peaks. Finally, a spreadsheet that contained for each feature the abundance in the individual samples and, if available, the corresponding peptide identification (feature report). For following statistical calculations, we used normalized and logarithmized (^{2}log) abundances.

Statistical analysis

Statistical comparison was performed using an unpaired two tailed Wilcoxon test. A p-value of <0.01 indicated statistical significance. Two types of analysis were carried out designated as Analysis 1 and Analysis 2. In the first analysis (Analysis 1), pregnancy associated longitudinal changes from third trimester of pregnancy to first postpartum period (ratio between abundances) were determined for 8 controls and for 31 MScl patients separately.

In the second analysis (Analysis 2), disease associated peptides were determined by comparing the longitudinal change from third trimester of pregnancy, and first postpartum period between 31 MScl patients, and 8 controls. First, we determined to what extend and significance, peptides abundance levels change during (third trimester), and after the end of pregnancy (delivery). For this, we calculated the protein abundance ratios (third trimester/postpartum) for each individual and subsequently the mean ratio per group and the difference (significant or not-significant) of the proteins identified (third trimester vs. postpartum). To determine the level of false discovery, sample groups were permutated in a repetitive way for 1000 times. Here, the false discovery was related to statistical significance in the comparison between the groups and the background determined by permutation was analyzed by scrambling/randomizing all samples. We permutated the complete set of 1699 observations (peptides) between four sample groups (MScl: third trimester of pregnancy; MScl: postpartum period; controls: third trimester of pregnancy and controls: postpartum period). This permutation test allowed for determining a background mean value including a standard deviation (SD) for the 1000 experiments performed. For these computations and to generate figures of the obtained data the R statistics package (R version 3.0.2, http://www.R-project.org) (16) was used. For other statistical tests and graphics, Microsoft Excel (version 2010), GraphPad Prism (version 5.00, www.graphpad.com) and SPSS were used.

RESULTS

Clinical characteristics

The main characteristics of the 31 MScl and 8 normal women included in the study are summarized in Table 1. Maternal age (at onset), duration of gestation, number of total pregnancies, deliveries in the past, birth weight, and gender of the baby did not show significant differences (p>0.01) between the two groups (Table 1). Six (out of 31) patients had experienced relapse at the first postpartum period i.e. in first 3 months. One of these 6 had relapse also during the third trimester. Urine creatinine levels did not show any significant differences (p>0.01) between groups. During measurement,

Table 1. Patient characteristics. Table illustrates the pregnancy and delivery characteristics of pregnant women with MScl and controls. Data is shown as median with the range between brackets; mean ± SD is also indicated. The patient and controls characteristics were compared using a Wilcoxon test. Table shows no significant difference (p>0.01) between controls and MScl women.

	Controls (n=8)		MScl patients (n=31)		p value
	Mean ± SD	Median (Range)	Mean ± SD	Median (Range)	Control vs. MScl
Age at onset (Years)	32 ± 5	34 (25 – 37)	32 ± 4	31 (25 – 39)	0.63 (ns)
Caesarean section	2	N/A	5	N/A	N/A
Duration of gestation (in weeks)	37 ± 3	38 (32 - 40)	39 ± 1	39 (37 – 42)	0.02 (ns)
Time to 1st symptom (weeks)	N/A	N/A	7 ± 5	6 (1 – 17)	N/A
Time to diagnosis (weeks)[a)]	N/A	N/A	4 ± 3	3 (1 – 10)	N/A
Relapse during 3rd trimester[b)]	N/A	N/A	2	N/A	N/A
Relapse in first three months after delivery[b')]	N/A	N/A	6	N/A	N/A
Number of total pregnancies	2 ± 1	2 (1 – 5)	2 ± 1	1 (1 – 5)	0.04 (ns)
Deliveries/abortions in past[c)]	0.87 ± 0.99	1 (0 – 3)	0.40± 0.72	0 (0 – 3)	0.06 (ns)
Vacuum	0	N/A	5	N/A	N/A
Birth weight of baby (in gram)	3269 ± 673	3280 (2050 – 4050)	3424 ± 394	3400 (2610 – 4490)	1 (ns)
Gender of baby[d)]	3 M, 6 F	N/A	18 M, 13 F	N/A	0.17 (ns)
Creatinine concentration Mean ± SD	8.89 ± 5.57	9.2 (2.1 - 21.2)	9.34 ± 5.87	8.3 (0.9-29)	0.06 (ns)

Definition: A relapse was described as an episode of neurological disorder for which causative lesions are possibly to be inflammatory and demyelinating in nature and remain for more than 24 hour.
a) Time to diagnosis was available for 8 patients only. b) Only one MScl patient had a relapse during the third trimester of pregnancy; b') Out of 31 MScl patients, 6 had relapse at the first-post-partum period. From these 6 MScl patients, one also had relapse at the third trimester.
c) Out of 31 MScl women; 17 MScl women had first pregnancy (no delivery in past), seven had second pregnancy (one delivery in past), four had second pregnancy (no delivery and one abortion in past), one had third pregnancy (no delivery and two abortions in past), one had fourth pregnancy (two delivery and one abortion in past), and one had fifth pregnancy (three delivery and one abortion in past).
Out of 8 control women; one control women had first pregnancy (no delivery in past), four had second pregnancy (one delivery in past), two had second pregnancy (no delivery and one abortion in past), and one had fifth pregnancy (three delivery and one abortion in past).
d) One control women had twins (one male and one female).
Abbreviations: F, female; M, male; MScl, multiple sclerosis; N/A, not applicable; ns, not significant; SD, standard deviation; Data was analyzed using the Mann Whitney test.

no significant difference (p>0.01) was observed between groups in LC-MS quantified peptide area (UV at 214 nm) and also, at the individual level in MS/MS spectral counts. In conclusion, no significant clinical chemistry differences were found in the two groups related to the parameters investigated (creatinine and protein concentration).

Technical variability was estimated from data coming from the quality control samples (as indicated in method section). These quality control samples were randomly divided into two parts. The mean (±SD) number of proteins identified in all quality control samples was 247±26, in the first group of quality control samples 258±25, and in the second group was 237±22. We did not observe a significant difference between first and second group of the quality control samples (p=0.16) i.e. technical variation in time is not changing significantly.

LC-MS ANALYSIS

Combining results from label-free LC-MS quantifications and peptide identifications yielded a set of 1699 unique peptides that relate to 409 proteins present in all samples of MScl patients and controls.

Analysis 1

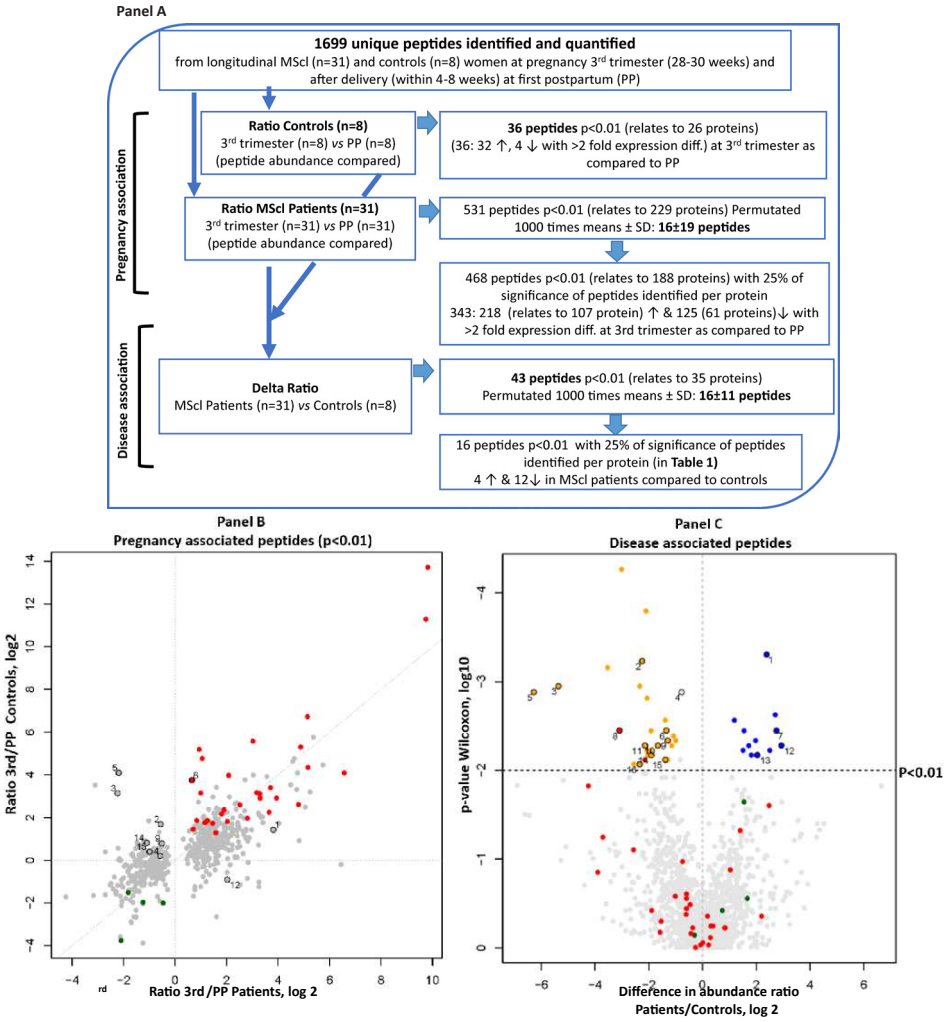
Investigation of pregnancy association (i.e. longitudinal changes between third trimester of pregnancy and postpartum period) in controls (n=8), resulted in 36 differentially expressed tryptic peptides ($p<0.01$) with a mean (±SD) background of 12±12 obtained after 1000 permutations which is far less than the combinations that exist and large enough to exclude only outliers. The number of 36 is significantly different from the value obtained by permutations using a t-test. From the 36 peptides, 32 peptides were increased (related to 22 proteins) and 4 (related to 4 proteins) were decreased more than 2 fold during the third trimester of pregnancy compared to postpartum (Analysis 1) (Figure 1, Panel A). Of these 32 peptides, the top 5 (based on fold expression difference) was: pregnancy-specific beta-1-glycoprotein 1, pregnancy-specific beta-1-glycoprotein 11, poliovirus receptor-related protein 3, pregnancy-specific beta-1-glycoprotein 9 and angiotensinogen. Examples of proteins that decreased at third trimester compared to postpartum in control subjects were: annexin A1, periplakin and protein HEG homolog.

Analog to this, analysis in the set of 31 MScl patients, resulted in total, of 531 differentially expressed peptides ($p<0.01$) with the mean (± SD) rate of permutation 16 ± 19. Of these 531, 468 peptides (relates to 188 proteins) were identified with $p<0.01$ and at least 25% of the significance of peptides identified per protein.

Of these 468 peptides, 343 were observed with > 2 fold intensity difference. Among them (from 343) 218 peptides (related to 107 proteins) showed increased and 125 (related to 61 proteins) decreased in abundance during the third trimester relative to the postpartum period in MScl patients (Figure 1, Panel A).

The most striking peptides elevated at third trimester pregnancy-specific beta-1-glycoprotein 1, pregnancy-specific beta-1-glycoprotein 11, pregnancy-specific beta-

Figure 1. (A) Summary of pregnancy and diseases associated peptide identification. The flowchart shows a summary of univariate analysis results.We tested 1699 peptides. First, we identified pregnancy associated peptides by compared abundance of peptide measured during 3rd trimester with the abundance of peptide measured at first postpartum in the same healthy women (n = 8) (ratio controls). The analysis resulted into 36 significant peptides. Then, we performed a similar comparison in 31 women with MScl (ratio patients) that resulted into 531 peptides. Next, to identify disease associated peptides, we compared difference in abundance ratio between controls and MScl patients that resulted into 43 peptides.
(B) Pregnancy dependent peptides in MScl patients and controls. All peptides plotted were associated with pregnancy in controls and MScl patients ($p < 0.01$). On the Y axis, the ratio of 3rd trimester of pregnancy/postpartum period for 8 controls is monitored. On the X axis, the ratio of 3rd trimester of pregnancy/postpartum period in 31 MScl patients is indicated. The diagonal line coincides or corresponds when the pregnancy association between controls and patients correlates ideally. Filled data points in red indicate peptides with significantly increased and those in green indicate a decrease in abundance identified during the 3rd trimester of pregnancy compared with the postpartum period. Filled circles in gray represent pregnancyassociated increased or decreased abundant peptides in MScl patients and controls.
(C) Volcano plot of all peptide difference ratios, displaying the relationship between significance (p value) versus fold change between MScl patients and controls. A volcano plot of peptides (n = 1699) showing the distribution of fold change and significance simultaneously, from this proteomics analysis. Each point represents a peptide and the difference in abundance ratio between MScl patients and controls plotted against the level of significance. The Y axis represents p values log 10 transformed from the Wilcoxon test between control ratios n = 8 (3rd trimester versus postpartum) and patient ratios n = 31 (3rd trimester versus postpartum). The X axis shows the difference in abundance ratio (MScl patients/controls log 2 transformed) between MScl patients and controls. The dashed horizontal line is drawn at p = 0.01. Below the dashed horizontal line: All data points show a nonsignificant difference in abundance ratio compared between MScl patients and controls. Here red labeled circles were found with increased abundance at the 3rd trimester as compared with postpartum in controls and green were found with decreased abundance at the 3rd trimester of pregnancy compared with the postpartum period. Such pregnancy associated peptides were not found to be associated with the disease when difference in abundance ratio between MScl patients and controls were compared. Above dashed horizontal line: Data points shows significantly increased or decreased abundance ratio ($p<0.01$) in patients. Toward right side of vertical dashed line: Blue filled circles show peptides found with increased delta ratio in MScl patients (right side of the vertical dashed line) compared with controls. Blue filled circles with label show peptides identified with 25% of significance per protein. Toward left side of vertical dashed line: Orange filled circles shows peptides identified with decreased delta ratio in MScl patients compared with controls; labeling is shown for those who were identified with 25% of significance per protein. Red circles were pregnancy-associated as well as disease-associated. Labeled circles are those that had 25% of significance of peptides per protein: (1) trefoil factor 3, (7) eukaryotic translation initiation factor 6, (12) lysosome-associated membrane glycoprotein 2, (13) hemoglobin subunit alpha, (2) collagen alpha-1(XII) chain, (3) plasma glutamate carboxypeptidase, (4) G-protein coupled receptor family C group 5 member B, (5) Ig mu chain C region, (6) desmoglein-3, (8) fibrillin-2, (9) receptor-type tyrosine-protein phosphatase, (10) 14–3–3 protein sigma, (11) Ig kappa chain V–III region, (14) osteoclast-associated immunoglobulin-like receptor, (15) dermokine, and (16) phosphatidylethanolamine-binding protein 1. Abbreviations: ↑, with increased abundance; ↓, with decreased abundance; 3rd, third trimester during pregnancy; PP, first postpartum period. Numbering between brackets in legend is numbering in the figure (panel B and C).



1-glycoprotein 2, and choriogonadotropin subunit beta variant 1. Also, glutaminyl-peptide cyclotransferase, trefoil factor 3 and poliovirus receptor-related protein 3 were observed to be elevated in the third trimester. Examples of top-listed peptides observed with decrease abundance at MScl third trimester compared to MScl post-partum were: Ig gamma-1 chain C region, matrix metalloproteinase-9, tyrosine-protein kinase receptor, Ig gamma-3 chain C region.

Resampling and permutation

Proteins associated with pregnancy were overwhelmingly found in MScl patients compared to controls. Another permutation test was carried out to determine the effect of sample size difference between control and MScl patients. We compared random 8 MScl

patients at third trimester (n=8) with postpartum (n=8) samples. We took repetitively 8 MScl patients from the 31 and determined the mean and SD for pregnancy related peptides. Thereafter, we determined if pregnancy related peptides in controls (36) are significantly different as compared to MScl from the found mean using the SD obtained by iterative analysis. The first time we took 8 patients from the 31 MScl, determined 59 (permutated mean ± SD, 12±14) different peptides ($p<0.01$) (third trimester versus postpartum), the second time taking at random another 8 from the 31 MScl patients resulted in 143 (12±15) peptides differentially expressed. Next, we repeated this for a third time, here we found 157 (11±13) and a fourth time 240 (11±14) differentially expressed peptides. This way, mean number of pregnancy related peptides obtained in MScl was 150 (11±7). Although the variation in the number of differentially abundant peptides appears large, this is a representation of the biological variation due to the heterogeneous character of MScl. Technical variation was assessed based on the identical quality control samples measured repetitively during the same measurement sequence and was not statistically significant ($p=0.16$).

In comparison with controls, significantly more pregnancy related peptides were found in MScl ($p<0.01$). Therefore, analysis showed that by taking the same sample size in MScl and controls, the number of pregnancy related peptides were highly elevated in MScl as compared to the control group. Thus, the large number of pregnancy-associated peptides found in MScl patients is not only because of the larger number of MScl samples analyzed.

Analysis 2

Comparison of longitudinal differences between third trimester of pregnancy and the postpartum period between MScl (n=31) and controls (n=8), resulted in 43 MScl associated peptides (related to 35 proteins) with a change in abundance during the third trimester and postpartum compared to controls. From these 35 proteins, two (pro-epidermal growth factor and serum albumin) had 50% differentially abundant peptides expressed in the same direction) and for the rest, all differentially abundant peptides for same protein followed the same trend of expression (increased or decreased). Of these 43 tryptic peptides with >2 fold difference in abundance ratio, 12 peptides were increased during pregnancy and 28 were stronger decreased in MScl patients compared to controls. Peptides with increased (out of 12) ratio in MScl patients compared to controls were: Lysosome-associated membrane glycoprotein 2, eukaryotic translation initiation factor 6, inter-alpha-trypsin inhibitor heavy chain H4, serum albumin, trefoil factor 3, hemoglobin subunit alpha, keratin, type II cytoskeletal 6A, pro-epidermal growth factor, keratin, type I cytoskeletal 19, cubilin and involucrin IV.

Examples of differentially abundant peptides (28) with a decreased difference in the abundance ratio in MScl patients relative to controls were: Ig mu chain C region, plasma

glutamate carboxypeptidase, uromodulin, fibrillin-2, glutaminyl-peptide cyclotransferase, hemopexin, phosphatidylethanolamine-binding protein 1, collagen alpha-1(XII) chain, beta-2-microglobulin, Ig kappa chain V-III region.

Sixteen disease associated peptides (out of these 43) which were identified with $p<0.01$ and at least 25% of the significance of peptides identified per protein are listed in Table 2 A and Table 2 B. Furthermore, the peptides per differentially expressed proteins followed the same trend of expression either, increased or decreased. Four (of 16) peptides had an elevated level in MScl (trefoil factor 3), whereas 12 peptides had a decreased ratio in MScl (Ig mu chain C, plasma glutamate carboxypeptidase, phosphatidylethanolamine-binding protein and Ig kappa chain V-III region etc.). Moreover, in Figure 1, Panel C, delta fold expression difference between MScl patients and controls and statistical significance are plotted simultaneously for illustration in a volcano plot. When, we determined the FDR (related to statistical significance) by permutation the complete set of 1699 observations (peptides) between four sample groups (MScl: third trimester of pregnancy; MScl: postpartum period; controls: third trimester of pregnancy and controls: postpartum period), analysis indicated identification of 43 disease specific peptides significant. The mean (±SD) rate of permutation was 16±11 (median 11), whereas the true hits, i.e. MScl associated identified peptides were significantly elevated to 43.

Table 2. MScl Disease-Associated Peptides: (A) 4 Peptides for Which Longitudinal Difference Ratios between 3rd and Postpartum Were Significantly Increased in Patients (n = 31) in Comparison with Controls (n = 8) and (B) 12 Peptides that had Significantly Decreased Abundance Ratios in Patients As Compared with Controls[a].

2 A.

Protein	Sequence	Description	p-value	Fold change (log 2)	Identified p<0.01/total
Q07654	IPGVPWCFKPLQEAECTF	Trefoil factor 3	0.0005	2.38	1/4
P56537	TSIEDQDELSSLLQVPLVAGTVNR	Eukaryotic translation initiation factor 6	0.004	2.75	1/1
P13473	GILTVDELLAIR	Lysosome-associated membrane glycoprotein 2	0.005	2.93	1/2
P69905	TYFPHFDLSHGSAQVK	Hemoglobin subunit alpha	0.007	2.03	1/4

2 B.

Protein	Sequence	Description	p-value	Fold change (log 2)	Identified p<0.01/total
Q99715	WDPAPGPVLQYR	Collagen alpha-1(XII) chain	0.0005	−2.25	1/2
Q9Y646	VGALASLIR	Plasma glutamate carboxypeptidase	0.001	−5.36	1/2

2 B. (continued)

Protein	Sequence	Description	p-value	Fold change (log 2)	Identified p<0.01/total
Q9NZH0	ETAFEEDVQLPR	G-protein coupled receptor family C group 5 member B	0.001	−0.78	1/1
P01871	GVALHRPDVYLLPPAR	Ig mu chain C region	0.001	−6.28	1/4
P35556	SATAGSEGGFLAPEYREEGAAVASR	Fibrillin-2	0.004	−3.09	1/2
P32926	VPDFNDNCPTAVLEK	Desmoglein-3	0.004	−1.36	1/3
Q12913	TPSSTGPSPVFDIK	Receptor-type tyrosine-protein phosphatase eta	0.005	−1.30	1/3
P01620	LLIYGASSR	Ig kappa chain V-III region	0.005	−1.79	1/3
P31947	SNEEGSEEKGPEVR	14-3-3 protein sigma	0.005	−1.79	1/2
Q8IYS5	EGVAAPLQYR	Osteoclast-associated immunoglobulin-like receptor	0.007	−2.34	1/3
Q6E0U4	VSEALGQGTR	Dermokine	0.008	−0.80	1/2
P30086	NRPTSISWDGLDSGK	Phosphatidylethanol-amine-binding protein 1	0.009	−1.74	1/4

[a]The Table shows a total 16 peptides identified with significant ($p < 0.01$) increased or decreased abundance ratio in MScl patients (n = 31) in comparison with controls (n = 8). Proteins had at least 25% significant ($p = 0.01$) differentially abundant peptides.

DISCUSSION

To our knowledge, this is the first LC-MS proteomics based work of MScl in pregnant women. We identified and quantified 1699 unique peptides in longitudinal urine samples from 31 MScl patients and 8 controls. In the current investigation, we have used urine as a sample because of its ease of availability during pregnancy. In addition, it is relatively less complex in protein content than serum (17), and relatively stable in protein content if stored in a protocolized way (18,19). Also, the peripheral pathological and physiological processes that occur outside the CNS, and triggers the MScl relapses (14) are likely to be reflected in body fluids. Such peripheral inflammatory and demyelination related factors might result in a systematic change in the abundances of specific proteins. The demyelination involves several types of immune cells (e.g., T cells, B cells, and macrophages/monocytes) and inflammatory mediators (e.g., cytokines and chemokines). The LC-MS method determines levels of peptide abundance, and can provide insight into the activity state of relevant proteins under different physiological/disease conditions in the sample chosen. Most of the previous studies have reconsidered those proteins which are already known to be increased during pregnancy for example, estrogen and progesterone (7). Complementary to

previously performed longitudinal studies to analyze PBMCs immune molecules/ monocytes (13, 20) at the RNA level (in blood) using microarray approaches, we directly investigated at the protein level using proteomics method. The transcriptomics based study on immune cells (13) is not directly comparable to the current study in terms of clinical parameters (see Table 1), because of differences in gestational age, and a time window at sampling (13). In the context of above, Gilli and colleagues found 347 differentially expressed genes in non-pregnant MScl patients compared to non-pregnant controls (13). We did not observe any overlap with this study, probably also because differentially regulated genes at the mRNA level often cannot directly be translated into protein abundance (21). Also, Neuteboom and colleagues analyzing, monocyte transcriptomics during pregnancy on six patients showed an increase of CD64 during the third trimester compared with baseline (20). This study indicates that enhanced innate immune functions can be in effect in MScl. This observation is in agreement with the current study.

Pregnancy association

First, we determined just the pregnancy association in controls and MScl patients (Analysis 1). We observed an altered expression of a large number of 531 peptides (Figure 1, Panel A) during the third trimester of pregnancy, as compared to the postpartum period. In agreement with a previous study in serum (22), we have also found an order of magnitude increase in abundance of placenta derived pregnancy-associated immunoregulatory proteins (pregnancy specific beta-1-glycoprotein 1, 9 and 11) at MScl third trimester compared to postpartum period compared to controls. Pregnancy specific glycoproteins are known to modulate the innate immune system by inducing secretion of anti-inflammatory cytokines by human monocytes (23), and also suggested as one of the most important immunosuppressive substances (23). Interestingly, in our study, we also observed increased abundance of choriogonadotropin subunit beta variant 1, which was previously described at transcript level only. It is essential for normal implantation and placental development (24, 25). This particular protein is known to be noncoding beta subunit of human chorionic gonadotropin (25), which is considered as an immunosuppressive. On the other hand, notable peptides with high fold decreased abundance at MScl third trimester as compared to postpartum period was linked with inflammation related molecules, such as Ig gamma-1 chain C region, Ig gamma-3 chain C region and matrix metalloproteinase-9. The highest activity of matrix metalloproteinase-9 in serum was observed to correlate with short duration relapsing and active forms of MScl (26). In another study, the high level of metalloproteinase-9 was found to be associated with MScl pathogenesis and contributes to blood–brain barrier disruption and entry of T-lymphocyte into the CNS (27). Here the findings of the above studies in serum and CNS were in agreement with our results in urine.

In both MScl and control, the most discriminating peptides with increased abundance at third trimester (compared with postpartum) were pregnancy related glycoproteins (Figure 1 A). In control subjects, only 4 peptides were found with decreased abundance at third trimester as compared to postpartum (Figure 1A). From the previously described pregnancy association result, it can be concluded that the pregnancy derived proteins with immunomodulatory function are abundant at third trimester ($p < 0.01$) relative to the postpartum period in MScl, and that these pregnancy related proteins are more abundant than in control subjects. Most likely, these proteins have functions during pregnancy third trimester, and contribute to a significant reduction of 70% in MScl relapse rate which is described previously (2, 5). Previous studies in serum also suggest that the increased levels of pregnancy derived factors might be responsible for suppression of disease state via T cell regulations (28). On the other side, peptides with decreased abundance at third trimester (compared to postpartum period), i.e. molecule elevated at postpartum might be explained by altering most likely diminished immunomodulatory responses due to disease aggravation.

The increased number of pregnancy associated peptides in MScl as observed in Analysis 1 is not due to a larger sample size as compared to control group. The result of permutation analysis showed a decreased permutated mean of 16 ± 19 as compared to the original observation of 531 pregnancy associated peptides, which indicated an expected difference related to pregnancy. The increased number of pregnancies associated peptides identified in both MScl and control during third trimester (as compared to postpartum) is probably the result of different physiological states due to pregnancy (Figure 1A). The observed elevation of pregnancy related proteins in MScl as compared to control group could reflect involvement in the reduction of MScl disease activity.

Disease association

Altered protein expression during third trimester compared to postpartum can be associated with MScl, but it can also be due to pregnancy or even a combination of both. Therefore, we compared longitudinally MScl and controls (Figure 1A). Peptides observed with increased abundance ratio in MScl compared to control were 12. The data were scrutinizing by using a filter of 25% of significant peptides identified per protein. We ended up with four peptides (related to 4 proteins,

Table 2 A), two related to immunomodulatory proteins: trefoil factor 3 and lysosome associated membrane glycoprotein 2 (LAMP 2), the other two (eukaryotic translation initiation factor 6 and hemoglobin subunit alpha) have no relation with immunomodulatory response during the third trimester of pregnancy. Trefoil factor 3 was significantly elevated ($p < 0.01$) at third trimester compared to postpartum, and this was on average three times higher in MScl than in control (higher fold difference). This protein is known to be significantly elevated (up to 47 times higher abundant) in serum during

gestation (29). Trefoil factor 3 has been shown to induce peptides related to innate immune defense such as defensins in breast milk, indicating a possible relation to both pregnancy (30) as well as MScl (31). This is in agreement with the data observed in this study.The direct evidence of LAMP 2 in relation to immune defense is not yet described in literature. However, a variant of LAMP 2 (LAMP 1) is linked to immune response (32), therefore it is worthwhile to investigate the role of trefoil factor 3 and LAMP 2 associated with MScl.

Decreased abundance ratio in MScl compared to controls

Peptides observed with decreased abundance ratio in MScl compared to control subjects were in number 31. The data were further filtered (by using the same parameter as described in the above section) and 12 peptides remained (Figure 1, Panel A and C). Among these 12, the Ig mu chain C region plays an important role in primary defense. Its decreased abundance in CSF of MScl patients is described after 1 year of Natalizumab treatment (33). The inhibition of glutamate carboxypeptidase II activity is known for the treatment of cognitive impairment in MScl (34), which is a homolog of plasma glutamate carboxypeptidase (carboxypeptidase Q). Another protein named as phosphatidylethanolamine-binding protein, inhibits nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation, NF-κB plays a critical role in the regulation of immune and inflammatory responses and has been shown in MScl pathogenesis (35), it is considered as a potential therapeutic target for the treatment of MScl. Next, immune molecules, for example, Ig kappa chain V-III region, and osteoclast-associated immunoglobulin-like receptor have been linked to inflammation.

We also observed that beta-2 microglobulin passed the criteria for significance $p<0.01$ but not for 25% of the significance of the peptides identified per protein (1 peptide identified at <0.01 out of 5), which has been previously described as a MScl related marker (36) in the urine of non-pregnant MScl patients. In addition, increased (as compared to controls) urinary free light chains kappa in MScl have been previously described for the activity of immunological processes (18).

Altogether, by using the high throughput and sensitive proteomics platform (LC-MS based proteomics), we have identified tryptic peptides with an increased abundance ratio that was related to the innate immune system. Peptides with a decreased abundance ratio were related to disease relapse. Also the accompanying biological function of differentially expressed pregnancy related proteins found in the current investigation in MScl pregnant women might have an advantageous effect on the reduction of disease activity in MScl disease.

CONCLUSION

Pregnancy is the only known natural modifier of disease course in MScl (1, 2). In the current study, we identified proteins that are particularly expressed during this most inactive disease period of a MScl patient. Moreover, we have compared MScl and control during and after pregnancy and found disease associated peptides. The LC-MS based proteomic profiling of urine samples during MScl pregnancy revealed a significant number of disease associated peptides. In addition, peptides that relate strongly to pregnancy, are linked with or involved in innate and adaptive immune response either directly or indirectly or were immunosuppressive. Apparently, the pregnancy associated proteins and their accompanying biological function might have an advantageous effect on MScl disease suppression. It is surprising that a disease primarily occurring in the CNS can be detected in body fluids as remote as urine. The finding of our current investigation may open a new window for further manipulation of identified peptide related to MScl disease association. It may also help to give more insight to understand further the pathogenesis, prognosis and treatment of MScl disease.

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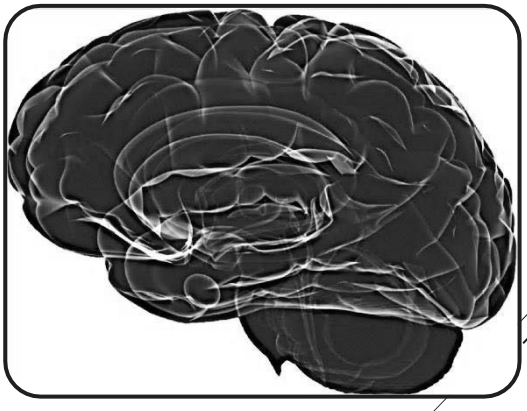
Chapter 7

Discussion



In this thesis we describe the mass spectrometry (MS) based proteomics investigations to identify the multiple sclerosis (MScl) associated potential biomarkers in body fluids. Developments in MS technology have made MS-based proteomics a promising tool for protein profiling and biomarker discovery. MScl is a complex disease whose pathogenesis is highly heterogeneous (1, 2) and due to heterogeneity it is doubtful that single biomarker will be found for pathological processes or to predict progression of the disease. We have applied unbiased discovery driven approach to screen body fluid proteome and measure expression of proteins using sensitive and high-throughput measuring platform. We have used a data dependent, label free quantitative method in a wide variety of samples (CSF, CSF IgG autoantibodies and urine) from a range of MScl patients. Differential expression analysis was performed and an effort was made to identify and evaluate potential MScl associated biomarkers involved in biological pathways.

The most important findings, and conclusions of chapter 2-6 are combined and discussed in this chapter. At the end of this chapter the challenges and future research prospects of MScl are highlighted.

PROTEOMICS COMPARISON OF CEREBROSPINAL FLUID OF RELAPSING REMITTING AND PRIMARY PROGRESSIVE MULTIPLE SCLEROSIS

In chapter 2, we have described the difference between two commonly believed different clinical subtypes (based on disease course) of MScl. We found that the proteome profiles of CSF in PP (n=10) and RR MScl (n=11) patients share many similarities. This is in agreement with the absence of clear-cut differences between the two main clinical MScl sub-groups, at genetic, immunological, imaging and pathological levels (3). However, proteomics comparison between the PP and RR MScl group revealed seven differentially abundant proteins. In the same context, one other group (4) using proteomics analysis (MALDI-TOF) on CSF showed 11 differentially regulated peptides in both serum and CSF between relapse-onset MScl (n=41) and PP MScl (n=13) patients, these peptides do not directly correlate with our data.

Next, two differential proteins were further validated by independent methods in validation cohort (containing of 10 RR MScl and 10 PP MScl samples), these were jagged-1 (abundance decreased in PP MScl compared to RR) and vitamin D-binding protein (only detected in the RR MScl). Jagged-1 protein mediates Notch signaling (role during T cell development)[5] and also previously shown to be associated with MScl (6) and have immunomodulatory properties, thus can be considered as a new therapeutic target for MScl (7). Vitamin D regulates the differentiation, activation of CD4+ T-lymphocytes (8) and can inhibit the development of autoimmune processes, therefore it might have

MAIN FINDINGS

- The proteome profile of CSF in primary-progressive (PP) and relapsing-remitting (RR) multiple sclerosis (MScl) patients overlap largely, still a low number of significant differences could be observed and validated. Protein jagged 1 (three times less abundant in PP compare to RR MScl) and vitamin D binding protein (detected in RR MScl only) were elevated in RR MScl. These two proteins deserve further study to be used as potential markers to differentiate between PP and RR MScl.
- CSF of childhood onset MScl is associated with significantly increased abundance of CNS grey matter proteins (for example contactin-2 and neurofascin) and decreased abundance of innate immunity related proteins in comparison to children presenting with monophasic acquired demyelinating syndromes (ADS). Interestingly, contactin-2 and neurofascin are previous autoimmune targets in MScl.
- CSF immunoglobulin G proteins shares common characteristic in the antigen binding region among different RR MScl patients and not in controls. This may indicate a common antigen for B-cell activation. Right now these sequences cannot be used as a marker and more validation is required.
- Pregnancy-related proteins were significantly elevated in MScl patients compared with controls (emerged from comparing third trimester with the postpartum period). When comparing the longitudinal differences, we identified 43 MScl associated peptides with increased (example trefoil factor 3) or decreased (such as Ig mu chain C region and plasma glutamate carboxypeptidase) abundance ratio in MScl compared to controls.
- Monitoring the effect of Natalizumab therapy in MScl patients before and after one year of treatment revealed differentially abundant and validated proteins, for example, Ig mu chain C region, haptoglobin, and chitinase 3 like protein 1. All these three proteins were significantly less abundant in CFS of MScl patients after one year of treatment.

neuroprotective role in MScl. Proteins identified with differential abundance were also studied using Ingenuity Pathways Analysis in order to place them in biological context. As a result, six of the seven were found to be related with the neurological disease. Further, when level of these proteins were measured for sensitivity and specificity, it was not high, however when analysed together sensitivity increases to 0.8 and specificity increases to 0.75, still value remain low for confident separation between two MScl types. Though we analysed only a low number of samples, still our study is of importance from MScl pathological point of view. Moreover, the findings of our study might also be useful in terms of future characterization of MScl patients group.

Future research

For better understanding of function, these proteins should be individually evaluated in relevance to MScl. Future step should be to validate the disease protein targets: Jagged-1 protein and vitamin D-binding protein i.e. target's relevance to MScl pathology must be determined. This includes more details for functional characterization, additional evidence for pathway or network assignment and modulating the protein's activity to determine its connection to the disease phenotype.

GRAY MATTER RELATED PROTEINS ARE ASSOCIATED WITH CHILDHOOD-ONSET MULTIPLE SCLEROSIS

In chapter 3 we studied the first event of CNS demyelination in CSF samples from 39 children with initial acquired demyelinating syndromes (ADS), of them 18 were detected with MS and 21 with monophasic disease course. Currently, an early adequate diagnosis of MScl in children with an initial acquired demyelinating syndrome is a challenge because combination of clinical features, CSF findings and MRI criteria (9-11) are not enough to predict the disease course at first event. Protein markers that help to differentiate between children with monophasic ADS and those subsequently diagnosed with MScl are desirable.

In this chapter, using high resolution nano liquid chromatography online coupled to a high-resolution mass spectrometer (LC-MS) we observed a total of 2260 peptides (relates to 318 proteins). Statistical analysis showed striking difference between childhood onset MScl and monophasic ADS. Interestingly, 14 proteins were identified with increased and seven with decreased abundance in childhood onset MScl compared to monophasic ADS, which was later evaluated by permutation analysis as described in chapter 3. Among 14 proteins with increased abundance in MScl, 12 were CNS structure and functions related proteins especially to the grey matter. These proteins were amyloid-like protein 2, neurofascin, contactin-2, amyloid beta A4 protein, brevican core protein, carboxypeptidase E, neuronal growth regulator 1, tyrosine-protein phosphatase non-receptor type substrate 1, neuronal cell adhesion molecule, disintegrin and metalloproteinase domain-containing protein 22, dickkopf-related protein 3 and kallikrein-6. Localization for some of these proteins in CNS structure are shown in Figure 1. Similar to the previous findings, our study also showed absence of (12-15) compact myelin antigens (myelin forms a layer around the axon of a neuron). We found this particularly striking because according to the traditional way of thinking myelin is viewed as potential disease targets in MScl (white matter disease) (16). However, the lack of myelin in our data might be related to physiochemical property of the hydrophobic myelin components and probably different pathway of elimination (17). Grey matter presence may merely represent damage by inflammation, and thus identified molecules might provide better clue to understand presumed inflammation induced neurodegeneration.

A similar study involving 19 CSF samples from children has been previously reported, however they had a different sample handling protocol (12) In the same context, we found some overlap for example carboxypeptidase E with increased abundance in childhood onset MScl group. Furthermore, our finding was also showed overlap with another previous study (18) which was performed on clinically isolated syndrome (CIS, individual's first neurological episode) versus established RR MScl patients and

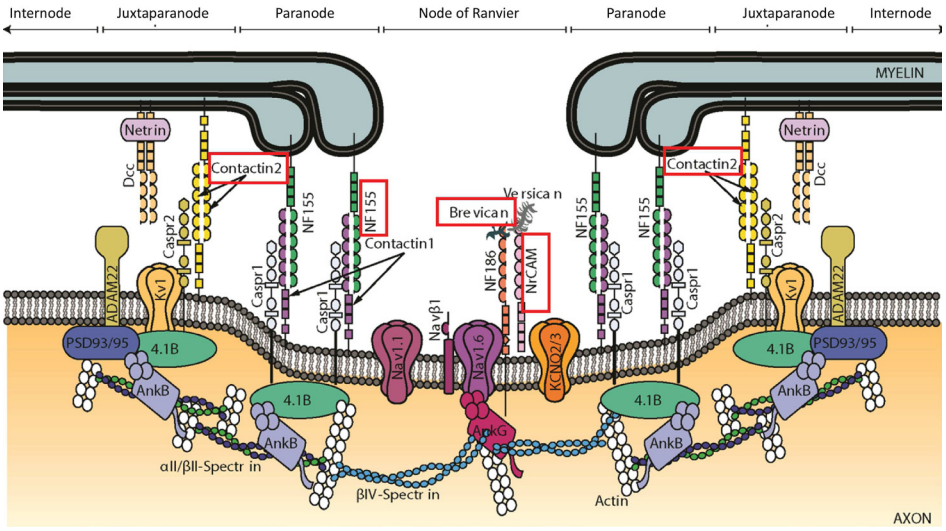


Figure 1. Proteins located at the node of Ranvier, paranode and juxtaparanode are shown in the figure. Red box indicates example of proteins which were identified with increased abundance in childhood onset MScl patients as compared to monophasic ADS group in our study cohort. These domains are the location for neurofascin, contactin 2, contactin-associated protein (cell adhesion molecules), brevican and NrCAM protein (extracellular matrix proteins). (*Reproduced with permission; Acta Neuropathol (2014) 128:161–175*)

showed elevated levels of contactin-2, kallikrein-6 and dickkopf-related protein 3 in first attack MScl patients (compared to established RR MScl). In addition, compared to children with monophasic ADS, we found increased abundance of proteins (six of 14) that localize to the axoglial apparatus (the area of contact between the myelinating cell and the underlying axon) for example neurofascin, contactin-2, brevican core protein, disintegrin and metalloproteinase domain-containing protein 22, neuronal cell adhesion molecule and neuronal growth regulator 1 (12, 19, 20).

In a previous study, neurofascin has been identified as a novel target for autoantibody-mediated axonal injury (21) whereas, contactin-2 was identified as an autoantigen targeted by T-cells and autoantibodies in MScl patients (22).

On the other hand, six out of seven proteins detected with decreased abundance in MScl group included those related to innate immune function such as haptoglobin, C4b-binding protein and monocyte differentiation antigen CD14.

Presence of differentially expressed proteins with over as well as under-representation in MScl indicated disturbances in the physiology of axoglial apparatus. Some areas yet to be addressed in this study was the inclusion of an age matched cohort however, our analysis did not show any influence on the identified differentially abundant proteins. In conclusion our study indicate that CNS grey matter proteins elevated in CSF of childhood onset MScl are pathologically relevant and might be part of the early mechanism of the disease.

Further clinical verification and validation may provide clinically valuable marker to differentiate children with monophasic ADS from children with MScl. Moreover, knowing the beginning of the disease could help to initiate an early treatment with disease modifying therapies.

Future research

In future, it would be interesting to further validate our findings with independent tools such as ELISA, SRM, Western blotting etc. and also in an independent group. Further insights into the role of these proteins could be useful for disease process understanding. In addition, correlation of serum antibodies against axoglial proteins such as contactin-2 and neurofascin might be important for understanding the disease pathology.

CEREBROSPINAL-FLUID-DERIVED IMMUNOGLOBULIN G OF DIFFERENT MULTIPLE SCLEROSIS PATIENTS SHARES MUTATED SEQUENCES IN COMPLEMENTARITY DETERMINING REGIONS

In this part, the main findings and conclusions of the study to identify common mutated antigen binding regions of IgG exclusively present in MScl, described in chapter 4 is discussed.

B lymphocytes play a crucial role in MScl pathology (23), possibly via both antibody dependent and independent pathways. Intrathecal synthesis of Igs (OCBs) restricted to the CSF i.e. not present in serum is a hallmark of MScl (24) (however, OCB is not specific) and it is produced by clonally expanded B cell populations. In this study in order to investigate CSF IgG antibodies clonality, we performed proteomic analysis on purified CSF IgG antibodies from untreated RR MScl patients (n=29) and non-neurological controls (n=30). The study was performed without antigen panels. Using LC-MS measurements of tryptically digested Ig bands (heavy (IgH) and light chain (IgL)) and the database search we found 1086 peptides for IgH and 920 peptides for IgL. Subsequently, within the IgH variable domain we could assign 41 peptides to the CDR1, 128 to the CDR2 and 171 to the CDR3. Within IgL variable domain, we assigned 78 peptides to the CDR1, 233 to CDR2 and 51 to CDR3.

We found over-representation ($p=0.03$) of peptide sequences of the VH4 family in MScl patients compared to controls. In addition, we observed a trend towards increased VH3 family usage in MScl ($p = 0.05$). We also found the increased κ/λ ratios in the MScl group, which was in line with previous study (25).

Further, we found five final CDR peptides that were shared between three or more MScl patients and not seen in any controls. The results were highly significant as shown

by permutation analysis. Moreover, at lower threshold i.e. shared in at least two MScl patients (and not in controls) we found 14 IgH and 13 IgL CDR peptides. None of the previous studies have made such an attempt for MScl, however one study described overlap between Ig B cell (CSF) transcriptomes and proteomes in four individual MScl cases, without interindividual overlapping sequences but without comparison between patients and controls (26).

Remarkably, one of the mutated CDR2 QDGSE**T**YYVDSVK (homologous to IGHV 3 – 7 gene, mutation from germline is shown in **bold**,) shared use was observed in seven different MScl patients, interestingly this particular CDR peptide had different mutations (T, E, and N) or insertion (F) at the same spot. Next, the IDWDDDKYYSTSLK, a CDR2 peptide (homologous to IGHV 2-70 *01) was shared exclusively in five MScl patients. Next, the YNSAPLTFGGGTK, CDR3 peptide (homologous to IGKV 1-27 *01 and IGKJ 4 *01) was identified exclusively in three MScl patients. Finally, the LLIHGASNR peptide was identified solely in three MScl patients. We found overlap with a few CDR peptides identified in other studies that investigated B-cell clonality in the CSF of MScl patients at the nucleic-acid level (26-28). Whether, such common B-cell responses are indeed driven by autoantigens remains to be determined. It will be of interest to study common V-region use in known auto reactive IgGs that appear to play a role in MScl.

Usually, the antigen specificity of B cells is determined via random mechanisms, and so one would guess different sequences of the antigen binding CDR in different individuals. This notion has recently been challenged by different studies, that suggest that the development of IgGs are not a random process, but selection do occur during immune response and this selection is shared by different patients. Earlier studies that confirm shared sequences observed in known antigen-specific antibodies are: human immunodeficiency virus (HIV) infection (29), Ro52 autoimmunity in Sjogren syndrome (30), and anti-Hu paraneoplastic disease (31). In line with these, our study in MScl patients appears to follow the similar pattern.

Our study indicates that under (auto) antigenic pressure there may be common selection mechanisms for the production of intrathecal Ig production by B lymphocytes. Although the reason remains uncertain, it would not be hard to assume that some CDR sequences might better survive the clonal selection process than others, possibly due to a stronger binding of the three-dimensional structure of the CDR to the antigen.

A better understanding of the common selection mechanisms for (auto) antibodies in MScl and the identification of inter-individually shared specific CDR sequences might even deliver markers for subgroup identification.

Compared to the other studies, a drawback of our study was that we do not know the antigen specificity of the V - regions identified here. In fact we do not show complete Ig sequences, due to the fact that trypsin digestion is needed for this approach. In light of the shared sequences observed in known antigen-specific antibodies in HIV infection,

anti-Hu paraneoplastic disease and Ro52 autoimmunity it would be relevant to investigate the possible use of public V - region sequences in purified specific antibodies against MScl candidate antigens such as anti MOG (32), anti neurofascin (21) and anti KIR 4.1 (33).

This is the first study at protein level that showed that peptides generated from the variable part (CDR) of IgG antibodies are shared between MScl patients, which might be used to differentiate patients and control cohort.

Future research

The next step would be to confirm the sequence of the marker CDR peptides using synthetic references by means of SRM method. Further, it might be interesting to examine the possible use of public V-region sequences in purified specific antibodies against MScl candidate antigens such as anti- MOG, anti-neurofascin, and anti- KIR 4.1 (exclusively expressed by glial cells especially oligodendrocytes and astrocytes). In a recent study (33), serum IgG antibodies against KIR4.1 were observed in 47% MScl patients, 1% other neurologic disease patients and 0% healthy donors. In addition, anti-KIR4.1 autoantibodies were also detected in the CSF of 63% MScl patients. Similarly, axoglial proteins such as neurofascin and contactin-2 (autoantigen that is targeted by both T cells and autoantibodies), around the node of Ranvier, were identified as potential targets for autoantibodies in MScl. In addition, this approach can also be used for some other autoantibody targets in the MScl brain as shown in Figure 2.

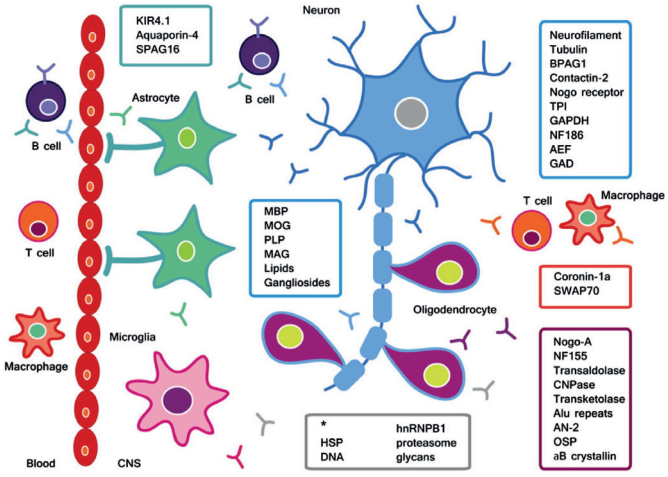


Figure 2. Location of targets of autoantibody in the MScl brain. Autoantibodies (in MScl) target different antigens in myelin, neurons, oligodendrocytes, astrocytes and immune cells. The color of the target cells matches with the color of the text boxes with the identified antigens. Some ubiquitous antibody targets (*) present in virtually all cells are also shown. (*Reproduced with permission; Autoimmunity Reviews (2014) 13:1126–1137*).

In this chapter, bottom-up (peptide based) method was used to characterize IgG variable region and CDR peptide (amino acid sequences) (34) using the highly effective and well studied liquid chromatography technique. MS method used in our study is highly sensitive (34) but it has some disadvantages as well, for example: a) it is hard to identify peptides from highly random CDR, and b) it is difficult to link a peptide back to the rest of the V-region it came from. In this way, digestion of protein into peptides increases complexity. Therefore, to address the above difficulty, it would be even better to use top-down (protein-based) proteomics (35), where we look at intact protein (or large fragments) using Fourier Transform Ion Cyclotron Resonance MS (a high resolution MS).

In chapter 4 we did not find many sequenced peptides recognized as CDR3. This is because the CDR3 is usually preceded by a conserved Lysine (K) or Arginine (R) amino acid and after tryptic cleavage, the 'random' CDR3 peptide is hard to align to a reference. Trypsin enzyme (cuts after K or R) is used as default because it has good specificity and compatibility with ionization in MS. To improve the CDR3 identification/ overcome this, an alternate enzyme that do not cleave at K and/or R can be used, this changes the products of protein digestion. As an example of an alternate enzyme: a) AspN can be used that cuts before Aspartic acid (D) but it is less specific (than trypsin) and may cleave additional amino acids from C-terminal end of peptides. b) LysN can be used that cuts before K; it may be useful in combination with Electron-transfer dissociation (ETD) but with no added benefit.

The coverage of proteins in LC-MS experiments can be improved. The coverage of proteins is often incomplete because: a) More or less peptides do not ionize well and therefore are not seen at all. b) In IgG for identification of CDR3 peptides, some peptides cut at an unfortunate place c) few peptides are too large and some are too small to be observed. a, b and c might be addressed by using a different protease other than (or in addition to) trypsin (as described above). Furthermore, sequence coverage of CDRs can be improved by increasing the spectral quality and mass accuracy.

EFFECTS OF NATALIZUMAB TREATMENT ON THE CEREBROSPINAL FLUID PROTEOME OF MULTIPLE SCLEROSIS PATIENTS

In chapter 5 we examined the effects of Natalizumab therapy on the CSF proteome of RR MScl patients. Natalizumab is directed against the α 4 component of the α4β1 integrin (CD49d;VLA4) expressed on the surface of lymphocyte and monocyte cells and by binding to α4β1 integrin (mediator of trans endothelial leukocyte migration) it blocks the migration of T-cells in to the CNS (36). The proposed mode of action of Natalizumab comprises inhibition of leukocyte infiltration into the CNS, hence re-

ducing the local inflammatory response (37). Hence, we anticipated that this analysis might potentially provide CSF markers relevant for CNS pathology, and therapeutic efficacy. Accordingly, we investigated how biologically relevant markers change in response to Natalizumab therapy in RR MScl patient before and after 12 months of treatment. In this chapter we used hypothesis free proteomic approach and attempted to mine entire CSF proteome to explore the effects of Natalizumab treatment. Using label-free LC-MS analysis (semi quantitative analysis) 301 proteins were identified by two or more peptides and showed a number of biomarkers that were significantly differentially abundant between the before and after treatment groups. These three proteins were found in lower abundance in CSF of MScl patients after one year of the treatment. Remarkably, in a new independent sample set, differential expression of three proteins Ig mu chain C region, haptoglobin and Chitinase-3-like protein 1 (secreted by activated macrophages) were subsequently confirmed using SRM (a different quantitative mass spectrometry for absolute quantification). Chitinase-3-like protein 1 was further confirmed by ELISA. Observation of decreased abundance of inflammation related proteins in CSF (after treatment), was in agreement with the proposed mechanism of action of the drug (37).

Also in agreement with the proposed mechanism of Natalizumab is the lack of significant differentially abundant neurodegenerative proteins after treatment however an effect on neurodegeneration was not directly expected to be observed here (according to drug mechanism). More interestingly, inflammatory response-related proteins with a known higher abundance in CSF of MScl patients such as Igs and haptoglobin (38) were found to be down regulated after treatment that inhibits the access of inflammatory cells into the CNS. As described in chapter 5 a similar study was performed by Ottervald et al, where they had CSF samples from 6 months after the start of Natalizumab treatment (39). Due to ethical reasons, we could not follow up the control group of patients without the Natalizumab treatment. The possibility of a placebo effect on biomarkers or other unknown factors theoretically remains. In spite of this deficiency, the treatment-related differences in this study group were prominent, the statistical thresholds were relatively high, and the findings have a biological basis.

In conclusion, chapter 5 demonstrated that response to Natalizumab is associated with the down regulation of inflammation-related proteins in CSF.

Most interestingly, we observe a decrease in the CSF levels of Chitinase 3-like protein 1, which is a potential marker (have predictive capacity) for conversion of CIS to clinically definite MScl (40).

Future research

Future research could be directed towards the detection and fractionation of low abundance but potentially informative proteins by more in depth analysis or the depletion of high-abundance proteins such as albumin from the CSF samples.

PROTEOMICS URINE ANALYSIS OF PREGNANT WOMEN SUFFERING FROM MULTIPLE SCLEROSIS

In chapter 6 we performed LC-MS based proteomics to detect alteration of the urinary proteome during third trimester and after pregnancy at the first postpartum period. A longitudinal study on 31 MScl afflicted women (untreated RR MScl patients) and 8 controls was carried out. We anticipated identifying MScl specific proteins that are potentially related to the changed frequency of relapses and remissions during pregnancy. We hypothesized that the investigation of the natural protein modifiers (pregnancy) of the MScl could shed more light in understanding the biological mechanisms of this altered disease course.

We found that pregnancy related peptides were significantly increased in MScl patients compared to controls (531 peptides in MScl group and 36 in controls higher abundant in the third trimester compared to postpartum). Most discriminating proteins reported were different types of pregnancy-specific glycoproteins (PSGs), which are synthesized in the syncytiotrophoblast of the placenta (41,42) and these proteins are known to have the immunoregulatory properties. Presumably, these proteins might have functions during pregnancy, which contribute to a significant decline of relapse in MScl women patients, as shown by our analysis and previous reports.

Our finding was also in line with a previous study that indicated that PSGs regulate T-Cell function and inflammatory autoimmune (MScl) disease during pregnancy and thus they suppress MScl (43).

The modified peptide abundances during (compared to after) pregnancy could therefore be linked with MScl or even more likely a combination of both. For this reason, we also compared the pregnancy related changes in a longitudinal way between MScl patients and controls. When comparing the longitudinal differences, we found 43 (related to 35 proteins) disease associated peptides with increased or decreased ratio. This was further evaluated by permutation analysis. Four distinguished proteins were found (on applying restrictive criteria) with increased difference in abundance ratio, for example trefoil factor 3 and lysosome associated membrane glycoprotein 2, both of which have immunomodulatory properties. These proteins is are recognized to be significantly elevated (up to 47 times higher abundant) in serum during gestation (44), moreover it has been shown to induce peptides associated to innate immune

defense such as defensins (45). Further, we found 12 disease associated peptides with decreased abundance ratio in MScl (compared to controls). For example, Ig mu chain C region, which has a role in primary defense, was reported to have decreased abundance in the CSF of MScl patients after 1 year of Natalizumab treatment (46). Other examples were: a) glutamate carboxypeptidase II, whose activity inhibition is known for the treatment of cognitive impairment in MScl (47) b) phosphatidylethanolamine-binding protein, which inhibits the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), NF-κB plays a critical role in the regulation of immune and inflammatory responses. NF-κB was found to be important for MScl pathogenesis and is even considered as a potential therapeutic target for the treatment of MScl (48). Furthermore, we also found Ig kappa chain V-III region (49), osteoclast-associated immunoglobulin-like receptor (both linked to inflammation) and beta-2 microglobulin (50), all of which before has been previously described as MScl related marker in the urine of non-pregnant MScl patients.

We found that the peptides with an increased ratio were related to the innate immune system whereas the peptides with a decreased ratio related to the disease relapse. Further, the accompanying biological functions of differentially expressed pregnancy related proteins found in the current investigation in MScl pregnant women might have an advantageous effect on the reduction of disease activity in MScl patients.

In the present research, we worked with urine samples because it is readily accessible (compared to CSF and brain tissue) for longitudinal sampling, and might be the ideal biofluid source for potential biomarkers of MScl (49). Another logical reason was that disease bouts in MScl primarily depend on events outside of the CNS without affecting the cells of CSF (51), which highlights the importance of studying the changes in serum and urine. In conclusion we identified tryptic peptides with increased abundance ratio that was related to the innate immune system. Peptides with a decreased abundance ratio were related to disease relapse.

This is the first study where a high throughput and sensitive proteomics has been performed at the protein level in pregnancy associated MScl. We performed discovery driven research using high throughput and sensitive proteomics platform on a compartment that is remote from the site of its generation and has inherently undergone proteomics modification. Our results using proteomic research can help expand our understanding of MScl pathophysiology.

In this chapter, we showed that peptides which relate strongly to pregnancy are also linked either directly or indirectly with innate and adaptive immune responses or were immunosuppressive. Apparently, the pregnancy associated proteins and their accompanying biological functions might have a positive effect on MScl inflammation/disease suppression. The outcome of our research is useful for future research directed towards the discovery of novel MScl treatments strategies.

Future research

The next step of the study, would be the confirmation and absolute quantification of marker peptides using other independent methods (absolute quantification) such as ELISA or SRM using the same or another dataset. It would also be interesting to validate the identified disease associated proteins in patient's serum (same or external).

CONCLUDING REMARKS

The designated research work here emphasized the identification of biomarkers in MScl patients using a discovery driven proteomic approach. We have executed high throughput differential protein expression profiling, comparing MScl with control samples, to identify the biomarker proteins that are likely contributors to the MScl by their increased or-decreased (or no detection) abundance. Indeed, we have identified potential MScl related targets. By comparing the CSF proteins, we first made an effort to differentiate between the PP and RR subtypes of MScl. Next we discriminated childhood onset-MScl and monophasic ADS. We also made an attempt to identify markers for MScl modifying (Natalizumab) therapy. Further, we explored clonality of CSF IgG autoantibodies in RR MScl patients and showed that IgG share common characteristics in the antigen binding regions among different MScl patients. Finally, we investigated pregnancy MScl and detected proteins responsible for decreased (during pregnancy) and increased (postpartum period relapse) disease activity. We have validated some of our proteins using absolute quantification method as described in chapter 2 and 5. At this point we are not claiming any therapeutic value to the above biomarkers, before we make further analytical and clinical verifications. However, the identified MScl related targets might assist with the diagnosis, prediction of the disease course, or identification of treatment response for Natalizumab. The findings of the current research would be critical for the detailed understanding of the biological pathways related to the neuropathology of the disease. These biomarkers may also help to identify interesting therapeutic values that and could be further investigated. Parts of these pathways might be suitable for new targeted therapies that might slow or stop the disease. In addition, these biomarkers may help to identify therapeutic values that new research can study. In future, the potential value of above identified biomarkers might serve as a curtain raiser the in depth understanding and treatment of MScl. Undoubtedly, the current findings adds a new chapter for further elucidation of MScl and provide valuable clues for increased understanding, treatment and cure for the MScl, and will help to tailor patient's best care.

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Summary

Multiple sclerosis (MScl) is an inflammatory, autoimmune, demyelinating disease of the central nervous system (CNS). The disease occurs most commonly in young adults, it is increasingly being diagnosed in children. Unfortunately, there is no cure for MScl and diagnosis remains challenging because the disease is biologically very complex. Symptoms of the disease vary from patient to patient, different subtypes occur and other disease mimic mainly in the initial stages of disease progression, like Acute Disseminated Encephalomyelitis, Neuromyelitis Optica, Optic Neuritis and Sjogren's syndrome (autoimmune diseases). Identification of biological markers in body fluids such as cerebrospinal fluid (CSF), and urine are of paramount. It helps with diagnosis, prediction of disease course, or identification of response outcome to treatments (such as Natalizumab) and to further understand the pathophysiology and therapeutic choices in various subtypes of MScl. Current advances in the field of proteomics allow large-scale, high-throughput, sensitive and accurate investigation for the detection, identification, and functional investigation of a proteome in a comparatively short period of time. Moreover progresses in protein fractionation and labeling techniques have improved protein identification including low abundant proteins. We used these approaches to find biomarkers of MScl from large numbers of well-characterized patient samples.

Thesis describes the identification of potential MScl biomarker research, with emphasis on distinguishing subtypes, first event of CNS demyelination in children, pathogenic IgG antibodies and pregnancy MScl.

Chapter 1.1 summarizes the current knowledge about the proteomics technologies for biomarker discovery in MScl. In this chapter, we have discussed biological samples, available mass spectrometry based proteomics technologies, sample selection considerations and future prospect of MScl research.

Chapter 1.2 describes the general understanding of the disease pathology, the different subtypes, childhood-onset, CSF B cells and pathogenic IgG antibodies, pregnancy and the disease modifying therapy (Natalizumab).

Chapter 2 highlights the differences and similarities of CSF proteome profile between primary-progressive (PP) (10–15% patients) and relapsing- remitting (RR) MScl (85–90% patients). Our research indicated that the proteome profiles of these two major subtypes of MScl overlap to a large extent. However, few differences were seen and confirmed by independent techniques for two proteins- Protein jagged-1 (more abundant in RR MScl as compared to PP MScl) and Vitamin D-binding protein (only detected in the RR MScl samples). Protein jagged-1 is related to remyelination of MScl

lesions. Vitamin D regulates the differentiation, activation of CD4+ T-lymphocytes and can prevent the development of autoimmune processes.

In **Chapter 3**, study aimed at the identification of protein markers linked to diagnosis of MScl after the first demyelinating event. Twenty one proteins differentiated childhood-onset MScl and children presenting with monophasic acquired demyelinating syndromes (ADS). Proteomics analysis revealed that the CSF of childhood onset MScl is associated with the increased abundance of 14 CNS grey matter related proteins (for example neurofascin, contactin-2 and brevican core protein) as compared to the children presenting with monophasic ADS. Whereas, seven proteins related to the innate immune system were abundant in children with monophasic ADS (such as haptoglobin and CD14). Further validation of differentially abundant protein is required.

In **Chapter 4**, we focused on humoral immune responses in CSF of MScl patients. We hypothesized, that the antigen binding region of IgG specific for certain antigens, are frequently shared between different MScl individuals. This hypothesis was tested by proteomic method by sequencing antigen-binding regions (CDR) of heavy and light chain of IgG antibodies. In this study, our main objective was to identify specific proteomic profiles of mutated complementarity determining regions (CDR) of IgG present in MScl patients but absent in controls. We found five CDR shared in three or more patients and not in controls. Interestingly, one CDR with a single mutation was found to be in common in six patients. In addition, as compared to controls, in CSF IgG of MScl patients, we showed a skew in kappa to lambda ratio (light chain of IgG) and in the usage of certain variable heavy region which was previously being detected at the transcriptome level. The result of the study provides leads concerning the question of whether common antigenic stimuli are accountable for the recruitment of intrathecal B cells in MScl.

In **Chapter 5**, we performed a study to monitor the effect of Natalizumab treatment on MScl patients. This drug targets α4 integrins and leukocyte trafficking. We addressed its effects on CSF proteome before and after one year of the treatment. This study revealed a number of proteins that were significantly differentially abundant between the before and after treatment groups. Subsequently, three proteins (Ig mu chain C region, haptoglobin and chitinase-3-like protein 1) were validated in an independent sample set and by means of an independent method. Interestingly chitinase-3-like protein 1 is described as a presumed biomarker for more rapid progression from a first clinically isolated syndrome to clinically definite MScl. Our study showed significantly lower abundance of three inflammation-related proteins in CSF after one year of treatment.

In **Chapter 6**, we performed proteomics approach on alterations of the proteome of urine from MScl patients during third trimester of pregnancy and at first postpartum period. This study was motivated by the fact that pregnancy is the natural modifier of

MScl course. We found that pregnancy related peptides, were significantly elevated in MScl patients compared to controls. When comparing the pregnancy relate changes, we found 43 disease associated peptides identified with increased (such as trefoil factor 3 and lysosomal associated membrane protein-2) or decreased (for instance Ig mu chain C region and osteoclast associated immune like receptor) difference ratio in MScl compared to control. Our findings indicated that the peptides that relate strongly to pregnancy are linked with or involved in innate and adaptive immune response either directly or indirectly or were immunosuppressive. It seems that the pregnancy associated proteins and their accompanying biological function might have a beneficial effect on MScl suppression. Therefore, these promising urinary proteins deserve further investigation in the field of MScl.

Studies presented in this thesis have successfully applied modern mass spectrometry based proteomics approaches to identify potential MScl biomarker. In CSF, we identified differentially abundant proteins that distinguished two clinical subtypes, showed therapy markers for Natalizumab treatment and distinguished childhood-onset MScl from children presented with monophasic ADS. In addition, we applied this method and identified proteins in urine related to underlying biological mechanisms of MScl improvement during pregnancy. Furthermore, we successfully used this method to identify a panel of CDR peptides that discriminated samples of MScl patients from controls. This was a first indication that peptides generated from the variable part of IgG antibodies in CSF were shared between different MScl patients and such CDR were not detected in control groups. Our studies show that we add new information to MScl biomarker field and such biomarkers are of substantial interest with respect to the disease pathology, general understanding, diagnosis and for novel therapeutic targets. However these biomarkers need further validation for clinical use. Future developments in method will create new possibilities and will help to achieve this goal.

List of abbreviations

2-DGE	2-dimensional gel electrophoresis
ACN	acetonitrile
AGC	automatic gain control
BBB	blood brain barrier
BLAST	basic local alignment search tool
BLASTp	basic local alignment search tool for protein
C	constant region
CAD	collision activated dissociation
CDR	complementarity determining regions
CNS	central nervous system
CSF	cerebrospinal fluid
D	diversity element of immunoglobulin sequence
Da	dalton
EAE	experimental autoimmune encephalomyelitis
ESI	electrospray ionization
FA	formic acid
FT-ICR	Fourier transform ion cyclotron resonance
FDRs	false discovery rates
HIV	human immunodeficiency virus
ICAT	isotope coded affinity tags
ID	internal diameter
Ig	immunoglobulin
IgH	heavy chain
IgL	light chain
IMGT	international ImMunoGeneTics information system
ITRAQ	isobaric tags for relative abundance and quantitation
J	joining element of immunoglobulin sequence
LC	liquid chromatography
LC - MS	liquid chromatography-mass spectrometry
MALDI	Matrix-assisted laser desorption/ionization
MOG	myelin oligodendrocyte glycoprotein
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MScl	multiple sclerosis

OBs	oligoclonal bands
ppm	parts-per-million
RR	relapsing remitting
SRM	selective reaction monitoring
TFA	trifluoroacetic acid
V	variable element of immunoglobulin sequence
VH	variable region of immunoglobulin heavy chain
VK	variable region of immunoglobulin light chain kappa
κ	immunoglobulin kappa light chain
λ	immunoglobulin lambda light chain

Acknowledgements

"The strength of the team is each individual member... the strength of each member is the team" (Coach Phil Jackson)

First and foremost I thank my supervisor, **Prof. dr. Rogier Q. Hintzen** and co-supervisor **Dr. Theo M. Luider** for accepting me as a PhD candidate. It has been an honor for me to be your student. Your frequent intellectual input into the projects, valuable guidance, work discussions, constructive suggestions, patience and encouragement were main motivations throughout my PhD.

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Dear Theo, You have been an excellent supervisor and given me so much of your valuable time and freedom to work. You are leading a wonderful proteomics and mass spectrometry facilities at the Erasmus MC. You have helped me right from the beginning personally and professionally. I really appreciate your vast knowledge and your guidance in experiment designing, writing manuscripts and all presentations. I am truly thankful for your steadfast integrity and selfless dedication to my academic development.

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I would like to dedicate this thesis to my **mother** and my **father** (1952-2013) for their endless support, love and encouragement.

I thank my: sisters (Bhavya and Saumya), brothers (Utkrisht, Rajesh and Vijay) and Bittu (my niece) and entire family (cousins, aunties and uncles). I am so grateful to my beloved son Arlen, who shared all the stress together during my thesis drafting.

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List of publications

Singh V, Van Pelt DE, Stoop MP, Stingl C, Ketelslegers IA, Neuteboom RF, Catsman-Berrevoets CE, Luider TM, Hintzen RQ. Gray Matter Related Proteins are Associated with Childhood Multiple Sclerosis. *Neurology: Neuroimmunology & Neuroinflammation. in press*

Singh V, Stingl C, Stoop MP, Zeneyedpour L, Neuteboom RF, Smitt PS, Hintzen RQ, Luider TM. Proteomics Urine Analysis of Pregnant Women Suffering from Multiple Sclerosis. *J Proteome Res. 2015;14:2065-7*

Stoop MP, Singh V, Stingl C, Martin R, Khademi M, Olsson T, Hintzen RQ, Luider TM. Effects of Natalizumab treatment on the cerebrospinal fluid proteome of multiple sclerosis patients. *J Proteome Res. 2013;12:1101-7*

Singh V, Stoop MP, Stingl C, Luitwieler RL, Dekker LJ, Vanduijn MM, Kreft KL, Luider TM, Hintzen RQ. Cerebrospinal fluid derived immunoglobulin G of different multiple sclerosis patients share mutated sequences in complementarity determining regions. *Mol Cell Proteomics. 2013;2:3924-34*

Singh V, Hintzen RQ, Luider TM, Stoop MP. Proteomics technologies for biomarker discovery in multiple sclerosis. *J Neuroimmunol. 2012;48:40-7*

Stoop MP, Singh V, Dekker LJ, Titulaer MK, Stingl C, Burgers PC, Sillevis Smitt PA, Hintzen RQ, Luider TM. Proteomics comparison of cerebrospinal fluid of relapsing remitting and primary progressive multiple sclerosis. *PLoS One. 2010;5:e12442*

Portfolio

Name PhD student: Vaibhav Singh
Erasmus MC Department: Neurology, Clinical and Cancer Proteomics
Research School: Erasmus Postgraduate school Molecular Medicine
PhD period: 2009-2014
Promotor: Prof. Dr. Rogier Q. Hintzen
Co-promotor: Dr. Theo M. Luider

PhD training

	Year	Workload
General courses and academic skills		**ECTS**
Laser Capture Micro Dissection Workshop, Erasmus MC, the Netherlands	2009	
Adobe Photoshop and Illustrator CS4, Erasmus MC, the Netherlands	2010	0.3
Basic Introduction Course on SPSS, Erasmus MC, the Netherlands	2010	0.6
Biomedical Research Techniques IX, Erasmus MC, the Netherlands	2010	1.6
Short Introductory Course on Statistics & Survival Analysis for MD's, Erasmus MC, the Netherlands	2010	0.4
Workshop Writing Successful Grant Proposal Workshop, Erasmus MC, the Netherlands	2011	0.4
Ensembl workshop, Erasmus MC, the Netherlands	2011	0.5
Biomedical English Writing and communication, Erasmus MC, the Netherlands	2011	4
Course on Presentation skill, Erasmus MC, the Netherlands	2012	0.4
Advanced course on Molecular Immunology, Erasmus MC, the Netherlands	2012	3
Access course, Erasmus MC, the Netherlands	2013	0.4
Workshop on InDesign CS6, Erasmus MC, the Netherlands	2014	0.4
Courses at United Europeans for the Development of PHArmacogenomics in Multiple Sclerosis Network (UEPHA* MS)	2009-2012	
Collaborative IRB Training Initiative Human Subjects Training Program (on-line training course)	2009	1
FACS training, Department of Immunology, University Medical Center Hamburg, Germany	2011	1
Entrepreneurship and concepts on intellectual property, Humboldt Graduate School, Berlin, Germany	2012	1
Progenika Biopharma company visit, Bilbao, Spain	2012	1
Summer school on "Technologies for Biomarker Discovery" School of Medicine, Toulouse, France	2009	
Training course on statistical aspect of genomics research, Toulouse, France	2009	0.4

Oral Presentations		
Proteomics meeting, Antibody meeting and Multiple Sclerosis Research Meeting, Department of Neurology, Erasmus MC, the Netherlands;	2009-2013	10
UEPHA* MS Summer Schools; Toulouse (2009), Barcelona (2010), Berlin (2011) and Bilbao (2012)	2009-2012	4
Invited speaker, UEPHA*MS Proteomics Workshop, Rotterdam, the Netherlands	2010	1
Poster Presentations		
10th International Congress of Neuroimmunology, Sitges, Spain	2010	1
4th Summer Course on Mass Spectrometry in Biotechnology and Medicine at the center for Advanced Academic Studies, Dubrovnik, Croatia	2010	1
5th Joint Terminal Congress of the European and America Committee for Treatment and Research in Multiple Sclerosis, Amsterdam, Netherlands	2011	1
Annual MS Research Days of the Dutch MS Research Foundation, Nijmegen, Netherlands	2012	1
Other activities		
Chaired one session in UEPHA* MS meeting of up to 20 academic and research staff working on multiple Sclerosis at Humboldt Graduate School, Berlin, Germany	2012	1
Total ECTS		**36.4**

Curriculum vitae

ABOUT THE AUTHOR

Vaibhav Singh was born on December 1st, 1980 in Mirzapur, India. She completed her bachelor's degree in Chemistry and Zoology. After finishing her graduation, she began her pharmacy diploma at Government Girls Polytechnic (Allahabad). In order to pursue her interests in biological sciences, she later enrolled for Masters of Science in Zoology program at the Faculty of Science, Department of Zoology, in Banaras Hindu University (Varanasi), India. During the course she attained proficiency in Animal Science. She is specialized in Molecular and Applied Endocrinology. After obtaining her Master's degree in 2006, she joined as a research assistant in Centre for Cellular and Molecular Biology (CCMB) at Hyderabad, India. She conducted a research project on the molecular basis of sperm motility in asthenozoospermic patients under the supervision of Dr. S. Shivaji at the CCMB. While working at CCMB, she was awarded with the Marie Curie research fellowship, funded by European Community's Seventh Framework Programme ([FP7/2007-2013] under grant agreement number 212877 in the United Europeans for the development of PHArmacogenomics in multiple sclerosis network (UEPHA* MS). She was one of the nine early stage researchers recruited by the UEPHA*MS consortium which is a Europe-wide network. She interacted with Prof. Dr. Rogier Q. Hintzen (Professor in neurology and immunology) and with Dr. Theo M. Luider (Head of Laboratory of Neuro-Oncology, Clinical & Cancer Proteomics), and started her PhD training in 2009 on the "Identification of potential biomarkers in multiple sclerosis" under supervision of Prof. Dr. R. Q. Hintzen and Prof. Dr. Theo M. Luider at Erasmus Medical Centre, Rotterdam, The Netherlands. The results of her PhD research and published research work on multiple sclerosis biomarker are documented in the current thesis.

Stellingen bij het proefschrift van
Propositions associated with the thesis

Identification of Potential Biomarkers in Multiple Sclerosis

1) Complementarity determining regions of immunoglobulins in cerebrospinal fluid share sequences between individual multiple sclerosis patients and not with controls. This may reflect local intrathecal B cell responses that are driven by common (auto-) antigens in different multiple sclerosis patients.

2) The proteome profiles of cerebrospinal fluid in primary progressive and relapsing remitting multiple sclerosis patients overlap to a large extent, but are not exactly the same. Differences can be observed in the presence jagged-1 and vitamin D-binding protein.

3) Natalizumab treatment of multiple sclerosis patients is associated with a decrease in the levels of inflammation-related proteins in the cerebrospinal fluid, such as chitinase 3-like protein 1.

4) Cerebrospinal fluid in pediatric multiple sclerosis contains significantly increased abundance of grey matter proteins such as for example contactin-2 and neurofascin, compared with monophasic acquired demyelinating syndromes. This indicates that early axoglial damage is important in the neuropathology of multiple sclerosis.

5) The 3rd trimester versus postpartum differences of urine concentrations of several potential disease modifying proteins were more exaggerated in multiple sclerosis patients versus healthy pregnant controls. Knowledge in such pregnancy-related physiological mechanisms to counter-act the disease will help to develop novel strategies to therapeutically manipulate multiple sclerosis.

6) The effect of pregnancy on multiple sclerosis has fascinated researchers so much that there are more review articles available on this topic than original studies.

7) The verification of potential multiple sclerosis biomarkers is necessary for the development of clinically applicable biomarkers.

8) Data do not speak for themselves - they need context, and require evaluation.

9) If the only tool you have is a hammer, you tend to see every problem as a nail. *(Abraham Maslow)*

10) Science is not about why... it's about why not?

11) A signature always reveals a man's character - and sometimes even his name. *(Evan Esar)*

Vaibhav Singh
Rotterdam, 29 september 2015

UEPHA*MS

United Europeans for development of PHArmacogenomics in Multiple Sclerosis

SEVENTH FRAMEWORK PROGRAMME

MARIE CURIE ACTIONS