Proteomics of cerebrospinal fluid for biomarker discovery in multiple sclerosis

The discovery of reliable biomarkers, which are eligible for the prediction of both disease progression and response to treatment, means a great challenge in the management of multiple sclerosis (MS), a devastating disease of the central nervous system. The results of recent proteomic findings from the cerebrospinal fluid of MS patients hold promise of finding ideal biomarkers in the near future.

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Multiple Sclerosis

Multiple sclerosis is a demyelinative disorder of the central nervous system that affects mainly young adults. It has a great impact on quality of life, social and family life, and the careers of the patients.

In the majority of cases the disease starts with a relapsing–remitting (RR) phase. After a variable period of time it turns into a secondary progressive (SP) phase characterized by the gradual accumulation of residual symptoms. In 10–15% of cases a continuous progression is observed from the very beginning, this is the primary progressive (PP) form. In very rare fulminant cases frequent relapses with incomplete remissions cause severe disability or even death in a short duration of time.

The diagnosis of multiple sclerosis is still mainly clinical, supported by MRI and cerebrospinal fluid (CSF) analysis findings. The revised McDonald Criteria [1] allow earlier diagnosis, especially in PP MS. The routine diagnostic CSF analysis in MS includes the detection of oligoclonal bands and quantitative IgG analysis. Isoelectric focusing (IEF) on agarose gels followed by immunoblotting is considered the 'gold standard' for detecting the presence of oligoclonal bands [2]. The sensitivity of the method is above 95% and the specificity is more than 86%. An increased IgG index and the presence of oligoclonal bands in the CSF support an MS diagnosis.

Although the diagnosis is quite straightforward in most cases, taking into account clinical findings and paraclinical tests, there are still no specific biomarkers to confirm the diagnosis nor do we have any validated prognostic markers to follow the progression of the disorder.

At the time of diagnosis, major problems include the identification of the different clinical forms of the disease and the identification of patients with a potential rapid progression before disability evolves; the differential diagnosis of clinically isolated syndrome (CIS) with optic neuritis as the presenting symptom, where neuromyelitis optica (NMO) spectrum disorder may be an alternative diagnosis. Markers of disease progression are needed to distinguish CIS patients with a high probability to develop clinically definite MS.

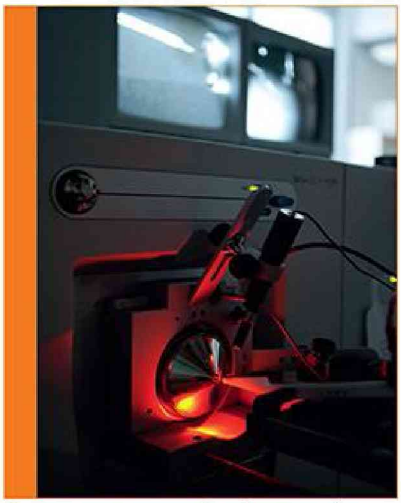
There is also a need for biomarkers of response to treatment and biomarkers for better understanding the underlying pathological processes of the disease. This is especially important with the growing variety of treatment options: now it is possible to change therapy in the case of an inadequate treatment response and to escalate MS treatment to more aggressive alternatives. In the near future individualized treatment choices need better classification of patient characteristics.

In order to discover new biomarkers in MS, one should analyse the whole protein content of body fluids, preferentially CSF. Because of its proximity to the central nervous system (CNS), CSF may reflect changes in the CNS that may help differentiate normal and pathological conditions.

Proteomics

Proteomics is the study of protein expression in an organism. There are excellent reviews on proteomic approaches [3–5], so we will discuss here only certain aspects of these methods relevant to multiple sclerosis biomarker research. Mass-spectrometry (*MS in Italic* to distinguish from multiple sclerosis in this paper) based protein identification strategies include whole-protein analysis ('top-down' proteomics) and analysis of enzymatically produced peptides ('bottom-up' proteomics) [4]. The latter is the standard for large-scale or high-throughput analysis of highly complex samples, and digestion with trypsin is the most common method. The separation of peptides and proteins is an important element of both approaches.

Mass spectrometry measures the mass-to-charge ratio (m/z) of ionized molecules, and, as multiple distinct peptides can have very similar or identical molecular masses, it can be difficult to identify the overlapping peptides [3]. The use of separation techniques, therefore, reduces the cases of coincident peptide masses simultaneously introduced into the mass spectrometer. One of the most commonly used separation techniques is high-performance liquid chromatography (HPLC) with a capillary column. Peptides of similar molecular mass but different hydrophobicity elute



The mass spectrometer: the advances within high resolution liquid chromatography and Fourier transform ion cyclotron resonance mass spectrometry has lead to ground breaking protein discoveries in many biological as well as clinical applications (Photo credit: Mikael Wallerstedt)

The central ion trap: combining forefront technology with complex clinical samples is the driving force for professor Bergquist's research group (Photo credit: Mikael Wallerstedt)

from the LC column and enter the mass spectrometer at different time points, no longer overlapping in the initial *MS* analysis. Liquid chromatography coupled to mass spectrometry reduces the complexity of the sample and allows more precise protein identification.

In order to limit the risk of systematic errors and achieve a high sample throughput, labelling by means of isobaric tags for relative and absolute quantification (iTRAQ) may be used [6]. Multiple samples may be processed in parallel with this multiplexed approach. The main advantage is that the samples are analysed under exactly the same conditions. The relative abundance of labelled peptides indicates relative changes in protein expression.

LC-*MS* experiments generate an enormous amount of data, making data analysis one of the most challenging parts of proteomic analysis. Protein identification and quantification is achieved by database searching. Programs, such as Mascot etc., compare observed spectra to predicted spectra for candidate peptides from a protein database. In a recent study Schutzer *et al.* established a database of the normal human CSF proteome [7].

Proteomics in multiple sclerosis

In recent years a number of papers appeared describing proteomic analysis of CSF or brain tissue of multiple sclerosis patients [8–12]. The first papers in the field analysed pooled samples from a relatively small group of patients [8, 9]. Hammack *et al.* [8] reported the analysis of a pooled sample of three relapsing–remitting MS patients and a pooled sample of three patients with non-MS inflammatory CNS disorders using two-dimensional gel electrophoresis (2-DE) and peptide mass fingerprinting. They identified four proteins in the gels containing MS CSF that were not reported previously in normal human CSF: CRTAC-1B (cartilage acidic protein), tetranectin (a plasminogen-binding protein), SPARC-like protein (a calcium binding cell signalling glycoprotein) and autotaxin t (a phosphodiesterase).

In the study of Dumont *et al.* [9] CSF samples from five MS patients (4 RR, one SP) were analysed by 2-DE followed by liquid chromatography tandem mass spectrometry. With this method 15 proteins have been identified that were not previously observed in non-multiple sclerosis CSF 2-DE gels. These proteins were: psoriasin, calmodulin-related protein NB-1, annexin 1, EWI-2, Niemann–Pick disease type C2 protein (NPC-2), semenogelin 1 (SEM1), semenogelin 2 (SEM2), complement factor H-related protein 1 (FHR-1), procollagen C-proteinase enhancer protein (PCPE), aldolase A, *N*-acetyllactosaminide β-1,3-*N*-acetylglucosaminyl-transferase, tetranectin, cystatin A, superoxide dismutase 3 and glutathione peroxidase.

Later, publications started to focus on the differentiation of the clinical forms of the disease. Lehmensiek *et al.* compared CSF samples from RR MS and clinically isolated syndrome (CIS) patients with controls using two-dimensional difference gel electrophoresis (2-D-DIGE) and matrix-assisted laser desorption/ionization – time of flight (MALDI-TOF) mass spectrometry [10]. In RR MS Ig kappa chain NIG93 protein was increased in concentration, while transferrin isoforms, alpha 1 antitrypsin isoforms, alpha 2-HS glycoprotein, Apo E and transthyretin decreased. In a study of Stoop *et al.* [11] significant differences were observed comparing the peak lists of spectra from CSF of MS patients and patients with other neurological diseases (OND), and also clinically isolated syndrome (CIS) vs OND. Three differentially expressed proteins were identified in the CSF of MS patients compared to CSF of patients with OND: chromogranin A, clusterin and complement C3.

The same group compared proteome profiles of CSF from RR and PP multiple sclerosis and found that they overlap to a large extent [13]. The main detected difference was that protein jagged-1 was less abundant in PP MS compared to RR MS, whereas vitamin D-binding protein was only detected in the RR MS CSF samples. Ottervald *et al.* found an increased CSF level of vitamin-D-binding protein in SP MS compared to the control [14]. Recently, impaired vitamin D homeostasis has been linked to multiple sclerosis [15]: high serum levels of 25-hydroxyvitamin D correlated with a reduced risk of MS [16] and vitamin D supplementation was proposed as an add-on therapy [17].

Biomarkers of disease progression are emerging as new targets of proteomics. In our recently published paper we analysed the CSF of a rare fulminant case of MS and compared it with RR MS and control samples [18]. The aim of this study was to identify proteins related to rapid progression. The presented bottom-up strategy, based on isobaric tag labelling in conjunction with enzymatic digestion followed by nanoLC coupled off-line to MALDI TOF/TOF *MS* resulted in the identification of 78 proteins. Seven proteins were found to be upregulated in both fulminant MS samples but not in the relapsing–remitting case compared to the control. These proteins included Ig kappa and gamma-1 chain C region, complement C4-A, fibrinogen beta chain, serum amyloid A protein, neural cell adhesion molecule 1 and beta-2-glycoprotein 1. These proteins are involved in the immune response, blood coagulation, cell proliferation and cell adhesion.

"The search for biomarkers that are able to identify patients at high risk of rapid progression becomes increasingly important with the appearance of more aggressive treatment possibilities."

Disease progression may be examined by analysing CSF samples from CIS patients who remain CIS and CIS patients who convert to clinically definite multiple sclerosis. Comabella *et al.* [19, 20] analysed pooled CSF samples with isobaric labelling and mass spectrometry.

They found that chitinase 3-like 1, ceruloplasmin and vitamin D-binding protein were upregulated in CSF of patients converted to clinically definite MS. In order to validate their results, the authors determined the levels of these selected proteins by enzyme-linked immunosorbent assay (ELISA) in individual CSF samples. Only chitinase 3-like 1 was validated. In a second validation step CSF chitinase 3-like 1 levels were measured in an independent CIS cohort and its level was again significantly increased in CIS patients who later converted to MS, compared to patients who remained as CIS. High CSF levels of this protein significantly correlated with the number of gadolinium enhancing and T2 lesions on baseline brain MRI scans and disability progression during follow-up.

The search for biomarkers that are able to identify patients at high risk of rapid progression becomes increasingly important with the appearance of more aggressive treatment possibilities. In another ongoing study we currently analyse LC-Fourier transform ion cyclotron resonance (FTICR) *MS* [20–22] data of a larger set of CSF samples from a variety of clinical forms of MS and matched controls.

Despite the increasing number of studies investigating potential biomarkers of MS disease progression and response to therapy, there is still no protein that is repeatedly identified and validated by different groups. This may be due to the relatively small sample sizes and the heterogeneity of the methods applied. Large scale multicentre projects using standard methods for collecting, storing and analysing the samples are necessary to validate these preliminary results and integrate candidate biomarkers into the pathomechanism of the disease.

A great step in this direction is the BIOMS project, which aims a standardized sample collection, storage and processing during the preanalytical steps to rule out the differences occurred by sample preparation [23–25] and test the different methods and hypotheses on a great sample number in multiple centres to shed light on the sources of errors using different methods. One of these initiatives was the neurofilament validation study, which is a candidate biomarker in multiple sclerosis [26]. Another validation study tested two different methods of detecting the neutralizing antibodies against interferon-beta therapy, which is a biomarker of therapy in MS [27].

In the future multi-centre studies on standardized samples and methods can bring us closer to solve the questions regarding the pathological processes and the classification of patients to the most appropriate therapy.

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References

1. Polman CH, *et al.* Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. Ann Neurol 2011; 69: 292–302.
2. Freedman MS, *et al.* Recommended standard of cerebrospinal fluid analysis in the diagnosis of multiple sclerosis: a consensus statement. Arch Neurol 2005; 62: 865–870.
3. Karpievitch YV, *et al.* Liquid Chromatography Mass Spectrometry-Based Proteomics: Biological and Technological Aspects. Ann Appl Stat 2010; 4: 1797–1823.
4. Han X, et al. 3rd Mass spectrometry for proteomics. Curr Opin Chem Biol 2008; 12: 483–490.
5. Becker CH, Bern M. Recent developments in quantitative proteomics. Mutat Res 2011; 722: 171–182.
6. Ross PL, *et al.* Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. Mol Cell Proteomics 2004; 3: 1154–1169.
7. Schutzer SE, *et al.* Establishing the proteome of normal human cerebrospinal fluid. PLoS One 2010; 5: e10980.
8. Hammack BN, *et al.* Proteomic analysis of multiple sclerosis cerebrospinal fluid. Mult Scler 2004; 10: 245–260.
9. Dumont D, *et al.* Proteomic analysis of cerebrospinal fluid from multiple sclerosis patients. Proteomics 2004; 4: 2117–2124.
10. Lehmensiek V, *et al.* Cerebrospinal fluid proteome profile in multiple sclerosis. Mult Scler 2007; 13: 840–849.
11. Stoop MP, *et al.* Multiple sclerosis-related proteins identified in cerebrospinal fluid by advanced mass spectrometry. Proteomics 2008; 8: 1576–1585.
12. Han MH, *et al.* Proteomic analysis of active multiple sclerosis lesions reveals therapeutic targets. Nature 2008; 451: 1076–1081.
13. Stoop MP, *et al.* Proteomics comparison of cerebrospinal fluid of relapsing remitting and primary progressive multiple sclerosis. PLoS One 2010; 5: e12442.
14. Ottervald J, *et al.* Multiple sclerosis: Identification and clinical evaluation of novel CSF biomarkers. J Proteomics 2010; 73: 1117–1132.
15. Cantorna MT, Mahon BD. Mounting evidence for vitamin D as an environmental factor affecting autoimmune disease prevalence. Exp Biol Med 2004; 229: 1136–1142.
16. Raghuwanshi A, *et al.* Vitamin D and multiple sclerosis. J Cell Biochem 2008; 105: 338–343.
17. §Myhr KM. Vitamin D treatment in multiple sclerosis. J Neurol Sci 2009; 286: 104–108.
18. Füvesi J, *et al.* Proteomic analysis of cerebrospinal fluid in a fulminant case of multiple sclerosis. Int J Mol Sci 2012; 13: 7676–7693.
19. Comabella M, *et al.* Cerebrospinal fluid chitinase 3-like 1 levels are associated with conversion to multiple sclerosis. Brain 2010; 133: 1082–1093.
20. Bergquist J. FTICR mass spectrometry in proteomics. Curr Opin Mol Ther 2003; 5: 310–314.
21. Ramstrom M, *et al.* Protein identification in cerebrospinal fluid using packed capillary liquid chromatography Fourier transform ion cyclotron resonance mass spectrometry. Proteomics 2003; 3: 184–190.
22. Ramstrom M, *et al.* Cerebrospinal fluid protein patterns in neurodegenerative disease revealed by liquid chromatography-Fourier transform ion cyclotron resonance mass spectrometry. Proteomics 2004; 4: 4010–4018.
23. Teunissen CE, *et al.* A consensus protocol for the standardization of cerebrospinal fluid collection and biobanking. Neurology 2009; 73: 1914–1922.
24. Teunissen CE, *et al.* Short commentary on 'a consensus protocol for the standardization of cerebrospinal fluid collection and biobanking'. Mult Scler 2010; 16: 129–132.
25. Tumani H, *et al.* Cerebrospinal fluid biomarkers in multiple sclerosis. Neurobiol Dis 2009; 35: 117–127.
26. Petzold A, et al. Neurofilament ELISA validation. J Immunol Methods 2010; 352: 23–31.
27. Bertolotto A, *et al.* Development and validation of a real time PCR-based bioassay for quantification of neutralizing antibodies against human interferon-beta. J Immunol Methods 2007; 321: 19–31.

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