markers is die aangevraagd wordt in de differentiaaldiagnostiek van patiënten met erytrocytose. Dit verklaart ook waarom er geen significant verschil werd gezien in de Hb-concentratie van de Jak2-mutatie-negatieve versus positieve subgroep (respectievelijk 11,2 ± 0,6 mmol/l en 11,5 ± 1,1 mmol/l). Wel werd bij de Jak2-mutatie-positieve subgroep een significant hogere Ht-waarde en lagere MCV-waarde aangetroffen ($p = 0{,}002$ voor beide), passend bij primaire erytrocytose (PV). Ook werd in de Jak2-mutatie-positieve subgroep de frequent bij PV-patiënten geobserveerde verhoogde trombocyten- en leukocytenaantallen significant meer gezien ten opzichte van de Jak2-mutatie-negatieve subgroep (respectievelijk $p < 0{,}001$ en $p = 0{,}002$) (8). Dit bevestigt het klonale karakter van PV, waarbij de Jak2-mutatie in de multipotente hematopoëtische stamcel aanwezig is.

Naast het feit dat de Jak2-V617F-mutatie wordt gezien in vrijwel alle patiënten met PV, wordt deze mutatie ook in ongeveer de helft van de patiënten met ET aangetroffen (1, 2, 7). Binnen ons laboratorium werd bij 52% (34/65) van de patiënten waarbij de Jak2-mutatiestatus werd aangevraagd op basis van de aanwezigheid van een trombocytose, de Jak2-mutatie aangetoond in het perifere bloed. Net als beschreven door o.a. Campbell et al. (7) werden ook binnen ons onderzoek verschillen zichtbaar in perifere hematologische parameters tussen Jak2-V617F-mutatie-negatieve en -positieve patiënten (zie tabel). Ten opzichte van de Jak2-mutatie-negatieve subgroep werden in de Jak2-mutatie-positieve groep significant hogere Hb-, Ht- en leukocytenwaarden gezien (respectievelijk $p < 0{,}001$, $p < 0{,}001$ en $p = 0{,}005$), terwijl geen verschil in trombocytenaantallen werd aangetroffen tussen de twee subgroepen. Dit duidt op een aantal PV-achtige kenmerken in Jak2-mutatie-positieve ET-patiënten (5, 7). Uit de literatuur blijkt daarbij dat een transformatie van ET naar PV significant vaker plaatsvindt bij Jak2-V617F-mutatie-positieve patiënten en dat zij gevoeliger zijn voor de behandeling met hydroxyureum ten opzichte van Jak2-mutatie-negatieve patiënten (7). Hoewel de Jak2-mutatiestatus ET duidelijk in twee groepen scheidt, blijft het moeilijk de Jak2-mutatiestatus van een persoon te voorspellen op basis van routinematig beschikbare klinische en laboratoriumbevindingen (5, 7).

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Proteomic profiling of cerebrospinal fluid to detect potential biomarkers for multiple sclerosis

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Introduction

Multiple sclerosis (MS) is an inflammatory, demyelinating disease of the central nervous system of unknown aetiology. Pathological manifestations are perivascular infiltration of lymphocytes and macrophages in the brain stem, optic nerves and spinal cord, followed by myelin loss, resulting in inflammatory plaques in the white and gray matter (1). The extended criteria indicate that the diagnosis of MS is not straightforward. The most important reason is a lack of reliable serological or cerebrospinal fluid (CSF) tests for the diagnosis of MS (2). Effective immunomodulatory therapy is available but a large range of conditions can mimic MS. This is exactly the reason why it is important to identify biological markers that reliably distinguish

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MS from other inflammatory neurological diseases. Proteomic analysis has recently been used to identify proteomic fingerprints for specific disorders both for diagnostic purposes and for further understanding of the disease process (3).
The aim of this pilot study is focused on the detection of potential CSF biomarkers for the diagnosis and prognosis of MS.

Materials and Methods

CSF samples: Samples were collected from 5 female relapsing-remitting multiple sclerosis (RR-MS) patients (aged 41.9 ± 6.3 years) and 5 female controls (aged 44 ± 6.2 years) with no history, symptoms or signs of neurological disease. Lumbar puncture was performed by standard procedure as a part of the diagnostic evaluation. MS diagnosis was performed according to diagnostic criteria described by McDonald. CSF samples were centrifuged at 500 *g* (4 ºC) for 10 min, aliquoted and stored at a -80 ºC freezer pending analysis. All participants gave informed consent for participation in the study.
Surface-Enhanced Laser Desorption/Ionisation Time-of-flight Mass Spectrometry (SELDI-TOF-MS) analysis: Following optimalisation experiments, CSF samples were profiled on an immobilized metal affinity capture coupled with copper (IMAC-Cu^{2+}) array with a binding buffer of pH 7 (Na-phosphate buffer 50 mM, 0.5 M NaCl) and a cation-exchange (CM10) array with a binding buffer of pH 4 (Na-acetate). Protocols from the manufacturer were followed for applying and washing samples. All samples were analysed at low, mid and high mass range. Sinapinic acid was used as the energy absorbing matrix. The arrays were analysed in the Protein Biosystem IIc instrument (Bio-Rad). Mass-to-charge (*m/z*) ratios were detected using Ciphergen Express Data manager 3.0.6. The biomarker wizard clusters were exported to Biomarker Patterns 5.0.2 (Bio-Rad) for further analysis.
Two-dimensional gel elektrophoresis (2-DE): CSF samples were precipitated using ice-cold acetone and the resulting air-dried pellet was dissolved in rehydratation buffer (8 M urea, 2% CHAPS, 65 mM DTT, 0.5% Biolyte 3-10). CSF proteins were separated first by means of isoelectric focusing. Fifty µg of protein was loaded on a 11 cm immobilized pH gradient strip with a non-linear pH range of 3-10 by in-gel rehydratation under low voltage overnight at 20ºC on an IPG-Phor (GE Healthcare). The voltage was then raised step-wise and the focusing was completed at 20 000 Vh. After focusing, proteins were separated by their molecular weight (MW) on 12.5% Criterion Tris–HCl gels. Subsequently, gels were stained using Sypro Ruby Protein gel stain and image analysis was performed using PDQuest 2-D analysis software (BioRad). The digestion and identification procedures were performed as described in the study of Bouwman et al. (4) with minor modifications.

Results

Using SELDI-TOF-MS, three proteins were found to be significantly up-regulated in the RR-MS group (table 1). The most promising discriminating peak (*m/z* 6215) was capable to separate the RR-MS patients from the controls with mean intensities of 13.4 and 9.6 for RR-MS patients and healthy controls, respectively (figure 1A). In future studies, attempts will be undertaken to identify the peak at *m/z* 6215.
Furthermore, using the 2-DE technology, the intensity of 7 protein spots was found to be significantly altered in the CSF of RR-MS patients compared to controls (table 1). One spot was significantly increased in the CSF of RR-MS patients. This spot was successfully identified as immunoglobulin G (IgG) light chain. We found six spots, which were significantly decreased in CSF of RR-MS patients. We were able to identify four of them. One spot, identified as a clusterin isoform, was expressed about 3 times less in RR-MS versus control group. Three other spots were also identified as clusterin isoforms. By combining these four spots, a consistently down-regulation of clusterin isoforms was achieved in all RR-MS patients (figure 1B).

Conclusion

This pilot supports the hypothesis that CSF protein profiles can discriminate healthy persons from RR-MS patients. Here, we show that, based upon information in SELDI-TOF CSF spectra and 2-DE gel images, potential classifiers could be found for the detection of RR-MS.

Table 1. Summary of proteins significantly ($p<0.05$) altered, at least two times up or down regulated, in CSF from RR-MS patients compared to controls

Up/down regulation RR-MS vs control	*m/z* value or MW	Mean peak intensity/Spot volume ± SD RR-MS vs control	Identity
SELDI-TOF-MS			
↑	*m/z* 6215	13.4 ± 1.4 vs 9.6 ± 1.0	Unknown
↑	*m/z* 13 341	13.4 ± 2.9 vs 9.5 ± 1.3	Unknown
↑	*m/z* 12 712	32.0 ± 4.5 vs 25.9 ± 4.4	Unknown
2-DE			
↓	50 kDa	11 ± 10 vs 41 ± 24	Unknown
↓	38 kDa	51 ± 36 vs 129 ± 46	Clusterin isoform
↓	37 kDa	54 ± 36 vs 143 ± 39	Clusterin isoform
↓	37 kDa	98 ± 83 vs 230 ± 45	Clusterin isoform
↓	36 kDa	119 ± 50 vs 275 ± 45	Clusterin isoform
↓	36 kDa	117 ± 64 vs 259 ± 28	Unknown
↑	27 kDa	146 ± 49 vs 64 ± 28	IgG light chain

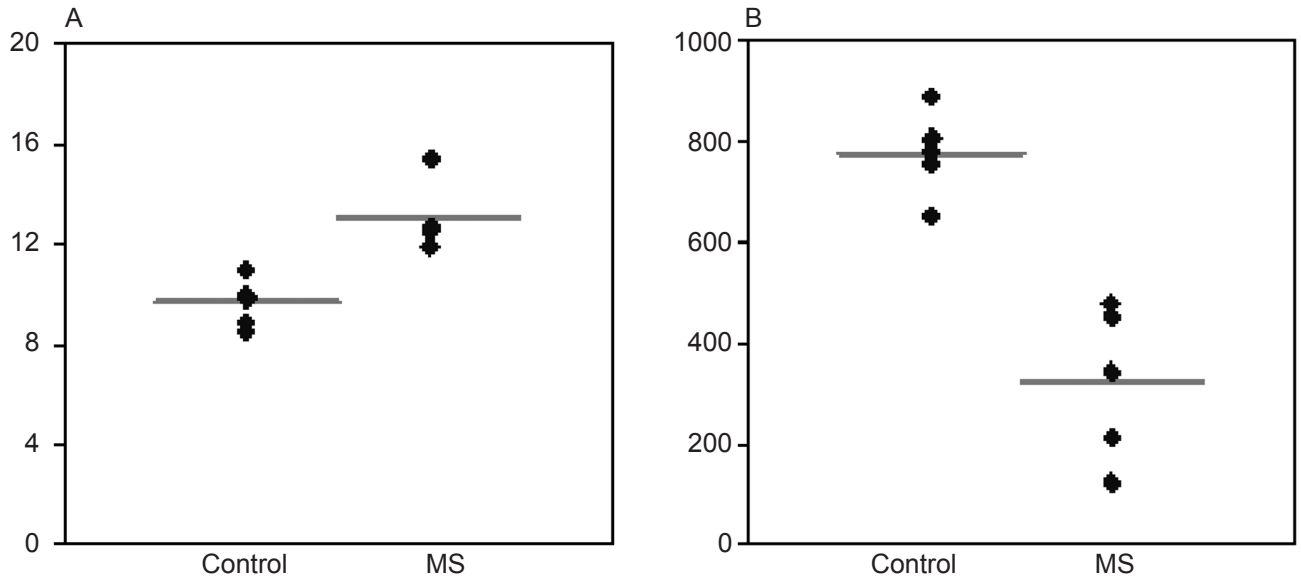

Figure 1. The cluster plots of the peak intensities at *m/z* 6215 (A) and the total volume of four clusterin isoforms (B). The groups are indicated on the x-axis and the normalized intensities/spot volumes are indicated on the y-axis. The red horizontal bars present the mean intensities/spot volumes for the control and RR-MS group.

The first potential marker (*m/z* 6215) found with SELDI-TOF-MS, was up-regulated in RR-MS patients. There was great absolute difference between the mean intensities of both groups, resulting in a proper discrimination between both groups. The second potential marker, a group of four spots on a single 2-DE gel, was consistently down-regulated in all five RR-MS patients in comparison to all 5 gender and age matched controls, respectively. These down-regulated spots in CSF were identified as clusterin isoforms. Polihronis et al. (5) performed a study measuring the CSF clusterin concentration in MS patients and other patients with or without neurological disease using an ELISA. Their results showed for 12 out of 15 patients, with clinically definite or probable MS, an over expression of total CSF clusterin compared to controls. Their control population consisted of 21 patients, undergoing spinal anaesthesia, without known neuropathology. The disparity between the study of Polirhonis et al. and our study might be due to the more sensitive detection of different isoforms of clusterin by 2-DE. Furthermore, IgG light chain was found to be over expressed in the RR-MS group. It is known that RR-MS is correlated with an increased production of IgG, but an elevated IgG level is not specific, nor sensitive for MS (6). Recently, Irani et al. (2), compared CSF samples from patients with MS or clinically isolated syndromes (CIS) and other neurological diseases. In two thirds of MS and CIS samples a 12.5 kDa peak was found, which was absent in controls with other neurological diseases. This 12.5 kDa protein was identified as a cleavage product of full length cystatin C. According to Del Boccio et al. (7) the marker found by Irani et al. (2) is a result of long-term storage at -20 ºC and this degradation product is absent at storage at -80 ºC. Dumont et al. (8) constructed a database of 2-DE separated CSF proteins from MS patients. This database can be used to study differential protein expression in MS patients.

In conclusion, our study has demonstrated, in a small cohort of samples, that the peak at *m/z* 6215 and clusterin are potential biomarkers for RR-MS. A larger cohort of samples would have the power to determine and confirm the established proteomic profiles for discrimination of RR-MS patients and healthy controls, which could be used as a screening tool. The detected biomarkers will also be validated in a blind sample set. In future studies, serum protein profiles will also be obtained, because the collection of blood is much less invasive compared to the collection of CSF.

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