

# Cerebrospinal fluid chitinase 3-like 1 levels are associated with conversion to multiple sclerosis

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In most patients with multiple sclerosis, the disease initiates with a first attack or clinically isolated syndrome. At this phase, magnetic resonance imaging is an important predictor of conversion to multiple sclerosis. With the exception of oligoclonal bands, the role of other biomarkers in patients with clinically isolated syndrome is controversial. In the present study, we aimed to identify proteins associated with conversion to multiple sclerosis in patients with clinically isolated syndrome. We applied a mass spectrometry-based proteomic approach (isobaric labelling) to previously collected pooled cerebrospinal fluid samples from patients with clinically isolated syndrome, who subsequently converted to clinically definite multiple sclerosis ( $n=30$ ) and patients who remained as having clinically isolated syndrome ( $n=30$ ). Next, three of the most represented differentially expressed proteins, i.e. ceruloplasmin, vitamin D-binding protein and chitinase 3-like 1 were selected for validation in individual cerebrospinal fluid samples by enzyme-linked immunosorbent assay. Only chitinase 3-like 1 was validated and cerebrospinal fluid levels were increased in patients who converted to clinically definite multiple sclerosis compared with patients who continued as clinically isolated syndrome ( $P=0.00002$ ) and controls ( $P=0.012$ ). High cerebrospinal fluid levels of chitinase 3-like 1 significantly correlated with the number of gadolinium enhancing lesions and the number of  $T_2$  lesions observed in brain magnetic resonance imaging scans performed at baseline, and were associated with disability progression during follow-up and shorter time to clinically definite multiple sclerosis (log-rank  $P$ -value=0.003). Cerebrospinal fluid chitinase 3-like 1 levels were also measured in a second validation clinically isolated syndrome cohort and found to be increased in patients who converted to multiple sclerosis compared with patients who remained as having clinically isolated syndrome ( $P=0.018$ ). Our results indicate that patients who will convert to clinically

definite multiple sclerosis could be distinguished from those patients who will remain as clinically isolated syndrome by proteomic analysis of cerebrospinal fluid samples. Although protein levels are also increased in other disorders characterized by chronic inflammation, chitinase 3-like 1 may serve as a prognostic biomarker for conversion to multiple sclerosis and development of disability which may help to improve the understanding of the aetiopathogenesis in the early stages of multiple sclerosis.

**Keywords:** clinically isolated syndrome; multiple sclerosis; proteomics; chitinase 3-like 1; cerebrospinal fluid

**Abbreviations:** CHI3L1 = chitinase 3-like 1; CIS = clinically isolated syndrome; ELISA = enzyme-linked immunosorbent assay; iTRAQ = isobaric tag for relative and absolute quantitation

## Introduction

In most patients who later develop multiple sclerosis, the disease usually initiates with an acute or subacute episode of neurological disturbance known as a clinically isolated syndrome (CIS). At this stage, MRI is an important tool, both to predict conversion to clinically definite multiple sclerosis and development of disability (Brex *et al.*, 2002; Tintoré *et al.*, 2006; Fisniku *et al.*, 2008). In addition, the presence of oligoclonal bands was found to be a risk factor for conversion to multiple sclerosis independently of baseline MRI findings (Tintoré *et al.*, 2008). With the exception of oligoclonal bands, the role of other biomarkers in CIS patients is controversial (Berger *et al.*, 2003; Kuhle *et al.*, 2007; Pelayo *et al.*, 2007). Biomarker discovery in CIS patients at the time of disease onset may be useful (i) to differentiate CIS patients who will convert to multiple sclerosis from patients in whom CIS is due to a separate disorder; (ii) to develop prognostic factors for disease progression and development of disability; and (iii) to understand the pathogenesis underlying the early stages of the disease better.

Proteomics is well suited for biomarker discovery, and mass spectrometry-based approaches have been widely applied for protein identification. Proteomic studies in patients with multiple sclerosis are rapidly emerging and have helped to identify proteins that may be potential disease-specific markers (Hammack *et al.*, 2003; Dumont *et al.*, 2004; Noben *et al.*, 2005; Irani *et al.*, 2006; Lehmsiek *et al.*, 2007; Chiasserini *et al.*, 2008; D'Aguanno *et al.*, 2008; Stoop *et al.*, 2008; Qin *et al.*, 2009; Tumani *et al.*, 2009). These studies have analysed the proteome profile present in CSF samples from patients with multiple sclerosis as well as other inflammatory and non-inflammatory neurological disorders. Its proximity to inflammatory lesions in the CNS makes CSF ideal for the identification of biomarkers related to the underlying disease.

In a first phase of the study, we applied a mass spectrometry-based approach to identify proteins associated with conversion to clinically definite multiple sclerosis in pooled CSF samples from patients with CIS who converted to clinically definite multiple sclerosis and patients who remained as CIS during the follow-up period. In a second phase of the study, selected proteins were validated by an independent technique in individual CSF samples. Our data show that the CSF proteome profile is different between CIS patients who will convert to multiple sclerosis and CIS patients who will not convert, and propose a candidate marker

associated with conversion to clinically definite multiple sclerosis in CIS patients.

## Materials and methods

### Patients

#### Screening phase

For the screening phase, 60 patients with CIS recruited at the Centre d'Esclerosi Múltiple de Catalunya from 1995 onwards were selected based on the following criteria: Group 1—no conversion to clinically definite multiple sclerosis, IgG oligoclonal band negative and normal brain MRI after 5 years of follow-up ( $n=30$ ); and Group 2—conversion to clinically definite multiple sclerosis, presence of oligoclonal bands and 3 or 4 Barkhof criteria at baseline brain MRI ( $n=30$ ). The study was approved by the Ethics Committee of Vall d'Hebron University Hospital. Clinical and MRI assessments have been previously described elsewhere (Tintoré *et al.*, 2006). Briefly, brain MRI scans were performed at baseline and after 1 and 5 years of follow-up on a 1.0 or 1.5 T magnet with a standard head coil. MRI included the following sequences: transverse proton-density and T<sub>2</sub>-weighted conventional spin echo, and contrast-enhanced T<sub>1</sub>-weighted spin-echo. The number of Barkhof criteria (Barkhof *et al.*, 1997; Tintoré *et al.*, 2008), number of T<sub>2</sub> lesions, number of gadolinium enhancing lesions and number of new T<sub>2</sub> lesions were scored. Disability was evaluated according to the Expanded Disability Status Scale score in each visit and only Expanded Disability Status Scale performed during stability periods were considered. Clinically definite multiple sclerosis was diagnosed when there was a second attack with a new neurological abnormality that was confirmed by examination (Poser *et al.*, 1983). Time of follow-up was computed as the difference between the date of the last visit and the date of the CIS event. A summary of demographic and clinical characteristics of patients with CIS included in the screening phase is depicted in Table 1.

#### First validation phase

Totally 84 patients with CIS recruited at the Centre d'Esclerosi Múltiple de Catalunya were selected for a first validation phase. Forty-eight patients fulfilled Group 2 criteria, and 20 patients strictly satisfied Group 1 criteria. An additional group of 16 patients with less stringent Group 1 criteria, namely no conversion to clinically definite multiple sclerosis, negative oligoclonal bands and normal brain MRI after 1 year follow-up was also included. Clinical and MRI assessments in these 84 patients were comparable to those described in the screening phase. Additionally, a control group of 20 individuals with other

**Table 1** Clinical information and CSF characteristics of CIS patients included in the screening phase

Characteristics	Group 1 No conversion	Group 2 Conversion to multiple sclerosis	P-values
<i>n</i>	30	30	–
Age (years) <sup>a</sup>	29.9 (8.9)	27.1 (6.6)	0.371
Female/male (% female)	23/7 (76.7)	23/7 (76.7)	1.0
Follow-up time (years) <sup>a</sup>	6.0 (3.0)	5.5 (2.0)	0.294
Clinical presentation			
Optic neuritis	19 (63.3%)	9 (30.0%)	0.073
Brainstem	4 (13.3%)	8 (26.7%)	
Spinal	4 (13.3%)	9 (30.0%)	
Others	3 (10.0%)	4 (13.3%)	
Time between CIS and CSF extraction <sup>b</sup>	51.0 (13.5–114.5)	47.0 (24.0–85.0)	0.877
Leukocytes <sup>b</sup>	2.0 (0–4.0)	2.0 (0–7.0)	0.367
Red cells <sup>b</sup>	2.0 (0–12.5)	0 (0–23.0)	0.993
Protein concentration (g/l) <sup>b</sup>	0.310 (0.250–0.430)	0.350 (0.260–0.440)	0.166
Albumin concentration (g/l) <sup>b</sup>	0.169 (0.135–0.231)	0.186 (0.139–0.244)	0.522
IgG concentration (g/l) <sup>b</sup>	0.021 (0.016–0.030)	0.032 (0.023–0.060)	0.020

a Data are expressed as mean (SD).

b Data are expressed as median (interquartile range). P-values were obtained following comparisons between Groups 1 and 2 by means of chi-square test (gender and clinical presentation) and Mann–Whitney's U-test (remaining variables).

neurological disorders was included in the study. Demographic and clinical characteristics of CIS patients and controls are shown in Table 2. Eighteen patients (50.0%) from Group 1 and 25 patients (52.1%) from Group 2 were also included in the screening phase of the study (Table 2).

### Second validation phase

A second independent cohort comprised of 52 new CIS patients recruited at the Hospital Ramón y Cajal (Madrid, Spain) was used for validation of candidate proteins. Group 1 included 26 CIS patients who remained as CIS during the follow-up, with negative oligoclonal bands and 0 (11 patients; 42.3%), 1 (11 patients; 42.3%), or 2 (four patients; 15.4%) Barkhof criteria at baseline brain MRI. Group 2 included 26 CIS patients who converted to multiple sclerosis (by the Poser criteria in 24 patients and McDonald criteria in two patients; McDonald *et al.*, 2001), with positive oligoclonal bands and 3 or 4 Barkhof criteria at baseline brain MRI. The median time (interquartile range) between the CIS event and CSF extraction was 36.5 days (9.5–111.3 days) in patients from Group 1 and 26.0 days (10.0–78.8 days) in patients from Group 2. Sixteen individuals with inflammatory neurological disorders were used as controls. Table 2 describes demographic and clinical characteristics of CIS patients and controls included in the second validation cohort.

### CSF sampling and pooling strategy

CSF samples were collected at baseline by lumbar puncture and centrifuged for 5 min at 1500 rpm to remove cells. Samples were subsequently used for routine CSF diagnostics that included biochemistry and determination of IgG oligoclonal bands by agarose isoelectric focusing combined with immunoblotting and immunoperoxidase staining. The remaining volume of the samples was aliquoted and conserved at –80°C until used. CSF characteristics of patients included in Groups 1 and 2 are shown in Table 1.

A CSF pooling strategy was designed in the screening phase to identify proteins differentially expressed between patients belonging to Groups 1 and 2. Twelve CSF pools were created, six per group,

each pool containing CSF from five different patients, and each patient contributing with 300 µl to a final volume of 1.5 ml. Pools between groups were sex- and age-matched, and patients between pools were different. An illustration of the pooling design is depicted in Supplementary Fig. S1.

### Proteomic analysis

Supplementary Fig. S2 exemplifies the workflow followed for proteomic analysis, which comprised the following steps.

#### Sample depletion and preparation

After thawing, CSF samples were first concentrated and then albumin and IgG depleted with the ProteoPrep Immunoaffinity Albumin and IgG Depletion Kit (Sigma-Aldrich, St. Louis, Missouri, USA) following the manufacturer's recommendations. Subsequently, samples were precipitated by adding four volumes of ice-cold acetone overnight, centrifuged and the protein pellet diluted in water. Finally, protein concentration was determined using the Bradford's protein quantification method (Bio-Rad Protein Assay, Bio-Rad Laboratories GmbH, Munich, Germany).

#### Isobaric tag for relative and absolute quantitation labelling

CSF pools were analysed by isobaric tag for relative and absolute quantitation (iTRAQ) as follows. Three independent 4-plex experiments were performed, and each experiment contained two pools from each group. Fifty micrograms of protein were transferred to a sample tube and dried in a speedvac. After re-dissolving samples in the Sample Buffer-Plasma following the manufacturer's recommendations (iTRAQ<sup>TM</sup> Reagents Application Kit-Plasma Protocol, Applied Biosystems, Foster City, CA, USA), samples were reduced, alkylated and enzymatically digested with porcine trypsin (Promega, Madison, WI, USA) as per the manufacturer's protocol. The resulting peptides were then labelled with the iTRAQ<sup>TM</sup> reagents and pooled following the manufacturer's protocol. Due to the complexity of the peptide mixture, a strong cation exchange chromatography was carried out.

Peptides were eluted using salt steps and seven fractions finally collected using a KCl range from 25 mM to 1 M.

### Nanoflow liquid chromatography and tandem mass spectrometry analysis

Samples were run on a Q-Star Pulsar i (Applied Biosystems) instrument fitted with a nano-ESI source, previous nano-liquid chromatography separation in an Ultimate II system (LCPackings). Prior to liquid chromatography-mass spectrometry analysis, tryptic peptide mixtures were desalted and concentrated on reverse phase-C18 columns (Spec C18, Varian). The reverse phase chromatography was performed in an Atlantis dC18 NanoEase Column, 75 µm × 150 mm (Waters), using a linear gradient of 5–55% acetonitrile in water 0.1% formic acid over 120 min. A data dependent analysis was performed using software Analyst QS 1.1 (Applied Biosystems).

### Data processing and selection of differentially expressed proteins

Protein identification and quantification was performed by the Paragon™ Algorithm in thorough search mode implemented in the ProteinPilot™ Software 2.0. Proteins were identified by searching in the UniProt/Swiss-Prot or NCBI nr databases.

For the analysis, six-pool comparisons were performed as follows: Pool 1 from Group 1 was directly compared with Pool 1 from Group 2, Pool 2 from Group 1 was compared with Pool 2 from Group 2, and so forth. From each pool comparison, differentially expressed proteins between Groups 1 and 2 ( $P < 0.05$ ) were selected, and the number of pool comparisons in which a selected protein was differentially expressed was counted. Proteins with changing directions in their expression, i.e. up-regulated and down-regulated in different pool comparisons, were not considered in the analysis.

### Enzyme-linked immunosorbent assay

In the validation phase, baseline levels of selected proteins were determined in CSF and serum samples using commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturers' recommendations. CSF samples were collected as described before, and serum was obtained after centrifugation of the clotted blood and stored frozen at  $-80^{\circ}\text{C}$  until used. Levels of chitinase 3-like 1 (CHI3L1) were measured with the METRA, EIA kit (Quidel Corporation, San Diego, USA) in undiluted CSF and serum following a 1:2 dilution factor. Levels of ceruloplasmin were measured by quantitative competitive sandwich ELISA (AssayPro, St Charles, USA) in diluted CSF (1:2) and serum (1:400) samples. Levels of vitamin D-binding protein were detected using a sandwich ELISA (Immunodiagnostik AG, Germany) following CSF (1:100) and serum (1:40 000) dilutions. All samples were measured in duplicate. The intra-assay variability was 8.4% for CHI3L1, 5.3% for vitamin D-binding protein and 12.2% for ceruloplasmin. The inter-assay variability was 15.0% for CHI3L1, 20.0% for vitamin D-binding protein and 22.7% for ceruloplasmin.

### Statistical analysis

Statistical analysis was performed by using the Statistical Package for the Social Sciences 15.0 package (SPSS Inc, Chicago, IL) for MS-Windows. A Mann-Whitney's test was used to test for significant differences in CSF and serum levels of selected proteins between Group 1 and Group 2 CIS patients, and between CIS patients and

**Table 2** Clinical information of CIS patients and controls included in the validation phase

Characteristics	Second validation phase			
	First validation phase		Group 2	
	Group 1	Group 2	Group 1	Group 2
<i>n</i>	36	20	26	16
Percentage in screening phase <sup>a</sup>	18 (50.0)	—	—	—
Age (years)	28.7 (9.4)	35.3 (10.0)	36.5 (11.1)	47.0 (14.7)
Female/male (% female)	29/7 (80.6)	12/8 (60.0)	18/8 (69.2)	11/5 (68.8)
Follow-up time (years)	4.1 (3.1)	—	2.3 (1.5)	—
Clinical presentation	22 (61.1%) ON 4 (11.1%) brainstem 7 (19.4%) spinal 3 (8.3%) others	5 diplopia, 4 headache, 4 vertigo, 4 papillitis, 1 facial pain, 1 neck pain, 1 ↓ visual acuity	8 (30.8%) ON 11 (42.3%) brainstem 4 (15.4%) spinal 3 (11.5%) others	7 systemic autoimmune disorders, 2 PS, 2 THS, 2 bilateral ON, 1 RE, 1 myelitis, 1 recurrent ON
		Conversion to multiple sclerosis	No conversion	Conversion to multiple sclerosis
		48 (52.1%)	26	26
		25 (52.1%)	—	—
		26.8 (6.6)	36.5 (11.1)	33.6 (8.9)
		35/13 (72.9)	18/8 (69.2)	19/7 (73.1)
		5.5 (2.3)	2.3 (1.5)	4.9 (3.0)
		15 (31.3%) ON 11 (22.9%) brainstem 15 (31.3%) spinal 7 (14.7%) others	8 (30.8%) ON 11 (42.3%) brainstem 4 (15.4%) spinal 3 (11.5%) others	4 (15.4%) ON 6 (23.1%) brainstem 10 (38.5%) spinal 6 (23.1%) others
		Other neurological disorders	Other neurological disorders	Other inflammatory neurological disorders

ON = optic neuritis; PS = paraneoplastic syndrome; THS = Tolosa-Hunt syndrome; RE = Rasmussen's encephalitis. Patients with systemic autoimmune disorders included Sjögren's syndrome ( $n = 2$ ), vasculitis ( $n = 2$ ), systemic lupus erythematosus ( $n = 1$ ), neurosarcoidosis ( $n = 1$ ) and Behçet's disease ( $n = 1$ ).

<sup>a</sup> Refers to number and percentage of patients that were also part of the screening phase.

<sup>b</sup> As described in the 'Materials and methods' section, in two patients conversion to multiple sclerosis was defined by the McDonald criteria. Data are expressed as mean (SD) unless otherwise stated.

control individuals. Correlations between CHI3L1 levels and radiological and clinical variables in CIS patients were assessed by the Spearman rank correlation coefficient. Development of clinically definite multiple sclerosis according to baseline CSF levels of CHI3L1 was assessed by Kaplan–Meier survival analysis with Log Rank test and Cox proportional hazard regression.

## Results

### Screening phase: clinical information and CSF characteristics

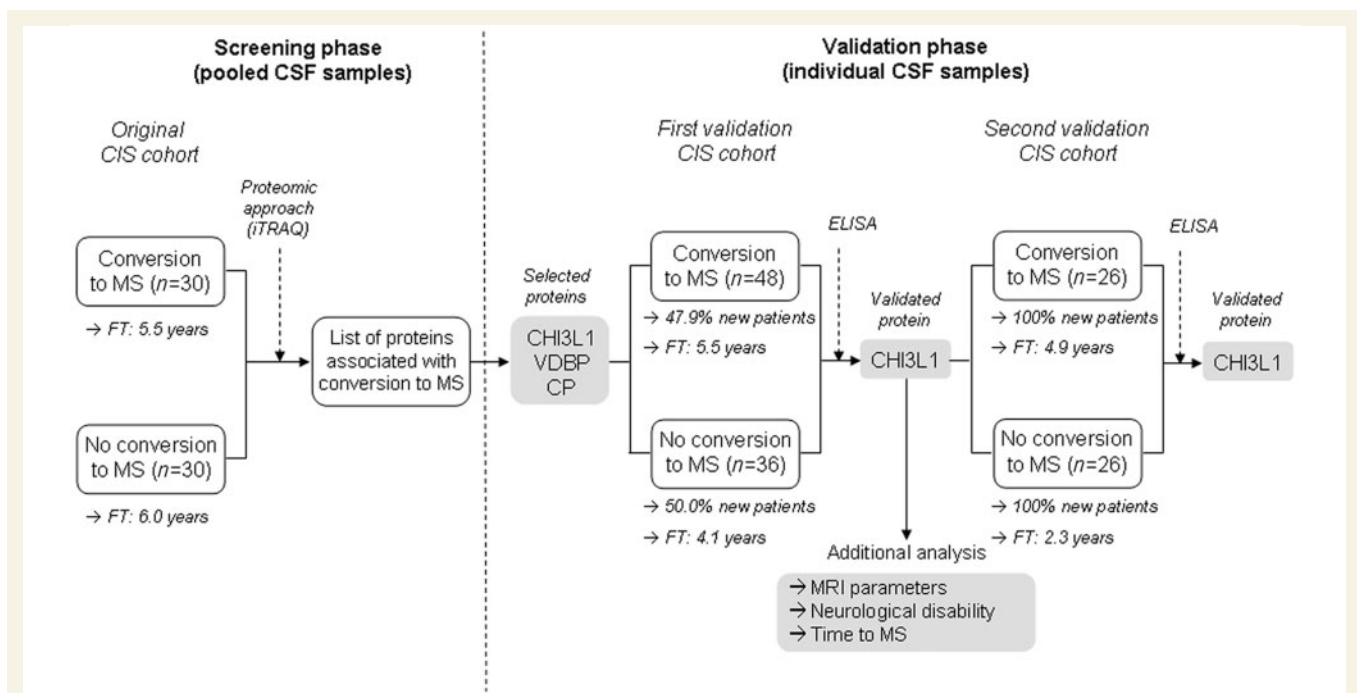
A schematic flow chart summarizing the main steps performed in study design is represented in Fig. 1. At baseline, demographic and clinical characteristics were comparable between CIS patients who did not convert to clinically definite multiple sclerosis (Group 1) and CIS patients who converted to clinically definite multiple sclerosis (Group 2) (Table 1). A higher number of CIS patients presenting with optic neuritis was observed in Group 1 compared with Group 2, although overall differences in clinical presentation were not statistically significant. As shown in Table 1, the median time between the CIS event and CSF collection was similar

between Groups 1 and 2 (51.0 and 47.0 days, respectively). Comparisons of CSF characteristics between groups only revealed a statistically significantly higher IgG concentration in Group 2 patients, which is associated with the IgG oligoclonal bands that are present in these patients.

### Screening phase: proteins associated with conversion to multiple sclerosis

In order to identify proteins associated with conversion to clinically definite multiple sclerosis, pooled CSF samples from 30 CIS patients fulfilling Group 1 criteria and 30 CIS patients satisfying Group 2 criteria were compared using the iTRAQ proteomic technique, as described in the 'Materials and methods' section. Only 4 proteins out of 267 identified (1.5%) showed up-regulation or down-regulation in different pool comparisons: alpha-1 antitrypsin precursor, which is a contaminant from the trypsin enzymatic digestion of proteins, and serum albumin precursor, serotransferrin precursor and plasma retinol-binding precursor, which all are common blood contaminants of CSF.

Table 3 displays the 23 proteins that were found to be differentially expressed between Groups 1 and 2 in three or more pool comparisons. Seventeen proteins (73.9%) were up-regulated and



**Figure 1** Flow chart summarizing the different steps undertaken in the study design. In a first phase of the study (screening phase), a mass spectrometry-based proteomic approach (iTRAQ) was applied to pooled CSF samples from 30 patients who converted to multiple sclerosis and 30 patients who remained as CIS (original CIS cohort) in order to identify proteins associated with conversion to multiple sclerosis. In a second phase of the study (validation phase), three proteins, CHI3L1, vitamin D-binding protein (VDBP) and ceruloplasmin (CP) were selected for validation in individual CSF samples by means of ELISA. CSF levels for these proteins were measured in a partially overlapping CIS cohort comprised of 48 patients who converted to multiple sclerosis and 36 patients who continued as CIS (first validation cohort). Only CHI3L1 was validated and an additional analysis was performed in order to evaluate the association between CSF levels of CHI3L1 and MRI parameters, Expanded Disability Status Scale and time to multiple sclerosis. Finally, CSF CHI3L1 levels were determined in a totally independent CIS cohort comprised of 26 patients who converted to multiple sclerosis and 26 patients who remained as CIS (second validation cohort). CHI3L1 was also validated in this new CIS cohort. The follow-up time (FT) and the percentage of new patients included in the different CIS cohorts are indicated. MS = multiple sclerosis.

**Table 3** Differentially expressed proteins identified in the screening phase in 3 or more CSF pool comparisons between Group 1 and 2 patients

Protein	Accession number	Expression <sup>a</sup>	Number of pools <sup>b</sup>
Ceruloplasmin	P00450	up	6
Vitamin D-binding protein	P02774	up	6
Apolipoprotein A-I	P02647	up	6
Chitinase-3-like protein 1	P36222	up	5
Semaphorin-7 A	O75326	down	4
Beta-Ala-His dipeptidase	Q96KN2	down	4
Plasminogen	P00747	up	4
Vitronectin	P04004	up	4
Apolipoprotein A-IV	P06727	up	4
Ig alpha-1 chain C region	P01876	up	4
Ig kappa chain C region	P01834	up	4
Ig mu chain C region	P01871	up	4
Ig lambda chain C regions	P01842	up	4
Brevican core protein	Q96GW7	down	3
Secretogranin II	P13521	down	3
Proactivator polypeptide	P07602	down	3
Thy-1 membrane glycoprotein (CD90 antigen)	P04216	down	3
Alpha-1-antichymotrypsin	P01011	up	3
Serum paraoxonase/arylesterase 1	P27169	up	3
Corticosteroid-binding globulin	P08185	up	3
Haptoglobin	P00738	up	3
Heparin cofactor 2	P05546	up	3
Alpha-2-antiplasmin	P08697	up	3

a Refers to the direction in protein expression: up = up-regulated in Group 2 (conversion to clinically definite multiple sclerosis) versus Group 1 (no conversion to clinically definite multiple sclerosis); down = down-regulated in Group 2 versus Group 1.

b Refers to the number of pool comparisons in which a protein was found to be differentially expressed in the same direction (up or down).

6 (26.1%) proteins down-regulated in Group 2 compared with Group 1. These results indicate that patients who converted to clinically definite multiple sclerosis could be distinguished from those patients who remained as CIS by proteomic analysis of CSF samples collected at the time of a CIS event.

## First validation phase: CSF CHI3L1 levels are increased in patients who converted to clinically definite multiple sclerosis

To rule out the presence of false positive results associated with the pooling methodology and proteomic technique, three of the most represented differentially expressed proteins were selected for validation in individual CSF samples using an independent analytical method. Ceruloplasmin and vitamin D-binding protein were selected among the three proteins found differentially expressed in all the pool comparisons, and CHI3L1 was selected as the unique protein identified in five-pool comparisons. CSF and serum levels of selected proteins were determined by ELISA in a first validation cohort comprised of 36 CIS patients who remained as CIS (Group 1) and 48 CIS patients who converted to clinically definite multiple sclerosis (Group 2). CSF and serum levels for these proteins were also determined in 20 individuals with other neurological diseases. As shown in Fig. 2, only CHI3L1 was validated. CSF CHI3L1 levels were significantly higher in CIS patients who converted to clinically definite multiple sclerosis compared with

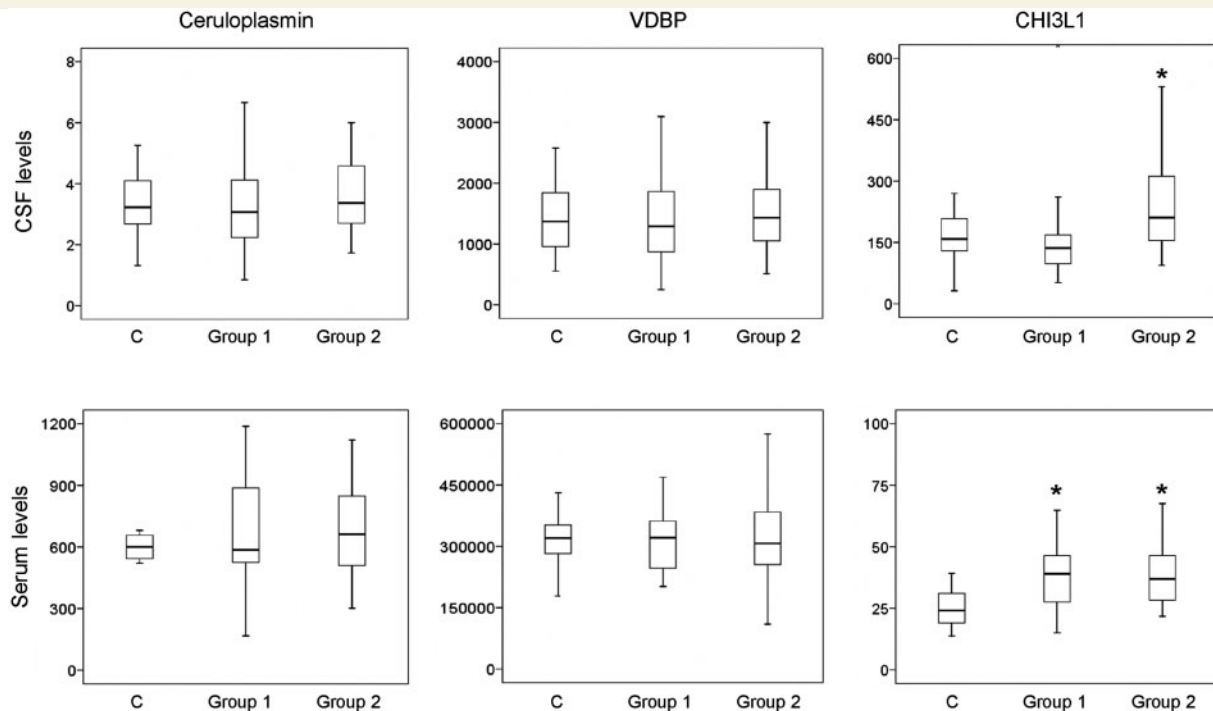
patients who did not convert ( $P=2.3 \times 10^{-5}$ ) and controls with other neurological disorders ( $P=0.012$ ). This finding was restricted to CSF samples, as CHI3L1 serum levels were similar between Groups 1 and 2. However, CHI3L1 serum levels were higher in CIS patients than in controls (Group 1 versus controls,  $P=0.048$ ; Group 2 versus controls,  $P=0.003$ ). CSF and serum levels of ceruloplasmin and vitamin D-binding protein were similar between patients who converted to clinically definite multiple sclerosis and patients who did not convert and comparable to the levels found in controls ( $P>0.05$  for all the comparisons).

These results point to CHI3L1 as the CSF biomarker that more reliably and best discriminated between patients who converted to clinically definite multiple sclerosis and those who continued as CIS.

## CSF CHI3L1 levels are associated with brain MRI abnormalities at baseline and disability progression during follow-up

We next investigated whether levels of CHI3L1 in CSF correlated with brain MRI-derived metrics in patients with CIS at the time of disease onset. As shown in Table 4, baseline CSF CHI3L1 levels significantly correlated with the number of gadolinium enhancing lesions and the number of T<sub>2</sub> lesions observed in brain MRI scans performed at baseline.

To determine whether baseline CSF CHI3L1 levels are associated with MRI abnormalities and disability progression during



**Figure 2** CSF and serum levels of selected proteins in CIS patients and controls included in the first validation phase of the study. Levels of ceruloplasmin ( $\mu\text{g/ml}$ ), vitamin D-binding protein (VDBP;  $\text{ng/ml}$ ) and CHI3L1 ( $\text{ng/ml}$ ) were determined by ELISA in CSF and serum samples from CIS patients who did not convert to clinically definite multiple sclerosis (Group 1,  $n=36$ ), CIS patients who converted to clinically definite multiple sclerosis (Group 2,  $n=48$ ), and individuals with other neurological disorders (C; Controls,  $n=20$ ). CSF CHI3L1 levels were significantly higher in Group 2 compared with Group 1 and controls ( $*P=2.3 \times 10^{-5}$  and  $P=0.012$ , respectively, Mann–Whitney U test). Serum CHI3L1 levels were higher in CIS patients compared with controls (Group 1 versus controls,  $*P=0.048$ ; Group 2 versus controls,  $*P=0.003$ ). Boxes represent the 75th and 25th percentiles divided horizontally by the median. Whiskers are drawn to the nearest value not beyond a standard span ( $1.5$  and  $3 \times$  the interquartile range) from the 75th and 25th percentiles. VDBP: vitamin D binding protein; CHI3L1: chitinase 3-like 1.

**Table 4** Correlations between CHI3L1 CSF levels and clinical and radiological parameters at baseline and during follow-up

	Baseline MRI		Follow-up MRI				Follow-up				
	NGD ( $n=44$ )	NT2L ( $n=44$ )	1 year		5 years		Expanded Disability Status Scale				
			NGD ( $n=24$ )	NT2L ( $n=25$ )	NGD ( $n=18$ )	NT2L ( $n=18$ )	1 year ( $n=44$ )	2 years ( $n=39$ )	3 years ( $n=41$ )	4 years ( $n=37$ )	5 years ( $n=29$ )
CHI3L1	<b>0.32</b> (0.037)	<b>0.44</b> (0.003)	-0.23 (0.274)	0.07 (0.756)	0.12 (0.636)	-0.05 (0.833)	<b>0.34</b> (0.025)	<b>0.40</b> (0.012)	<b>0.47</b> (0.002)	<b>0.38</b> (0.022)	0.29 (0.131)

Data are expressed as Spearman correlation coefficient ( $P$ -value). Number of patients available for each comparison is shown in parenthesis. NGD = number of gadolinium enhancing lesions; NT2L = number of  $T_2$  lesions. Statistically significant correlations are shown in bold.

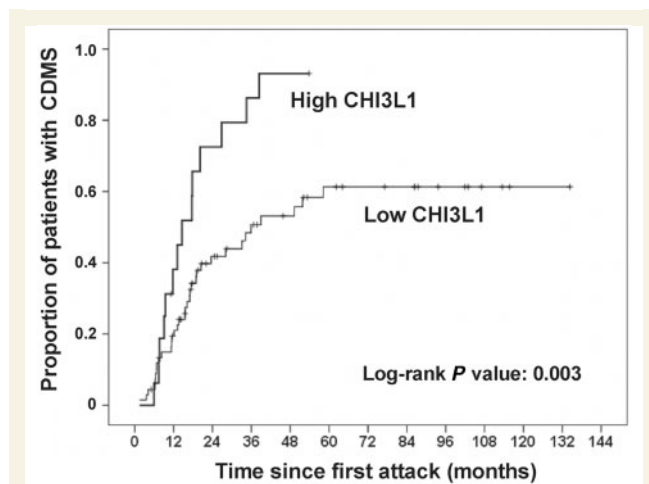
follow-up, analysis was restricted to those patients who will convert to multiple sclerosis (Group 2). As shown in Table 4, significant correlations between baseline CSF CHI3L1 levels and brain MRI findings were lost at 1- and 5-year follow-up, most likely reflecting the small number of patients from whom MRI data were available. Of note, baseline CSF CHI3L1 levels were associated with disability progression at follow-up, as reflected by the statistically significant correlations observed between CSF CHI3L1 levels and Expanded Disability Status Scale during Years 1 through 4 (Table 4).

These results indicate that CSF levels of CHI3L1 at the time of a CIS event are associated with the amount of CNS inflammation and  $T_2$

lesion burden, and more interestingly, that CHI3L1 may be used as a prognostic marker for disability progression in patients who will later convert to clinically definite multiple sclerosis.

### Baseline high CSF levels of CHI3L1 in patients with CIS are associated with shorter time to clinically definite multiple sclerosis

In order to evaluate the association between baseline levels of CHI3L1 in CSF and time to clinically definite multiple sclerosis,



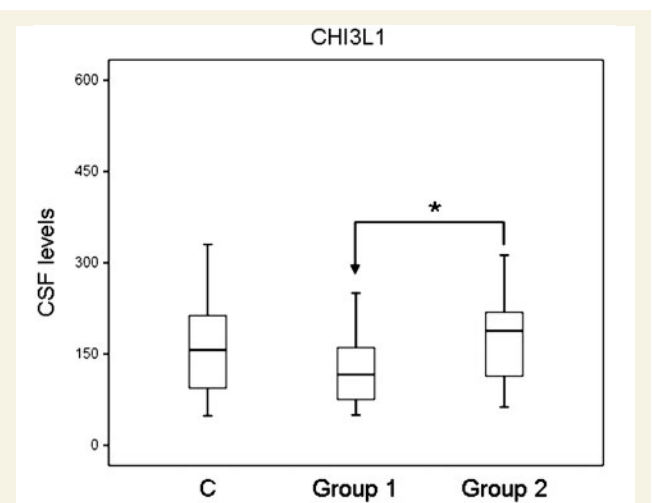
**Figure 3** Kaplan-Meier curves for time to clinically definite multiple sclerosis according to baseline CSF levels of CHI3L1 in patients with CIS. Levels of CHI3L1 were categorized into low and high as described in the 'Results' section. CIS patients with high CHI3L1 levels had a significantly shorter time to clinically definite multiple sclerosis (CDMS) compared with patients with low CHI3L1 (log-rank  $P$ -value = 0.003). Median survival time (95% CI) in patients with high and low levels of CHI3L1 in CSF is 17.6 months (11.6–23.7) and 34.2 months (16.9–51.5), respectively.

patients were classified into two groups (high/low) based on a cut-off value in the CSF CHI3L1 levels of 287.9 mg/ml. This cut-off value was calculated from the mean CSF CHI3L1 levels + 2 SDs obtained in the group of patients with other neurological disorders. Nineteen (22.6%) CIS patients with CHI3L1 levels in CSF above the cut-off value comprised the 'high' group, and the remaining 65 (77.4%) patients comprised the 'low' group. Interestingly, the time to clinically definite multiple sclerosis was significantly shorter in patients with high levels of CHI3L1 in CSF at baseline compared with patients with low protein levels (log-rank  $P$  = 0.003) (Fig. 3). The median survival times (95% confidence interval) between the CIS event and the second relapse in patients with high and low CSF CHI3L1 levels were 17.6 months (11.6–23.7) and 34.2 months (16.9–51.5), respectively. When the CSF CHI3L1 levels were incorporated into a univariate Cox regression model, the presence of high baseline CHI3L1 levels were associated with an increased risk of conversion to clinically definite multiple sclerosis (hazard ratio: 2.5; 95% confidence interval: 1.3–4.7;  $P$  = 0.004).

These results indicate that high baseline CSF levels of CHI3L1 may be used as a prognostic marker for conversion to clinically definite multiple sclerosis.

## Determination of CSF CHI3L1 levels in a second validation cohort of patients with CIS

Finally, in a second validation phase, CSF levels of CHI3L1 were also determined by ELISA in a totally independent validation cohort comprised of 52 new CIS patients classified based on



**Figure 4** CSF levels of CHI3L1 in CIS patients and controls included in the second validation phase of the study. Levels of CHI3L1 (ng/ml) were measured by ELISA in CSF samples from a second independent validation cohort comprised of CIS patients who did not convert to multiple sclerosis (Group 1,  $n$  = 26), CIS patients who converted to multiple sclerosis (Group 2,  $n$  = 26), and controls with inflammatory neurological disorders (C; Controls,  $n$  = 16). CSF CHI3L1 levels were significantly increased in Group 2 compared with Group 1 ( $*P$  = 0.018, Mann-Whitney  $U$ -test).

conversion to multiple sclerosis, as described in the 'Materials and methods' section. Sixteen individuals with inflammatory neurological disorders were also included as a control group. Similar to the findings observed in the first validation cohort, CSF CHI3L1 levels were found to be significantly increased in CIS patients who later converted to multiple sclerosis compared with patients who remained as CIS ( $P$  = 0.018; Fig. 4). Although CSF levels of CHI3L1 were also higher in patients who converted to multiple sclerosis compared with controls with other inflammatory neurological disorders, the difference did not reach statistical significance ( $P$  = 0.351). These results reinforce the potential of CHI3L1 to discriminate between CIS patients who convert to multiple sclerosis and patients who remain as CIS.

Given the contradictory results observed between the iTRAQ and ELISA techniques for proteins that were differentially expressed in all pool comparisons using the proteomic approach, CSF levels of vitamin D-binding protein were also measured by ELISA in the second validation cohort. Mean values of vitamin D-binding protein were similar between groups ( $P$  = 0.752 for comparison between Group 2 and Group 1;  $P$  = 0.711 for comparison between Group 2 and controls; Mann-Whitney  $U$ -test) (data not shown). These results confirm the lack of association between CSF vitamin D-binding protein levels and conversion to multiple sclerosis observed in the first validation cohort, and point to a false positive result of the iTRAQ technique for this protein.

## Discussion

Mass spectrometry has been widely applied in proteomics for protein identification. Recently, new strategies based on 2D liquid



chromatography with tandem mass spectrometry have been developed in order to quantify differences between distinct physiological or pathological stages. The advantages of mass spectrometry-based protein quantification are precision, sensitivity, throughput and convenient automation (Boehm *et al.*, 2007). Stable isotopic-labelling approaches rely on the covalent attachment of stable isotope tags to specific amino acid residues of proteins or peptides. iTRAQ is one of the chemical-labelling techniques that has received more attention (Zieske, 2006; Wiese *et al.*, 2007) and the performance and limitations of this technique have been described elsewhere (DeSouza *et al.*, 2005; Wu *et al.*, 2006). In this approach, peptides are labelled at the N-terminus and the lysine side chains. MS/MS fragmentation produces signature ions (114, 115, 116 and 117 for four states) that are used to determine relative amounts of each protein in each sample. The quantization of proteins is based on the ratio observed in tryptic peptides identified for each protein. Then, in order to quantify one protein, at least three peptides from this protein should be quantified with good *P*-values (statistically significant); in consequence, not all proteins identified can be confidently quantified.

We took advantage of the iTRAQ methodology to identify proteins associated with conversion to multiple sclerosis in CIS patients classified based on stringent criteria. Interestingly, by applying this proteomic approach to CSF samples collected at the time of a CIS event, patients who converted to clinically definite multiple sclerosis could be distinguished from those who remained as CIS. In this methodology, relative quantitation of proteins in the CSF relies on the assumption that the expression levels for most proteins do not change in response to disease.

Three of the most represented proteins that were differentially expressed in the pool comparisons were selected for a first validation phase in individual CSF samples using a different analytical approach. To give more strength to the validation process, approximately 50% of the CSF samples used for validation came from new CIS patients not included in the initial CSF pools. Although vitamin D-binding protein and ceruloplasmin were differentially expressed in all pool comparisons, differences were not validated by ELISA. It is important to remark that iTRAQ is an appropriate tool for biomarker discovery, but results always need to be validated by more sensitive and independent analytical approaches. Vitamin D-binding protein is an immunomodulatory factor that binds vitamin D and monomeric actin (Niino *et al.*, 2002). In two previous proteomic studies, vitamin D-binding protein was decreased in patients with multiple sclerosis and CIS compared with individuals with other neurological disorders (Lehmensiek *et al.*, 2007; Qin *et al.*, 2009). Ceruloplasmin is an antioxidant protein that was previously identified in pooled CSF from multiple sclerosis patients but not in samples from patients with other inflammatory disorders (Hammack *et al.*, 2003). In our study, CSF levels of vitamin D-binding protein and ceruloplasmin determined by ELISA were similar between CIS patients who converted to multiple sclerosis and patients who continued as CIS, and comparable to individuals with other neurological disorders. Furthermore, the lack of association between CSF levels of vitamin D-binding protein and conversion to multiple sclerosis was also confirmed in a second validation cohort. Differences in the study design may explain the lack of reproducibility

between studies. For instance, in previous studies CIS patients were analysed as a whole without drawing a distinction between patients who will convert to multiple sclerosis and patients who will remain as CIS.

In five out of six-pool comparisons, CHI3L1 was overexpressed in CIS patients who converted to clinically definite multiple sclerosis. Interestingly, proteomic results were confirmed by ELISA in two independent validation cohorts, and CSF CHI3L1 levels were increased in this group of patients. The fact that both validation cohorts included less stringent classification criteria of conversion to multiple sclerosis compared with the original cohort used in the screening phase, i.e. number of Barkhof criteria at baseline MRI and follow-up times, indicates that CHI3L1 can be applied to a broad spectrum of CIS patients to discriminate between patients who will later convert to multiple sclerosis and patients who will continue as CIS. CHI3L1, also known as YKL40, is a member of the glycoside hydrolase 18 chitinase family that binds chitin of different lengths but lacks chitinase activity (Renkema *et al.*, 1998). It is mainly secreted by activated macrophages, chondrocytes, neutrophils, vascular smooth muscle cells and some tumour cells. CHI3L1 has been found to be up-regulated in several human cancers, in which increased serum levels have been associated with disease severity, poorer prognosis and shorter survival (Jensen *et al.*, 2003; Johansen *et al.*, 2004; Diefenbach *et al.*, 2007; Kucur *et al.*, 2008; Mitsuhashi *et al.*, 2009). In addition, increased serum levels of CHI3L1 have been found in non-neoplastic disorders characterized by chronic inflammation and tissue remodelling, such as rheumatoid arthritis, osteoarthritis, inflammatory bowel disease and sarcoidosis; and protein levels again correlated with disease severity (Vos *et al.*, 2000; Koutroubakis *et al.*, 2003; Johansen *et al.*, 2005; Erzin *et al.*, 2008). Hence, CHI3L1 was not expected to be specific for multiple sclerosis and, in fact, differences of CSF CHI3L1 levels between CIS patients who converted to multiple sclerosis and individuals with inflammatory disorders were not statistically significant. In our study, increased CSF CHI3L1 levels correlated with baseline brain MRI findings that reflect the degree of brain inflammation and lesion burden, and with development of disability progression at follow-up. Interestingly, high baseline CSF protein levels paralleled poorer prognosis, as they were associated with shorter time to clinically definite multiple sclerosis.

For the first validation phase, CHI3L1 levels were measured in both CSF and serum samples. CSF represents better the local events occurring in the brain but is invasive, whereas serum is simpler to collect. However, differences in CHI3L1 levels between CIS patients were only observed in CSF samples. Of note, CHI3L1 serum levels were increased overall in CIS patients compared with individuals with other neurological disorders, a finding that increases the specificity of the differences observed in the CSF protein levels between patients who converted to multiple sclerosis and patients who continued as CIS. Although its precise physiological role is unclear and the identity of its ligand remains unknown, our data point to CHI3L1 not only as a reliable protein associated with conversion to multiple sclerosis but also as an attractive prognostic marker for the disease. CHI3L1 expression has been shown to be induced by strongly proinflammatory cytokines such as interleukin-1 $\beta$  and tumour necrosis factor- $\alpha$

(Recklies *et al.*, 2005). This finding may indicate that, in CIS patients, CHI3L1 is secreted by activated macrophages in the context of the inflammatory environment that is taking place in the CNS. In our study, CSF levels of CHI3L1 significantly correlated with the number of gadolinium enhancing lesions and the number of T<sub>2</sub> lesions. Furthermore, CHI3L1 is proposed as a candidate autoantigen in rheumatoid arthritis, and CHI3L1-mediated immune responses in these patients are shifted toward an interferon-gamma- $\gamma$ -dominated proinflammatory phenotype (van Bilsen *et al.*, 2004). If similar responses are operating in patients with multiple sclerosis, CHI3L1 might contribute to the Th1 type immune responses observed in these patients.

Interestingly, enzyme activity of another member of the chitinase family, the chitotriosidase, was increased in patients with multiple sclerosis compared with controls (Sotgiu *et al.*, 2006; Comabella *et al.*, 2009), which further reinforces an important role of the chitinase family in the aetiopathogenesis of multiple sclerosis.

Although they need to be validated, the remaining proteins identified in our study represent potential candidates associated with conversion to multiple sclerosis. Apolipoprotein A-I was up-regulated in all pool CSF comparisons. Apolipoprotein A-I has been shown to block the T cell-dependent contact-mediated activation of monocytes (Bresnihan *et al.*, 2004), and it was up-regulated in a previous study in CIS and multiple sclerosis patients compared with controls (Lehmensiek *et al.*, 2007). However, given its presence in blood contaminated CSF samples (You *et al.*, 2005), apolipoprotein A-I was not considered for validation in the present study.

Of note, among the proteins that were differentially expressed in four pool CSF comparisons, semaphorin-7A and beta-Ala-His dipeptidase were down-regulated in patients who later converted to clinically definite multiple sclerosis. Semaphorin-7A is a membrane-anchored member of the semaphorin family that is involved in immune responses and plays an important role in axon growth and guidance (Pasterkamp *et al.*, 2003). Beta-Ala-His dipeptidase, also known as carnosinase, is an enzyme with diverse biological functions such as intracellular buffer, metal-chelator (particularly copper), antioxidant and free radical scavenger. Interestingly, serum carnosinase activity was decreased in patients with multiple sclerosis compared with control individuals (Wassif *et al.*, 1994).

The remaining proteins identified in four-pool comparisons were up-regulated in patients who developed multiple sclerosis. Plasminogen is a circulating zymogen that is converted to the active enzyme plasmin, the main function of which is to dissolve fibrin clots. Interestingly, tissue plasminogen activators have been implicated in multiple sclerosis pathogenesis (Gveric *et al.*, 2003). Vitronectin is a plasma and extracellular matrix glycoprotein involved in pleiotropic functions such as cell adhesion, coagulation and phagocytosis. Of note, in the animal model of multiple sclerosis, experimental autoimmune encephalomyelitis, vitronectin deposition in brain tissue directly promoted microglial activation and matrix metalloproteinase-9 expression (Milner *et al.*, 2007). Apolipoprotein A-IV is a major circulating apolipoprotein with

physiological functions that are not completely understood. It was previously identified in CSF from multiple sclerosis patients but not from controls (Hammack *et al.*, 2003). In a recent study (Tumani *et al.*, 2009), apolipoprotein A-IV was overexpressed in CSF samples from CIS patients who remained as CIS compared with patients who converted to multiple sclerosis. Differences in the methodology, CSF characteristics and criteria to classify CIS patients may account for the discrepancies observed between the two studies.

Finally, the overexpressed IgG heavy and light chains are most likely associated with intrathecal immunoglobulin synthesis (Fischer *et al.*, 2004), and related to one of the classification criteria applied to patients who converted to multiple sclerosis (presence of IgG oligoclonal bands).

In summary, the iTRAQ proteomic approach carried out in the present study facilitated identification of CSF proteins associated with conversion to clinically definite multiple sclerosis that may help to understand the aetiopathogenesis of early stages of multiple sclerosis better. In particular, CHI3L1 is proposed as an attractive and strong prognostic biomarker for disease conversion and development of disability in a patient with CIS.

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## Supplementary material

Supplementary material is available at *Brain* online.

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