

Markers for different glial cell responses in multiple sclerosis: clinical and pathological correlations

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Summary

Disease progression in multiple sclerosis occurs within the interface of glial activation and gliosis. This study aimed to investigate the relationship between biomarkers of different glial cell responses: (i) to disease dynamics and the clinical subtypes of multiple sclerosis; (ii) to disability; and (iii) to cross-validate these findings in a post-mortem study. To address the first goal, 51 patients with multiple sclerosis [20 relapsing remitting (RR), 21 secondary progressive (SP) and 10 primary progressive (PP)] and 51 neurological control patients were included. Disability was assessed using the ambulation index (AI), the Expanded Disability Status Scale score (EDSS) and the 9-hole PEG test (9HPT). Patients underwent lumbar puncture within 7 days of clinical assessment. Post-mortem brain tissue (12 multiple sclerosis and eight control patients) was classified histologically and adjacent sites were homogenized for protein analysis. S100B, ferritin and glial-fibrillary acidic protein (GFAP) were quantified in CSF and brain-tissue homogenate by ELISA (enzyme-linked immunosorbent assay) techniques developed in-house. There was a significant trend for increasing S100B levels from PP to SP to RR multiple sclerosis ($P < 0.05$). S100B was significantly higher in RR multiple sclerosis than in control patients ($P < 0.01$), whilst ferritin levels were significantly higher in SP multiple sclerosis than in control patients ($P < 0.01$). The S100B : ferritin ratio

discriminated patients with RR multiple sclerosis from SP, PP or control patients ($P < 0.05$, $P < 0.01$ and $P < 0.01$, respectively). Multiple sclerosis patients with poor ambulation (AI ≥ 7) or severe disability (EDSS > 6.5) had significantly higher CSF GFAP levels than less disabled multiple sclerosis or control patients ($P < 0.01$ and $P < 0.001$, respectively). There was a correlation between GFAP levels and ambulation in SP multiple sclerosis ($r = 0.57$, $P < 0.01$), and between S100B level and the 9HPT in PP multiple sclerosis patients ($r = -0.85$, $P < 0.01$). The post-mortem study showed significantly higher S100B levels in the acute than in the sub-acute plaques ($P < 0.01$), whilst ferritin levels were elevated in all multiple sclerosis lesion stages. Both GFAP and S100B levels were significantly higher in the cortex of multiple sclerosis than in control brain homogenate ($P < 0.001$ and $P < 0.05$, respectively). We found that S100B is a good marker for the relapsing phase of the disease (confirmed by post-mortem observation) as opposed to ferritin, which is elevated throughout the entire course. GFAP correlated with disability scales and may therefore be a marker for irreversible damage. The results of this study have broad implications for finding new and sensitive outcome measures for treatment trials that aim to delay the development of disability. They may also be considered in future classifications of multiple sclerosis patients.

Keywords: brain tissue; CSF; GFAP; ferritin; S100B

Abbreviations: 9HPT = 9-hole PEG test; AI = ambulation index; AL = acute lesion; BSP = brain-specific proteins; CL = chronic lesion; EDSS = Expanded Disability Status Scale; GFAP = glial fibrillary acidic protein; GM = grey matter; NAWM = normal appearing white matter; PP = primary progressive; RR = relapsing remitting; SAL = subacute lesion; SP = secondary progressive

Introduction

In his remarkable 1868 papers, Charcot (1868) distinguished three steps in the pathology of the disease he first described, '*la sclérose en plaques*' (multiple sclerosis). (i) Initial astrocytic and microglial activation: '*la multiplication des noyaux et l'hyperplasie concomitante des fibres réticulées de la névroglie sont le fait initial*'; (ii) secondary neuro-axonal degeneration: '*l'atrophie dégénérative des éléments nerveux est secondaire*'; and (iii) astrogliosis: '*la névroglie fait place au tissu fibrillaire*', which he considered to represent the anatomical substrates of progressively impaired locomotor activity, '*est considérée à juste titre comme le substratum anatomique de l'ataxie locomotrice progressive*'.

Of these three steps, axonal damage has become one of the most intensely studied aspects of recent multiple sclerosis research. The clinical relevance of the glial response has, however, received less attention despite recent evidence that glial pathology can precede secondary axonal degeneration (Griffiths *et al.*, 1998).

During the glial response different cell-type-specific proteins are released, which can be measured in the CSF (Thompson and Green, 1998). The CSF concentration of these brain-specific proteins (BSP) depends upon the synthesis, catabolism and cellular integrity of astrocytes and microglia. We decided to analyse several BSP in the CSF of multiple sclerosis patients. The BSP chosen to be quantified in this study was S100B for astrocytic activation (Green *et al.*, 1997b), ferritin for microglial activation (Keir *et al.*, 1993) and glial fibrillary acidic protein (GFAP) for astrogliosis (Rosengren *et al.*, 1995). Astrocytic and microglial activation describe the immediate cellular response to any CNS challenge (Streit *et al.*, 1988; Eng and Ghirnikar, 1994; Barron, 1995) and astrogliosis is defined as the fibrinoid scar that replaces lost tissue (Charcot, 1868; Eng *et al.*, 1970). GFAP was first isolated from multiple sclerosis plaques and subsequently found in normal astrocytes (Eng *et al.*, 1970). S100B has been used for many years as a marker for astrocytic proliferation. Interest in the role of S100B in neurological diseases has recently focused on its ability to exhibit both neuroprotective and neurotoxic properties (Donato, 2001). Ferritin has been widely used by histologists for staining microglial cells. The designs of previous studies did not allow sufficiently detailed analysis of both disease subtype and disability in relation to CSF BSP levels (Rosengren *et al.*, 1995; Jongen *et al.*, 1997, 1998; LeVine *et al.*, 1999). Consequently, most authors did not show any statistically significant difference between clinical subtypes or any direct correlation with disability. Confirmation of some results has been hampered by the use of tests that have only been available to the original laboratory (Rosengren *et al.*, 1992, 1995). To overcome this problem we have developed a new ELISA (enzyme-linked immunosorbent

assay) technique for quantification of GFAP using commercially available reagents.

This is the first study quantifying CSF levels of S100B, GFAP and ferritin in well defined clinical multiple sclerosis subtypes, which are not heavily biased by patients having acute relapses. This is important because release of biomarkers during the acute phase of disease is slanted towards relapse-related tissue destruction.

This cross-sectional study aimed to investigate the relationship between the concentration of biomarkers for glial reaction in the CSF with the clinical subtypes and the degree of disability in multiple sclerosis patients. Assumptions that CSF BSP levels are related to pathology in multiple sclerosis brains were tested in a post-mortem brain tissue study comparing multiple sclerosis with control brains. The hypotheses underlying the study were: (i) CSF BSP levels are influenced by the dynamics of disease and can be used to distinguish different multiple sclerosis subtypes; (ii) CSF BSP levels relate to the degree of disability; and (iii) BSP levels are related to histopathological features of CNS lesions. Results from this study may be exploited to try to establish new outcome measures for treatment trials that aim to delay the development of disability in multiple sclerosis.

Methods

Patients

102 patients with neurological disease were included in the study. In response to an article in the *Journal of the Dutch Society of Multiple Sclerosis*, 65 multiple sclerosis patients volunteered to undergo lumbar puncture. Fifty-one patients in whom a diagnosis of clinically definite multiple sclerosis could be made were included in the study. Multiple sclerosis patients were classified as having relapsing remitting (RR), secondary progressive (SP) or primary progressive (PP) disease according to previously published criteria (Lublin and Reingold, 1996).

The control group consisted of 51 patients with the following conditions: one patient had aphasia, one ataxia, one back pain, one benign intracranial hypertension, one chorea, two cerebral infarction, two dementia, one dysphagia, 12 headache, four motor symptoms, two peripheral neuropathies, one sarcoid, one transient ischaemic attack, and 21 non-specific sensory symptoms presumably with a functional basis. These samples were obtained from a CSF library from patients undergoing diagnostic lumbar punctures at the National Hospital for Neurology and Neurosurgery, London. The CSF samples were coded and made anonymous in accordance with the MRC (Medical Research Council) guidelines on the ethical use of biological specimen collections in clinical research.

Patient demographics and baseline characteristics are shown in Table 1.

Clinical assessment

The Amsterdam group assessed all the multiple sclerosis patients. An ambulation index (AI) (Amato and Ponziani, 1999), an Expanded Disability Status Scale score (EDSS) (Kurtzke, 1983) and a 9-hole PEG test (9HPT) for both hands (Amato and Ponziani, 1999; Kalkers *et al.*, 2000) were performed on all patients within 1 week of the lumbar puncture. The AI classified the gait on a scale ranging from 0 (no impairment) to 9 (restricted to wheelchair without independent transfer). The 9HPT is a measure of upper limb motor function. The 9HPT was performed twice with each hand. The quickest performance for each hand was taken to calculate an average value (Kalkers *et al.*, 2000). Samples of CSF were obtained by routine lumbar puncture. Aliquots of CSF were stored at -70°C until assayed. Approval for the study was obtained from the Ethics Committee of The VU Medical Centre and The Joint Medical Ethics Committee of The Institute of Neurology and The National Hospital for Neurology and Neurosurgery. Written informed consent was obtained from all multiple sclerosis patients.

Brain tissue preparation

Material

Post-mortem unfixed brain tissue was obtained from 12 clinically and histologically definite multiple sclerosis patients and eight controls. These specimens were kindly provided by the Multiple Sclerosis Society Tissue Bank at the Institute of Neurology. All multiple sclerosis cases were classified as SP with significant disability (Gveric *et al.*, 2001). The mean age (and range) of the multiple sclerosis patients was 48.6 (29–65) years, with a mean disease duration of 19.5 (7–43) years and a post-mortem interval of 30.2 (9–52) h. The mean age in the control group was 56.7 (37–71) years and the mean post-mortem interval 26.9 (1–40) h. The brain tissue from multiple sclerosis patients was histologically classified into normal-appearing white matter (NAWM), acute lesions (AL), subacute lesions (SAL), chronic lesions (CL) and grey matter (GM) using previously published criteria (Li *et al.*, 1993). Control grey and white

matter (WM) was obtained from normal subjects without neurological diseases. Adjacent pieces of each type of tissue were excised and homogenized for BSP analysis.

Immunohistochemistry

For immunohistochemistry, sections were immunoperoxidase stained with antibodies directed against GFAP (Newcombe *et al.*, 1986), 14E for oligodendrocytes and reactive astrocytes (Newcombe *et al.*, 1992). Cryostat sections were fixed in methanol (-20°C , 10 min), incubated with primary antibody overnight (4°C) and stained using a three-step peroxidase method.

Protein extraction

Snap-frozen blocks of brain and spinal cord from multiple sclerosis and control cases (0.5–1 g wet weight) were finely cut and resuspended at 1 : 5 g/ml in Tris-HCl buffer (100 mM Tris pH 8.1 with 1% Triton X-100). Samples were homogenized on ice by sonication, triturated three times through 19 and 21 gauge needles, and spun at 20 000 g. The supernatant was stored at -70°C . Total protein concentration was determined using the Lowry method.

Assays

Brain-specific proteins

S100B (Green *et al.*, 1997b), ferritin (Keir *et al.*, 1993) and GFAP (A.Petzold *et al.*, unpublished) were measured using in-house ELISA techniques. Albumin in CSF and serum concentrations was determined by a standard Laurell 'rocket' electro-immunoassay.

GFAP

Ninety-six-well microtitre plates were coated with SMI26 (Sternberg Monoclonals) in 0.05 M carbonate buffer. The plates were washed with 0.67 M barbitone buffer containing 5 mM EDTA, 0.1% BSA (bovine serum albumin) and 0.05% Tween. The plates were blocked with 1% BSA and washed.

Table 1 Demographic and clinical data [median (range, number)]

	Control	Multiple sclerosis	Clinical classification		
			RR	SP	PP
Age (years)	41 (27–63), 51	46 (27–65)	40 (27–55)	46 (28–65)	51 (43–55)
Gender (M/F)	37/14	28/23	11/9	10/11	7/3
AI	NA	4.5 (0–10), 44	1.5 (0–10), 18	6.5 (1–9), 18	4 (1–9), 8
EDSS	NA	3.5 (0–8), 51	2 (0–6.5), 20	6 (1–8), 21	6 (2–8), 10
			— $P < 0.001$ —		
9HPT	NA	25 (17–84), 49	20 (18–29), 18	29 (17–84), 21	26 (17–36), 10
			— $P < 0.01$ —		

Multiple sclerosis patients are classified into clinical subtypes. NA = not applicable, F = female, M = male.

CSF was diluted in 0.67 M barbitone buffer containing {3-[(3-cholaminodopropyl)dimethylammonio]-1-propanesulfonate, CHAPS} and EDTA. The plate was incubated with a horseradish peroxidase (HRP)-conjugated cow polyclonal anti-GFAP (Dako, Denmark) diluted in barbitone buffer containing 5 mM EDTA. After washing, the TMB colour reaction was stopped with 1 M hydrochloric acid. Absorbance was read at 450 and 600 nm. All samples were processed in duplicate. The antigen concentration was calculated from an internal standard curve ranging from 0 to 100 pg/ml. The inter-assay coefficient of variation was <10%.

Oligoclonal bands

CSF and serum oligoclonal immunoglobulin G (IgG) bands were detected using isoelectric focusing (Keir *et al.*, 1990; Andersson *et al.*, 1994).

Statistical analysis

All statistical analyses and graphs were done using SAS software (SAS Institute, Inc., Cary, NC, USA). All mean values are given ±SD or SEM as appropriate. The box (median and 25–75% cumulative frequency) and whisker (1–100% cumulative frequency) are shown in the graphs. The linear relationship between continuous variables was evaluated using the Spearman correlation coefficient ($\alpha = 0.05$). Linear regression analysis was performed using the least-squares method. Independent variables were compared using the non-parametric two-sample exact Wilcoxon rank-sum test or the unbalanced two-way ANOVA (general linear model) for more than two groups (Cody and Smith, 1997). Trend analysis was done using the Mantel–Haenszel (M–H χ^2) test (Cody and Smith, 1997). For small sample sizes, levels of significance revealed by either non-parametric method were checked on a categorical level by the Fisher’s exact test ($\alpha = 0.05$). The cut-off for categorical data analysis was set to the 100% cumulative frequency of the indicated control group. *P* values of <0.05 were considered significant.

Results

CSF study

Oligoclonal bands

The CSF and serum isoelectric focusing patterns were classified according to whether the patients had evidence of intrathecal IgG synthesis (OCB+), evidence of a systemic oligoclonal response with matched bands in the serum and CSF (OCB*), i.e. a ‘mirror pattern’, or no evidence of an intrathecal or systemic oligoclonal ‘IgG’ response (OCB–). Forty-six out of 51 multiple sclerosis patients (90%) were classified as OCB+, four (8%) were OCB* and one (2%) was OCB–. All of the control patients were OCB–.

Brain-specific proteins

CSF levels of BSP did not correlate with age, age at onset of disease, disease duration or time from last relapse in the multiple sclerosis patients. In the subgroup analysis, CSF ferritin levels in SP multiple sclerosis patients correlated with disease duration ($r = 0.46, P < 0.05$). The CSF to serum albumin ratio was normal.

Clinical subtypes

Relapsing disease

RR multiple sclerosis patients had higher CSF S100B and GFAP levels compared with the other clinical subtypes.

S100B was significantly different between the clinical subtypes and controls [$F(3,105) = 2.77, P < 0.05$; Table 2]. The post-hoc analysis showed that this significance originated from higher levels present in RR multiple sclerosis patients when compared with control patients ($P < 0.01$). Mean S100B levels in RR patients were nearly 2-fold higher compared with PP and 1.5-fold higher compared with SP multiple sclerosis patients, but these differences did not reach statistical significance (data not shown). There was, however, a trend for a stepwise increase of S100B levels from PP to SP to RR multiple sclerosis patients. None (0 out of 10) of the PP, 14% (3 out of 21) of the SP and 35% (7 out of 20) of the RR

Table 2 CSF levels of S100B, ferritin and GFAP in RR, SP and PP multiple sclerosis patients [median (range, number)]

	Control	Multiple sclerosis	Clinical classification		
			RR	SP	PP
S100B (ng/ml)	0.25 (0–0.4), 51 — $P < 0.05$ — — $P < 0.01$ —	0.3 (0.1–2), 51	0.3 (0.1–2), 20	0.27 (0.2–1.4), 20	0.25 (0.1–0.4), 10
Ferritin (ng/ml)	5 (3–7), 51 — $P < 0.01$ —	5 (1–20), 51	4.5 (1–20), 20	6 (1–19), 21	5.5 (3–13), 10
GFAP (pg/ml)	1 (0–13), 51	3 (0–16), 51	3 (0–11), 20	2 (0–16), 21	0.5 (0–11), 10

S100B significantly distinguishes between multiple sclerosis and control patients [$F(3,98) = 3.09, P < 0.05$], with RR multiple sclerosis patients being the main contributor (post-hoc analysis). SP multiple sclerosis patients have significantly higher ferritin levels than control patients [post-hoc analysis only; $F(3,98)=2.27$, not significant].

multiple sclerosis patients had S100B levels above the cut-off of 0.39 ng/ml. This trend for linear increase was significant (M-H $\chi^2 = 5.633$, $P < 0.05$). Importantly, S100B did not correlate with time from last relapse in either clinical subtype.

Progressive disease

SP patients had the highest CSF ferritin levels of the clinical subtypes. Ferritin levels in progressive patients (SP and PP) were higher than in RR multiple sclerosis patients, which is the inverse of the levels observed for S100B. Consequently a ratio of S100B : ferritin was able to distinguish significantly between clinical subtypes [$F(3,98) = 6.45$, $P < 0.001$]. The S100B : ferritin ratio was significantly higher in RR (1.0 ± 0.8) than in SP (0.7 ± 0.6), PP (0.5 ± 0.3) or control (0.5 ± 0.2) patients ($P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively; Fig. 1). None (0 out of 10) of the PP, 19% (4 out of 21) of the SP and 45% (9 out of 20) of the RR multiple sclerosis patients had a S100B : ferritin ratio above the cut-off. The trend analysis revealed a significant linear increase of the S100B : ferritin ratio from PP to SP to RR multiple sclerosis patients (M-H $\chi^2 = 7.7$, $P < 0.01$).

In PP patients, ferritin and GFAP were slightly elevated but the difference from the control patients did not reach statistical significance. S100B in PP multiple sclerosis patients was similar to the control group.

Controls

CSF S100B was significantly higher in multiple sclerosis patients compared with control patients ($P < 0.05$; Table 2). CSF GFAP levels did not distinguish significantly between

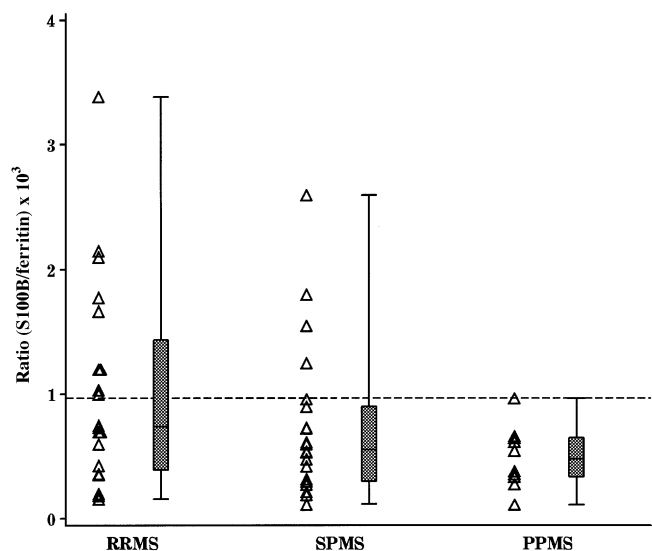


Fig. 1 Scatter and box-whisker plot for the CSF (S100B : ferritin) $\times 10^{-3}$ ratio, which was significantly higher in RR than in SP ($P < 0.05$) or PP ($P < 0.01$) multiple sclerosis patients. Zero per cent of PP, 19% of SP and 45% of RR multiple sclerosis patients had S100B levels above the cut-off of 0.97 (dotted line). The linear stepwise increase is significant ($P < 0.01$).

control and multiple sclerosis patients. CSF ferritin levels were generally higher in multiple sclerosis patients compared with control patients, but this was not significant.

Disability

Patients were categorized according to the frequency distribution on the clinical scales. The distribution of the AI and EDSS was trimodal. Patients were therefore classified accordingly into those with good (AI ≤ 2), moderate (3–6) and poor (≥ 7) ambulation. The EDSS was classified into patients with mild (0–3), moderate (3.5–6.5) and severe disability (7–10).

AI

There was a significant difference in CSF GFAP levels between multiple sclerosis patients classified according to the AI and control patients [$F(3,64) = 5.49$, $P < 0.001$; Table 3]. In the post-hoc analysis, multiple sclerosis patients with poor ambulation had significantly higher CSF GFAP levels than control patients ($P < 0.001$) or multiple sclerosis patients with good ambulation ($P < 0.05$). The subgroup analysis revealed that this significance was due to the nine SP multiple sclerosis patients with poor ambulation; these patients had nearly 6-fold elevated median GFAP levels when compared with control patients ($P < 0.05$; Table 3). Significantly elevated GFAP levels were also present in poorly ambulating RR multiple sclerosis patients compared with control patients ($P < 0.01$), but not in poorly ambulating PP multiple sclerosis patients.

In SP multiple sclerosis patients, disability measured by the AI correlated with levels of GFAP ($r = 0.57$, $P < 0.01$; Fig. 2). The 100% cumulative frequency (2 pg/ml) of the CSF GFAP levels of patients with good ambulation was taken as cut-off for the trend analysis. No (0 out of 4) patients with good ambulation, 40% (2 out of 5) of patients with moderate ambulation and 78% (7 out of 9) of patients with poor ambulation had CSF GFAP levels above this cut-off. The trend analysis revealed a significant linear increase within these three AI categories (M-H $\chi^2 = 6.6$, $P < 0.01$).

EDSS

There was a significant difference in CSF GFAP levels between multiple sclerosis patients classified according to the EDSS and control patients [$F(2,57) = 5.06$, $P < 0.01$; Table 3]. Severely disabled multiple sclerosis patients had significantly higher GFAP levels than control patients ($P < 0.01$). The post-hoc analysis revealed that this was caused by the approximate 6-fold elevation in median GFAP levels in severely disabled SP multiple sclerosis patients when compared with control patients ($P < 0.01$). The post-hoc analysis also revealed significantly elevated GFAP levels in severely disabled multiple sclerosis patients when compared with moderately disabled patients ($P = 0.05$). There was no linear

Table 3 CSF GFAP levels (pg/ml) in control, multiple sclerosis patients and clinical subtypes [median (range, number)]

Clinical subtype	GFAP (pg/ml)			
	Control	Ambulation index		
		<2	2–6	>6
Multiple sclerosis	1 (0–13), 51	2 (0–11), 19	0 (0–11), 8	6 (0–16), 17
		$P < 0.001$ —		
SP		0 (0–2), 4	0 (0–22), 5	6 (0–16), 9
		$P < 0.05$ —		
PP		0 (0–11), 3	4.5 (0–9), 2	1 (0–10), 3
RR		3 (0–10), 12	0 (0), 1	6 (3–11), 5
		$P < 0.01$ —		
	Control	EDSS		
		<3.5	3.5–6.5	>7
	Multiple sclerosis	3 (0–11), 27	0 (0–11), 15	6 (0–16), 9
		$P < 0.01$ —		
SP		2 (0–10), 5	0 (0–11), 9	6 (0–16), 7
		$P = 0.05$ —		
PP		0 (0–11), 4	4.5 (0–10), 4	5.5 (1–10), 2
RR		3.5 (0–11), 18	0 (0), 2	NA
		$P < 0.01$ —		

GFAP significantly distinguishes grades of disability in clinical subtypes (post-hoc analysis) from control patients [AI: $F(3,64) = 5.49$, $P < 0.001$; EDSS: $F(2,57) = 5.06$, $P < 0.01$].

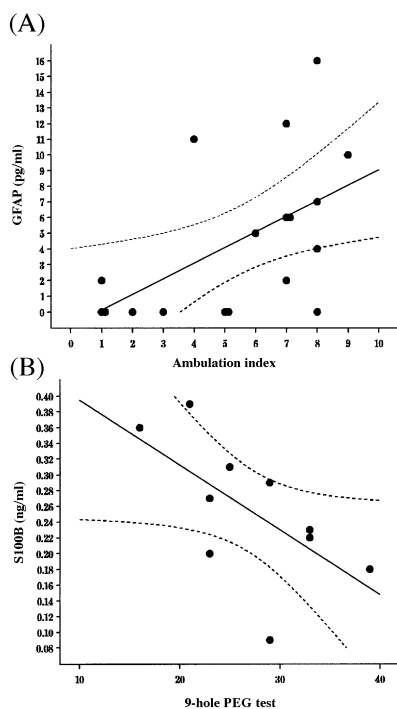


Fig. 2 Disability. (A) CSF GFAP levels correlated significantly with the AI in SP multiple sclerosis patients ($r = 0.57$, $P < 0.01$). Data points are placed adjacent to each other if observations overlapped. (B) CSF S100B levels correlated significantly with the 9HPT of the dominant hand in PP multiple sclerosis patients ($r = -0.85$, $P < 0.01$). The linear regression line, and the 5% lower and 95% upper confidence curves are shown.

correlation between the EDSS and GFAP levels and the trend analysis was negative.

In SP multiple sclerosis patients, ferritin correlated with the EDSS ($r = 0.45$, $P < 0.05$). Because of the previously indicated correlation between disease duration and ferritin levels, a partial correlation correcting for disease duration was performed that abolished the correlation between EDSS and ferritin.

9HPT

CSF ferritin was ~2-fold higher in patients with a test performance >55 s (12.3 ± 5.1 ng/ml) than in ‘quick’ (6.2 ± 4.8 ng/ml) patients ($P < 0.05$, Wilcoxon rank sum test). Because there are only four ‘slow’ patients the results were checked by the Fisher’s exact test, and no significance could be demonstrated. CSF S100B correlated negatively with the 9HPT in PP multiple sclerosis patients ($r = -0.85$, $P < 0.01$; Fig. 2).

Brain tissue study

Grey matter

GFAP and S100B were elevated 2–3-fold in multiple sclerosis GM compared with control GM (Fig. 3; Table 4). Significantly more multiple sclerosis GM samples had S100B and GFAP levels above the cut-off when compared with control GM ($P < 0.001$ and $P < 0.05$, respectively).

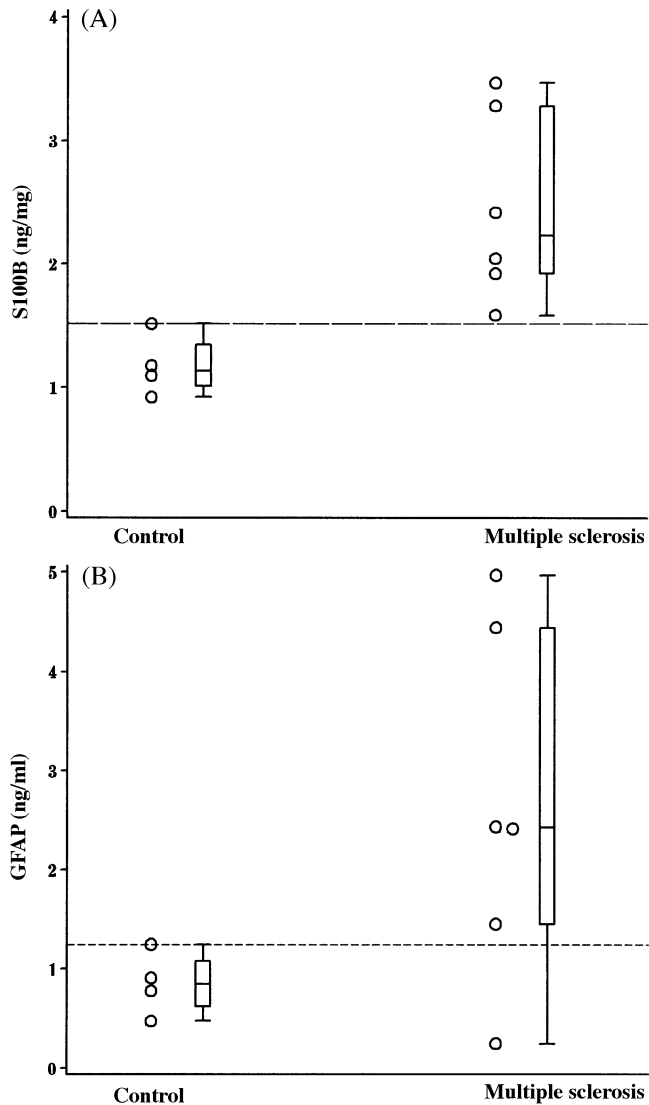


Fig. 3 (A) When comparing levels of GM S100B in cortical multiple sclerosis patients with controls, a 2-fold increase is observed. Significantly more cortical samples from multiple sclerosis patients than controls had S100B levels above the cut-off (dotted line; $P < 0.001$). (B) When comparing levels of GM GFAP in cortical multiple sclerosis patients with controls, a 3-fold increase is observed. Significantly more cortical samples from multiple sclerosis patients than controls had GFAP levels above cut-off (dotted line; $P < 0.05$).

Ferritin levels were higher in multiple sclerosis GM than in control GM. There were, however, no differences between multiple sclerosis GM and multiple sclerosis WM, or control GM and control WM ferritin levels.

White matter

S100B levels were ~2-fold higher in acute plaques than in SAL (Fig. 4A). Significantly more AL than SAL lesions had S100B levels above the cut-off ($P < 0.001$).

Ferritin levels were higher in all multiple sclerosis lesion types compared with controls (Fig. 4B). A significantly higher proportion of samples from NAWM had ferritin levels above cut-off when compared with control WM tissue ($P < 0.05$).

Discussion

Clinical subtypes

Our findings show that biomarkers for astrocytic (CSF S100B) and microglial (CSF ferritin) activation have the potential to distinguish between patients with RR and progressive (PP and SP) multiple sclerosis. There was a significant trend for increasing S100B levels from PP to SP to RR multiple sclerosis patients, with RR multiple sclerosis patients having significantly higher S100B levels than control patients. Ferritin levels were significantly higher in SP patients than in control patients (Table 2). Consequently, a S100B/ferritin ratio was capable of distinguishing RR from SP multiple sclerosis patients. This suggests that astrocytic and microglial activation predominate in relapsing and progressive disease, respectively (Fig. 1).

Multiple sclerosis patients had significantly elevated CSF S100B levels compared with control patients, which was principally due to the high S100B levels in RR multiple sclerosis patients. This confirms the results of most previous studies (Michetti *et al.*, 1980; Massaro *et al.*, 1985, 1997; Lamers *et al.*, 1995). The median relapse-free interval in our study was 15 months in RR and 77 months in SP multiple sclerosis patients. Therefore, CSF S100B levels represent relapse-independent astrocytic activity. *In vitro* astrocytes have been shown to increase their expression of S100B after exposure to adrenocorticotrophic hormone (Suzuki *et al.*, 1987), which has proved to be an effective drug in the treatment of multiple sclerosis (Filippini *et al.*, 2000). In line with these findings would be the high S100B levels in NAWM, which further supports the concept of relapse-independent disease activity. The dynamics of S100B clearance from the extracellular space via the CSF are, however, unknown, but one would assume that due to dilution the extracellular concentration at the site of release would be much higher than that in the CSF. Nanomolar levels of S100B have neurotrophic properties, while micromolar S100B levels have cytotoxic properties (Donato, 2001). Nave's group described how glial pathology precedes axonal degeneration, but the mechanism of progress from one to the other is not fully understood (Griffiths *et al.*, 1998). MRI techniques (Brex *et al.*, 1999; Tourbah *et al.*, 1999) and immunohistochemistry (Trapp *et al.*, 1998; Bjartmar *et al.*, 2001) provide compelling evidence of disease activity in NAWM. The pathological features underlying changes in NAWM need to be clarified. In a post-mortem study combining MRI and histology studies, de Groot *et al.* (2001) suggest that such changes could indicate '(pre)reactive' lesions. Filippi (1998) recently presented evidence that quantitative changes in

Table 4 Levels of S100B, GFAP and ferritin (in µg/mg protein) in homogenized brain tissue

Protein (µg/ml)	White matter				
	Control	NAWM	AL	SAL	CL
S100B	2.4 (1.5–5.6), 5	4.8 (2.6–7.1), 5	5.2 (4.0–6.4), 6	3.4 (2.4–4.1), 4	3.9 (2.0–8.0), 7
GFAP	1.7 (1.1–5.9), 5	1.4 (0–6.0), 5	5.3 (2.6–6.7), 6	3.9 (2.5–5.4), 4	4.0 (0.4–11.4), 7
Ferritin	3.6 (2.5–4.7), 5	7.0 (4.8–9.4), 5	5.2 (3.7–11.2), 6	5.1 (3.9–7.0), 4	5.7 (4.0–13.2), 7
	— <i>P</i> < 0.001—				
	— <i>P</i> < 0.05—				
Protein (µg/ml)	Grey matter				
	Control	Multiple sclerosis			
S100B	1.1 (0.9–1.5), 4	2.2 (1.6–3.5), 6			
GFAP	0.8 (0.5–1.2), 4	2.4 (0.2–5.0), 6			
Ferritin	3.1 (1.3–4.1), 4	4.8 (2.5–12.3), 6			
	— <i>P</i> < 0.001—				
	— <i>P</i> < 0.05—				

The median (range, number) values for WM control tissue, NAWM, AL, SAL and CL, and values for GM from control and multiple sclerosis tissue are shown.

magnetization transfer can be observed weeks before the development of enhancing lesions.

It is of note that elevated levels of S100B due to head injury or rapid parenchymal destruction have been reported in patients with epilepsy (Steinhoff *et al.*, 1999), Creutzfeldt–Jakob disease (Otto *et al.*, 1997), stroke (Aurell *et al.*, 1991; Wunderlich *et al.*, 1999) and acute brain injury (Herrmann *et al.*, 2000). These levels should, however, be distinguished from those in slowly progressive diseases where S100B might play a different pathogenic role, i.e. by modulating the inflammatory response through stimulation of inducible nitric oxide synthase (Adami *et al.*, 2001; Donato, 2001) or modify disease progression by yet unknown mechanisms, e.g. in Down’s syndrome or Alzheimer’s disease (Griffin *et al.*, 1989; Green *et al.*, 1997a; Donato, 2001). Although extracranial sources of S100B, such as adipose tissue, testis and skin, are known (Hidaka *et al.*, 1983; Takahashi *et al.*, 1984; Michetti *et al.*, 1985) and are potential confounding factors in the interpretation of serum S100B levels (Donato 2001; Jackson *et al.*, 2001), this is not likely to affect reported levels of CSF in this study because the blood–brain barrier (as determined by CSF/serum albumin ratio) was intact and all multiple sclerosis patients were beyond acute relapse.

In our study, CSF ferritin is significantly higher in SP multiple sclerosis patients than in the control group. To our knowledge only one other study has measured ferritin levels in multiple sclerosis patients and found them to be elevated in progressive patients (LeVine *et al.*, 1999). This result is supported by the results of our brain tissue study. Ferritin concentrations were higher in all lesion types of progressive multiple sclerosis brains when compared with control WM. This was significant for NAWM versus control WM (Fig. 4B). As NAWM contributes the bulk of brain tissue equilibrating with the CSF, this result is not surprising. Interestingly, Hulet *et al.* (1999) found decreased ferritin

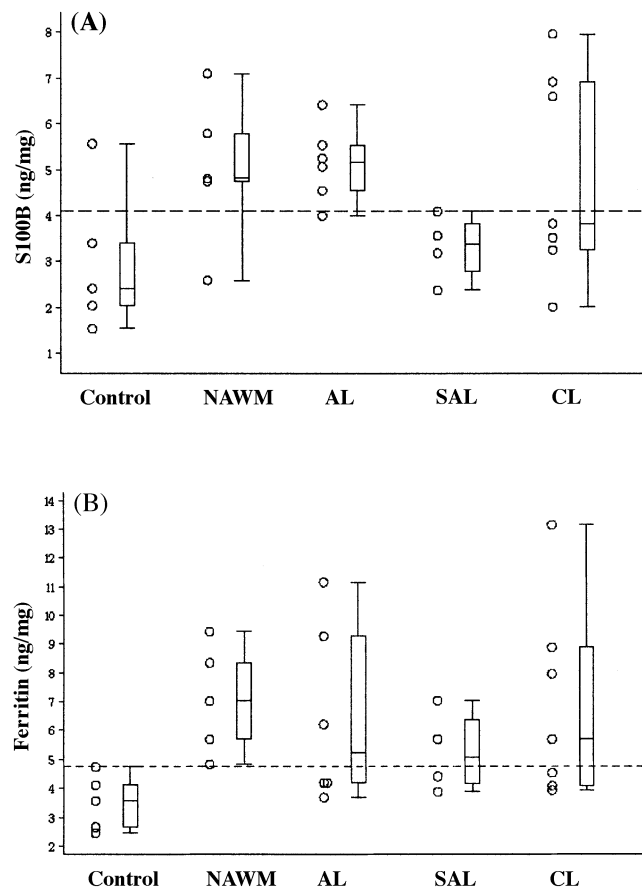


Fig. 4 (A) White matter S100B levels. Significantly more samples from acute plaques than from subacute plaques have S100B levels above the cut-off (dotted line; *P* < 0.001). (B) White matter ferritin levels. Significantly more samples from NAWM had ferritin levels above the cut-off (dotted line) compared with control white matter (*P* < 0.001).

binding to white matter within a multiple sclerosis lesion. The oligodendrocyte requires iron for the synthesis of myelin (Connor and Menzies, 1996), therefore upregulated ferritin levels in multiple sclerosis brain could reflect a physiological reaction to decreased binding and metabolic needs.

In contrast to one study using the CSF of five healthy volunteers as controls (Rosengren *et al.*, 1995), we and others (Albrechtsen *et al.*, 1985; Noppe *et al.*, 1986) found no overall significant difference between CSF GFAP levels in multiple sclerosis patients and a control group, consistent with patients with other neurological disorders. However, significantly elevated GFAP levels compared with our controls were found in multiple sclerosis patients with poor ambulation (AI ≥ 7) or severe disability (EDSS > 6.5). GFAP has also been found to be elevated in dementia (Eng and Ghirnikar, 1994), normal pressure hydrocephalus (Albrechtsen *et al.*, 1985), asphyxiated newborns (Blennow *et al.*, 1995), post head injury (Missler *et al.*, 1999), brain infarction (Aurell *et al.*, 1991) Lyme-borreliosis (Dotevall *et al.*, 1999), trypanosomiasis (Lejon *et al.*, 1999) and multiple sclerosis (Rosengren *et al.*, 1995). GFAP should therefore be regarded as a non-specific biomarker of CNS tissue injury.

Disability

Patients with poor ambulation had significantly higher CSF GFAP levels than patients with good ambulation and control patients (Table 3). Also severely disabled patients had significantly higher CSF GFAP levels compared with mildly disabled patients. This is suggestive of increased astrogliosis within the spinal cord of poorly ambulating or disabled patients. Compared with the control group, only these patients had significantly higher GFAP levels. The subgroup analysis revealed that this was most marked within patients with SP multiple sclerosis. This study revealed a significant correlation between GFAP and individual AI scoring for SP multiple sclerosis patients ($r = 0.57$; Fig. 2). The lack of correlation in PP multiple sclerosis may relate to the small number of cases studied. We interpret the results as demonstrating a direct relationship between GFAP and astrogliosis, which is expressed clinically as disability.

The reason why a direct correlation was found between GFAP and individual points on the AI but not with the EDSS can be explained by the physiological basis of these clinical scales. The AI essentially measures gait. The EDSS, on the other hand, includes other neurological functions that are outside (rostral) the anatomical parts of the CNS that equilibrate with the CSF in the lumbar sac. This 'CSF analytical brain' consists of the inner half of the telencephalon, the basal cortex, the cerebellum, the brain stem and the spinal cord (Felgenhauer and Beuche, 1999). Each lost axon innervating the lower limb could potentially be replaced by a gliotic scar of ~1 m in length (Kreutzberg, 1995), which is the source of GFAP release, and would parallel the decline in ambulation. Thus, almost all changes measured by the AI, but

only some assessed by the EDSS, would be reflected in a change in the level of lumbar CSF GFAP.

This was also demonstrated by the study of Rosengren *et al.* (1995), who studied serial CSF samples in 10 RR multiple sclerosis patients. The scale applied to assessing disability, the RFSS (regional functional score system), includes visual and mental functions. Contradictory changes in the RFSS and lumbar CSF GFAP levels were observed in eight of the 10 patients studied. Importantly, this study of serial CSF samples (seven lumbar punctures per patient over a 2-year period) did not reveal any relationship between the level of CSF GFAP and the time from relapse.

It is difficult to explain the strong negative correlation between S100B and the 9HPT, which has not previously been observed in PP multiple sclerosis patients. The neurotrophic role of S100B in nanomolar concentrations, however, is well described (Haglid *et al.*, 1997; Donato, 2001). One might speculate on whether there is an association between the treatment response to adrenocorticotrophic hormone in multiple sclerosis (Fillippini *et al.*, 2000) that upregulates S100B excretion *in vitro* (Suzuki *et al.*, 1987). Certainly moderately elevated S100B levels in multiple sclerosis could be an indicator for moderate astrogliosis, which might be beneficial. In this context, high levels would have to be associated with relapse and possible toxic effects, while low levels would be related to 'burn out'. These results need to be confirmed in other groups to assess whether the correlation is a consistent finding.

Brain tissue study

The levels of all BSP appear to be increased in multiple sclerosis GM (Fig. 3). This was significant for S100B and GFAP. This finding is particularly relevant for studies focusing on the cognitive and neuropsychiatric aspects of multiple sclerosis. The results of studies examining BSP in GM, however, should be interpreted with caution. The cortex does not form part of the 'CSF analytical brain' (Felgenhauer and Beuche, 1999) since cortically derived BSP will flow into the CSF and will be absorbed by the rostral arachnoid villi. It is unlikely that BSP released from the cortex will be detectable in the lumbar CSF. It might, however, be possible to measure changes in cortical BSP in other body fluids (Thompson and Green, 1998).

The significantly higher levels of S100B in AL compared with SAL suggest that S100B expression is predominantly upregulated in the acute phase of the disease and returns to normal in at least half of all patients (Fig. 4A). In contrast, ferritin levels are consistently higher in multiple sclerosis than in control brain tissue and were found to be significantly higher in multiple sclerosis NAWM compared with control WM (Fig. 4B). This is paralleled by S100B but not by GFAP, supporting the idea that GFAP might be more relevant as a biomarker for damaged tissue.

The results of the S100B and ferritin analysis point to early astrocytic activation, which may return to normal despite

continuing microglial activation in multiple sclerosis WM (Fig. 4), as well as the striking elevation in the cortex of S100B and GFAP (Fig. 3).

Conclusion

The first hypothesis of this cross-sectional study, that there is a relationship between biomarkers for glial reaction and clinical subtypes, was confirmed. In the relapsing phase of the disease, S100B is elevated, while high CSF ferritin levels are observed in all phases. The second hypothesis, that BSP levels relate to disability, is true for GFAP. GFAP correlates with the AI and EDSS. Finally it was shown that BSP levels relate to histopathological features of CNS lesions, which allows us to draw certain conclusions regarding CSF findings. The question of whether these biomarkers prove useful as outcome measures in future treatment trials needs to be addressed in further prospective studies.

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