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

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DOI: 10.1093/brain/awf165

Key words: S100B, GFAP, ferritin, CSF, brain tissue, multiple sclerosis.

Abbreviations: AI = ambulation index, EDSS = expanded disability status scale, 9HPT = nine hole PEG test, GFAP = glial fibrillary acidic protein, BSP = brain-specific proteins, MS = multiple sclerosis, RR = relapsing remitting, SP = secondary progressive, PP = primary progressive, CSF = cerebrospinal fluid, IEF = isoelectric focusing, ELISA = enzyme linked immunosorbant assay, OCB = oligoclonal bands, NAWM = normal appearing white matter, AL = acute lesion, SAL = subacute lesion, CL = chronic lesion, GM = grey matter, CTRL = control.

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Abstract

Glial cells are most sensitive to changes in CNS homeostasis. In multiple sclerosis (MS) disease progresses within the interface of glial cell activation and astrogliosis. This study aimed (1) to investigate the relations between biomarkers for different glial responses with clinical subtypes of MS and disability and (2) to cross-validate these findings in a post-mortem study.

51 patients with multiple sclerosis (20 relapsing remitting, 21 secondary and 10 primary progressive) and 51 neurological control patients were included into this cross-sectional study. Disability was assessed using the ambulation index (AI), EDSS and 9 hole PEG test (9HPT). Patients underwent MRI and lumbar puncture within 7 days from clinical assessment. Post-mortem brain tissue (12 MS and 8 control patients) was classified histologically and adjacent slides were homogenised. S100b, ferritin and glial-fibrillary acidic protein (GFAP) were quantified in CSF and brain-tissue homogenate by in-house developed ELISA techniques.

There was a significant trend for increasing S100b levels from PP to SP to RR MS ($p<0.05$). S100b was significantly higher in RR MS than in control patients ($p<0.01$), whilst ferritin levels were significantly higher in SP MS than in control patients ($p<0.01$). In pathological terms, elevated S100b levels are significantly higher in the acute than in subacute plaques ($p<0.01$), whilst ferritin levels are elevated in all MS lesion stages. The $\frac{S100\beta}{ferritin}$ ratio discriminates significantly RR MS patients from SP, PP or control patients ($p<0.05$, $p<0.01$ and $p<0.01$, respectively). MS patients with poor ambulation ($AI\geq7$) or severe disability ($EDSS \geq 6.5$) had significantly higher CSF GFAP levels than MS patients who do better or control patients ($p<0.01$, $p<0.001$, respectively). There was a correlation between GFAP levels and ambulation in SP MS ($R=0.57$, $p<0.01$) and S100b and 9HPT in PP MS patients ($R=-0.85$, $p<0.01$). Both GFAP and S100b levels were significantly higher in the cortex of MS than in control brain homogenate ($p<0.001$, $p<0.05$, respectively).

We found that S100b is a good marker for the acute phase of the disease as opposed to ferritin which is present throughout the entire course. GFAP correlated with 2 disability scales (AI and EDSS), S100b with the 9HPT and ferritin did not. The results of this study have broader implications for finding new and sensitive outcome measures for treatment trials which aim to delay the development of disability. They might also contribute to future classification of MS pa-

tients along the lines of evidence-based diagnostic-criteria, which will become particularly important for modern, quantitative CSF analytical methods.

1 Introduction

In his remarkable 1868 papers Charcot distinguished three steps in the pathology of his disease, *la sclse en plaques* (multiple sclerosis): (1) initial astrocytic and microglial activation: *la multiplication des noyaux et l'hypertroplasie concomitante des fibres rcul de la noglie sont le fait initial*, (2) secondary neuro-axonal degeneration: *l'atrophie dnerative des ments nerveux est secondaire* and (3) astrogliosis: *la noglie fait place au tissu fibrillaire*,[1] which he considered to represent the anatomic substrate of progressively impaired locomotor activity: *"est conside ste titre comme le substratum anatomique de l'ataxie locomotrice progressive."*[1]

Out of these, axonal damage has become one of the most intensely studied aspects of recent MS research. The clinical relevance of the glial response has however received little attention despite recent evidence that glial pathology can even precede secondary axonal degeneration.[2]

During the glial response there is successive release of different cell type specific proteins, which can be measured in the cerebrospinal fluid (CSF).[3] The CSF concentration of these brain-specific proteins (BSP) depends upon the synthesis, catabolism and cellular integrity of astrocytes and microglia. The BSP chosen to be quantified in this study were S100b for astrocytic activation,[4] ferritin for microglial activation[5] and GFAP for astrogliosis.[6] Astrocytic and microglial activation describe the immediate cellular response to any condition challenging the CNS[7–9] and astrogliosis is defined as the fibrinoid scar which replaces lost tissue.[10,11] GFAP was first isolated from MS plaques and subsequently found also in normal astrocytes.[11] S100b has been used for many years as a marker for astrocytic proliferation, possibly due to its ability to chelate calcium. Ferritin has been widely used by histologists to mark microglial cells. The design of previous studies did not allow sufficiently detailed analysis of the relevance both, disease subtype and disability have for interpreting CSF BSP levels.[6,12–14] Consequently most authors had difficulties in showing 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 which have only been available to the original laboratory.[6,15]

We have developed a new ELISA technique for quantification of GFAP which is entirely based on commercially available reagents. This is the first study quantifying CSF levels of S100b, GFAP and ferritin in well defined clinical MS subtypes, which are not heavily biassed by the acute relapse

related phase. This is important because release of biomarkers during the acute phase of disease are slanted towards relapse-related tissue destruction. This is also the first study to apply the same methods to the analysis of CSF and brain-tissue homogenate.

This cross-sectional study aimed to investigate the relation between the concentration of biomarkers for glial reaction in the CSF with the clinical subtypes and the degree of disability in MS patients and their clinical subtypes. The assumption that CSF BSP levels are related to pathology in MS brains was tested in the post-mortem brain-tissue study comparing MS with control brains. The hypotheses underlying the study were: (1) CSF BSP levels are influenced by the dynamics of disease manifestation and can be used to distinguish different MS subtypes and (2) CSF BSP levels relate to the degree of disability. The results of this study might be exploited to choose a surrogate marker for drug activity (ie, indicator of biological activity as opposed to functional outcome). A surrogate marker would not only provide an assessment of a therapy's direct impact on a targeted pathological process relapse/gliosis, but may also accelerate drug development and reduce cost.

2 Methods

Patients and methods One-hundred and two patients with neurological disease were included in the study. In response to an article in the journal of the Dutch Society of MS 65 MS patients volunteered to undergo MRI study and lumbar puncture. Fifty-one patients in whom a diagnosis of clinically definite MS could be made were included in the study. MS patients were classified as having relapsing remitting (RR), secondary progressive (SP) or primary progressive (PP) disease according to previously published criteria.[16]

The control group consisted of 51 patients with the following conditions: One patient had aphasia, 1 ataxia, 1 back pain, 1 benign intracranial hypertension (BIH), 1 chorea, 2 cerebral infarction, 2 dementia, 1 dysphagia, 12 headache, 4 motor symptoms and 21 sensory symptoms presumably of a functional basis, 2 peripheral neuropathies, 1 sarcoid and one transient ischaemic attack. 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 anonymised in accordance with the MRC 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 MS patients. An ambulation index (AI),[17] an expanded Disability Status Scale score (EDSS)[18] and a 9 hole PEG test (9HPT) for both hands[?,17] were performed on all patients within one 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 measures the time to stick an item 9 times in and out of a hole. The 9HPT was performed twice with each hand. The quickest performance in each hand was taken to calculate an average value used for further analysis.[?] Samples of CSF were obtained by routine lumbar puncture. Aliquots of CSF were stored at -70C until studied. Approval for the study was obtained from the local Ethics Committee (Dutch). Written informed consent was obtained from all MS patients.

Brain tissue preparation Material: post-mortem unfixed brain tissue was obtained from 12 clinical and histological definite MS patients and 8 controls. These specimens were kindly provided by the MS Society Tissue Bank at the Institute of Neurology. All MS cases where classified as secondary progressive MS with significant disability.[?] The mean age of the MS 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) hours. The mean age in the control group was 56.7 (37–71) years and the mean post-mortem interval 26.9 (1–40) hours. The brain tissue was histologically classified into normal appearing white matter (NAWM), acute lesions (AL), subacute lesions (SAL), chronic lesions (CL) and grey matter (GM) from MS patients and controls (CTRL). 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 (mouse monoclonal antibody),[19] 14E for oligodendrocytes and reactive astrocytes.[20] Cryostat sections were fixed in methanol (-20C, 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 MS and control cases, weighing between 0.5 and 1g wet weight, were finely cut and re-suspended at 1:5 g/mL in Tris-HCl buffer (100mM Tris, pH 8.1 with 1% Triton X-100). Samples were homogenized on ice by sonication, tritu-

rated 3 times through 19 and 21 gauge needles and spun at 20,000g. The supernatant was stored at -70 C. Total protein concentration was determined using the Lowry method.

2.0.1 Assays

Brain-specific proteins Nf-H, GFAP, S100b and ferritin were measured using in-house ELISA techniques.[?,4,5]

GFAP 96-well microtiter plates were coated with 0.05 M carbonate buffer containing monoclonal anti GFAP (SMI26, Sternberger Monoclonals). The plates were washed with 0.67 M barbitone buffer containing 5 mM EDTA, 0.1% BSA and 0.05% tween. The plates were blocked with 1% BSA and washed. 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 HRP-conjugated cow polyclonal anti GFAP diluted in barbitone buffer containing 5 mM EDTA. After washing the colour was developed with TMB colour reaction was stopped with 1 M hydrochloric acid. The absorbance 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 pg/mL to 100 pg/mL with a inter-assay CV of less than 10%.

Oligoclonal bands CSF and serum oligoclonal immunoglobulin (IgG) bands were detected using isoelectric focusing (IEF).[21,22]

Statistical Analysis All statistical analyses and graphs were done using SAS software (SAS Institute, Inc., Cary, North Carolina, USA). All mean values are given ± the standard deviation (SD) or the standard error 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 (α=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.[23] Trend analysis was done using the Mantel-Haenszel (M-Hχ^2) test.[23] 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 (α=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 as significant.

3 Results

3.1 CSF study

Oligoclonal bands The CSF and serum IEF 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 response (OCB-). 46 out of 51 MS patients (90%) had intrathecal oligoclonal IgG synthesis. 4 (8%) out of the MS patients had a mirror pattern OCB(*). 1 (2%) out of the MS patients had no evidence of local IgG synthesis in the CSF. None of the control patients had evidence of intrathecal IgG synthesis.

Brain-specific proteins The CSF BSP levels of all MS patients were analysed and compared to (1) disease subtypes and control patients and (2) disability. No overall correlation between age, age at onset of disease, disease duration or time from last relapse in the MS patients reached statistical significance. In the subgroup analysis CSF ferritin levels of SP MS patients correlated with disease duration (R=0.46, p<0.05).

3.1.1 Clinical subtypes

Relapsing disease RRMS patients had higher CSF S100b and GFAP levels if compared to the other clinical subtypes.

S100b was significantly different between the clinical subtypes and controls ($F_{3,105}$=2.77, p<0.05). The post-hoc analysis showed that this significance originated in the significantly higher levels present in RR MS if compared to control patients (p<0.01). S100b levels in RR patients were nearly 2-fold higher compared to PP and 1.5-fold compared to SP MS patients, but this difference did not reach statistical significance. There was however a trend for stepwise increase of S100b levels from PP to SP to RR MS patients. None of the PP, 14% (3/21) of the SP and 35% (7/20) of the

RR MS patients had S100b levels above the cut-off of 0.39 ng/mL (figure 1). This trend for linear increase was significant (M-Hχ^2=5.633, p<0.05). Importantly S100b did not correlate with time from last relapse in either clinical subtypes.

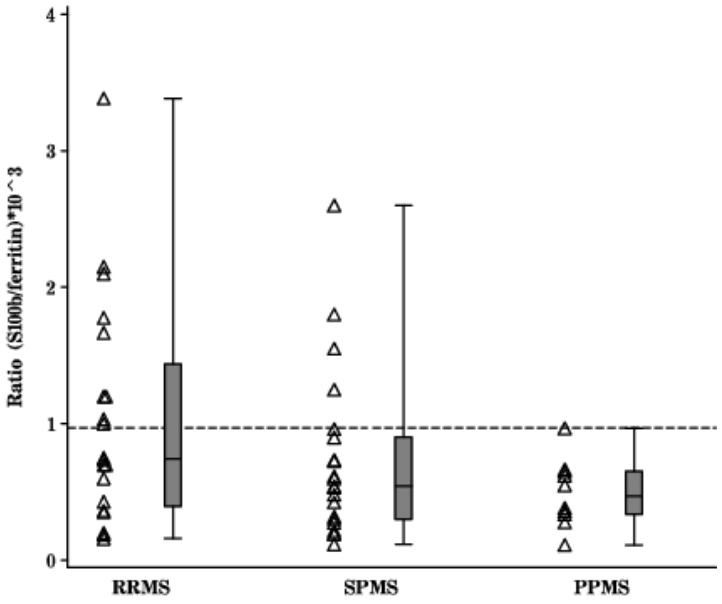

Figure 1: Scatter and box-whisker plot for the CSF $\frac{S100b}{ferritin}$ [$\times 10^3$] ratio. The $\frac{S100\beta}{ferritin}$ ratio was significantly higher in RR than in SP ($p<0.05$) or in PP MS ($p<0.01$) patients. 0% of PP, 19% of SP and 45% of RR MS patients have S100b levels above cut-off. The trend for linear stepwise increase is also significant ($p<0.01$).

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 MS patients, which is inverse to the levels observed for S100b levels. Consequently a ratio of $\frac{S100\beta}{ferritin}$ was able to distinguish significantly between clinical subtypes ($F_{3,98}=6.45$, $p<0.001$). The $\frac{S100\beta}{ferritin}$ ratio was significantly higher in RR (1.0 ± 0.8) than in SP (0.7 ± 0.6) or in PP (0.5 ± 0.3) and control (0.5 ± 0.2) patients ($p<0.05$, $p<0.01$ and $p<0.001$, respectively, figure 1 b). None (0/10) of the PP, 19% (4/21) of the SP and 45% (9/20) of the RR MS patients had a $\frac{S100\beta}{ferritin}$ ratio above cut-off. The trend analysis revealed a significant linear increase of the $\frac{S100\beta}{ferritin}$ ratio from PP to SP to RR MS patients (M-H$\chi^2=7.7$, $p<0.01$).

In PP patients ferritin and GFAP are slightly elevated, but the difference to the control patients did not reach statistical significance. S100b in PP MS patients is similar to the control group.

Controls CSF S100b was significantly higher in MS compared to control patients ($p<0.05$, table 2). CSF GFAP levels did not distinguish significantly between control and MS patients. CSF ferritin levels were generally higher in MS if compared to control patients, but this did not reach statistical significance.

3.1.2 Disability

The clinical scales were categorised according to their distribution. The distribution of the AI is trimodal. Patients were classified accordingly into patients with good (AI $\leq$2), moderate (3–6) and poor ($\geq$7) ambulation. The EDSS was classified into patients with mild (0–3), moderate (3.5–6.5) and severe disability (7–10). In the 9HPT 4 patients with test performance time $\geq$ 55 seconds were observed. All had SPMS with over 10 years disease duration and were severely disabled.

EDSS, AI and 9HPT were correlated with disease duration, age at onset, age and time from last relapse. A correlation with disease duration was found for the EDSS ($R=0.61$, $p<0.001$) and the 9HPT ($R=0.51$, $p<0.001$). A negative correlation with age at onset was found for the AI ($R=-0.37$, $p<0.05$) and the 9HPT ($R=-0.37$, $p<0.01$). Age and time from last relapse correlated only with the EDSS ($R=0.32$, $p<0.05$ and $R=0.43$, $p<0.01$, respectively).

Clinical subtypes differed significantly in their EDSS ($F_{2,48}=19.92$, $p<0.001$) and 9HPT ($F_{2,46}=6.26$, $p<0.01$, table 1). RR MS patients had a significantly lower EDSS than PP ($p<0.001$) and SP MS patients ($p<0.001$). RR had a significantly quicker test performance time than SP MS patients in the 9HPT ($p<0.01$). No significant difference was found for the AI ($F_{2,41}=2.2$, N.S.).

Ambulation index There was a significant difference of CSF GFAP levels between MS patients classified according to the AI and control patients ($F_{3,64}=5.49$, $p<0.001$, table 3). In the post-hoc analysis MS patients with bad ambulation had significantly higher CSF GFAP levels than control patients ($p<0.001$) or MS patients with good ambulation ($p<0.05$). The subgroup analysis revealed that this significance is due to the 9 SP MS patients with bad ambulation, which had nearly 3-fold elevated GFAP levels if compared to control patients (table 3). Significantly elevated GFAP levels were also present in badly ambulated RR MS patients if compared to control patients ($p<0.01$), but not in badly ambulated PP MS patients. Only for the SP MS

subtype poorly ambulating patients had significantly higher GFAP levels compared to patients with good ambulation ($p<0.05$).

For the SPMS patients disability measured by the AI also correlated with the levels of GFAP ($R=0.57$, $p<0.01$, figure 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. According to this cut-off 0% (0/4) of patients with good, 40% (2/3) of patients with moderate and 78% (7/2) of patients with poor ambulation had CSF GFAP levels above cut-off. The trend analysis revealed a significant linear increase within these 3 AI categories (M-H$\chi^2=6.6$, $p<0.01$).

EDSS There was a significant difference of CSF GFAP levels between MS patients classified according to the EDSS and control patients ($F_{2,57}=5.06$, $p<0.01$). Severely disabled MS patients had significant higher GFAP levels than control patients ($p<0.01$). The post-hoc analysis revealed that this was caused by the about 3-fold elevated GFAP levels in severely disabled SPMS patients if compared to control patients ($p<0.01$). The post- hoc analysis also revealed significantly elevated GFAP levels in severely disabled MS patients if compared to moderately disabled patients ($p=0.05$). But there was no simple correlation between the EDSS and GFAP levels and the trend analysis was negative.

For the SPMS patients ferritin correlated with the EDSS ($R=0.45$, $p<0.05$). Because of the previously revealed significant correlation between disease duration with ferritin levels and also with the EDSS a partial correlation correcting for disease duration was performed, which abolished the correlation between EDSS and ferritin.

9HPT CSF Ferritin was about 2-fold higher in patients with a test performance < 55 seconds (12.3 ± 5.1 ng/mL) than in "quick" (6.2 ± 4.8 ng/mL) patients ($p<0.05$, Wilcox rank sum test). Because there are only 4 "slow" patients the results were checked by the Fisher's exact test and no significance could be demonstrated.

CSF S100 correlated negatively in PPMS patients with the 9HPT ($R=-0.85$, $p<0.01$, figure 2).

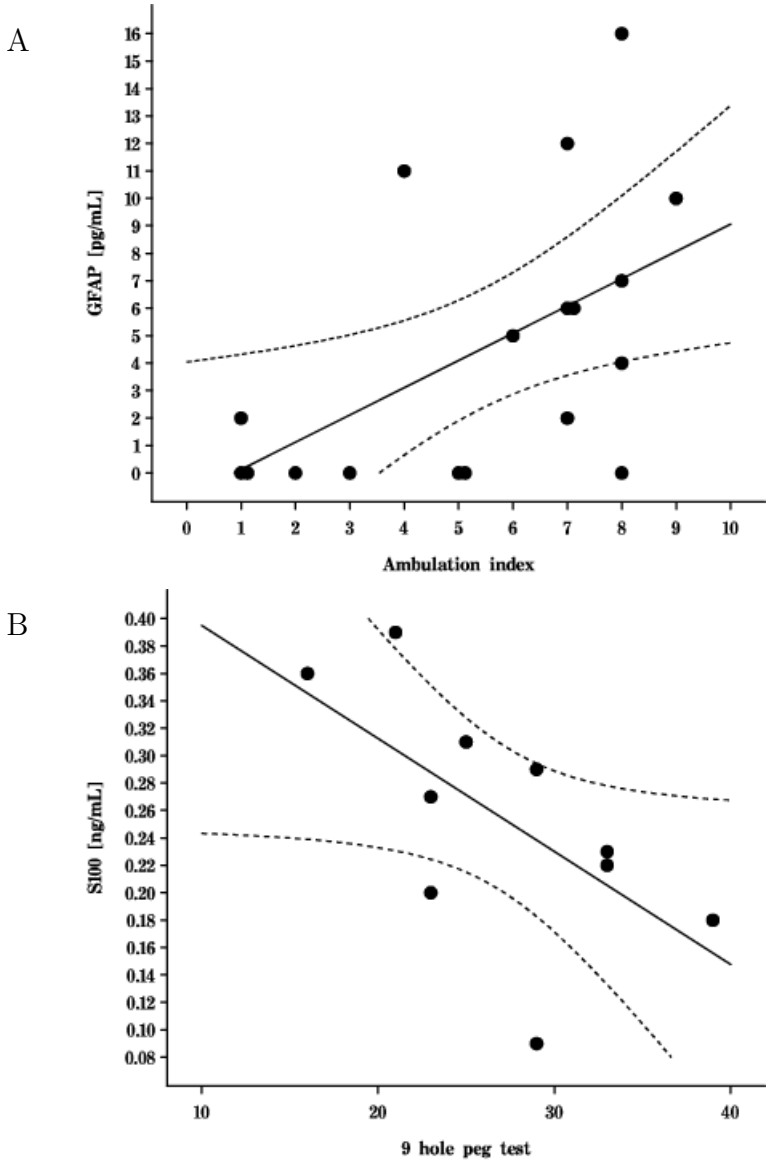

Figure 2: Disability: (A) CSF GFAP levels correlated significantly with the AI in SP MS patients (R=0.57, p<0.01). MS patients are jittered if observations occurred in duplicates. The linear regression line, the 5% lower and 95% upper confidence curves are shown. (B) CSF S100b levels correlated significantly with the 9HPT of the dominant hand in PP MS patients (R=-0.85, p<0.01).

3.2 Brain tissue study

Grey matter GFAP and S100b were 2- to 3-fold elevated in MS grey matter (GM) compared to control GM. Significantly more MS GM samples had S100b and GFAP levels above the cut-off if compared to control GM (figure 3, $p<0.001$, $p<0.05$, respectively).

Ferritin levels were higher in MS GM than in control GM and ferritin levels GM and WM were similar within each group.

White matter S100b levels were approximately 2-fold higher in acute plaques than in SAL. Significantly more AL than SAL lesions had S100b levels above the cut-off (figure 4, $p<0.001$).

Ferritin levels were higher in all MS lesions types compared to controls (table 2). Ferritin was significantly higher in NAWM from MS patients when compared to control white matter in the post-hoc analysis ($p<0.05$). This result was confirmed comparing numbers of lesions above and below cut-off (figure 4, $p<0.05$).

4 Discussion

4.1 Clinical subtypes

Our findings show that biomarkers for astrocytic (CSF S100b) and microglial (CSF ferritin) activation can distinguish between relapsing remitting (RR) and progressive (SP and PP) disease in MS patients. There is a significant trend for increasing S100b levels from PP to SP to RR MS patients and RR MS patients have significantly higher S100b levels than control patients. SP MS patients have significantly higher ferritin levels than control patients (table 2). Consequently a $\frac{S100\beta}{ferritin}$ ratio distinguishes significantly RR from SP MS patients. This suggests that astrocytic activation predominates in relapsing and microglial activation in progressive MS patients (figure 1).

MS patients had significantly elevated CSF S100b levels compared to control patients, which was principally due to the high S100b levels in RRMS patients. This result confirms most previous studies[24–27] although Sindic et al. were not able to measure CSF S100b levels in MS patients.[28] The median relapse free interval in our study was 15 months in the RR and 77 months in the SP MS patients. Therefore the CSF S100b levels represent underlying disease activity independent from relapse. This notion is supported by the

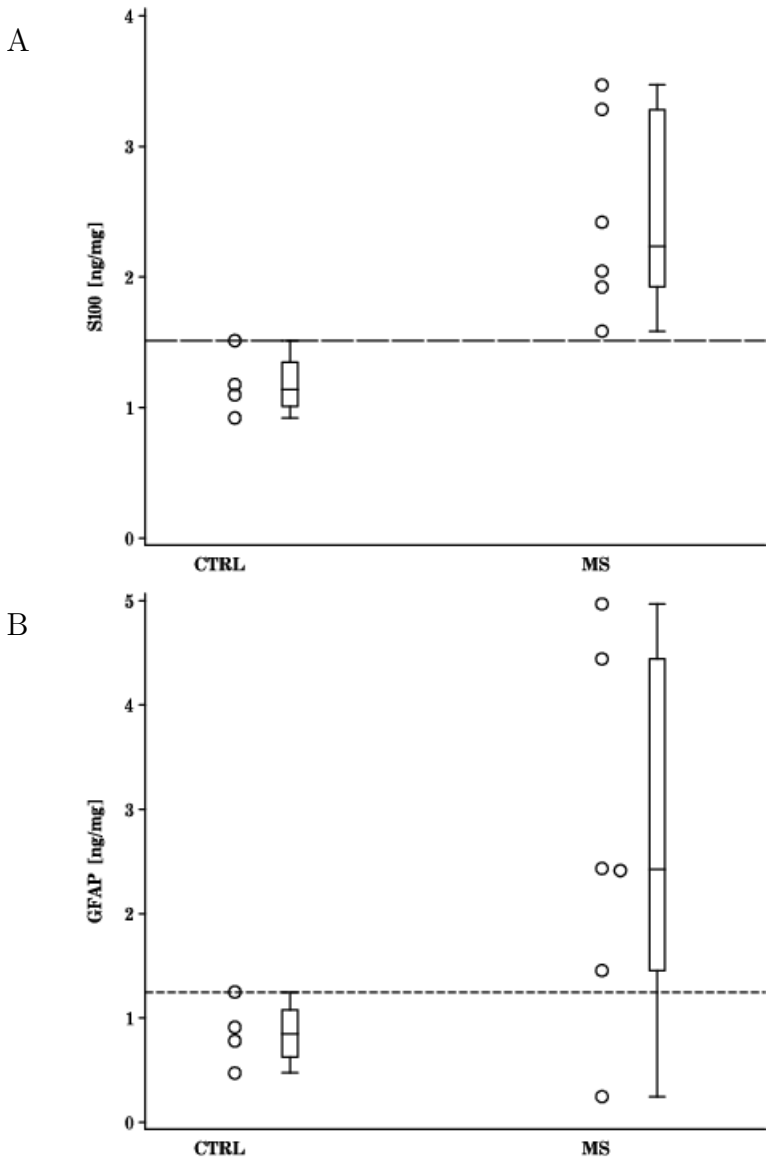

Figure 3: (A) *Grey matter S100b: comparing levels of S100b in cortical MS versus controls a 2-fold increase is observed. Significantly more cortical samples from MS than controls had S100b levels above cut-off (dotted line) suggestive of widespread cortical astrocytosis in MS brain tissue ($p<0.001$). (B) Grey matter GFAP: comparing levels of GFAP in cortical MS versus controls a 3-fold increase is observed. Significantly more cortical samples from MS than controls had GFAP levels above cut-off (dotted line) suggestive of widespread cortical astrogliosis in MS brain tissue ($p<0.05$).*

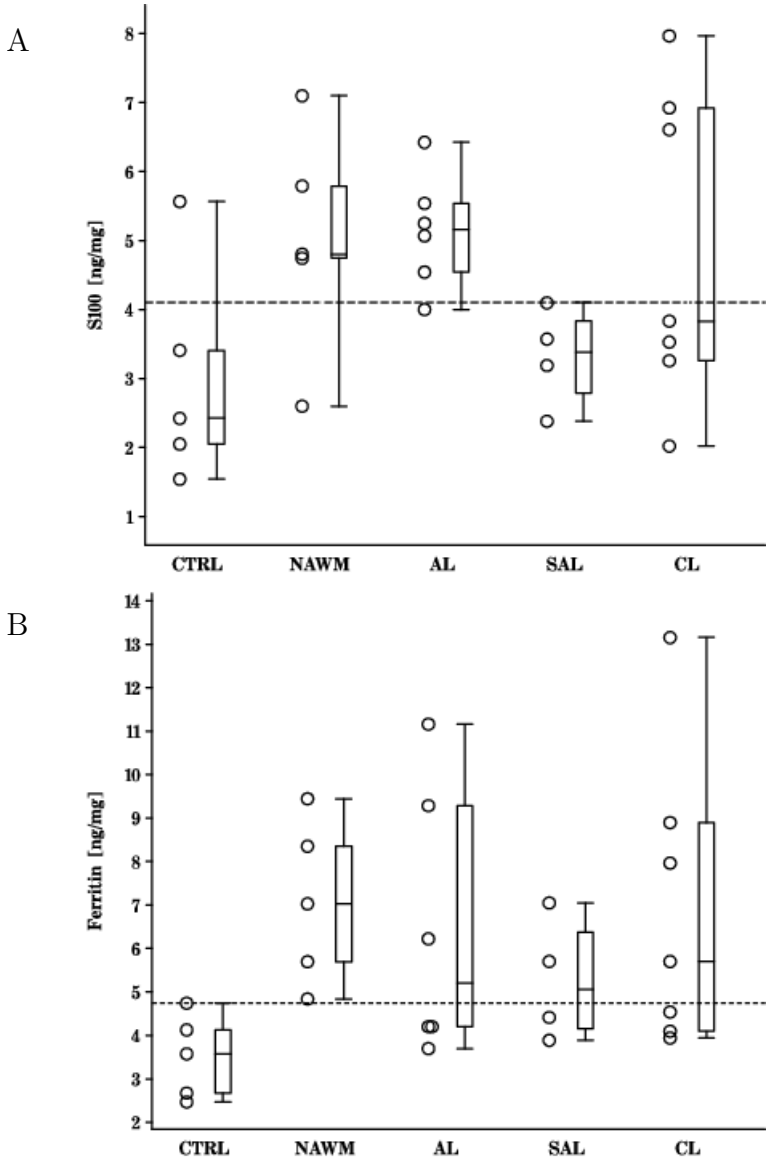

Figure 4: (A) *White matter S100b levels: the brain tissue was from CTRL=control, NAWM=normal appearing white matter, AL=acute lesion, SAL=subacute lesion and CL=chronic lesions. The dotted line represents the cut-off for control S100b values. Significantly more samples from acute plaques (AL) have S100b levels above cut-off (dotted line) than subacute plaques (SAL) (p<0.001). (B) Ferritin in white matter brain tissue homogenates from MS patients: the dotted line represents the cut-off for control ferritin values. Significantly more samples from NAWM had "high" ferritin levels when compared to control white matter (p<0.001).*

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brain-tissue study which shows similar high S100b levels in NAWM as in AL (figure 4). The group of Nave described how glial pathology can precede axonal degeneration.[2] MRI techniques[29,30] and immunohistochemistry[31] provide compelling evidence for underlying disease activity in NAWM. It needs to be clarified if NAWM changes precede disease exacerbation or represent a spurious finding. De Groot et al. suggest in a post-mortem study combining MRI and histology that such changes could indicate "(p)reactive" lesions.? The results of this study might be exploited to contribute to future classification of MS patients along the lines of evidence-based diagnostic-criteria.[32] This will be particularly important for integrating modern, quantitative CSF analytical methods.

It is noteworthy that elevated S100b levels are a biomarker for astrocytic activation which is also present in Alzheimer's disease,[33] Creutzfeldt-Jakob disease,? epilepsy,[34] stroke,[35,36] acute brain injury.[37,38] The sensitivity of these biomarker changes is stunning because elevated S100b levels can be measured after prolonged running.[39]

In our study CSF ferritin is significantly higher in SPMS patients than in the control group. To our knowledge only one study measured ferritin levels in MS patients and found them to be elevated in progressive patients.[14] This result is supported by the results of our brain tissue study. Ferritin concentrations were higher in all lesion types of progressive MS brains if compared to control WM. This was significant for NAWM versus control WM (figure 4 b). NAWM contributes the bulk of brain tissue equilibrating with the CSF. Interestingly Hulet et al. found decreased ferritin binding to white matter within a MS lesion.[40] Because the oligodendrocyte requires iron for the synthesis of myelin[41] upregulated ferritin levels in MS brain could reflect a physiological reaction to decreased binding and metabolic needs. If this is the case, high ferritin levels in MS patients could indicate a subgroup of patients which would possibly benefit from treatment strategies aiming to enhance remyelination. The interpretation of CSF ferritin concentrations needs to consider the possibility of an intracranial haemorrhage, which leads always to elevated levels.[42–45]

In contrast to one study using the CSF of 5 healthy volunteers as controls[6] we and others[46,47] did not find an overall significant difference between CSF GFAP levels in MS and a control group consistent of patients with other neurological disorders. Significantly elevated GFAP levels compared to our controls were however found in MS patients with poor ambulation (AI) or severe disability (EDSS). GFAP has found to be elevated in dementia,[8] nor-

mal pressure hydrocephalus,[46] asphyxiated newborn- infants,[48] head injury,[49] brain infarction[36] Lyme-borreliosis,[50] trypanosomiasis[51] and MS.[6] GFAP has to be regarded as a biomarker related to total CNS tissue involvement causing the neurological deficit.

4.2 Disability

Patients with poor ambulation had significantly higher CSF GFAP levels than patients with good ambulation and control patients. Also severely disabled patients had significantly higher CSF GFAP levels compared to mildly disabled patients. This is suggestive of increased astrogliosis within the spinal cord of badly ambulated / disabled patients. Compared to the control group only these patients had significantly higher GFAP levels. The subgroup analysis revealed that this was most marked within patients with SPMS. This study revealed a significant correlation between GFAP and individual scoring on the AI for SPMS patients (R=0.57). We interpret this as a direct relationship between GFAP and astrogliosis, which is clinically expressed in disability.

The reason why a direct correlation between GFAP and individual points on the AI but not with the EDSS was found can be explained by the physiological basis of these clinical scales. The AI measures essentially gait. The EDSS in contrast includes tests of cognitive and visual functions which are outside (rostral) the anatomical parts of the brain which equilibrate with the CSF that descends into the lumbar sac. This "CSF analytical brain" consists of the inner half of the telencephalon, the basal cortex, the cerebellum, the brainstem and the spinal cord.[52] Each lost axon innervates the lower limb could potentially be replaced by a gliotic scar of about one metre length.[?] Therefore a considerable amount of gliotic tissue would form the source of GFAP release and would hence 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 of lumbar CSF GFAP levels.

This was also demonstrated by the study of Rosengren et al. who studied serial CSF samples in 10 RRMS patients.[6] The scale applied for assessing disability, the regional functional score system (RFSS) includes visual and mental functions. Contradicting changes in the RFSS and lumbar CSF levels can be observed in 8 out of 10 patients. Importantly this study on serial CSF samples (seven lumbar punctures per patient over a 2 year period) did not reveal any relationship between CSF GFAP levels and the time from relapse.

We find it difficult to explain the strong negative correlation between S100b and the 9HPT in PPMS patients. S100b might have a neuroprotective effect through its ability to chelate calcium, which can cascade much secondary damage via multiple pathways. Certainly another group of patients is needed to assess whether the above correlation is a consistent finding and it may thus represent a failure to initiate glial processes needed for neuro-axonal protection/regeneration.

4.3 Brain tissue study

The levels of all BSP appear to be increased in MS GM (figure 3). This was significant for S100b and GFAP. This finding is particularly relevant for studies focusing on the cognitive and neuro-psychiatric aspects of MS. Interpretation of the results on BSP in GM needs to be done with caution. The cortex does not form part of the "CSF analytical brain",[52] since cortically derived BSP will flow into the CSF and will be absorbed by the rostral arachnoid villi. It is unlikely that they will be detectable in the lumbar CSF. It might however be possible to measure changes of cortical BSP in other body fluids.[3]

The significantly higher levels of S100b in AL versus SAL suggest that S100b expression is predominatly up-regulated in the acute phase of the disease and returns to normal at least in about half of the patients (figure 4 a). In contrast ferritin is consistently higher in MS than in control brain tissue and was significantly higher in MS NAWM compared to control WM (figure 4 b). This "acute phase" elevation of S100b is in keeping not only with our own results of S100b in RR versus SP and PP MS patients, but also the findings of others.[25,26]

The results of the S100b and ferritin analysis are suggestive of early astrocytic activation which may return to normal in spite of continuing microglial activation in MS white matter (figure 4) as well as the striking elevation in the cortex of S100b and GFAP (figure 3).

Acknowledgments This study was devised as part of a study into biomarkers for neurodegeneration supported by the Multiple Sclerosis Society of Great Britain and Northern Ireland (AP, GG), the BR Kirk Fund of the Institute of Neurology (AP, ET), the Wellcome Trust (DG, LC) and the Multiple Sclerosis Society of the Netherlands (JE, BU).

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			Clinical classification		
	CTRL	**MS**	**RR**	**SP**	**PP**
Age (yrs)	41(27–63,51)	46(27–65)	40(27–55)	46(28–65)	51(43–55)
Gender (f, m)	37, 14	28, 23	11, 9	10, 11	7, 3
AI	N/A	4.5 (0–10,44)	1.5 (0–10,18)	6.5 (1–9,18)	4 (1–9,8)
EDSS	N/A	3.5 (0–8,51)	1.75 (0–6.5,20) **|– p<0.001 ————-|** **|– p<0.001 —|**	6 (1–8,21)	6 (1.5–8,10)
9HPT	N/A	25 (17–84,49)	20 (18–29,18) **|– p<0.01 —|**	29 (17–84,21)	26 (17–36,10)

Table 1: Demographic and clinical data: median (range, number). MS patients are classified into clinical subtypes: PP = primary progressive, SP = secondary progressive, RR = relapsing remitting disease. AI = ambulation index, EDSS = Kurtzke's extended disability status score, 9HPT = 9-hole PEG test (see text).

			Clinical classification		
	CTRL	**MS**	**RR**	**SP**	**PP**
S100b					
[ng/mL]	0.25(0–0.4,51)	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)
	|— **p<0.05** ———|				
	|— **p<0.01** ——————————|				
Ferritin					
[ng/mL]	5(3–7,51)	5(1–20,51)	4.5(1–20,20)	6(1–19,21)	5.5(3–13,10)
	|— **p<0.01** ——————————————————|				
GFAP					
[pg/mL]	1(0–13,51)	3(0–16,51)	3(0–11,20)	2(0–16,21)	0.5(0–11,10)

Table 2: CSF levels of S100β, ferritin and GFAP in RR, SP and PP MS patients: median (range, number). S100β distiguishes significantly between MS and control patients ($F_{3,98}$=3.09, p<0.05) with RR MS patients being the main contributor (post-hoc analysis). SP MS patients have significantly higher ferritin levels than control patients (post-hoc analysis only, $F_{3,98}$=2.27, N.S.).

GFAP [pg/mL]		**Ambulation index**		
	CTRL	**< 2**	**2–6**	**> 6**
MS	1(0–13,51)	2(0–11,19)	0(0–11,8)	6(0–16,17)
		|——————— **p<0.001** —|		
			|————- **p<0.05** —|	
SP		0(0–2,4)	0(0–11,5)	6(0–16,9)
		|——————— **p<0.001** —|		
			|————- **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** —|		

	EDSS		
	< 3.5	**3.5–6.5**	**> 6.5**
MS	3(0–11,27)	0(0–11,15)	6(0–16,9)
	|——————— **p<0.01** —|		
		|—— **p=0.05** —|	
SP	2(0–10,5)	0(0–11,9)	6(0–16,7)
	|——————— **p<0.01** —|		
PP	0(0–11,4)	4.5(0–10,4)	5.5(1–10,2)
RR	3.5(0–11,18)	0(0,2)	N/A

Table 3: *CSF GFAP levels in control, MS patients and clinical subtypes: median (range, number). GFAP distinguishes significantly 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).*