**IgM to S-nitrosylated protein is found intrathecally in relapsing-remitting multiple sclerosis**

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**Abstract**

This study has established the presence of IgM against S-nitrosylated proteins in cerebrospinal fluid (CSF) of multiple sclerosis (MS) patients using S-nitrosocysteine epitope (anti-SNOcys) as previously shown in serum. Anti-SNOcys IgM increased significantly in CSF during relapsing-remitting MS compared to milder neurological conditions. Evidence from albumin, IgG and IgM suggest that the production of anti-SNOcys IgM is intrathecal rather than the result of ingress from serum. Two correlations during relapse: between CSF level of anti-SNOcys IgM and time elapsed since relapse onset; and between CSF and serum anti-SNOcys IgM levels, suggest that this antibody may have potential as a biomarker.

**1. Introduction**

Intrathecal synthesis of immunoglobulin was reported some thirty years ago in the cerebrospinal fluid (CSF) of multiple sclerosis (MS) patients by means of oligoclonal band detection in two-dimensional electrophoresis (Walsh et al., 1985). Oligoclonal bands of IgG isotype are found in around 90% of MS patients (Compston and Coles, 2008), although its presence varies in different parts of the world, to peak in 95% of Western patients (Fredrikson, 2010). Oligoclonal IgG detection and calculation of the IgG index is routinely performed to confirm a diagnosis of clinically definite MS (Polman et al., 2011). By contrast, the predictive value of IgM oligoclonal bands to confirm a definite diagnosis in clinically isolated syndrome, though not a recent concept (Sharief and Thompson, 1991a), is still
unclear (Schneider et al., 2007). However, recent studies with a large number of patients have confirmed the initial observation (Bosca et al., 2010; Garcia-Barragan et al., 2009). Although IgM oligoclonal bands are found in only 35% to 75% relapsing-remitting patients (RRMS), a body of literature associates them with a more severe disease course (Mandrioli et al., 2008; Sharief and Thompson, 1991b; Thangarajh et al., 2008; Villar et al., 2003).

It is clearly established that nitric oxide (NO) is abundantly produced during MS attacks and generates footprints: high levels of nitrite and S-nitrosothiol in the CSF, proteins with nitrotyrosine residues in lesions, and diffuse S-nitrosothiols in normal-appearing white matter (Bizzozero et al., 2005; Brundin et al., 1999; Calabrese et al., 2002; Hooper et al., 1997). S-nitrosocysteine (SNOcys) is a ubiquitous post-translational modification of proteins (Hess et al., 2005). We previously reported levels of IgM directed against SNOcys, at the exclusion of IgA or IgG isotypes, in the serum of MS patients (Boullerne et al., 1995), that were correlated to more intense clinical activity (Boullerne et al., 2002). The objective of the present pilot study was therefore to determine whether IgM against SNOcys could be detected in the CSF of MS patients, and examine whether it has potential value as a biomarker for diagnosis or clinical activity. The search for a biomarker in MS has intensified over the past decade but faces many challenges and remains restricted to the crude IgG oligoclonal bands (Graber and Dhib-Jalbut, 2011).

2. Materials and Methods

Patients and samples

CSF and serum were obtained concomitantly from patients as part of the diagnostic procedure at the Department of Neurology, University Hospital of Trondheim (St. Olav's Hospital), Norway. Twenty two patients with a diagnosis of MS according to the McDonald criteria (Polman et al., 2011) were included: 12 during remission and 10 patients during relapse. A relapse was defined as a worsening of function in the absence of fever or infection, lasting at least 24 hours and followed by improvement. Patients were not treated by corticosteroid at the time of relapse. No patient was suffering from influenza or cold at the time of sampling, and none was under immunosuppressive treatment or MS-specific drug. All patients showed brain lesions by MRI (T2 imaging with contrast); according to the criteria at least 9 lesions in the brain, or 5-6 brain lesions plus 2 lesions in the spinal cord. All MS patient CSF demonstrated at least five extra oligoclonal bands of IgG, and typically more than ten. MS patients showed moderate signs of central nervous system inflammation with occasional elevated protein above the normal range up to 0.5 g/L (Regeniter et al., 2009), and sometimes a cell count above 5 cells/μL (Freedman et al., 2005).

The subjects in the control group (n=17) displayed general symptoms (numbness, headache, dizziness, glaucoma, or asthenia), and four were subsequently diagnosed with facial paresis (n=2) or vestibular neuritis (n=2). They hence qualified as milder neurological controls. All had a CSF cell count, protein, glucose (2.2-4.4 mM), albumin (0.1-0.4 g/L), and serum albumin (30-56 g/L) within the physiological range as referenced by diagnostic
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laboratory standards (Fischbach and Dunning, 2009), and showed neither lesions by MRI, nor oligoclonal bands. None has subsequently developed MS.

There was a skew in gender between the control and MS groups: males and females were balanced in the MS groups, but there was a strong female preponderance in the control group. This situation arose purely by chance during identification of MS patients and suitable controls among available patient samples (approximately 300 pairs of CSF and serum). However, we did not expect the gender imbalance to affect immunoglobulin levels, which are not significantly altered in adults by age or gender. The physiological range is 7-15 g/L for serum IgG; up to 50 mg/L for CSF IgG; and 0.6-3 g/L for serum IgM (Fischbach and Dunning, 2009).

All patients gave signed, informed consent. The study was approved by the Regional Committee for Medical Research Ethics for central Norway in accordance with Norwegian law, and was in compliance with the ethical regulations of the University of Illinois at Chicago. The demographic data are shown in Table 1.

CSF was obtained by lumbar puncture performed at the level L3/L4 or L4/L5 with the patient lying on their side in a horizontal position, placed directly in ice-water, and frozen within 30 minutes. Patients with an accidental bleeding during puncture were excluded. Serum was obtained concomitantly from venous blood and centrifuged at room temperature for 10 min., 1500 g within one hour of sampling. CSF cells, glucose and protein were measured by the routine procedures of the University Hospital of Trondheim, as were also albumin and IgG in serum. The albumin quotient was calculated according to the ratio of the concentration in CSF relative to serum. An albumin quotient above 4 to 7 is considered to be an indication of a damaged blood brain barrier (BBB) depending on the age (Tibbling et al., 1977; Blennow et al., 1993). The IgG index was calculated from the concentrations of IgG in CSF and serum according to the ratio [(CSF IgG / serum IgG) / (CSF albumin / serum albumin)], and considered normal up to 0.7 (Fredrikson, 2010). CSF and serum samples for further analysis were stored at -80°C.

Detection of anti-S-nitrosylated protein IgM

To circumvent a lengthy search for S-nitrosylated targets, we synthesized a generic S-nitrosocysteine epitope (SNOcys) that we linked with glutaraldehyde to the bovine serum albumin (BSA) carrier, as described previously (Boullerne et al., 1995). We used a SNOcys epitope with the optimum ratio of 6 cysteines per BSA molecule throughout the assays including competitive ELISA. Because the BSA carrier alone treated with glutaraldehyde (BSA-g) is immunogenic, it served as decoy in the antibody detection assay. We hence detected a specific response against S-nitrosylation. All chemicals were from Sigma-Aldrich (Saint Louis, MO) unless specified otherwise. Anti-SNOcys IgM was measured in CSF and serum samples by indirect ELISA protected from light in 96-well plates (Nunc, Roskilde, Denmark). The SNOcys antigen and BSA-g decoy were plated overnight at 4 °C in carbonate buffer, pH 9.6. Plates were incubated for 1 h at 37 °C in PBS-Tween with 0.1% BSA and 10%
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glycerol (blocking buffer). Plates were rinsed 5 times before incubating the pairs of serum and CSF in blocking buffer containing 0.1% BSA-g. Serum diluted 1:2,000 and CSF diluted 1:2 were incubated in triplicate overnight at 4°C. After rinsing, rabbit antiserum to human IgM-HRP (Dako, Carpinteria, CA) was incubated for 1 h at 37 °C. After rinsing, a tetramethylbenzidine (TMB) solution was incubated for 30 min. at 37 °C before adding HCl to stabilize the optical density (OD). OD was read at 450 nm against a 620 nm reference and the background obtained with BSA-g subtracted. Repeated testing showed a 2-5 % inter-well variation for serum and CSF and a day-to-day variation of 19 % for serum and 11 % for CSF.

Competitive ELISA was carried out to assess the binding specificity of IgM to the S-nitrosocysteine epitope. Soluble SNOcys antigen at concentrations of 10⁻⁶ to 10⁻¹¹ M was incubated overnight at 4°C with CSF samples calibrated to generate a 0.5 to 2.5 OD in sample buffer. After centrifugation at 10,000 g for 30 min., supernatants were tested in triplicate in plates coated with SNOcys following the same indirect ELISA. The resulting OD revealed the competition between liquid phase SNOcys that displaced IgM binding to immobilized SNOcys. Avidity curves were plotted by normalizing the average OD of triplicates at a given SNOcys concentration with OD at the lowest SNOcys concentration virtually identical to absence of SNOcys. CSF samples were tested repeatedly and at different dilutions along with pairs of CSF-serum.

Total IgM content quantification

Quantification of total IgM content in serum was carried out by immunoturbidity, outsourced to a company servicing routine laboratory hospital tests (Quest Diagnostics, Schaumburg, IL). Quantification of total IgM content in CSF was performed by capture ELISA. Briefly, 96-well plates (Nunc) were coated overnight at 4 °C in basic carbonate buffer with a capture antibody to human IgM (BD Pharmingen, San Jose, CA). Plates were blocked for 3 h at room temperature by direct addition of blocking buffer. After 5 rinses, each plate was incubated overnight at 4 °C with CSF samples diluted in blocking buffer from 1:5 to 1:500 (triplicate), and a standard curve prepared with commercial human IgM (MP Biomedicals, Irvine, CA). After rinsing, plates were incubated with antiserum to human IgM-HRP (Dako) for 1 h at 37 °C. After rinsing, a TMB solution was incubated for 30 min. at 37 °C, stopped by HCl, and the absorbance read at 450 nm against a 620 nm reference. OD values were converted into IgM concentration using the standard curve. In this assay, OD was linear against concentration up to an OD value of 3. All CSF IgM concentrations were measured 2 - 4 times. Because the physiological range of IgM concentration in CSF varies greatly in the literature, we adopted a conservative approach to define its normalcy. The upper level of 0.4 mg/L IgM was based on a large study of over 100 healthy subjects whose age (18-88 year-old) spans the entire adulthood (Blennow et al., 1996). We hence considered the normal range of CSF IgM to be 0.1-0.4 mg/L, as in Jongen et al. (2008). The IgM index was calculated similarly as the IgG index, and considered normal up to 0.07 (Sharief and Thompson, 1991b).
Statistical analysis

Results were analyzed using non-parametric statistics (IBM SPSS versions 19 and 20). Graphs were constructed using GraphPad Prism version 5. The Kruskal-Wallis test was first applied for detecting group differences (controls, relapse and remission) with \( P < 0.05 \) considered significant. Where significant, a Mann-Whitney post-hoc analysis between two groups with \( P \) set at \(< 0.017\) was performed to take account of multiple comparisons across the 3 groups. Correlations were carried out with Spearman's correlation coefficient \((r_s)\), \( P < 0.05\). \( P \) was chosen as two-sided in all tests.

3. Results

The levels of anti-SNOcys antibodies in CSF and serum of RRMS patients were compared to milder neurological controls, along with other basic immunological parameters, to evaluate the relationship between this new IgM antibody, the BBB permeability and intrathecal synthesis. The summary of statistical differences is presented in Table 2. Despite the small number of samples, we found highly significant differences between the control group, and RRMS patients during both relapse and remission.

Anti-SNOcys IgM is present in the CSF of MS patients

Anti-SNOcys IgM was identified in the CSF of MS patients. We have previously reported this IgM antibody in serum (Boullerne et al., 1995; Boullerne et al., 2002) and found it in serum also in the present study. Strikingly, in the control group affected by other milder neurological diseases, CSF anti-SNOcys IgM was detected only at background level (Fig. 1a). The specificity of anti-SNOcys IgM detection in CSF was established by running serial dilutions from 1:4 to 1:256 (Fig. 1b), and by competitive binding (Fig. 2). Because of the extremely low OD values obtained in the control samples, the cut-off for least detection of anti-SNOcys IgM in CSF was set as the mean plus 5 standard deviations of the control group, which is more conservative than the usual mean plus 3 standard deviations. Based on this conservative cut-off (OD=0.102), we found 13 of 22 (59%) MS patients (5 of 10 in relapse and 8 of 12 in remission) positive for this IgM antibody, but none of the neurological controls. Interestingly, two additional control patients excluded from the statistical analysis because of their impaired BBB were also negative for this IgM antibody (Supplementary Table 1).

Elevated IgM levels in the CSF of MS patients

As reported in the literature, we found elevated IgM levels in the CSF of MS patients. Only one (at 0.47 mg/L) of 17 milder neurological controls (6 %) was above the upper cut-off level of 0.4 mg/L based on healthy subjects (see Materials and Methods). In sharp contrast, 16 of 22 MS patients (73 %) were above cut-off level. As expected, this is a higher percentage.
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than for the IgM antibody marker. The CSF IgM levels in MS patients were up to 23-fold of that of controls, as shown by the scatter plots for each group in Fig. 3a. The neurological controls showed no significant correlations between total IgM level and anti-SNOcys IgM or other parameters in their CSF, which strikingly differed from the MS patients, as described below. Expectedly from their high CSF IgM levels, most RRMS patients (15 of 22, 68%), had an IgM index elevated above cut-off 0.07, while only two of 17 controls (12%) had an IgM index above normal.

Normalization of CSF anti-SNOcys IgM

Centrally, anti-SNOcys IgM correlated strongly with total IgM content in the RRMS group (r_s=0.81, P<0.0005, n=22). To address the possibility of a non-specific increase of anti-SNOcys antibodies when an IgM surge occurred in CSF, we normalized the OD antibody signal with the IgM concentration according to the ratio: OD/CSF IgM (Fig. 3b). Before normalizing, the anti-SNOcys IgM and total IgM content were both highly significantly elevated in the CSF of RRMS patients when compared to controls (P<0.0005). The statistical difference between the RRMS group (n=22) and the control group (n=17) was retained after normalizing the anti-SNOcys IgM OD (P=0.027), supporting their specific increase in the CSF.

CSF anti-SNOcys IgM correlated with serum level

In the case of serum, the Kruskal-Wallis test indicated no significant differences for any measured parameters: serum anti-SNOcys IgM, serum IgM, serum IgG, or serum albumin in any of the 3 controls and clinical MS groups. Correlations were found between serum anti-SNOcys IgM and serum IgM in all groups (Control: r_s=0.54, P=0.024, n=17; Relapse r_s=0.77, P=0.009, n=10; Remission r_s=0.60, P=0.039, n=12), as previously reported (Boullerne et al., 1995). Remarkably, serum anti-SNOcys IgM correlated positively with CSF anti-SNOcys IgM in relapsing patients (r_s=0.73, P=0.016, n=10), but disappeared during remission and was not seen in the milder neurological control group. CSF anti-SNOcys IgM did not correlate with any other serum parameters in relapsing patients, except predictably with serum IgM (r_s=0.71, P=0.022, n=10), owing to the tight correlation between serum IgM and serum anti-SNOcys IgM during relapse, as described above. Patients in remission and controls did not show any correlation whatsoever between CSF anti-SNOcys IgM and any serum parameters.

Intrathecal synthesis of CSF anti-SNOcys IgM

In the case of CSF, significant differences were found only between the control group and each of the two RRMS patient subgroups. There was a significantly elevated protein level and albumin quotient during relapse, in agreement with the known temporary BBB permeability. However, in both relapse and remission the IgG and IgM indices were
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significantly higher, and this is a sign of intrathecal synthesis for both immunoglobulins. Interestingly, CSF IgM correlated with CSF IgG but only during relapse ($r_s=0.67$, $P=0.033$, $n=10$), the correlation being lost during remission, and absent in the controls. This is in agreement with a temporary increase of IgM intrathecal synthesis during relapse. Additionally, we found a positive correlation in RRMS between CSF anti-SNOcys IgM and the IgM index signaling IgM intrathecal synthesis ($r_s=0.63$, $P=0.002$, $n=22$). This correlation was absent with IgG index (IgG intrathecal synthesis) or albumin quotient (BBB permeability), supporting further an intrathecal synthesis of anti-SNOcys IgM.

Temporal fluctuation of CSF anti-SNOcys IgM during relapse

Interestingly, we found an inverse correlation for the time from relapse onset to lumbar puncture relative to CSF anti-SNOcys IgM ($r_s=-0.66$, $P=0.04$, $n=10$), a correlation not observed for the other CSF parameters nor the IgM and IgG indices. This correlation between time elapsed from relapse onset and anti-SNOcys IgM was lost in serum, supporting a temporal relationship between central inflammation and CSF anti-SNOcys antibodies. However, the CSF anti-SNOcys antibodies did not correlate with EDSS, despite EDSS correlating strongly with disease duration ($r_s=0.70$, $P<0.0005$, $n=22$), attesting to the quality of our RRMS group.

4. Discussion

Improved ELISA revealed a clear difference between MS and controls

The indirect ELISA analysis used in the present study is an improved assay compared to that published in 2002 (Boullerne et al., 2002), where the presence of anti-SNOcys IgM in CSF remained in doubt. The new assay employed a more potent chromogen (TMB instead of $o$-phenylenediamine), which generates a 10-fold increase in signal, thus clearly revealing anti-SNOcys IgM in CSF. The present patient material is only small, but the clarity and consistency of many of the results are quite striking. The milder neurological controls used in the present study cannot be considered "healthy subjects" as they were referred to the Department of Neurology for diffuse neurological symptoms, and were subsequently selected for the present study based on absence of a serious neurological condition. The significant differences in anti-SNOcys IgM levels were restricted to the CSF. Regarding the level of the antibody in CSF from control patients, it was around, or barely above, the background of the assay.

High percentage of positivity for antibody of IgM isotype in MS CSF

We found 59% RRMS patients positive for our antibody in the CSF using a very conservative cut-off. Had we used the conventional level (control mean plus 3 standard
deviations), the percentage of RRMS positives would have been even higher. A survey of the literature extending back to the past twenty years revealed substantially lower percentages of specific IgM in the CSF of RRMS patients. It spans from a low 8% for anti-gangliosides (Marchiori et al., 1990), to 10-24% for anti-sulfatide (Ilyas et al., 2003; Haghghii et al., 2012), to 28-31% for anti-myelin lipids (Villar et al., 2005; Thangaraj et al 2008), to 33% for anti-myelin proteins (Mata et al., 1999), and peaks at 35% for anti-MBP (Annunziata et al., 1997). Our data are placed at the high-end of the spectrum next to IgG antibodies against common viruses (measles 55%, rubella 63%, varicella zoster 50%) being reported in MS (Kulakowska et al 2012).

A new epitope for oligoclonal bands?

The discovery of oligoclonal bands in MS (Walsh et al., 1985) spurred an intense investigation to identify the molecules triggering the immune response, in search of an etiology that remains elusive. Remarkably, despite numerous studies, very few antigens of intrathecal IgM have been identified in MS and most remain unknown. Myelin lipids such as phosphatidylcholine have been identified as part of IgM oligoclonal bands (Villar et al., 2005). Other IgM responses have been reported in MS CSF to include neuronal surface antigens and neurofilaments (Bartos et al., 2007; Beltran et al., 2012), myelin proteins cyclic nucleotide phosphodiesterase (Walsh and Murray, 1998) and myelin basic protein (Marchiori et al., 1990; Annunziata et al., 1997), proteasome (degrading non-lysosomal proteins) subunits (Mayo et al., 2002), gangliosides and cardiolipin (Marchiori et al., 1990; Stevens et al., 1992; Matà et al., 1999), and sulfatide (Matà et al., 1999; Ilyas et al., 2003; Haghghii et al., 2012). Molecules that are phylogenetically conserved, such as carbohydrates and lipids, trigger mainly innate IgM antibodies (Boes, 2000). The present study shows that S-nitrosylated proteins can be added to the list of molecules triggering an innate IgM response.

Protein S-nitrosylation is generated in MS brain by NOS2, the proinflammatory inducible nitric oxide synthase occurring downstream of toll-like receptor activation. Protein S-nitrosylation constitutes a large part of the ubiquitous influence of nitric oxide on cellular signal transduction. Accumulating evidence indicates important roles for S-nitrosylation both in normal physiology and in a broad spectrum of human diseases, with specific protein targets rather than a general cellular insult (Foster et al., 2009). The emerging picture for MS shows that pathophysiology correlates with hyper-S-nitrosylation, although the specific protein targets of S-nitrosylation remain to be identified. An experimental model of MS in rat allowed the identification of those spinal cord proteins with abnormally high S-nitrosylation: neurofilaments, NMDA receptors, alpha/beta-tubulin, beta-actin, GADPH, and neuronal-specific enolase (Bizzozero and Zheng, 2009).
Potential use of anti-SNOcys IgM as biomarker

We report for the first time a correlation between serum and CSF levels for a specific IgM antibody: anti-SNOcys IgM during relapse. This correlation disappeared during remission. To rule out the possibility that this correlation was due to passive transfer from serum to CSF, the individual data were matched against BBB permeability. Patients with no compromise of the BBB (as measured by the albumin quotient), had a strong likelihood that the CSF anti-SNOcys IgM came from an intrathecal source. Conversely, in four cases with slightly increased albumin quotient (8.6 to 13.6), there were no anti-SNOcys IgM antibodies detected in serum (background OD values), but a strong signal in CSF, indicating that in these patients the CSF anti-SNOcys IgM could not have originated in the circulation. In cases where the barrier was compromised concomitantly with elevated circulating anti-SNOcys IgM, it cannot be ruled out that there could have been some passive transfer into the CSF.

Additionally, we found an inverse correlation between time from relapse onset and CSF anti-SNOcys IgM levels which has never been formerly reported despite being investigated (CSF anti-MBP IgM, Annunziata et al., 1997). We also found a positive correlation between CSF anti-SNOcys IgM and IgM index. Altogether, these combined data suggest that, on the contrary, B-plasmablasts could be reactivated centrally during a relapse, leading to a transient increase of IgM in CSF, and could egress from CSF to blood. This raises the interesting potential for use of anti-SNOcys IgM in serum as a biomarker of clinical activity in RRMS. It might be used to assess how active an RRMS patient is in the course of their disease, considering one of the challenges faced by neurologists is to discriminate between real and subjective relapse and that most MRI activity is subclinical (Filippi and Agosta, 2010).

CSF IgM correlated with CSF IgG only during relapse, the correlation being lost during remission, suggesting that collectively, the data shows IgM immunological reaction during relapse. Although it should be reproduced with a larger cohort, given that most IgM antigens are unknown, it is possible that some of them have a temporal fluctuation. A few MS patients in this study had intrathecal IgM synthesis (higher IgM index) with no elevated anti-SNOcys IgM, which fits such a hypothesis.

Conclusion

Our results clearly indicate that antibodies to S-nitrosylated protein, measured as anti-SNOcys IgM with the help of the epitope SNOcys, are produced intrathecally as well as in serum, and are significantly increased in RRMS. This pilot study was carried out on a limited number of samples, and the use of such antibodies as a biomarker for RRMS activity, as well as its specificity for MS, as opposed to serious inflammatory disease in general, needs to be studied in more comprehensive cohorts.
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References


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Table 1. Demographic data of patients with relapsing-remitting multiple sclerosis (RRMS) and milder neurological control patients.

<table>
<thead>
<tr>
<th></th>
<th>Control (n=17)</th>
<th>RRMS relapse (n=10)</th>
<th>RRMS remission (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender ratio (F:M)</td>
<td>15:2</td>
<td>6:4</td>
<td>6:6</td>
</tr>
<tr>
<td>Age (y)</td>
<td>42 (23 – 56)</td>
<td>42 (26 – 50)</td>
<td>41 (26 – 57)</td>
</tr>
<tr>
<td>CSF free cells (10^6/L)</td>
<td>1 (0 – 3)</td>
<td>3 (0 – 20)</td>
<td>3 (0 – 11) ^a</td>
</tr>
<tr>
<td>CSF glucose (mM)</td>
<td>3.4 (2.8 – 3.7)</td>
<td>3.5 (3.2 – 3.9)</td>
<td>3.3 (3.0 – 4.0)</td>
</tr>
<tr>
<td>CSF protein (g/L)</td>
<td>0.28 (0.15 – 0.43)</td>
<td>0.42 (0.19 – 1.31) ^a</td>
<td>0.34 (0.21 – 0.94)</td>
</tr>
<tr>
<td>Duration of disease (y)</td>
<td>n.a.</td>
<td>3.0 (0.3 – 10)</td>
<td>5.5 (0.5 – 20)</td>
</tr>
<tr>
<td>EDSS at spinal tap</td>
<td>n.a.</td>
<td>1 (1.0 – 5.0)</td>
<td>2 (0.3 – 3.5)</td>
</tr>
<tr>
<td>Days from relapse to lumbar puncture</td>
<td>n.a.</td>
<td>10 (5 – 120)</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

Data are presented as the median and range. (EDSS = Expanded Disability Status Scale; CSF = cerebrospinal fluid). Comparisons between individual groups were carried out with the Mann-Whitney U-test. As there were no significant differences found between the relapse and remission subgroups, P values relate only to comparisons between each RRMS subgroup and the control group, with: ^a P <0.017 (P<0.05/ N=3 groups) to take account of multiple comparisons.
### Table 2. Immunological data from serum and cerebrospinal fluid (CSF) of patients with relapsing-remitting multiple sclerosis (RRMS) and milder neurological control patients.

<table>
<thead>
<tr>
<th></th>
<th>Control (n=17)</th>
<th>RRMS relapse (n=10)</th>
<th>RRMS remission (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum albumin (g/L)</td>
<td>43 (34 – 48)</td>
<td>42 (36 – 47)</td>
<td>43 (37 – 49)</td>
</tr>
<tr>
<td>CSF albumin (g/L)</td>
<td>0.19 (0.09 – 0.28)</td>
<td>0.29 (0.12 – 1.02)</td>
<td>0.26 (0.11 – 0.64)</td>
</tr>
<tr>
<td>Albumin quotient * 10^3</td>
<td>4.78 (2.25 – 6.51)</td>
<td>6.47 (3.33 – 24.88) (a)</td>
<td>6.05 (2.79 – 13.62)</td>
</tr>
<tr>
<td>Serum IgG (g/L)</td>
<td>9.6 (7.1 – 12.0)</td>
<td>11.1 (7.3-12.6)</td>
<td>10.2 (8.1 – 12.8)</td>
</tr>
<tr>
<td>CSF IgG (mg/L)</td>
<td>21 (13 – 31)</td>
<td>51 (19 – 188) (b)</td>
<td>65 (36 – 81) (b)</td>
</tr>
<tr>
<td>IgG index</td>
<td>0.47 (0.42 – 0.60)</td>
<td>0.65 (0.46 – 2.48) (b)</td>
<td>0.92 (0.55 – 2.76) (b)</td>
</tr>
<tr>
<td>Serum IgM (g/L)</td>
<td>1.12 (0.28 – 2.93)</td>
<td>1.15 (0.62 – 2.64)</td>
<td>1.09 (0.51 – 1.90)</td>
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<td>CSF IgM (mg/L)</td>
<td>0.22 (0.08 – 0.47)</td>
<td>1.07 (0.06 – 3.72) (b)</td>
<td>0.83 (0.22 – 5.19) (b)</td>
</tr>
<tr>
<td>IgM index</td>
<td>0.04 (0.03 – 0.08)</td>
<td>0.12 (0.01 – 0.23) (a)</td>
<td>0.11 (0.03 – 1.90) (a)</td>
</tr>
<tr>
<td>Serum anti-SNOcys IgM (OD)</td>
<td>0.242 (0.124 – 0.892)</td>
<td>0.401 (0.139 – 1.434)</td>
<td>0.261 (0.123 – 0.498)</td>
</tr>
<tr>
<td>CSF anti-SNOcys IgM (OD)</td>
<td>0.029 (0.011 – 0.074)</td>
<td>0.152 (0.022 – 1.395) (b)</td>
<td>0.202 (0.023 – 3.240) (b)</td>
</tr>
<tr>
<td>Normalized CSF anti-SNOcys IgM with CSF IgM (OD)</td>
<td>0.137 (0.047 - 0.654)</td>
<td>0.216 (0.067 - 1.337)</td>
<td>0.240 (0.106 - 1.875)</td>
</tr>
</tbody>
</table>

Data are presented as the median and range. Statistical differences between the groups were carried out with the Mann-Whitney U-test (significance level set at \(P<0.017\) to take account of multiple comparisons), with values of \(P\) indicated by: \(a\) \(P<0.017\), \(b\) \(P\leq0.003\). No significant differences between the relapse and remission subgroups were found, therefore, levels of significance relate only to comparisons between the RRMS subgroups and the control group.
Figure 1. (A) Optical density (OD) levels of IgM to S-nitrosocysteine (anti-SNOcys IgM) in the cerebrospinal fluid (CSF) of multiple sclerosis patients, comprising 10 in relapse and 12 in remission (RRMS, n=22) and milder neurological conditions (Controls, n=17). All CSF were tested at a dilution of 1:2. Horizontal lines indicate median values. When compared to Controls, CSF anti-SNOcys IgM were found to be significantly elevated in RRMS patients ($P<0.0005$). (B) CSF samples from 3 RRMS patients were serially diluted from 1:4 to 1:256 to extinguish their OD and assess the specificity of ELISA detection for anti-SNOcys IgM. OD values reached background at high dilutions, and displayed typical avidity curves of specific binding.
Figure 2. Competition avidity curves showed specific binding of anti-SNOcys IgM. Cerebrospinal fluid (CSF) and serum samples were preincubated with soluble SNOcys antigen from $10^{-6}$ to $10^{-11}$ M before testing on SNOcys bound to the plate. Samples were diluted to generate a starting OD between 0.5 and 2.5 at the lowest SNOcys concentration ($10^{-11}$ M). OD at a given SNOcys concentration was normalized with the starting OD. Normalized OD revealed the competition between liquid phase and immobilized SNOcys that displaced IgM binding. (A) Multiple sclerosis (MS) patient #5 was tested for both CSF (1:2) and serum (1:1,000). (B) CSF samples from three RRMS patients were investigated: MS#1 (1:8), MS#4 (1:16) and MS#5 (1:2).
Figure 3. (A) Total IgM content (mg/L) in the cerebrospinal fluid (CSF) of multiple sclerosis patients, comprising 10 in relapse and 12 in remission (RRMS, n=22) and milder neurological conditions (Controls, n=17). CSF IgM concentrations were found significantly elevated in RRMS patients compared to Controls ($P<0.0005$). (B) Optical density (OD) levels of IgM to S-nitrosocysteine (anti-SNOcys IgM) in the CSF tested at a dilution of 1:2 were normalized with CSF IgM content (mg/L) in multiple sclerosis patients (10 in relapse and 12 in remission, RRMS, n=22) and milder neurological conditions (Controls, n=17). When compared to Controls, CSF anti-SNOcys IgM normalized with IgM concentrations were found to be significantly elevated in RRMS patients ($P=0.027$). Horizontal lines indicate median values.
**Supplementary Table 1.** Demographic and immunological data from serum and cerebrospinal fluid (CSF) of six neurological controls with moderate central inflammation.

<table>
<thead>
<tr>
<th>Patient code</th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
<th>C4</th>
<th>C5</th>
<th>C6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>M</td>
<td>M</td>
<td>F</td>
<td>F</td>
<td>M</td>
<td>F</td>
</tr>
<tr>
<td>Age (y)</td>
<td>49</td>
<td>42</td>
<td>45</td>
<td>44</td>
<td>56</td>
<td>41</td>
</tr>
<tr>
<td>Diagnosis</td>
<td>Facial paresis</td>
<td>Optic neuritis</td>
<td>Facial paresis</td>
<td>Vestibular neuritis</td>
<td>Facial paresis</td>
<td>Vestibular neuritis</td>
</tr>
<tr>
<td>MRI</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Oligoclonal IgG</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>CSF cell count per µL</td>
<td>4</td>
<td>6</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>CSF glucose (mM)</td>
<td>3.6</td>
<td>4.7</td>
<td>3.4</td>
<td>3.2</td>
<td>3.2</td>
<td>3.5</td>
</tr>
<tr>
<td>CSF protein (g/L)</td>
<td><strong>0.63</strong></td>
<td>0.42</td>
<td>0.26</td>
<td>0.39</td>
<td>0.33</td>
<td>0.25</td>
</tr>
<tr>
<td>CSF albumin (g/L)</td>
<td><strong>0.48</strong></td>
<td>0.24</td>
<td>0.16</td>
<td>0.24</td>
<td>0.23</td>
<td>0.19</td>
</tr>
<tr>
<td>Albumin quotient *10^3</td>
<td><strong>11.71</strong></td>
<td><strong>6.15</strong></td>
<td>4.10</td>
<td>5.71</td>
<td>5.90</td>
<td>4.42</td>
</tr>
<tr>
<td>Serum IgG (g/L)</td>
<td>9.3</td>
<td>11.0</td>
<td>7.1</td>
<td>9.1</td>
<td>9.8</td>
<td>9.3</td>
</tr>
<tr>
<td>CSF IgG (mg/L)</td>
<td>49</td>
<td>29</td>
<td>13</td>
<td>27</td>
<td>27</td>
<td>19</td>
</tr>
<tr>
<td>IgG index</td>
<td>0.45</td>
<td>0.43</td>
<td>0.45</td>
<td>0.52</td>
<td>0.47</td>
<td>0.46</td>
</tr>
<tr>
<td>Serum IgM (g/L)</td>
<td>0.52</td>
<td>2.56</td>
<td>1.31</td>
<td>0.82</td>
<td>1.37</td>
<td>1.12</td>
</tr>
<tr>
<td>CSF IgM (mg/L)</td>
<td>0.292</td>
<td><strong>0.443</strong></td>
<td>0.134</td>
<td>0.365</td>
<td>0.255</td>
<td>0.171</td>
</tr>
<tr>
<td>IgM index</td>
<td>0.048</td>
<td>0.028</td>
<td>0.025</td>
<td><strong>0.078</strong></td>
<td>0.032</td>
<td>0.035</td>
</tr>
<tr>
<td>Serum anti-SNOcys IgM (OD)</td>
<td>0.133</td>
<td>0.567</td>
<td>0.315</td>
<td>0.145</td>
<td>0.277</td>
<td>0.194</td>
</tr>
<tr>
<td>CSF anti-SNOcys IgM (OD)</td>
<td>0.025</td>
<td>0.041</td>
<td>0.028</td>
<td>0.049</td>
<td>0.035</td>
<td>0.029</td>
</tr>
<tr>
<td>Normalized CSF anti-SNOcys IgM with CSF IgM (OD)</td>
<td>0.086</td>
<td>0.093</td>
<td>0.209</td>
<td>0.134</td>
<td>0.137</td>
<td>0.170</td>
</tr>
</tbody>
</table>

C1 and C2 were excluded from the study because of their impaired blood brain barrier, but C3 to C6 were part of the control group. Bold values are above normal range. Albumin quotient was calculated as CSF/serum concentration. The IgG and IgM indices were calculated according to the ratio [(CSF Ig/serum Ig) / (CSF albumin/serum albumin)]. The upper physiological limits are 5 cells/µL in CSF (Freedman et al., 2005); CSF protein up to 0.5 g/L (Regeniter et al., 2009); 2.2-4.4 mM glucose; 0.1-0.4g/L CSF albumin; 30-56 g/L serum albumin; 50 mg/L CSF IgG; 7-15 g/L serum IgG; 0.6-3 g/L serum IgM (hospital laboratory
Intrathecal IgM to S-nitrosocysteine in MS

standards from Fischbach and Dunning, 2009); 0.1-0.4 mg/L CSF IgM (Blennow et al, 1996; Jongen et al., 2008); albumin quotient of 4-7 depending the age (Tibbling et al., 1977; Blennow et al., 1993); IgG index above 0.7 (Fredrikson, 2010); IgM index above 0.07 (Sharief and Thompson., 1991b). Serum anti-SNOcys IgM cut-off was calculated as the average + 3 SD of neurological controls (OD above 0.838), and CSF anti-SNOcys IgM cut-off calculated as the average + 5 SD of neurological controls (OD above 0.102, see Results).