Sulfatide isoform pattern in CSF discriminates progressive MS from relapsing remitting MS

Lenka Novakova[1,#], Avadhesh K. Singh[2#], Clas Malmeström[1], Henrik Zetterberg[3,4,5,6], Marcus Ståhlman[7], Markus Axelsson[1], Martin Adiels[7,8], Jan Lycke[1], Susanna L. Cardell[2,*] and Maria Blomqvist[9]

Affiliations:

[1]Department of Clinical Neuroscience, Institute of Neuroscience and Physiology, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

[2]Department of Microbiology and Immunology, Institute of Biomedicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

[3]Department of Psychiatry and Neurochemistry, Institute of Neuroscience and Physiology, Sahlgrenska Academy, University of Gothenburg, Mölndal, Sweden

[4]Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Mölndal, Sweden

[5]Department of Molecular Neuroscience, UCL Institute of Neurology, Queen Square, London, UK

[6]UK Dementia Research Institute at UCL, London, UK

[7]Department of Molecular and Clinical Medicine and Wallenberg Laboratory, Institute of Medicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

[8]Health Metrics Unit, Sahlgrenska Academy, University of Gothenburg, Sweden

[9]Department of Clinical Chemistry and Transfusion Medicine, Institute of Biomedicine, Sahlgrenska Academy, University of Gothenburg, Sweden

[#]**These authors contributed equally**

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[*] **Correspondence**:

Susanna L. Cardell
Department of Microbiology and Immunology, University of Gothenburg,
Box 435, 405 30 Gothenburg, Sweden

E-mail: susanna.cardell@microbio.gu.se
Phone: +46 31 786 6228

Abstract

Background: MS is an inflammatory demyelinating disease of the CNS. Several biomarkers including proteins and lipids have been reported in MS CSF, reflecting different aspects of the pathophysiology particularly of relapsing–remitting MS (RRMS). Sulfatide, abundant in the myelin sheath and a proposed target for autoimmune attack in MS, has been reported altered in MS CSF.
Objective: To investigate the potential of CSF sulfatide and its isoforms as biomarkers in MS.
Methods: A highly sensitive and quantitative mass spectrometry method was employed to determine levels of sulfatide isoforms in CSF from RRMS and progressive MS (PMS) patients, and healthy donors (HD).
Results: Levels of total CSF sulfatide and C24:1, C26:1 and C26:1-OH isoforms were significantly increased in PMS compared with RRMS patients and HD, while C23:0-OH was significantly decreased in CSF from PMS patients compared to the other two groups. Multivariate discriminant analysis showed that CSF sulfatide isoform pattern in PMS patients was distinct and non-overlapping with that of RRMS patients and HD. Sulfatide levels did not correlate with tested biomarkers or clinical parameters.
Conclusion: CSF sulfatide levels may be used to discriminate the phenotype of MS and might play a role in the progression of the disease.

Introduction

MS is a chronic inflammatory disease of the CNS where the myelin sheath around nerve fibers is the target of an autoimmune attack. This leads to demyelination, axonal loss, and subsequent progressive neurologic functional deficits [1]. There are two major phenotypes of MS, relapsing-remitting (RRMS) with relapses mediated by inflammatory activity, and progressive MS (PMS) characterized by slowly evolving disability that exhibits degenerative features. PMS in turn can be divided into primary progressive MS (PPMS), if the clinical course is progressive from onset, or secondary progressive MS (SPMS), when RRMS subsequently transforms into a progressive state [1]. With few exceptions, the disease-modifying therapies of MS are approved for RRMS and not PMS, whereas biomarkers may reflect inflammatory activity as well as degeneration. The distinction between MS phenotypes is essentially based on clinical grounds and there is currently no convincing biomarker from body fluids that can differentiate between RRMS and PMS.

Sulfatide is a sulfated glycosphingolipid found at high concentrations in the myelin sheath. Over 70% of the myelin sheath is comprised of lipids, and around 30% of myelin lipids are sulfatide and its non-sulfated precursor galactosylceramide [2]. Sulfatide consists of a mixture of naturally existing isoforms with different physicochemical properties. The composition of sulfatide differs between organs, where the predominant isoforms in myelin are characterized by long (>20 carbon atoms) unsaturated and hydroxylated fatty acyl chains [3]. An increasing number of reports suggest that lipids are targeted in MS autoimmunity [4]. In particular, enhanced antibody response to sulfatide has been closely associated with CNS inflammation and demyelination [5, 6], and altered levels of sulfatide and anti-sulfatide antibody were found in MS [7-9] but also in patients with neurodegenerative disorders [10]. In addition, it was demonstrated that MS patients harbor an increased frequency of glycolipid-reactive T cells in peripheral blood compared to healthy individuals [11].

Myelin-associated isoforms of sulfatide act as endogenous antigens recognized by a subset of T lymphocytes in the context of the major histocompatibility complex (MHC) class I-like molecule, CD1d [12-16]. Moreover, sulfatide-reactive T lymphocytes are involved in the pathogenesis of autoimmune encephalomyelitis (EAE), the mouse model for MS [12]. Recently, we reported that $V\delta1^+$ T lymphocytes, a subset of γδ T lymphocytes that include sulfatide-reactive, CD1d-restricted T cells[13, 17], have

increased IFN-γ production in newly diagnosed RRMS patients, suggesting that they may be involved in the early stage of the immunopathogenesis of MS [18]. Sulfatide can also modulate inflammation independently of CD1d [19, 20].

Despite the abundance of myelin-associated sulfatide and its proposed roles in the pathogenesis of several CNS disorders, most studies on antigen targets in MS have focused on the myelin proteins. Previous publications suggest that during the autoimmune destruction of CNS myelin, sulfatide may be released and appear in elevated concentrations in CSF [9]. This could lead to increased activation of sulfatide reactive T lymphocytes that may contribute to the autoimmune process. Further, CNS inflammation and degeneration could lead to alterations of the composition of sulfatide isoforms in CSF, making it a possible marker of the disease process and MS phenotypes

In this study, we have used a recently developed, highly sensitive ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) [21], to investigate the concentrations of sulfatide isoforms in CSF from newly diagnosed RRMS and PMS patients and compared them to CSF obtained from healthy donors (HD).

Materials and methods

Study groups

All patients (n=42) fulfilled the revised McDonald diagnostic criteria of MS [22]. They were consecutively enrolled in the study at the MS center of Sahlgrenska University Hospital, Gothenburg, Sweden, and included newly diagnosed RRMS (n=29) and PMS (n=13; 6 primary PMS and 7 secondary PMS). RRMS patients had no concomitant neurological disease and none of the patients were treated with disease modifying drugs, however four PMS patients had previously been treated with interferon beta and/or glatiramar acetate but not within 42 months before sample collection. All MS patients were examined and their neurological disability was scored according to the Expanded Disability Status Scale (EDSS) [23] at the time of sampling. HD (n=16; 5 males and 11 females; range 24-50 years; mean ± SEM 32.6 ± 1.8), age-matched with RRMS patients, served as controls. None of the HD had any neurological signs or a history of neurological disease. Demographics and clinical characteristics of patients are shown in Table 1. All patients and HD provided written-informed consent and the Regional Ethics Committee of Gothenburg approved the study (T487-14).

Sample preparation

The spinal tap and the CSF samples were handled according to the consensus protocol of the BioMS-EU network for CSF biomarker research in MS [24]. Venous blood samples from patients were collected in heparinized tubes. Blood was diluted 1:1 with sterile phosphate-buffered saline (PBS; pH 7.2). Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using Ficoll-Paque (density: 1.077 g/mL; GE Healthcare). Cell viability was determined by Trypan Blue dye exclusion. CSF samples were collected in 10 ml polypropylene tubes and centrifuged; cell-free CSF samples were immediately stored at -80^0C in 0.5 mL aliquots for future analyses.

CSF sulfatide level detection

The quantification of sulfatide was performed as previously described [21]. Briefly, sulfatide was extracted from 100 µL CSF by using the BUME method [25] and further quantified by UPLC-MS/MS using C19:0 sulfatide as an internal standard. The lower

limit of quantification was at 0.1 nmol/L and intra- and inter-assay coefficients of variation were below 10% for the majority of the sulfatide species (except for three low abundant sulfatide species).

Neurofilament light chain (NFL) and glial fibrillary acidic protein (GFAP) analyses

NFL concentration in CSF was measured with a sensitive sandwich enzyme-linked immunosorbent assay (ELISA) (NF-light® ELISA kit, UmanDiagnostics AB, Umeå, Sweden). Intra- and inter-assay coefficients of variation were below 10%. The lower limit of quantification of the assay was 31 pg/mL. The GFAP concentration was measured by ELISA, as previously described [26]. The absorbance was read at 490 nm, and the sensitivity of the GFAP assay was 16 pg/mL. Both assays were conducted at the Clinical Neurochemistry Laboratory (Sahlgrenska University Hospital) according to protocols approved by the Swedish Board for Accreditation and Conformity Assessment.

Magnetic resonance imaging (MRI)

MRI scans of brain and cervical column were performed with 3.0 Tesla machine using 1-3 mm thicknesses. A standard protocol for MS was used with T1 3D weighted sequence following a dose of intravenous gadolinium contrast, T2 weighted sequence and 3D fluid-attenuated inversion recovery sequence. The number of T2 weighed and contrast enhancing T1 lesions were recorded (Table 1). Synthetic MRI (SyMRI) was used to assess brain atrophy with automatic quantification of brain parenchymal fraction (BPF). SyMRI is a novel, reproducible, and fast automatic method for calculating BPF, correlates strongly with a manual segmentation and has a low coefficient of variation (0.45%) [27].

Cell stimulation and flow cytometry

To stain for the intracellular cytokine IFN-γ, PBMC were stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) (Sigma-Aldrich) in the presence of Brefeldin A (eBioscience) for 4 hours at 37°C, as described before [18]. Stimulated cells were first incubated with Fc receptor binding inhibitor (eBioscience), and then stained with surface mAbs for 30 minutes at 4°C using the following fluorochrome-conjugated anti-human monoclonal antibodies (mAbs): anti-CD3ε (OKT3), anti-CD19 (HIB19) (both from BioLegend), anti-TCRγδ (B1) (eBioscience and BioLegend), and anti-

TCR Vδ1 (TS8.2) (Thermo Fisher Scientific). Then the cells were fixed and permeabilized using Intracellular Fixation and Permeabilization Buffer (eBioscience), and stained with anti-IFN-γ (4S.B3) (BioLegend). Cells were always stained with LIVE/DEAD® aqua stain (Life Technologies). Fluorescence minus one (FMO) was used as background control. Data were acquired using an LSRII (BD Biosciences) cytometer, and analyzed using FlowJo v.10 software (Tree Star).

Statistical analysis

CSF sulfatide levels were compared between RRMS, PMS and HD using one-way ANOVA with Tukey's post-hoc comparisons. Correlation analyses were performed using Spearman's nonparametric correlation. Results are presented as mean ± standard error of mean (SEM), and a two-sided *p*-value < 0.05 was considered to be statistically significant. All statistical tests were performed using GraphPad Prism Software, version 7 (La Jolla, CA, USA).

Multivariate discriminant analysis

Discriminant analysis of principal components (DAPC) was used to describe the between-group diversity of sulfatide isoform levels. For this analysis the two isoforms with lowest concentration in CSF (C26:0-OH and C26:1-OH) were omitted. DAPC is comprised of a principal component analysis and a linear discriminant analysis [28]. The number of principal components to retain was assessed by repeated (n=30) cross-validation (training set=90%). DAPC was performed using statistical computing software R (version 3.4.2) with package adegenet (version 2.1.0) [29].

Results

PMS patients had increased levels of sulfatide isoforms in CSF compared to RRMS patients and HD

Here we have investigated the levels of sulfatide isoforms in CSF from HD and patients with RRMS or PMS. Using UPLC-MS/MS, we identified twenty different sulfatide isoforms in CSF that were compared between the three groups (Figure 1 and Supplemental Figure 1). As shown before [21], the sulfatide isoforms carried a range of fatty acids, varying in chain length, hydroxylation and degree of unsaturation. The total sulfatide levels in CSF from PMS patients were significantly higher compared

with those determined in CSF from RRMS patients (97.8 ± 9.6 vs. 77.4 ± 3.8 nM; p = 0.044). However, there were no significant differences observed between PMS patients and HD, nor between RRMS patients and HD (Figure 1A). The HD were age matched with RRMS patients, while the age of PMS patients was slightly higher than that of the other two groups (see Materials and methods and Table 1). We exclude age as a determinant of sulfatide levels in CSF as we have previously shown, employing a cohort of HD with a broader age range, that sulfatide levels in CSF do not increase with age [21].

The comparison of individual isoforms of sulfatide in CSF showed that in all groups, C24:1 was the most abundant isoform, in agreement with previous studies. C24:1 together with C26:1 and C26:1-OH were found to be the statistically most significantly increased isoforms in CSF from PMS patients compared to CSF from HD and RRMS patients (Figure 1B and Supplemental Figure 1). In contrast, one of the isoforms, C23.0-OH, was reduced in CSF of PMS patients compared with the other two groups (Figure 1B). The levels of the other sulfatide isoforms identified in CSF did not differ significantly between the groups (Supplemental Figure 1).

The global isoform pattern of sulfatide in CSF distinguished PMS patients from RRMS patients and HD

We next performed multivariate discriminant analysis, taking into account the variance of the different isoforms of sulfatide in CSF, to obtain a global comparison of isoforms in CSF between HD and the two major phenotypes of MS. We found that retaining 8 principal components was enough to produce a clear separation. In a combined analysis of all three groups, we observed that PMS patients were distinct from the other two groups in terms of their sulfatide isoform distribution in CSF, while RRMS patients and HD did not exhibit a separate pattern. This is displayed in a one-dimensional plot in Figure 2A and in a two-dimensional plot in Figure 2B. The values from all PMS patients fall in an area that is clearly separated and not overlapping with values from RRMS and HD (Figure 2B). To investigate whether there was an underlying difference between RRMS and HD, not revealed in the comparison of all three groups, we performed a separate analysis of only the RRMS and HD groups. The result shows that RRMS and HD values were overlapping also in this analysis (not shown). Taken together, these observations suggest that the sulfatide isoform pattern in CSF can discriminate PMS from RRMS.

Sulfatide levels in CSF did not correlate with frequencies of IFN-γ producing Vδ1 T lymphocytes

Vδ1$^+$ T lymphocytes can recognize sulfatide presented on CD1d [17] and may be involved in the early phase of the immunopathogenesis of MS [18]. Further, the CSF carries antigens that activate immune cells. Therefore, we postulated that a high level of sulfatide in CSF might contribute to the increased activation of Vδ1$^+$ T cells in RRMS patients. However, our analysis did not reveal a positive correlation between frequencies of IFN-γ producing Vδ1$^+$ T lymphocytes and levels of total sulfatide in CSF, nor with the levels of individual isoforms C24:1 and C26:1 (Figure 3A and Supplemental Figure 2A).

No correlation of CSF sulfatide with other CSF biomarkers, MRI measures and disability in MS patients

In order to further explore the role of sulfatide in MS, we performed correlation analysis between sulfatide levels and other CSF biomarkers, MRI outcomes and clinical measures. However, no significant relationships were found between CSF concentrations of sulfatides and GFAP or NFL, the numbers of T2 or contrast-enhancing T1 lesions, the BPF as measure of brain volume on SyMRI, nor with the EDSS score of RRMS patients or PMS patients (Figure 3B, C and Supplemental Figure 2A, B and data not shown).

Discussion

Here we demonstrate that discriminant analysis of sulfatide isoforms in CSF distinguished PMS patients from RRMS patients with newly diagnosed disease. In contrast, there was no difference in sulfatide isoform composition in CSF from RRMS patients compared to HD. This is to our knowledge the first convincing biomarker from a body fluid that appears to differentiate, on the individual patient level, patients with PMS and RRMS. It will be important to validate these results with a larger cohort of patients, and further, to perform longitudinal studies to determine whether the altered distribution of sulfatide isoforms, as seen here in CSF from PMS patients, precedes the conversion of RRMS to secondary PMS. If that is the case, the composition of sulfatide isoforms in CSF should be evaluated as a prognostic biomarker for risk and/or timing of conversion of RRMS to SPMS.

We previously reported elevated levels of total sulfatide in CSF from MS patients [9]. The patient cohort in the previous study included an equal number of RRMS and PMS patients with a mean duration of disease of 16 years (14 years for the RRMS group). In contrast, the RRMS data presented here are from newly diagnosed patients. Together with the results in the previous study, this may suggest that longer disease duration with involvement of CNS degeneration in RRMS can lead to increased sulfatide levels in CSF, and that altered sulfatide composition in CSF might precede RRMS conversion to PMS. However, sulfatide levels (total and selected isoforms) in CSF of patients did not correlate with degenerative CSF biomarkers in RRMS or PMS disease, i.e. NFL indicating axonal damage and GFAP, a marker of astrocyte damage and astrogliosis. Nor was there a correlation with MRI measures such as T2 or T1 contrast enhancing lesions and brain volume, or with clinical assessment of neurological disability (EDSS score). Thus, these data suggest that CSF sulfatide level and isoform alteration is not a marker of disease activity, nor supports the concept that sulfatide is associated with degeneration in MS. However, it is possible that a shift in sulfatide isoform distribution in CSF from PMS patients reflects remyelination. It was recently shown that oligodendrocytes of different developmental stages contained sulfatides with different fatty acid chains, and sulfatide species had different distribution in the adult brain [30]. It was therefore suggested that each sulfatide variant might have different functions in myelin formation, function and maintenance.

Nevertheless, our data supports a discriminative role of sulfatide that may separate MS phenotypes from each other.

It is notable that a patient cohort with Alzheimer's disease demonstrated unaltered total levels of sulfatides in CSF, while compared to the control group, the ratio of hydroxylated to non-hydroxylated sulfatides was significantly increased in patients [21]. Thus, this was suggested to reflect grey matter pathology in Alzheimer's disease since sulfatide in grey matter has been shown to contain high proportions of hydroxylated sulfatide isoforms [29]. This pattern is somewhat different from what we find in CSF from PMS patients, indicating disease-specific alterations in sulfatide isoform composition. Sulfatide in the CNS is mainly produced by oligodendrocytes, and myelin is the major source of sulfatide, however, the origin of sulfatide in CSF is not well understood. Furthermore, the sulfatide fatty acid composition differs when comparing myelin-associated sulfatide enriched in very long chain fatty acids (C22/C26-sulfatide) to neurons and astrocytes where large amounts of C18:0-sulfatide are present [31]. Altered composition of CSF sulfatide in these diseases could potentially have different origins, including a modified sulfatide biogenesis induced by the pathological process, or disease-induced release into CSF of sulfatides with tissue/cell-specific sulfatide isoform composition [32]. Yet, further studies are warranted to identify the underlying processes that determine pathological changes of sulfatide isoform composition in CSF. A recent report demonstrates that there is also an increase in sphingomyelin and hexocylceramide in CSF in MS [33].

The CSF is a source of antigens that are targets of immune reactivity in RRMS. With the lack of an identified lymphatic system draining CNS, it was until recently unclear how CNS antigens were delivered to lymph nodes and antigen presenting cells that initiate immune reactivity. A meningeal lymphatic system has now been demonstrated that drains the CNS to the deep cervical lymph nodes, possibly via CSF [34, 35]. We reasoned that destruction of myelin during MS might increase sulfatide concentration in CSF, which could drain to lymph nodes and contribute to the increased immune reactivity to sulfatide seen in RRMS. However, concentration and composition of sulfatide in CSF from RRMS patients were not different from that of HD. It is possible that sulfatide from degraded myelin is transported to draining lymph nodes by antigen-presenting cells, and/or presented to T lymphocytes by local antigen-presenting cells in the CNS.

To the best of our knowledge, this is the first study to demonstrate that a body fluid biomarker, the isoform composition of CSF sulfatides, can discriminate between different phenotypes of MS. Further studies are encouraged to assess whether alterations of CSF precedes the conversion of RRMS to PMS, and the potential significance of such measurement as a biomarker for disease progression in MS.

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Declaration of conflicting interests

The authors declare that there is no conflict of interest.

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Figure legends

Figure 1. Sulfatide levels were increased in CSF from PMS patients. The levels of total CSF sulfatide (A) and its indicated isoforms (B) were compared between HD (n=16), RRMS (n=29) and PMS (n=13) patients. HD, healthy donors; RRMS, newly diagnosed MS; PMS, progressive MS; nM, nanomolar. Indicated *p* values were derived using the one-way ANOVA with Tukey's post-hoc comparisons.

Figure 2. Discriminant analysis of principal components revealed that sulfatide isoform pattern in CSF distinguished PMS from RRMS patients. One-dimensional (A) and two-dimensional (B) separation of HD (n=16), RRMS (n=29) and PMS (n=13) patients based on their CSF sulfatide levels using DAPC.

Figure 3. CSF sulfatide levels did not correlate with disease biomarkers or clinical parameters. Correlation analyses between the CSF sulfatide and IFN-γ production in Vδ1 cells from peripheral blood in RRMS patients (n=15) are shown (A). Total sulfatide levels in CSF from RRMS (n=29) (B) and PMS (n=13) (C) patients is correlated with CSF biomarkers GFAP and NFL, and clinical parameters MRI (Gd) and EDSS. NFL, neurofilament light chain; GFAP, glial fibrillary acidic protein; EDSS, Expanded Disability Status Scale. r is Spearman's correlation coefficient.

Supplemental Figure 1. Altered levels of individual isoforms of sulfatide in CSF from HD and patients with RRMS or PMS. A comparison of average concentrations of 20 different CSF sulfatide isoforms in CSF from HD (n=16), RRMS (n=29) and PMS (n=13) patients is shown. Isoforms with hydroxylated fatty acids are plotted to the right. Sulfatides that show significant difference between PMS and the other two groups are shown in bold. nM, nanomolar.

Supplemental Figure 2. CSF levels of sulfatide isoforms did not correlate with disease biomarkers or clinical parameters. Correlation analyses between the CSF sulfatide isoforms and IFN-γ production in Vδ1 cells from peripheral blood, CSF biomarkers GFAP and NFL, and clinical parameters MRI (Gd) and EDSS in RRMS (n=29) patients (A) and PMS (n=13) (B) are shown. NFL, neurofilament light chain; GFAP, glial fibrillary acidic protein; EDSS, Expanded Disability Status Scale. r is Spearman's correlation coefficient.