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Original Research Paper

Hexosylceramides as intrathecal markers of worsening disability in multiple sclerosis

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Abstract

Background: Sphingolipids are important components of neurons and the myelin sheath whose levels are altered in multiple sclerosis (MS).

Objectives: We aimed to determine if cerebrospinal fluid (CSF) sphingolipids can be used as markers of MS disease progression.

Methods: Using liquid chromatography tandem mass spectrometry, we analysed sphingolipids in CSF from 134 individuals. The MS group included 65 patients divided into 41 relapsing–remitting MS (RRMS) and 24 progressive MS (ProgMS). In addition, a group of 13 early MS/clinically isolated syndrome (EarlyMS) and two control groups consisting of 38 individuals with other neurological diseases (OND) and 18 OND with signs of inflammation (iOND) were analysed. A follow-up study included 17 additional RRMS patients sampled at two time points 4.7±1.7 years apart.

Results: Levels of sphingomyelin (SM)- and hexosylceramide (HexCer)-derived sphingolipids increased in the CSF of patients with MS independently of the fatty acid chain length in RRMS ($p<0.05$). Levels of palmitic acid (16:0)-containing HexCer (HexCer16:0) increased significantly in ProgMS compared with the OND ($p<0.001$), iOND ($p<0.05$) and EarlyMS ($p<0.01$) groups and correlated with Expanded Disability Status Scale in RRMS in both studies ($p=0.048$; $p=0.027$).

Conclusion: HexCer16:0 is a promising candidate marker of disease progression in MS, especially in RRMS.

Keywords: Multiple sclerosis, cerebrospinal fluid, inflammation, axonal damage, demyelination, mass spectrometry

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Introduction

Multiple sclerosis (MS) is an inflammatory disease, most probably autoimmune in character, which targets the central nervous system (CNS). Research on the molecular pathogenesis of MS has identified therapeutic targets effective during the relapsing–remitting phase of the disease, but the most effective therapies broadly target the immune system, with inherent risks for adverse events. There is a lack of insight into the pathobiology of the progressive phase of the disease and no proper therapy. Diagnosis is established from clinical evidence, supplemented by laboratory investigations, when disease activity is consistent with focal demyelination and has affected more than one part of the CNS on more than one occasion.[1] There has been intensive investigation to identify biomarkers for MS disease prediction and diagnosis, as well as determine disease activity and treatment response;[2] however, there is to date no validated biomarker in biological fluids capable of tracking worsening/progression of MS.

Lipids are important components of myelin that are targeted by T cells as well as autoantibodies during the course of MS.[3,4] Sphingolipids (SLs) are one of the dominant lipid species of the myelin sheath,[5] and are therefore a logical source for identifying biomarkers linked to the origin and/or worsening of the disease.[6] Ceramides (Cer) are the central hub of the SL pathway, which includes sphingomyelins (SM), gluco- and galactosylceramides, grouped as hexosylceramides

(HexCer), lactosylceramides (LacCer), sphingosine (Sph), dihydrosphingosine (DhSph) and dihydroceramides (DhCer) among other compounds. Each subclass is linked to one or more fatty acid alkyl chains, resulting in extensive structural diversity.[7] In order to test for associations between MS disease severity/progression and SL levels, we applied a liquid chromatography tandem mass spectrometry lipid profiling method to quantify SLs in cerebrospinal fluid (CSF) in multiple stages of MS.

Methods

Samples and subjects

All CSF samples were obtained from an in-house biobank collected during routine neurological diagnostic work-up in the Neurology Clinic at the Karolinska University Hospital. Following lumbar puncture, CSF obtained from each individual (10–20 ml) was collected in polypropylene tubes and centrifuged for 12 min at 350×*g*. The supernatants were immediately transferred to new polypropylene tubes, placed on dry ice and stored as cell-free CSF at −80°C until use. A total of 134 subjects were included in this study. MS patients (*n*=65) comprised relapsing–remitting (RRMS; *n*=41) and progressive (ProgMS; *n*=24) groups. The ProgMS group consisted of individuals with secondary progressive (SPMS; *n*=15) and primary progressive (PPMS; *n*=9) disease courses. A group of early MS / clinically isolated syndrome (CIS/EarlyMS; *n*=13) who had a first clinical relapse with one or more magnetic resonance imaging (MRI) lesions characteristic of MS[8] were also included. Two control groups consisted of 1) individuals with other neurological diseases (OND; *n*=38) with the following diagnoses: unspecified sensory disturbance (n=18), different psychological symptoms (*n*=10), dizziness (*n*=1), unspecified headache (*n*=2), chronic idiopathic fatigue (*n*=1), balance disturbance (*n*=1), visual disturbance (*n*=2), muscle diseases (*n*=2) and trigeminal neuralgia (*n*=1) and 2) other neurological diseases with signs of inflammation (iOND; *n*=18) including: systemic lupus erythematosus (*n*=6), neurosarcoidosis (*n*=6), rheumatoid arthritis (*n*=1), aseptic meningitis (*n*=1), myasthenia gravis (*n*=1), epilepsy (*n*=1), hypesthesia (*n*=1) and tropical spastic paraparesis (*n*=1). A follow-up study included 17 RRMS patients sampled at two different occasions with a mean time interval of 4.7±1.7 years. Demographic data for the patients and controls in both studies are presented in Tables 1 and 2. Clinical examinations were performed by specialists in neurology, and all patients diagnosed with MS fulfilled the McDonald criteria.[9] All patients with MS were evaluated clinically at time of sampling with the Expanded Disability Status Scale (EDSS) and the Multiple Sclerosis Severity Score (MSSS). SPMS was defined as an initial relapsing–remitting disease course followed by >12 months of continuous worsening of neurological function (≥0.5 EDSS point) not explained by relapses. At time of sampling, 10 patients with MS were receiving immunomodulatory treatment including interferon (IFN)-β1a (*n*=3), IFN-β1b (*n*=2), glatiramer acetate (*n*=3), corticosteroids (*n*=1) and natalizumab (*n*=1). Inclusion or exclusion of the treated individuals did not affect the results concerning treatment influence on measured SLs since there were only three (or fewer) patients for each therapy. Accordingly, no individuals were excluded.

Neurofilament ELISA study

Neurofilament-light chain (NFL) levels were measured in CSF with commercially available ELISA kits (NF-L: Uman diagnostics, Umeå, Sweden) according to the manufacturer's instructions. Measurements were performed in duplicates using 50 µl undiluted cell-free CSF per well with a detection limit of 31 ng/l.

MRI measurements

The standard MRI protocol included T1, T2 and flair sequences, and in most instances also T1 and T2 after gadolinium. The number of lesions indicates the number of lesions visible on T2/flair weighted images.

Sphingolipid extraction and mass spectrometry

CSF SLs were extracted using a modified Bligh and Dyer procedure.[10] Analysis was performed using two separate ultra high-performance liquid chromatography-coupled electrospray ionisation tandem mass spectrometry methods (UHPLC-MS/MS) on an Acquity UPLC separation module coupled to a Xevo TQ mass spectrometer (Waters, Milford, MS). For more details, see Supplementary Methods. The SL data are discussed in terms of the lipid class (e.g. SM, Cer) and the associated fatty acid chain (e.g. palmitic acid). The fatty acid nomenclature depends upon the length of the alkyl chain and degree of unsaturation. For example, palmitic acid contains a 16 carbon saturated alkyl chain (16:0) and nervonic acid possesses a 24 carbon alkyl chain with a single double bond (24:1).

Statistical analysis

SL values for each group are presented as mean ± SD. Normality was tested via Kolmogorov–Smirnov. Two

Table 1. Demographic data of the patients with MS, CIS/EarlyMS and controls.

Clinical / paraclinical parameters	RRMS	SPMS	PPMS	CIS/EarlyMS	iOND	OND
No. of subjects	*n*=41	*n*=15	*n*=9	*n*=13	*n*=18	*n*=38
Mean age (years)	35.0	49.7	50.9	36.1	37.1	34.3
(range)	(29–41)	(33–62)	(36–60)	(18–50)	(32–50)	(25–41)
Female / Male	(29 / 11)	(11 / 4)	(2 / 6)	(11 / 2)	(18 / 0)	(26 / 12)
Mean disease duration (years)	4.4	18.5	6.5	1.6	N/A	N/A
(range)	(1–13)	(7–37)	(1–17)	(1–4)	–	–
Mean EDSS	1.7	5.4	3.5	1.4	N/A	N/A
(range)	(0–3.5)	(2.0–7.0)	(1.0–6.0)	(0–3.0)	–	–
Mean MSSS	3.61	5.21	5.64	3.85	N/A	N/A
(range)	(0.24–9.08)	(0.90–8.91)	(2.44–8.54)	(0.53–7.06)	–	–
Mean IgG-index	0.98	0.82	0.69	1.04	0.54	0.47
(range)	(0.49–2.44)	(0.53–1.77)	(0.47–1.22)	(0.45–3.27)	(0.44–0.99)	(0.42–0.58)
Oligoclonal IgG bands (+ / - / NA)	36 / 4 / 0	14 / 1 / 0	6 / 2 / 0	9 / 3 / 1	3 / 14 / 1	1 / 37 / 0
Mean CSF albumin quotient	5.1	6.6	6.9	4.3	5.2	4.3
(range)	(2.3–9.9)	(2.6–12.0)	(4.0–12.4)	(2.2–6.5)	(2.4–8.9)	(2.1–8.8)
CSF albumin (mg/l)	228.1	278.7	318.8	203.3	312.2	189.9
(range)	(100–434)	(103–554)	(206–487)	(111–318)	(111–1540)	(88–429)
No. of CSF cell counts/l	6.7×10^6	2.6×10^6	5.7×10^6	6.2×10^6	4.6×10^6	1.1×10^6
(range)	($0–30 \times 10^6$)	($0–12 \times 10^6$)	($0–18 \times 10^6$)	($0–18 \times 10^6$)	($0–18 \times 10^6$)	($0–6 \times 10^6$)
Neurofilaments (ng/l)	915	878	971	511	630	316
(range)	(193-3377)	(302-3757)	(149-3734)	(213-2389)	(168-1827)	(141 –899)
No. of MRI lesions: 0–2 (%)	(8%)	(0%)	(0%)	(15%)	N/A	N/A
3–5 (%)	(8%)	(0%)	(0%)	(8%)	–	–
6–8 (%)	(22%)	(13%)	(17%)	(23%)	–	–
>9 (%)	(62%)	(87%)	(83%)	(54%)	–	–

Age (in years) refers to age at sampling time point. Disease duration (in years) refers to years from disease onset until sample date; RRMS: relapsing–remitting multiple sclerosis; SPMS: secondary progressive MS; PPMS: primary progressive MS; CIS/EarlyMS: clinically isolated syndrome/ EarlyMS; OND: other neurological diseases; iOND: other neurological diseases with signs of inflammation; EDSS: Expanded Disability Status Scale; MSSS: Multiple Sclerosis Severity Score; CSF: cerebrospinal fluids; MRI: magnetic resonance imaging of the brain; N/A: not available.

tailed Student's *t*-test or Mann–Whitney test were used to test differences between two groups normally and non-normally distributed respectively. One-way ANOVA with Dunnet's post-hoc comparisons and Kruskal–Wallis test with Dunn's post-hoc comparisons for each control group were used for multiple group comparisons of normally and non-normally distributed data, respectively. Partial correlations adjusted for age were calculated by first conducting the regression of the two variables on age and then determining Spearman's rank correlation coefficient of the residuals. A two-sided *p*-value <0.05 was considered as significant. The McNemar test was used to test the association between sphingolipids and EDSS. Paired samples were compared using two-sided Wilcoxon signed-rank test. Statistical analyses were done using Graph Pad Prism 5.0 for Windows (GraphPad Software, San Diego, CA) and SPSS 22.0 (SPSS Inc, Chicago, IL).

Standard protocol approvals, registrations, and patient consents

The ethical review board of the Karolinska Institute approved the study (Diary Number: 2009/2107-31-2) and written informed consent was obtained from all patients.

Results

Sphingolipids increase in CSF from progressive MS groups

The analysis of 34 different SLs was conducted in CSF of 134 patients, including 65 patients with different subtypes of MS, 13 patients with early MS, 38 patients with OND and 18 patients with iOND to serve as controls. Six compounds were under the limit of detection

Table 2. Demographic data of the patients with RRMS in the paired follow-up study.

Clinical / paraclinical parameters	Sample 1	Sample 2
No. of subjects	*n*=17	*n*=17
Mean age (years)	34.7	40.0
(range)	(23–54)	(27–59)
Female / Male	(13 / 4)	(13 / 4)
Mean disease duration (years)	4.0	8.6
(range)	(1–19)	(4–24)
Mean EDSS	1.9	2.3
(range)	(0–5.0)	(1.0–6.0)
Mean IgG-index	0.99	0.84
(range)	(0.44–2.15)	(0.47–1.22)
Oligoclonal IgG bands (+ / - / NA)	12 / 5 / 0	13 / 4 / 0
Mean CSF albumin quotient	5.0	5.1
(range)	(2.3–12.0)	(2.1–10.0)
No. of MRI lesions: 0–2 (%)	(6%)	(6%)
3–5 (%)	(12%)	(0%)
6–8 (%)	(0%)	(0%)
>9 (%)	(82%)	(94%)

Age (in years) refers to age at sampling time point. Disease duration (in years) refers to years from disease onset until sample date; RRMS: relapsing–remitting multiple sclerosis; EDSS: Expanded Disability Status Scale; CSF: cerebrospinal fluids; MRI: magnetic resonance imaging of the brain; N/A: not available.

(LOD) and eight did not fulfil the quality control criterion (CV<20%) and were excluded from further analysis. Some 20 compounds were consistently detected and included SM, Cer, HexCer and LacCer with a range of different fatty acid moieties present in their structure, varying in chain length and degree of unsaturation. Supplementary Table 1 presents the average values obtained for the different groups.

ANOVA and Kruskal–Wallis test showed differences between groups for all SM, HexCer and LacCer species (Supplementary Table 1). Among them, only $HexCer_{16:0}$ evidenced significantly increased levels ($p<0.05$) in the ProgMS group relative to iOND, OND and CIS/Early MS control groups (Figure 1). For example, $HexCer_{24:1}$ levels were non-significantly altered between ProgMS and the OND control group (Figure 1). Comparisons for the remainder of the compounds are presented in Supplementary Table 1. A follow-up analysis including RRMS patients measured longitudinally found increased levels for all analysed SM and HexCer species (including $HexCer_{16:0}$; Figure 2), while Cer and LacCer levels remained unchanged (Supplementary Table 2). The observed increase in albumin levels did not reach significance ($n=15$, $p=0.201$).

EDSS clinical score and neurofilaments correlate with hexosyceramide CSF levels sphingolipids

Next we assessed if the increase in SL levels was associated with worsening disease status. Levels of SLs were examined for correlations with two different clinical scores (EDSS and MSSS) as well as neurofilament levels. Moreover, correlations within the two particular phenotypes (RRMS or ProgMS) were also evaluated. Levels of SLs containing either palmitic or nervonic acid ($C_{16:0}$ and $C_{24:1}$ species, respectively) correlated with neurofilament levels when age was taken as a covariate (Supplementary Table 3), except for $Cer_{24:1}$ ($p=0.051$) and $LacCer_{16:0}$ ($p=0.126$). In the case of EDSS, only $HexCer_{16:0}$, $HexCer_{18:1}$ and $HexCer_{24:1}$ correlated (Figure 3). No correlations were observed for MSSS. Further inspection within the RRMS and ProgMS groups showed that correlations to EDSS were constrained to only RRMS patients (Supplementary Table 4). Results from the RRMS follow-up cohort (Supplementary Table 5) confirmed the correlation of $HexCer_{16:0}$ with EDSS, while correlation with $HexCer_{24:1}$ was barely significant ($p=0.052$). The McNemar test found a significant association between EDSS and $HexCer_{16:0}$ ($p=0.039$), but not between EDSS and $HexCer_{24:1}$ ($p=0.227$).

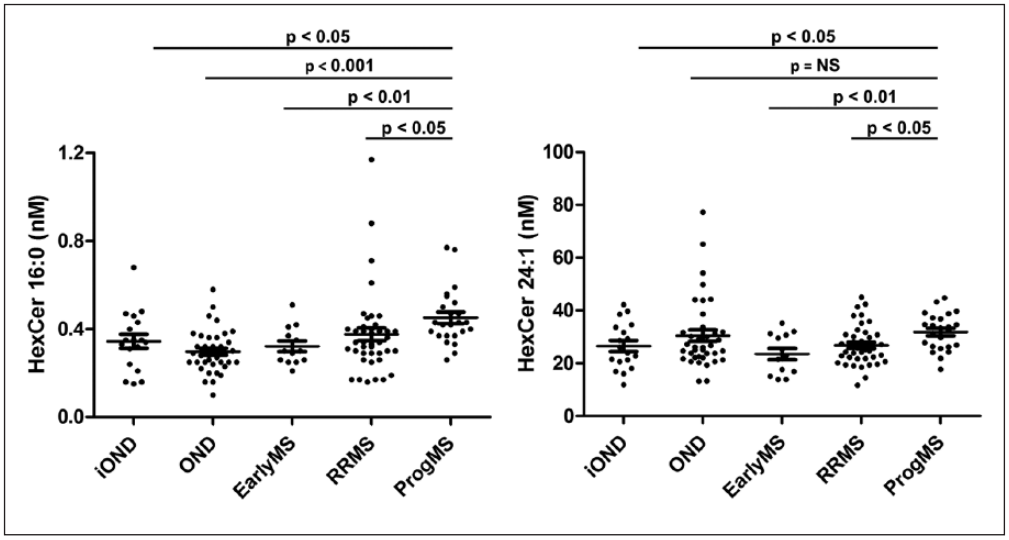

Figure 1. Levels of $HexCer_{16:0}$ and $HexCer_{24:1}$ in the CSF for the different MS and control groups. Circles represent individual samples. Dunn's post-hoc comparisons after significant Kruskal–Wallis test are shown. Data are provided as the geometric mean and standard error mean. CSF: cerebrospinal fluid; HexCer: hexosylceramide; MS: multiple sclerosis; NS: not significant.

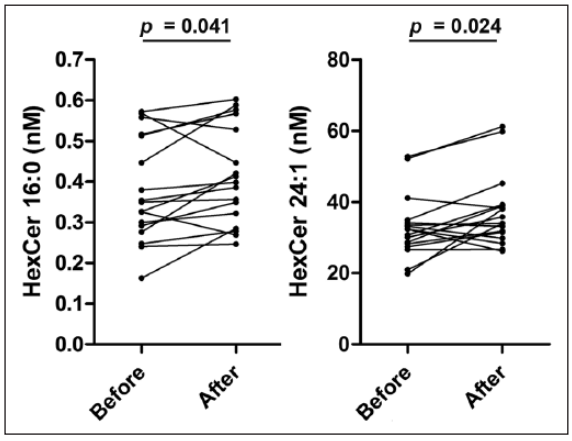

Figure 2. Longitudinal increase of $HexCer_{16:0}$ and $HexCer_{24:1}$ in patients with RRMS in the follow-up study. Connected circles represent samples from the same individual collected before and after an average interval period of 4.7 ± 1.7 years. CSF: cerebrospinal fluid; HexCer: hexosylceramide; MS: multiple sclerosis.

Increased levels of C16:0 sphingolipids in patients with >9 MRI lesions

Finally, SL levels were inspected in patients according to their number of brain MRI lesions (either <9 or >9). $C_{16:0}$ and $C_{24:1}$ species were increased in the >9 MRI lesions group. The case of $HexCer_{16:0}$ and $HexCer_{24:1}$ is presented in Figure 4. Results for the remaining compounds are reported in Supplementary Table 6.

Discussion

The role of SLs as markers of MS worsening in CSF has been investigated for over half a century with confounding results.[11-13] The primary reasons that may explain these discrepancies include the low abundance of these compounds in CSF compared with blood, the heterogeneous levels of these compounds even within the same class,[7,10] and the high diversity in the SL family.[7] In recent years, the significant improvement in mass spectrometry instrumentation has enabled some of these problems related to the detection of low abundance compounds to be addressed. The inverse correlation of potential markers in CSF and brain tissue has recently been shown for biomarkers such as Aβ42 in Alzheimer disease (AD),[14] suggesting that a similar relationship could exist in MS. This issue has been previously examined on a limited scale; however, these studies have generally examined a low number of samples[15,16] or only focused on the parent SL species.[12] While insightful, these approaches often overlook the structural variety of the different subclasses, which are generated via at least six different ceramide synthases (CerS),[17,18] potentially leading to confounding results.[7] In addition, MS patients are treated as a single group, which may mask possible different trends in SL content within various MS subclasses (e.g. CIS/EarlyMS, RRMS, ProgMS).

In MS, T cells and antibodies exhibit a strong reactivity to lipids, which constitute ~70% of the myelin

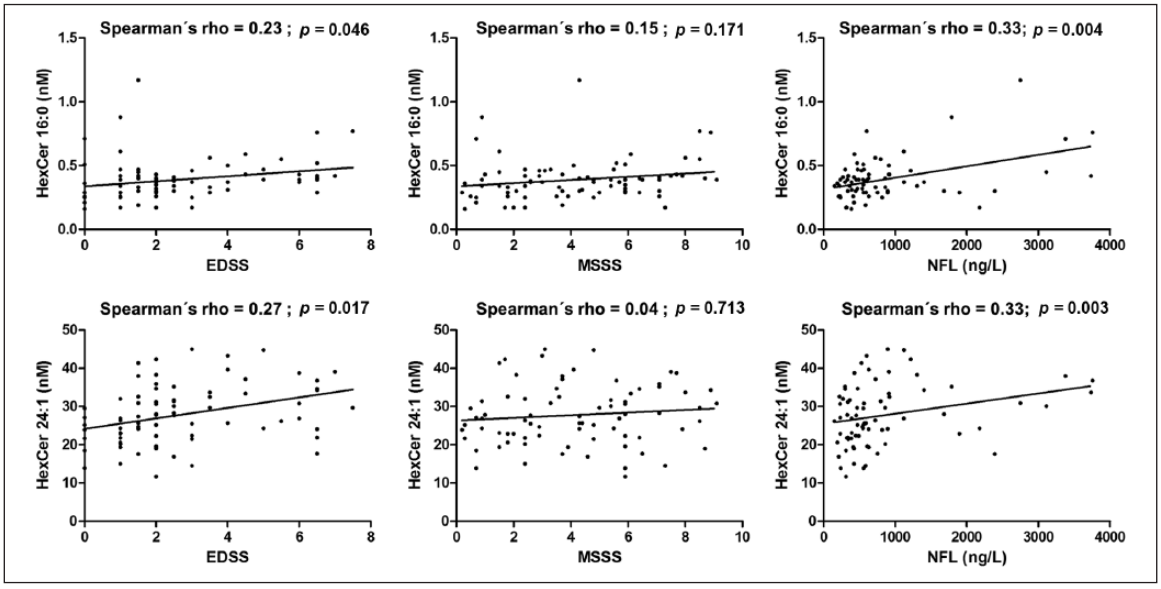

Figure 3. Correlation analyses of levels of $HexCer_{16:0}$ and $HexCer_{24:1}$ with EDSS and MSSS clinical scores and NFL. Circles represent individual samples. Partial correlations adjusted for age calculated by first conducting the regression of the two variables on age and then determining Spearman's rank correlation coefficient of the residuals are presented. CSF: cerebrospinal fluid; EDSS: Expanded Disability Status Scale; HexCer: hexosylceramide; MS: multiple sclerosis; MSSS: Multiple Sclerosis Severity Score; NFL: neurofilaments.

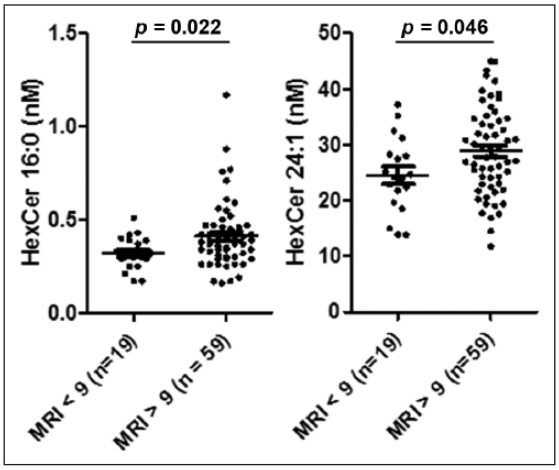

Figure 4. CSF $HexCer_{16:0}$ and $HexCer_{24:1}$ levels according to the number of MRI lesions in MS patients. Data are provided as the geometric mean and standard error mean. CSF: cerebrospinal fluid; HexCer: hexosylceramide; MRI: magnetic resonance imaging; MS: multiple sclerosis.

sheath.[5] Among these lipids, SMs and cerebrosides (HexCer in our study) are the primary SL components (7.9 and 22.7% of total content, respectively),[19] while Cer and LacCer abundances are low. We observed a general increase in the levels of the different SL subclasses in CSF of individuals with MS. There are multiple hypotheses for this observation, including dysfunction of the blood–brain barrier[13] and CNS destruction;[20] however, no studies to date have measured the influence of the fatty acid chain length, making interpretation challenging. We suggest that the increase is a reflection of the SL alterations in the CNS that occur as a result of the antibody reactivity of myelin SLs. Observed increases for SM during MS worsening mirror previously published decreases in white and grey matter SM in active MS regions.[21] From the common species measured in the current study and previous work,[21] the decrease in $SM_{16:0}$ and $SM_{24:0}$ in the CNS was reflected as an increase of the same species in CSF. In addition, none of the studies observed changes in $SM_{18:0}$. In the case of ceramides, $Cer_{16:0}$ was unaltered, while an increase of long-chain Cer ($Cer_{22:0}$ and $Cer_{24:0}$) in CSF corresponded to the decrease observed in the CNS. The only discrepancy between both studies is the reported decrease of $Cer_{18:0}$ levels in the CNS, which did not increase in the current CSF measurements. This observation may be explained by the higher relative abundance of $Cer_{18:0}$ in CSF, which represents ~50% of the total Cer content measured. It is more challenging to detect small changes in large abundance compounds, potentially masking the observation of an increase. Moreover, a recent study confirming the decreases of Cer in brain lesions showed that the ratios $Cer_{16:0}/Cer_{24:0}$ and $Cer_{18:0}/Cer_{24:0}$ are increased in MS brain lesions, suggesting that these ceramides are lost at a lower rate

because there is parallel astrocytic generation.[22] Unfortunately, no data regarding cerebrosides and LacCer were reported in these studies from the SL content of the CNS, but the high abundance of cerebrosides in brain and the lower abundance of LacCers in CSF could explain the differences observed for these compounds independently of the fatty acid moiety. Results from MRI lesions point towards that direction, with increases in $C_{16:0}$- and $C_{24:1}$-containing SLs in patients with >9 MRI lesions. Nonetheless, the number of patients presenting <9 MRI lesions in our cohort was low and results should be interpreted with caution.

Sphingosine-1-phosphate (S1P) is of potential interest in the study of MS, because it is associated with a range of normal physiological regulatory processes in the CNS;[23] however, it did not pass the quality control criteria in the current study. The S1P receptors 1 and 3 are increased in MS lesions[24] and are the main targets of fingolimod.[22] A previous study has shown increased intrathecal S1P levels,[25] but the levels observed in the current study were an order of magnitude lower (subnanomolar range), which may explain the high variability obtained for the quality controls.

Increased levels of NFLs, a marker for axonal damage, have also been suggested as a marker of MS disease progression, but their correlation with MS disease severity scales has led to controversial results.[26] This marker has the added benefit of being closely related to the main determinant of permanent disability in MS (i.e. neurodegeneration). Axonal loss is a major cause of permanent neurological disability in MS and, as suggested, the degree of NFL elevation probably reflects the degree of axonal transection. Thus, there is a clear rationale for NFL as a prognostic marker in MS.[27] In the present study, increased levels of NFL were found in MS groups (Table 1). No correlation between NFL and either EDSS or MSSS was observed, but separately NFL and EDSS correlated with $HexCer_{16:0}$ and $HexCer_{24:1}$, which was specific for RRMS patients (see Supplementary Table 4). The fact that this correlation is limited to patients with RRMS suggests the possibility of an alternative source contributing to SL increases in CSF in the later disease stages (e.g. impairment of the blood–brain barrier). The method employed for calculating EDSS is another possible explanation for these discrepancies. While the RRMS group comprises patients with disabilities in the range 0.0–3.5 (Table 1), ProgMS patients presented, as expected, higher disability scores (1.0–7.0). Because EDSS values below and above 4.5 refer to very different outcomes in terms of progression of the disease (non-walking impairment vs. walking impairment, respectively), it is possible that there simply are no correlations with high EDSS values.

Because of the high inter-individual variation in SL values, a focused subset of paired RRMS patients was investigated to confirm our findings. Results from this follow-up study supported the theory that the brain is the origin of increased SM and HexCer levels in CSF, with observed increases in these brain-enriched compounds while levels of Cer and LacCer were unaltered. Thus, it is possible that losses of SLs highly enriched in the brain (SMs and HexCers) are rapidly reflected in CSF, while increases in other less abundant compounds require larger time spans to be observed. In addition, levels of $HexCer_{16:0}$ correlated positively with EDSS in this additional cohort, with $HexCer_{24:1}$ presenting the same trend (although the correlation was not significant; p=0.052). Importantly, these findings confirmed the initial results (Supplementary Table 5). To the best of our knowledge, this is the first time that SL levels have been measured longitudinally in MS patients. The enzyme responsible for the synthesis of C16:0 SLs (CerS6) has been previously suggested as an inflammatory mediator in MS, and its levels were elevated in experimental autoimmune encephalomyelitis (EAE) mice.[15] $Cer_{16:0}$ was also increased in the CSF of MS patients relative to OND controls, but the number of patients was lower than the current study. Moreover, levels of CerS responsible for synthesizing other alkyl chain lengths were not altered and thus cannot explain the increase of SM and HexCer observed in CSF for patients with MS, which is independent of the chain length. Interestingly, $Cer_{16:0}$ was the only ceramide that correlated with NFL values in our cohort. Moreover, $Cer_{16:0}$ was the only compound presenting higher values in the CIS/EarlyMS group in patients that converted to MS within 3 years from sampling (Supplementary Figure 1). Thus, higher a priori levels may be the reason why no increments in this compound are detected between early MS and ProgMS in our cohort. While we propose a series of compounds that may serve as markers of axonal damage and worsening disease, their implication in its aetiology remains unclear. For example, a previous study showed that cerebrosides are unable to suppress myelin basic protein-specific T-cell proliferation and that their administration does not affect EAE (the animal model of MS).[5]

The current data indicate that some SL species increase during the course of MS; however, their suitability as biomarkers requires determination of specificity via comparison with other neurological diseases.

For example, the levels of $HexCer_{24:1}$ increased with worsening MS, but they were not significantly elevated relative to the OND control group. On the other hand, $HexCer_{16:0}$ was consistently increased with respect to OND, iOND and CIS/EarlyMS in the progressive group. Moreover, levels correlated with EDSS scores and NFL levels. Interestingly, results from a recent study showed higher relative levels of $HexCer_{16:0}$ in comparison with controls.[16] Although the study was performed with fewer individuals (13 MS and 10 controls), results from the HexCer fraction point to similar conclusions: levels of $HexCer_{16:0}$ are elevated in comparison with controls, whereas levels of $HexCer_{24:1}$ are not. Thus, even though both HexCer species evidenced an increasing trend with disease worsening, only increases in $HexCer_{16:0}$ were specific relative to the control groups. Thus, the data suggest that $HexCer_{16:0}$ appears to be a suitable marker for monitoring worsening disease status, at least for RRMS patients in which $HexCer_{16:0}$ levels correlate with EDSS. It would be of interest to examine the effect of treatment upon SL levels in CSF. For example, natalizumab has been shown to have some effects upon protein markers (e.g. osteopontin, neurofilaments)[28,29] and efficacy in disease management could potentially be reflected in shifted SL levels in CSF.

In conclusion, the current work represents the most comprehensive study to date employing targeted SL methods to assess the relationship between SL levels and worsening disability in MS. The results indicate that the levels of SM and HexCer increase in the CSF of MS patients with disease worsening. These findings suggest that CSF levels of $HexCer_{16:0}$ may be suitable to monitor worsening disability status in patients with RRMS, and that decreased brain SLs are the origin of the observed increases. Further studies examining $HexCer_{16:0}$ levels in patient groups of treatment responders and non-responders could confirm this hypothesis. In addition, further investigations are required to elucidate if there are other potential sources contributing to the CSF SL levels during the progressive MS phase, which may explain the lack of correlation of $HexCer_{16:0}$ levels with disease for ProgMS patients. This highlights the importance of disease sub-phenotyping when performing SL analysis.

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Conflict of interest

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