Development of an LC-MS/MS method to measure sphingolipids in CSF from patients with multiple sclerosis

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pwMS  patients with multiple sclerosis
NBV  normalized brain volume
NfL  neurofilament light
NfH  neurofilament heavy
PPMS  primary progressive multiple sclerosis
PCBV  percent change brain volume
RMS  relapsing multiple sclerosis
T2Vol  T2 lesion volume
Abstract

Multiple sclerosis is an inflammatory and degenerative disease characterized by different clinical courses including relapsing multiple sclerosis (RMS) and primary-progressive-multiple sclerosis (PPMS). A hallmark of patients with multiple sclerosis (pwMS) includes a putative autoimmune response, which results in demyelination and neuroaxonal damage in the central nervous system. Sphingolipids in cerebrospinal fluid (CSF) have been proposed as potential biomarkers reflective of disease activity in pwMS. Hence, sensitive methods to accurately quantify sphingolipids in CSF are needed. In this study, we report the development of a sensitive high-throughput multiplexed LC-MS/MS method to perform quantitation on 14 species of sphingolipids in human CSF. We applied this method to measure CSF sphingolipids in healthy controls (n=10), PPMS (n=27), and RMS (n=17) patients before and after ocrelizumab treatment. The median CSF levels of the 14 sphingolipids measured herein was higher in PPMS (17.2 ng/mL) and RMS (17.6 ng/mL) when compared to the healthy controls (13.8 ng/mL). Levels of sphingolipids were decreased by 8.6% at week-52 after-treatment with ocrelizumab in RMS patients, but not in PPMS patients. Specifically, C16 Glc Cer (-26%;P=0.004) and C18 Cer (-13%;P=0.042) decreased from baseline in RMS patients. Additionally, in PPMS patients C16 Glc Cer levels correlated with CSF neurofilament heavy levels at baseline (Rho:0.532;P=0.004) and after treatment (Rho:0.424;P=0.028). Collectively, these results indicate that CSF sphingolipid levels are altered in pwMS and that treatment with ocrelizumab results in significant shifts in the sphingolipid profile that may reflect a reduction in disease activity supporting further investigation into sphingolipids as tools to monitor disease state.
Significance statement

This study describes the development of a new method to measure 14 sphingolipid species in CSF. These results demonstrate that sphingolipids levels are elevated in CSF of pwMS when compared to healthy controls. Distinct sphingolipid signatures between patients with different clinical disease courses were observed and these lipid signatures changed after treatment with ocrelizumab, especially in RMS patients. This method enables further investigation into the role of sphingolipids as candidate biomarkers in pwMS and other CNS disorders.
Introduction

Multiple sclerosis is a chronic inflammatory, debilitating putative-autoimmune disease and it is the leading cause of acquired non-traumatic disability in young people (Koch-Henriksen & Sørensen, 2010). The main pathogenic mechanism associated with the early stages of multiple sclerosis is characterized by a cellular immune process with lymphocytes and macrophages infiltrating into the central nervous system (CNS) parenchyma (Bar-Or & Li, 2021). The pathological result of this local inflammation is myelin loss and apoptosis or necrosis of mature oligodendrocytes, resulting in demyelinated plaques, astrocytic scars, and neuroaxonal damage and loss that leads to neurodegeneration (Brück, 2005; Lassmann et al., 2001).

Multiple sclerosis is a progressive disease that presents as different clinical types including relapsing (RMS), and primary-progressive (PPMS) (Lublin et al., 2014). Briefly, RMS patients go through phases of relapses of neurological symptoms followed by periods of partial remissions and lesions are more likely to appear in the brain compared to the spinal cord. Around 85% of patients with multilpe sclerosis (pwMS) are diagnosed with RMS initially and the incidence of RMS is around 3 times higher among women than men (Wallin et al., 2019). Interestingly, unlike RMS, PPMS tends to affect men and women at equal rates (Coyle, 2021). Recent evidence suggests that common mechanisms underlying progressive biology occur in both RMS and PPMS clinical forms of pwMS. RMS biology is believed to be driven by activation and subsequent CNS migration of peripheral immune cells that results in focal acute perivascular inflammatory lesions and clinical relapses. In contrast, progressive biology appears related to insidious injury from ongoing
neurodegeneration and/or compartmentalized inflammation (smoldering lesions and chronic glial activation) (Absinta et al., 2021; Mahad et al., 2015). It has been reported that lipid metabolism imbalance plays an important role in the pathophysiology of neurodegenerative diseases and leads to clinical manifestations in the motor system (Dodge, 2017) and specifically in pwMS (Ferreira et al., 2020; Podbielska et al., 2022).

Sphingolipids are one of the most abundant lipid species in the brain with sphingomyelin species representing around 20% of total brain lipids (Colsch et al., 2015). Sphingolipids are bioactive lipids comprised of a sphingoid base (SPB) linked to various long-chain fatty acids (ceramides; Cer), phosphocholine (sphingomyelins; SM), and some species also contain one or more sugars (glycosphingolipids, cerebrosides) (Figure 1A, 1B). It has been reported that sphingolipids produced by activated glial cells may play an important role in the pathogenesis of pwMS by modulating multiple signaling pathways that result in a wide range of downstream effects including inflammation, cellular proliferation, and cell cycle arrest (De Wit et al., 2019).

Additionally, sphingosine-1 phosphate (SPBP) a phosphorylated sphingosine is a signaling lipid and has an integral role in immune cell trafficking, vascular permeability, and production of immune mediators and it has been shown to be dysregulated in pwMS (Strub et al., 2010). Interestingly, SPBP receptor modulators (e.g. fingolimod) regulate immune cell migration out of secondary lymph nodes and are used as pharmacotherapies in pwMS (Wingerchuk & Carter, 2014).

The CNS myelin sheath which provides nutrients and protection to axons is rich in lipids (70–85% of dry weight); it is mainly comprised of cholesterol,
glycerophospholipids, and glycosphingolipids at a ratio of 4:4:2 (Cermenati et al., 2015; O'Brien & Sampson, 1965). Glycosphingolipids are major components of oligodendrocyte plasma membranes and myelin and they are essential for the stability and maintenance of myelin during aging. The sugar attached to the sphingosine base determines the subclass of glycosphingolipid, for example, galactosylceramide (Gal Cer) contains galactose, glucosylceramide (Glc Cer) contains glucose, and lactosylceramide contains lactose (Lac Cer). Interestingly, Gal Cer species are enriched in myelin (~24%), and their accumulation in the brain has been used to measure myelin biogenesis (Schmitt et al., 2015). While sphingolipids are main structural components of myelin, sphingolipids play an important role in additional cellular pathways critical for CNS function. For example, Glc Cer species are ubiquitously present in cellular membranes and they are especially enriched in lipid rafts likely playing a key role in membrane organization (Varela et al., 2016). Other important Glc Cer functions include regulating endocytic trafficking and controlling cytoskeletal dynamics (Raju et al., 2015; Sillence et al., 2002).

CSF is the most disease-proximal biofluid that is accessible in pwMS and despite the important role sphingolipids are thought to play in demyelinating diseases, there are very few studies that have accurately quantified sphingolipids in CSF from this patient population. Several targeted quantitative approaches have found elevated levels of ceramides in CSF from pwMS, specifically C16 and C24 ceramides and C16 hexosylceramide (comprising Gal Cer and Glc Cer) (Checa et al., 2015; Vidaurre et al., 2014). Previous attempts to measure sphingolipids in pwMS have been hindered by sample size and their inability to resolve glucosyl and galactosyl isomers. A few reports
have been published using non-targeted approaches, however, these methods are not quantitative, and therefore biological interpretation of changes in disease is challenging (Nogueras et al., 2019; Oliveira et al., 2019; Péter et al., 2020; Pieragostino et al., 2015). Therefore, new methods with higher sensitivity and better resolution of isomeric lipid species are needed to inform the biological interpretations of changes in these lipid profiles in the context of this disease.

Here we report a highly sensitive, multiplexed, robust, single-injection method to quantify 14 sphingolipid species including ceramides, sphingosines, and glycosphingolipids. It is worth noting that this method has the ability to separate the Glc/Gal Cer and SPB structural isomers. We also describe a single plex method to quantify SPBP in CSF from PPMS, RMS, and healthy controls. These methods were utilized to characterize the CSF sphingolipid profile in pwMS before and after treatment with ocrelizumab, a B cell depleting monoclonal antibody targeting CD20 that is currently approved for treatment in both RMS and PPMS (Hauser et al., 2017; Montalban et al., 2017).

**Materials and Methods**

**Materials**

HPLC-analytical grade solvents, acetonitrile, and methanol were purchased from Midland Scientific, formic acid and ammonium formate were purchased from Sigma-Aldrich. Standards and internal standards for ceramides, hexosylceramides, and sphingosines were purchased from Avanti Polar Lipids (Listed in Supplementary Table 1). C16 lactosyl (β) ceramide d3 was purchased from Matreya LLC. C16 lactosyl (β)
ceramide was purchased from Cayman chemical. Standards and internal standard stock solutions were prepared at 1 and 100 µg/mL respectively in methanol and stored at -80°C for long-term storage. Analytical column HALO HILIC 4.6x 150 mm, 2.7 µm was purchased from Mac-Mod Analytical Inc.

**CSF samples**

In this study, we included patients with available CSF at baseline and week 52 after treatment with ocrelizumab: PPMS n=27 and RMS n=17 (Figure 1C). Patient demographic data for the baseline vs after treatment study is listed in Table 3. For the cross-sectional study, a subset of the PPMS (n=10) and RMS (n=10) baseline samples were included and CSF samples from healthy controls were procured from Precision Medicine. In healthy controls, the age range was 32-47 years old with a median of 42 and 40% were women. Patient data demographics for the cross-sectional study is listed in Supplementary Table 2. Both studies used samples from the Ocrelizumab Biomarker Outcomes Evaluation Study (OBOE) (manuscript under revision).

The OBOE study (NCT02688985) was conducted in accordance with the International Council for Harmonization E6 Guideline for Good Clinical Practice and principles of the Declaration of Helsinki. Study sites received local institutional review board approval and written informed patient consent was obtained. Key inclusion criteria were age 18 to 55 years and an Expanded Disability Status Scale (EDSS) score of 0 to 5.5 for RMS patients and 3.0 to 6.5 for PPMS patients. RMS patients were required to have ≥1 documented relapse and/or T1w Gadolinium positive enhancing lesions (Gd+ lesions) and/or new T2-weighted (T2 lesion) within the prior year. PPMS patients were
required to show elevated CSF immunoglobulin G (IgG) index and/or ≥1 CSF-restricted oligoclonal band.

OBOE patients underwent baseline lumbar puncture (LP) before their first ocrelizumab infusion and at week 52 after treatment. Patients received ocrelizumab as previously described (Hauser et al., 2017; Montalban et al., 2017) and underwent neurological examinations and EDSS assessment at screening, baseline, and weeks 12, 24, 48, and 52. MRI was used to assess lesion burden and changes in brain volume throughout the duration of the study. MRI scans were evaluated centrally by blinded readers at NeuroRx Research, Montreal, Quebec, Canada. For baseline brain volume measurements, the subject’s skull volume was used to obtain a scaling factor that was applied to normalize the brain volume for head size and normalized brain volume (NBV) and T2 lesion burden was determined using previously reported methods (Montalban et al., 2017). CSF Neurofilament heavy (NfH) levels were measured with a Simple Plex assay from ProteinSimple according to the manufacturer’s instructions. CSF Neurofilament light (NfL) levels were measured via a matrix-validated assay using Quanterix Simoa NF-light Advantage Kit according to manufacturer’s instructions.

14 plex sphingolipid method sample preparation

20 µL of CSF biofluid, standards, or quality controls were aliquoted into a 96-well plate and protein was precipitated with 120 µL of 97.5:2.5 (volume for volume, mobile phase A: mobile phase B (MPB) (see below section)) containing a mixture of internal standards at 0.5 ng/mL (Supplementary table 1). The mixture was vortexed for one minute and centrifuged at 3000 rpm for 5 min. The supernatant was transferred, and
dried-down at 45°C using a TurboVap (Biotage) under nitrogen. The dried samples were reconstituted with 100 µL of 97.5:2.5 MPB and analyzed by LC-MS/MS. Calibration curves were prepared from 204.8 ng/mL down to 0.1 ng/mL. Quality control samples were prepared in CSF and MPB at 0, 1.25, 5, 20, and 80 ng/mL and measured in triplicate to evaluate precision and accuracy.

**14plex sphingolipid analysis by LC-MS/MS**

LC-MS/MS analysis was performed on a Sciex 5500 operated in multiple reaction monitoring (MRM) mode coupled with a UPLC Nexera LC-30 AD Nexera X2 UHPLC system (Shimadzu, Kyoto, Japan). Sphingolipids were chromatographically separated using a HALO HILIC column (4.6 x 150 mm, 2.7 µm). The mobile phases consisted of 100:0.5% acetonitrile: formic acid (volume for volume, MPA) and 50 mM ammonium formate in 60:40:0.5 water: methanol: formic acid (volume for volume, MPB). The run time for this method was 6 min starting with a linear gradient from 1% to 25% of MPB in 2.7 minutes, followed by a linear gradient to 40% MPB from 2.7 to 3.5 minutes, a linear gradient to 45% MPB from 3.5 to 4.0 min and a linear gradient to 1% of MPB from 4.0 to 4.1 min, and a re-equilibrium step of 1.9 min to the initial 1% MPB (flow rate 1.0 mL/min). The injection volume was 25 µL using a column temperature of 50°C. The sphingolipids and deuterium-labeled internal standards were detected using MRM with a positive turbo spray ion drive mode with the following parameters curtain gas (CUR) 20, collision gas (CAD) high, ion spray voltage (IS) 5500 V, temperature (TEM) 500, ion source gas 1 (GS1) 40, and ion source gas 2 (GS2) 60. The Q1/Q3 transitions, dwell time, declustering potential, collision energy, entrance potential, and CXP for each analyte are listed in Supplementary Table 3.
**SPBP method sample preparation**

Briefly, 50 µL of sphingosine-1-phosphate d7 in 30% Acetonitrile was spiked into 50 µL of sample (CSF, surrogate matrix (MPAB 97:5:2.5), or standards), and the mixture was vortexed for 30 seconds. 25 µL of 10% of formic acid was added to all samples and vortexed for 60 seconds. Protein was precipitated from samples with 200 µL of 100% acetonitrile and vortexed for 10 min at 3000 rpm at room temperature. 250 µL of supernatant was transferred and dried down using TurboVap (Biotage) under nitrogen. The dried samples were reconstituted with 125 µL of 30:70 acetonitrile: water and 10 µL of 0.1% formic acid. The calibration curve range was from 0.980 to 1000 pg/mL. Quality control (QC) samples were prepared in CSF and surrogate matrix at 5.00 and 40.0 pg/mL and measured in triplicate to evaluate precision and accuracy.

**SPBP analysis by LC-MS/MS**

Analysis was performed on a Sciex QTRAP 6500+ coupled to a UPLC Nexera LC-30 AD Nexera X2 UHPLC system (Shimadzu, Kyoto, Japan). SPBP was chromatographically separated using a Venusil XBP C18 (L) column (2.1 x 50 mm, 3 µM). The mobile phases consisted of 100:0.1 deionized water: formic acid (volume for volume, mobile phase A2; MPA2) and 100:0.1 acetonitrile: formic acid (volume for volume, mobile phase B2; MPB2). The run time for this method was 4.5 min starting with 60% of MPA2 for 1 min, followed by a linear gradient from 60% to 10% of MPA2 in 2.5 min, maintenance of 10% MPA2 for 0.5 minutes, and equilibrated at 60% MPA2 from 3.1 minutes, and a holding step for 1.4 min to the initial 60% MPA2 (flow rate 0.7 mL/min). The injection volume was 110 µL using a column temperature of 60°C. SPBP and SPBP-d7 were detected using MRM in positive ion mode with the following
parameters CUR 20, CAD high, IS 5500 V, TEM 400, GS1 40, and GS2 60. The Q1/Q3 transitions, dwell time, declustering potential, collision energy, entrance potential, and CXP for each analyte are listed in Supplementary Table 3. The SPBP retention time was compared to the retention time for the deuterium-labeled internal standard SPBP-d7. The lower limit of quantification (LLOQ) was defined as the lowest concentration of analyte that could be measured with reproducibility of $\pm$ 25% and an accuracy of 75-125%.

**Statistical analysis**

The areas under the peaks generated for both analytes and internal standards were integrated using 1.4.2 MultiQuant software (Sciex). Peak areas for C24 Cer and C18 Cer were corrected for natural stable isotope M+2 contamination from the unsaturated species (C24:1 and C18:1 Cer). For C24 Cer the total peak area was corrected due to co-elution, and for C18 Cer, 35% of the total peak area was corrected since only partial co-elution is observed. Analyte concentration was calculated using the area under the curve and calibration curve for each analyte. Statistical differences between groups were determined by one-way ANOVA tests; paired t-tests were performed for baseline vs after treatment comparisons with p-values < 0.05 being considered statistically significant. Spearman correlation tests were used to analyze correlations between levels of sphingolipids and clinical variables. P-values < 0.05 were considered statistically significant and are reported as unadjusted values with no statistical adjustment for multiple comparisons unless otherwise specified. All statistical analyses were performed using GraphPad Prism 9.5.1 (733).
Results
Development of a multiplexed method to monitor sphingolipids in CSF

In this study, we developed a method using a multiplexed targeted 6-minute LC-MS/MS method to quantify 14 sphingolipids in CSF ceramides (C14, C16, C18, C20, C24, C18:1, and C24:1), hexosylceramides (C16 Glc Cer and C16 Gal Cer), lactosylceramides (C16 Lac Cer and C18 Lac Cer), and sphingosines (SPB, Glc SPB, and Gal SPB). A representative chromatogram (standards in buffer) showing retention times for the 14 analytes is shown in Figure 2A. All analytes were chromatographically baseline resolved including the hexosylceramide isomers C16 Glc Cer and C16 Gal Cer, and sphingosine isomers Glc SPB and Gal SPB (Figure 2A). Calibration curves were analyzed covering analyte concentration ranges from 0.1-250 ng/mL and this method was shown to have high accuracy and a good fit to the curve ($R^2 > 0.96$ for all analytes) over the entire concentration range tested (Table 1). The lower limit of quantitation (LLOQ) for all 14 analytes is listed in Table 1. A fit for purpose accuracy ($87.58-153.81\%$) and precision (Coefficient Variation (CV) %: 0.56-22.71) was observed for all analytes.

Development of a method to measure sphingosine-1-phosphate (SPBP)

A targeted LC-MS/MS MRM method was developed to quantify the abundance of SPBP in CSF samples. A representative chromatogram of SPBP and the internal standard SPBP-d7 is shown in Figure 2B. Calibration curves for SPBP were established covering the concentration range 0.98 pg/mL -1000 pg/mL and the method demonstrated good linearity and high accuracy ($R^2 > 0.98$, Table 2). The LLOQ was determined to be 0.01 ng/mL. A fit for purpose accuracy and precision were observed with the percent deviation <35% and CV% <17%.
Sphingolipids are elevated in CSF from pwMS compared with healthy controls

To assess changes in sphingolipid levels in CSF based on disease status, levels of sphingolipids were measured in CSF from n=10 RMS patients, n=10 PPMS patients, and n=10 healthy controls (Table 2). All sphingolipids species were detected, except for SPB which was below the detectable range for most samples, and therefore values were not reported for this study. Sphingolipid abundances observed among patients in all groups are listed in Table 2. C16 Lac Cer was the most abundant sphingolipid species across all groups followed by C18 Cer and C18 Lac Cer. No difference in the rank order of analyte abundance was observed among the three different groups. Additionally, the levels of C16 Cer, C18 Cer, C20 Cer, C24 Cer, and C24:1 Cer were highly correlated with each other (P<0.05). Similarly, significant correlations were observed between C16 Glc Cer, C16 Gal Cer, C16 Lac Cer, and C18 Lac Cer (P<0.05) (Figure 3A). Based on the different sphingolipid abundances across groups described in Table 2, C16 Cer was found to be elevated in CSF of both RMS and PPMS patients (Figure 3B), and C16 Cer, C24:1 Cer, and C16 Glc Cer, were found to be elevated specifically in CSF from PPMS patients compared to healthy controls (ANOVA P<0.05) (Figure 3C, 3D). Levels of C16 Gal Cer followed the same trend (Figure 3E) although they were not significant (ANOVA= 0.102). Among healthy controls, age was positively correlated with C24:1 Cer (Rho= 0.697; P=0.031, Supplementary table 2). In contrast, in PPMS and RMS patients no significant associations were observed between sphingolipid levels and age or BMI.

Longitudinal analysis of CSF from ocrelizumab treated patients identifies changes in sphingolipid profiles
In this cohort, we analyzed associations between baseline sphingolipid levels and patient demographics. Sphingolipids trended higher in males than in females in both PPMS and RMS however, only C16 Lac Cer in PPMS patients reached statistical significance (males = 0.48 and females = 0.41 ng/mL; P=0.007). Age was positively correlated with Cer 24 (Rho=0.45; P=0.019) and C16 Lac Cer (Rho=0.41; P=0.036) among RMS patients. In contrast, C14 Cer (Rho=0.52; P=0.034), C16 Cer (Rho=0.66; P=0.005), C24:1 (Rho=0.68; P=0.004), C16 Glc Cer (Rho=0.55; P=0.025), and C16 Gal Cer (Rho=0.59; P=0.015) were positively correlated with age in PPMS patients.

To understand the impact of B cell depletion therapy on the CSF sphingolipid profile, levels of sphingolipids were measured in samples from baseline and after 52 weeks of treatment with ocrelizumab in PPMS (n=27) and RMS (n=17) patients (Figure 1C, Table 4). Overall, a decreasing trend was observed in sphingolipid levels after treatment in RMS patients (-26 to 36% change) while relatively small changes were observed among PPMS patients (-10 to 74% change). C16 Glc Cer (-26%; P=0.004) and C18 Cer (-13%; P=0.042) were both significantly decreased in RMS patient CSF compared to baseline levels (Figure 4A).

**Sphingolipids correlate with clinical features of pwMS**

To explore the potential role that sphingolipids may play in clinical manifestations of pwMS, relationships between individual sphingolipid levels in CSF and clinical features of disease were evaluated in this cohort (Table 3, Figure 4B). Baseline and after treatment sphingolipid levels had no significant correlations with BMI, Gd+ lesions, T2 lesion volume (T2 LV) and CSF Neurofilament Light (NfL) (Supplementary Table 2). Among RMS patients C16 Glc Cer was correlated with CSF Neurofilament Heavy (NfH)
levels at baseline ($Rho= 0.664; P=0.008$) and this relationship was lost after treatment with ocrelizumab ($Rho= 0.288; P=0.318$). Conversely, this correlation was statistically significant in CSF from PPMS patients at baseline ($Rho= 0.532; P=0.004$) and after treatment ($Rho= 0.424; P=0.028$) (Figure 4C). In PPMS patients, CSF C16 Glc Cer was also observed to be associated with EDSS after treatment and not at baseline ($Rho= 0.532; P=0.004$). A similar trend was observed at baseline in RMS patients; however, it did not reach statistical significance (Figure 4D).

Normalized brain volume (NBV) was assessed by MRI and was shown to be negatively correlated with C14 Cer ($Rho= -0.527; P=0.038$), C16 Cer ($Rho= -0.658; P=0.007$), and C24:1 Cer ($Rho= -0.638; P=0.009$) in RMS patients at baseline, whereas no significant correlations were observed after treatment or among PPMS patients at either time point. Consistent with this, a negative trend was observed between the percent brain volume change (PBVC) and percent change C16 Cer in RMS patients ($Rho= -0.479; P=0.062$) and no trend was observed in PPMS patients (Supplementary Figure 1). Interestingly, among PPMS patients CSF NfL levels were positively correlated after treatment with C18 Cer ($Rho= 0.419; P=0.030$) and C18 Lac Cer ($Rho= 0.482; P=0.011$) but not at baseline or at either time point in RMS patients (Supplementary Table 4).

In RMS patients at baseline, there were significant correlations between C18 Cer, C24 Cer, and C16 Gal Cer levels and the albumin quotient ($\text{Alb-Quot} = \text{CSF albumin/serum albumin}$), an indicator of blood-brain barrier (BBB) impairment (Figure 4B). After treatment, correlations with Alb-Quot remained significant for the above-
mentioned species, and C14 Cer, C16 Cer, C20 Cer, C16 Glc Cer, and C16 Lac Cer became significantly correlated. Similarly, correlations between Alb-Quot and C24 Cer, and C16 Lac Cer were observed in PPMS patients at baseline, and after treatment correlations with Alb-Quot remained significant for the above-mentioned species, and C24:1 Cer, C16 Gal Cer, and C18 Lac Cer became significantly correlated as well (Supplementary Table 4).
**Discussion**

In this study, targeted LC-MS/MS methods were developed to measure 15 sphingolipids in CSF, and 14 sphingolipids were quantifiable in CSF samples from both healthy controls and pwMS. These methods were applied to two pwMS cohorts and relationships between sphingolipids and demographic and clinical features were characterized. Importantly, sphingolipid CSF profiles were determined to be different in pwMS versus healthy controls and in patients with PPMS compared to patients with RMS. These profiles changed after B cell depletion therapy, suggesting they could serve as biomarkers reflective of disease activity, and deserve further study.

Sphingolipids in the same class (ceramides, glycosphingolipids, and sphingosines) were highly correlated among each other, which is not surprising based on their shared biosynthesis pathway. While results from this study are in concordance with previous studies that indicate that hexosylceramides (consisting of Glc Cer and Gal Cer) are increased in pwMS (Checa et al., 2015), our findings provide additional depth of identification and clinical context to these results which may alter the biological interpretation of these changes. In contrast to the method presented herein, previous reports did not chromatographically resolve the isomeric species C16 Glc Cer and C16 Gal Cer. Thus, they may have incorrectly assumed that much of the CSF hexosylceramide peak was coming from Gal Cer species when, in fact, C16 Glc Cer appears to be much more abundant. It is important to have a method that allows specific quantitation of hexosylceramide isomers because Glc Cer is thought to only be a minor part of the myelin sheath compared to Gal Cer (Raju et al., 2015; Saadat et al., 2010). It is possible that other Gal Cer species or their metabolites not included in this
method may be more abundant in CSF, and further experiments are needed to fully test this hypothesis (Checa et al., 2015; Reza et al., 2021). The elevated levels of CSF Glc Cer observed in this study likely do not come from ongoing demyelination, but rather, could reflect other important disease relevant biology including local inflammation and cellular membrane damage.

In this study, baseline CSF levels of sphingolipids were elevated in RMS (0.7-2.4 fold) and PPMS (0.8-1.6 fold) when compared to healthy controls, specifically C16 and C24:1 Cer. An increase in ceramides in CSF from pwMS has been previously reported and is linked with neuronal mitochondrial dysfunction and neuroaxonal damage (Péter et al., 2020; Vidaurre et al., 2014). One hypothesis is that C16 Cer is being produced by astrocytes as C16 Cer has been shown to be elevated in this cell type in brain samples collected postmortem from patients with other neurological diseases (De Wit et al., 2019). This finding is supported by a previous study that demonstrated that sphingomyelinase, an enzyme that catalyzes the hydrolysis sphingomyelin to ceramide, has increased activity in pwMS (Pieragostino et al., 2018). Combined with our results, these findings implicate a dysregulation of sphingolipid signaling pathways with pwMS disease mechanisms and additional studies in pwMS cohorts with tissue samples should be further explored to examine associations between ceramide levels and lesions, lesion subtypes, and other clinically relevant metrics. Importantly, after treatment with ocrelizumab, both C16 and C24:1 ceramide species were decreased in both PPMS and RMS patients. All these findings together highlight that ceramides have the potential to reflect the pathophysiology of pwMS and that after treatment with ocrelizumab, the levels of these ceramides tend to be reduced. The implications for the
reduction of ceramides after treatment with Ocreluzimab should be further evaluated in larger independent cohorts.

C16 Glc Cer levels were also significantly correlated with levels of CSF NfH in both PPMS and RMS patients at baseline and in PPMS patients after treatment (Figure 4C). Neurofilaments such as NfL and NfH are highly specific neuroaxonal damage biomarkers and levels in CSF have been shown to correlate with disease activity and progression of disability (Khalil et al., 2018). Sphingolipids and NfH can both interact at the membrane level in the lipid rafts and regulate the stability of the neuronal cytoskeleton (Raju et al., 2015; Varela et al., 2016). Interestingly, there is less of reduction in C16 Glc Cer in PPMS patients and there is still a significant correlation between NfH and C16 Glc Cer after treatment. This could result from different disease biology mechanisms in PPMS patients compared to RMS patients, with more neuroinflammation in the latter population, thus suggesting sphingolipids may reflect ongoing progressive pathobiology in this more severe disabled patient population.

The EDSS score reflects a summary of disabilities among clinical functions in people with pwMS and is one of the main clinical variables tracked in pwMS. The EDSS has previously been shown to correlate with levels of hexosylceramides in CSF from RMS patients (Checa et al., 2015). In this study, while there was a correlation between EDSS and C16 Glc Cer at baseline, this correlation was abrogated after treatment with ocrelizumab as C16 Glc Cer levels significantly decreased. In contrast, PPMS patients experienced a slight increase in EDSS score during treatment and the C16 Glc Cer correlation with EDSS remained significant both before and after treatment. As the difference between RMS and PPMS are related to level of CNS inflammation, with RMS
patients having more neuroinflammation than PPMS patients, the level of CNS
neuroinflammation may underlie the differences in C16 Glc Cer correlations between
the two subtypes. C16 Glc Cer was elevated in PPMS patients and might be a good
marker of chronic neuroaxonal damage and neurodegeneration. While these results are
consistent with previous reports that have correlated CSF and serum glucosylceramide
levels with EDSS (Checa et al., 2015; Filippatou et al., 2021), this study is small and
further research is needed to fully understand this relationship.

One of the most common pathological features in pwMS is BBB impairment
(Iwasaki, 2017; Kanda, 2013). An important question that remains is whether the
sphingolipids measured in CSF are in fact derived from the CNS or whether they enter
CSF from the periphery through the impaired BBB. Alb-Quot measures the ratio of
albumin in CSF compared to plasma and can be used to indirectly assess the level of
BBB permeability. Various sphingolipids species were observed to have high correlation
with Alb-Quot at baseline and after treatment in both PPMS and RMS patients. The
origin of the sphingolipids measured in this study remains unclear and requires further
investigation with larger clinical cohorts. Similarly, the lesion load of most of the patients
in this study was minimal and evaluating patients with higher lesion burden would
enhance the significance of the findings. One of the limitations of this study is the small
sample size, although interesting trends were observed. We did not perform statistical
corrections for multiple correlation testing, and thus these findings should be considered
as exploratory and hypothesis generating to be further corroborated in a larger clinical
cohort.
The scope of this study was limited to the analytes described in this paper; we acknowledge that other isobaric species may be present in our samples and co-eluting with our analytes of interest. Hence, this may result in an overestimation of some of the species here measured.

In summary, this study has two important conclusions. One is that utilizing the LC-MS targeted approach described here can be used to accurately quantify 14 sphingolipids in human CSF. Among the main strengths of this method is its ability to accurately discern between the isomers Glc Cer and Gal Cer. Second, results from this study suggest that the CSF sphingolipid profile is altered by multiple sclerosis clinical subtype and treatment with a B cell-depleting monoclonal antibody. Altogether, these findings support further research of sphingolipids as potential biomarkers in neurodegenerative diseases and other CNS disorders. Specifically, C16 Glc Cer warrants further investigation as a fluid biomarker reflective of multiple sclerosis disease pathobiology. These results may help to understand the role of sphingolipids involved in multiple sclerosis relapsing and progressive disease biology and support use of sphingolipids to inform drug development of new multiple sclerosis treatments.

Acknowledgments
We acknowledge and thank all the patients and clinicians that participated in the OBOE study.

Data availability statement
The authors declare that all the data supporting the findings of this study are available within the paper and its Supplemental Data.
Authorship Contributions

*Participated in research design:* Anania, V.G., Mathews W.R., Harp C., Perez Paramo Y.X.

*Conducted experiments:* Perez Paramo Y.X., Dufield D., Veeramachaneni R. Parkhurst E.

*Contributed new reagents or analytic tools:* Dufield D, Veeramachaneni R., Parkhurst E.

*Performed data analysis:* Perez Paramo Y.X.

*Wrote or contributed to the writing of the manuscript:* Perez Paramo Y.X., Anania, V.G, Mathews W.R., Harp C., Winger R. C., Cross A.H., Gelfand J.M., Bar-Or Aθ Ramesh A., Dufield D

Conflicts of interest

The authors also report the following conflict of interest: Yadira X Perez-Paramo, Christopher Harp, Akshaya Ramesh, Ryan C. Winger, W. Rodney Mathews, and Veronica G. Anania are employees of Genentech, Inc. and shareholders of F. Hoffmann-La Roche Ltd. Dawn Dufield, Rathna Veeramachaneni, and Emily Parkhurst are employees of KCAS. Jeffrey Gelfand reports research support to his institution from Hoffman La Roche / Genentech, research support to his institution from Vigil Neurosciences, and consulting for Arialys. Anne H. Cross was supported in part by the Manny & Rosalyn Rosenthal – Dr. John L. Trotter Chair in Neuroimmunology, and received honoraria for consulting or serving on scientific advisory boards from Biogen, Bristol Myers Squibb, EMD Serono, Genentech, Horizon, Janssen (J&J), Novartis,
Octave, Roche, and TG Therapeutics, and contracted grants from Genentech Inc. and F. Hoffmann-LaRoche Ltd.
References


Footnotes

This work received no external funding.

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**Figure legends**

**Figure 1.** A) Multiple sclerosis disease biology illustration: immune cells and antibodies infiltrate into the central nervous system causing local inflammation and tissue damage resulting in neuroaxonal myelin damage and neurodegeneration. B) Sphingolipids *de novo* synthesis pathway highlighting the families of molecules quantified in this study in bold. C) Experimental study design for the cross-sectional study and longitudinal studies.

**Figure 2.** Representative LC-MS/MS chromatograms. A) 14-plex sphingolipid MRM method, each panel is a representative chromatogram (standards in buffer) of a sphingolipid family. B) **Representative chromatogram for the** SPBP method (standard in buffer). For each compound, the ratio of the analyte peak area to that of the deuterated standard (or the closest in RT) was used for quantification. The full list of analytes and their corresponding internal standards are listed in Supplementary Table 1.

**Figure 3.** Sphingolipid levels in the cross-sectional study. A) Spearman’s correlations among sphingolipid species across all CSF samples. Sphingolipid species levels (ng/mL) in PPMS, RMS, and healthy controls for B) C16 Cer; C) C24:1 Cer; D) C16 Glc Cer, and E) C16 Gal Cer are shown. The horizontal lines within each box plot are median values, while individual values are represented in each graph. One-way ANOVA Tukey’s multiple comparisons: unadjusted p values, * P<0.05, ** P<0.01, ***P<0.001, ****P<0.0001.
Figure 4. Sphingolipid levels observed in the longitudinal cohort. 

A) Percent change of sphingolipids after treatment with ocrelizumab in CSF from PPMS (n=27) and RMS (n=17) patients. 

B) Spearman correlations between sphingolipid levels and clinically relevant variables at baseline and after treatment with ocrelizumab in CSF from PPMS (n=27) and RMS (n=17) patients. 

C) Spearman coefficient correlation analysis between C16 Glc Cer and CSF NfH levels. 

D) Spearman coefficient correlation analysis between C16 Glc Cer and EDSS. Unadjusted p values: * P<0.05, ** P<0.01. There were no Gd+ lesions after treatment so no correlation was calculated.
Table 1. Method qualification parameters for multiplexed and single plex method.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Linearity</th>
<th>Accuracy</th>
<th>Precision</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LOD ng/ml</td>
<td>LOQ ng/ml</td>
<td>R2</td>
</tr>
<tr>
<td>C14 Ceramide</td>
<td>0.006</td>
<td>0.05</td>
<td>0.99</td>
</tr>
<tr>
<td>C16 Ceramide</td>
<td>0.01</td>
<td>0.05</td>
<td>0.98</td>
</tr>
<tr>
<td>C18 Ceramide</td>
<td>0.02</td>
<td>0.05</td>
<td>0.96</td>
</tr>
<tr>
<td>C20 Ceramide</td>
<td>0.02</td>
<td>0.05</td>
<td>0.98</td>
</tr>
<tr>
<td>C22 Ceramide</td>
<td>0.01</td>
<td>0.05</td>
<td>0.99</td>
</tr>
<tr>
<td>C24:1 Ceramide</td>
<td>0.05</td>
<td>0.05</td>
<td>0.97</td>
</tr>
<tr>
<td>C26:1 Ceramide</td>
<td>0.04</td>
<td>0.05</td>
<td>0.99</td>
</tr>
<tr>
<td>C14 Glucosyl (SC) Ceramide</td>
<td>0.01</td>
<td>0.05</td>
<td>0.96</td>
</tr>
<tr>
<td>C14 Galactosyl (SC) Ceramide</td>
<td>0.02</td>
<td>0.05</td>
<td>0.98</td>
</tr>
<tr>
<td>C14 Lactoyl (SC) Ceramide</td>
<td>0.02</td>
<td>0.05</td>
<td>0.99</td>
</tr>
<tr>
<td>C16 Lactoyl (SC) Ceramide</td>
<td>0.02</td>
<td>0.05</td>
<td>0.99</td>
</tr>
<tr>
<td>Sphingosine</td>
<td>0.1</td>
<td>0.05</td>
<td>0.99</td>
</tr>
<tr>
<td>C18:1 Glucosyl (SC) Sphingosine</td>
<td>0.05</td>
<td>0.05</td>
<td>0.99</td>
</tr>
<tr>
<td>C24:1 Galactosyl (SC) Sphingosine</td>
<td>0.05</td>
<td>0.05</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Sphingosine 1-phosphate:
- LOD ng/ml: 0.01
- LOQ ng/ml: 0.05
- R2: 0.98
- Accuracy: 8.4
- % CV: 135.4, 330.6, 113.1, 167.2, 306.4
- Precision: 17, 2.3, 1.6, 3.9, 3.4
Table 2. Sphingolipid levels in the cross-sectional study. PPMS n=10, RMS n=10, and healthy controls n=10. P values were determined using one-way ANOVA Tukey’s with multiple comparisons.

<table>
<thead>
<tr>
<th>Sphingolipid Type</th>
<th>PPMS Average</th>
<th>PPMS S. D.</th>
<th>RMS Average</th>
<th>RMS S. D.</th>
<th>Healthy Average</th>
<th>Healthy S. D.</th>
<th>p value***</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14 Ceramide</td>
<td>0.117</td>
<td>0.008</td>
<td>0.116</td>
<td>0.006</td>
<td>0.111</td>
<td>0.007</td>
<td>0.160</td>
</tr>
<tr>
<td>C16 Ceramide</td>
<td>0.692</td>
<td>0.113</td>
<td>0.694</td>
<td>0.106</td>
<td>0.540</td>
<td>0.129</td>
<td>0.018</td>
</tr>
<tr>
<td>C18 Ceramide</td>
<td>3.405</td>
<td>0.485</td>
<td>3.207</td>
<td>0.686</td>
<td>2.568</td>
<td>1.107</td>
<td>0.068</td>
</tr>
<tr>
<td>C20 Ceramide</td>
<td>0.812</td>
<td>0.105</td>
<td>0.857</td>
<td>0.194</td>
<td>0.701</td>
<td>0.176</td>
<td>0.109</td>
</tr>
<tr>
<td>C24 Ceramide</td>
<td>1.278</td>
<td>0.731</td>
<td>1.072</td>
<td>0.364</td>
<td>0.807</td>
<td>0.495</td>
<td>0.145</td>
</tr>
<tr>
<td>C18:1 Ceramide</td>
<td>0.019</td>
<td>0.008</td>
<td>0.015</td>
<td>0.005</td>
<td>0.020</td>
<td>0.006</td>
<td>0.145</td>
</tr>
<tr>
<td>C24:1 Ceramide</td>
<td>1.171</td>
<td>0.398</td>
<td>0.917</td>
<td>0.227</td>
<td>0.652</td>
<td>0.261</td>
<td>0.003</td>
</tr>
<tr>
<td>C16 Glucosyl (β) Ceramide</td>
<td>1.467</td>
<td>0.485</td>
<td>1.331</td>
<td>0.401</td>
<td>0.942</td>
<td>0.487</td>
<td>0.044</td>
</tr>
<tr>
<td>C16 Galactosyl (β) Ceramide</td>
<td>0.729</td>
<td>0.256</td>
<td>0.701</td>
<td>0.231</td>
<td>0.490</td>
<td>0.294</td>
<td>0.102</td>
</tr>
<tr>
<td>C16 Lactosyl (β) Ceramide</td>
<td>7.400</td>
<td>5.539</td>
<td>5.999</td>
<td>1.823</td>
<td>5.250</td>
<td>2.696</td>
<td>0.432</td>
</tr>
<tr>
<td>C18 Lactosyl (β) Ceramide</td>
<td>2.431</td>
<td>0.631</td>
<td>2.308</td>
<td>0.602</td>
<td>2.122</td>
<td>0.865</td>
<td>0.622</td>
</tr>
<tr>
<td>C18:1 Glucosyl (β) sphingosine</td>
<td>0.015</td>
<td>0.028</td>
<td>0.008</td>
<td>0.023</td>
<td>0.012</td>
<td>0.038</td>
<td>0.884</td>
</tr>
<tr>
<td>C18:1 Galactosyl (β) sphingosine</td>
<td>0.029</td>
<td>0.026</td>
<td>0.016</td>
<td>0.008</td>
<td>0.004</td>
<td>0.006</td>
<td><strong>0.007</strong></td>
</tr>
<tr>
<td>Sphingosine-1-phosphate**</td>
<td>0.031</td>
<td>0.024</td>
<td>0.024</td>
<td>0.005</td>
<td>0.026</td>
<td>0.008</td>
<td>0.569</td>
</tr>
</tbody>
</table>

Sphingolipid levels were BLOD for all samples

* Levels in ng/ml

** Levels in pg/ml

***One way ANOVA
**Table 3.** Patient demographic and clinical information at baseline and after treatment. Median values are shown (Interquartile Range). P values were determined using one-way ANOVA Tukey’s with multiple comparisons.

<table>
<thead>
<tr>
<th></th>
<th>PPMS</th>
<th></th>
<th></th>
<th></th>
<th>RMS</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>After treatment</td>
<td>p value*</td>
<td>Baseline</td>
<td>After treatment</td>
<td>p value*</td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>27</td>
<td>NA</td>
<td></td>
<td>17</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>44 (41-47)</td>
<td>NA</td>
<td></td>
<td>37 (32-43)</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI</td>
<td>27 (25-30)</td>
<td>NA</td>
<td></td>
<td>26.9 (25-29)</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female (%)</td>
<td>50</td>
<td>NA</td>
<td></td>
<td>70</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDSS**</td>
<td>3.5 (3.0-4.0)</td>
<td>3.5 (2.9-5.1)</td>
<td>&gt;0.999</td>
<td>2.3 (1.3-2.8)</td>
<td>1.5 (1.01-2.0)</td>
<td>&gt;0.999</td>
<td></td>
</tr>
<tr>
<td>Normalized brain volume, cm³</td>
<td>1405 (1381-1435)</td>
<td>1398 (1381-1430)</td>
<td>&gt;0.999</td>
<td>1457 (1376-1493)</td>
<td>1459 (1383-1484)</td>
<td>&gt;0.999</td>
<td></td>
</tr>
<tr>
<td>T2 lesion volume, mm³</td>
<td>6.5 (2.9-16)</td>
<td>7.3 (2.7-16)</td>
<td>&gt;0.999</td>
<td>3.2 (1.2-5.1)</td>
<td>2.9 (0.09-3.7)</td>
<td>&gt;0.999</td>
<td></td>
</tr>
<tr>
<td>Gd+ lesions present</td>
<td>0.0 (0-0)</td>
<td>0.0 (0-0)</td>
<td>&gt;0.999</td>
<td>0.0 (0-1)</td>
<td>0.0 (0-0)</td>
<td>&gt;0.999</td>
<td></td>
</tr>
<tr>
<td>CSF NfL, pg/mL</td>
<td>741 (607-1070)</td>
<td>599 (469-1017)</td>
<td>0.047</td>
<td>1205 (771-1882)</td>
<td>564 (390-716)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>CSF NfH, pg/mL</td>
<td>621 (489-913)</td>
<td>686 (475-807)</td>
<td>0.976</td>
<td>731 (595-900)</td>
<td>608 (447-784)</td>
<td>0.312</td>
<td></td>
</tr>
<tr>
<td>Albumin Quotient</td>
<td>3.6 (2.5-5.6)</td>
<td>4.0 (2.8-5.4)</td>
<td>&gt;0.999</td>
<td>4.1 (3.3-5.7)</td>
<td>4.3 (3.2-4.9)</td>
<td>&gt;0.999</td>
<td></td>
</tr>
</tbody>
</table>

*p value* Adjusted p value (One-way ANOVA)
** Expanded Disability Status Scale
Table 4. Levels of sphingolipids (mean) in CSF from PPMS (n=27) and RMS (n=17) patients at baseline and after treatment with ocrelizumab. Percent of change from baseline is shown and p values were determined using paired t-tests.

<table>
<thead>
<tr>
<th>Sphingolipid</th>
<th>PPMS</th>
<th>RMS</th>
<th>Baseline</th>
<th>After treatment</th>
<th>% change</th>
<th>p value</th>
<th>Baseline</th>
<th>After treatment</th>
<th>% change</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14 Ceramide</td>
<td>0.01600</td>
<td>0.01563</td>
<td>-2.3</td>
<td>&gt;0.0009</td>
<td></td>
<td></td>
<td>0.015</td>
<td>0.013</td>
<td>-10.9</td>
<td>0.9807</td>
</tr>
<tr>
<td>C16 Ceramide</td>
<td>0.458</td>
<td>0.437</td>
<td>-4.7</td>
<td>0.8028</td>
<td></td>
<td></td>
<td>0.446</td>
<td>0.398</td>
<td>-10.7</td>
<td>0.8973</td>
</tr>
<tr>
<td>C18 Ceramide</td>
<td>1.439</td>
<td>1.512</td>
<td>5.0</td>
<td>0.6516</td>
<td></td>
<td></td>
<td>1.685</td>
<td>1.463</td>
<td>-13.2</td>
<td>0.0721</td>
</tr>
<tr>
<td>C20 Ceramide</td>
<td>0.133</td>
<td>0.134</td>
<td>0.1</td>
<td>0.9954</td>
<td></td>
<td></td>
<td>0.145</td>
<td>0.136</td>
<td>-6.6</td>
<td>0.9386</td>
</tr>
<tr>
<td>C24 Ceramide</td>
<td>0.322</td>
<td>0.321</td>
<td>-0.1</td>
<td>0.9962</td>
<td></td>
<td></td>
<td>0.439</td>
<td>0.454</td>
<td>3.4</td>
<td>0.9004</td>
</tr>
<tr>
<td>C22:1 Ceramide</td>
<td>0.012</td>
<td>0.013</td>
<td>6.8</td>
<td>&gt;0.0009</td>
<td></td>
<td></td>
<td>0.012</td>
<td>0.012</td>
<td>-0.5</td>
<td>0.9986</td>
</tr>
<tr>
<td>C24:1 Ceramide</td>
<td>0.667</td>
<td>0.643</td>
<td>-3.6</td>
<td>0.8824</td>
<td></td>
<td></td>
<td>0.644</td>
<td>0.579</td>
<td>-10.0</td>
<td>0.801</td>
</tr>
<tr>
<td>C26 Glucosyl (B) Ceramide</td>
<td>0.679</td>
<td>0.611</td>
<td>-10.0</td>
<td>0.8716</td>
<td></td>
<td></td>
<td>0.590</td>
<td>0.435</td>
<td>-26.3</td>
<td>0.2079</td>
</tr>
<tr>
<td>C16 Galactosyl (B) Ceramide</td>
<td>0.451</td>
<td>0.448</td>
<td>-0.6</td>
<td>0.9871</td>
<td></td>
<td></td>
<td>0.393</td>
<td>0.353</td>
<td>-10.2</td>
<td>0.7445</td>
</tr>
<tr>
<td>C16 Lactosyl (B) Ceramide</td>
<td>3.127</td>
<td>3.371</td>
<td>7.8</td>
<td>0.1288</td>
<td></td>
<td></td>
<td>2.678</td>
<td>2.538</td>
<td>-5.2</td>
<td>0.2556</td>
</tr>
<tr>
<td>C18 Lactosyl (B) Ceramide</td>
<td>1.425</td>
<td>1.472</td>
<td>3.3</td>
<td>0.7693</td>
<td></td>
<td></td>
<td>1.344</td>
<td>1.298</td>
<td>-3.4</td>
<td>0.704</td>
</tr>
<tr>
<td>C18 Glucosyl [B] sphingosine</td>
<td>0.004--</td>
<td>0.007</td>
<td>78.3</td>
<td>0.3061</td>
<td></td>
<td></td>
<td>0.004--</td>
<td>0.006</td>
<td>36.2</td>
<td>0.6268</td>
</tr>
<tr>
<td>C18:1 Galactosyl (B) sphingosine</td>
<td>8.000</td>
<td>0.002--</td>
<td>NA</td>
<td>NA</td>
<td></td>
<td></td>
<td>8.000</td>
<td>8.000</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Sphingosine 1-phosphate</td>
<td>7.58</td>
<td>6.85</td>
<td>-10.6</td>
<td>NA</td>
<td></td>
<td></td>
<td>5.725</td>
<td>5.224</td>
<td>-8.8</td>
<td>0.566</td>
</tr>
</tbody>
</table>

CSF levels in ng/mL.
*Paired t-test for baseline/after treatment, 0.05 FDR corrected
**AOCG 0.005 ng/mL, but above LOQ
***CSF levels in µg/mL.