Distinctive sphingolipid patterns in chronic multiple sclerosis lesions

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Running title: Potential sphingolipid biomarkers in progressive MS

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The abbreviations used are: C1P, ceramide 1-phosphate; Cer, ceramide; cPLA2α, cytosolic phospholipase A2α; CSF, cerebrospinal fluid; dhCer, dihydroceramide; EAE, experimental autoimmune encephalomyelitis; GluCer, glucosylceramide; H&E, hematoxylin and eosin; HexCer, hexosylceramide; HPLC-MS/MS, high-performance liquid chromatography-tandem mass spectrometry; ISs, internal standards; LacCer, lactosylceramide; LFB, Luxol fast blue; MS, multiple sclerosis; Ac-MS, chronic active multiple sclerosis; In-MS, chronic inactive multiple sclerosis;
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NAWM, normal appearing white matter; nCNS, normal central nervous system; OND, other neurological diseases; I-OND, inflammatory other neurological diseases; NI-OND, non-inflammatory other neurological diseases; SL, sphingolipid; SM, sphingomyelin; Sph, sphingosine; SPT, serine palmitoyltransferase.

ABSTRACT
Multiple sclerosis (MS) is a CNS disease characterized by immune-mediated demyelination and progressive axonal loss. MS-related CNS damage and its clinical course have two main phases: active and inactive/progressive. Reliable biomarkers are being sought to allow identification of MS pathomechanisms and prediction of its course. The purpose of this study was to identify sphingolipid (SL) species as candidate biomarkers of inflammatory and neurodegenerative processes underlying MS pathology. We performed sphingolipidomic analysis by high-performance liquid chromatography-tandem mass spectrometry to determine the lipid profiles in post mortem specimens from the normal appearing white matter (NAWM) of normal CNS (nCNS), from subjects with chronic MS (active and inactive lesions) as well as from patients with other neurological diseases. Distinctive SL modification patterns occurred in specimens from MS patients with chronic inactive plaques with respect to NAWM from nCNS and active MS lesions. Chronic inactive MS lesions were characterized by decreased level of dihydroceramide (dhCer), ceramide, and sphingomyelin subspecies whereas level of hexosylceramide and ceramide-1-phosphate (C1P) subspecies was significantly increased in comparison to NAWM of nCNS as well as active MS plaques. In contrast, active MS lesions were characterized by a significant increase of major dhCer subspecies in comparison to NAWM of nCNS. These results suggest the existence of different SL metabolic pathways in the active versus inactive phase within progressive stage of MS. Moreover, they suggest that C1P could be a new biomarker of inactive MS progressive phase, and its detection may help to develop future prognostic and therapeutic strategies for the disease.
Supplementary keywords: brain lipids, central nervous system, ceramides, ceramide 1-phosphate, clinical lipidology, inflammation, lipidomics, neurodegeneration, neurological diseases, mass spectrometry.

INTRODUCTION

Multiple sclerosis (MS) is a polyphasic immune-mediated disorder characterized by multifocal inflammatory infiltrates (T cells, B cells, and macrophages) within CNS, with concomitant degradation of myelin sheath, oligodendrocytes and axons, along with reactive astrogliosis and activated microglia (1).

Multiple areas of myelin loss within CNS called “plaques” or “lesions” are the pathologic hallmark of MS. It is evident that MS lesions evolve differently during early/acute versus chronic phase of the disease and within each phase, different plaque types occur in particular stages of activity (2). Furthermore, it is well known that degradation of minor myelin proteins (myelin oligodendrocyte glycoprotein, myelin associated glycoprotein, 2',3'-cyclic-nucleotide 3'-phosphodiesterase) denotes early active plaques, whereas the presence of hydrophobic major myelin proteins (proteolipid protein, myelin basic protein) indicates late active lesions. Inactive lesions are infiltrated by macrophages that lack myelin debris, but may still contain empty vacuoles/or periodic acid-Schiff-positive degradation products; the results of the macrophages' inability to digest the myelin neutral lipid components (2). As the plaque progresses from acute /active to chronic/inactive, its edema resolves, inflammation decreases, and macrophages and microglia gradually disappear. Astrocytes produce glial scars that fill the demyelinated plaque. These characteristics prompted Charcot to name these lesions as sclerotic plaques (3), appearing as a major autopsy finding in MS subjects.

MS-related damage to CNS tissues has been found to include two main pathological processes: inflammatory myelin destruction (demyelination) and progressive, irreversible axonal loss (neurodegeneration). The underlying pathology of inflammatory component is generally believed to be associated with an autoimmune attack upon myelin antigens. However, extensive studies have not yet established the predominant target antigenic structures involved in the autoimmune response most relevant for MS background (4). Both processes were shown to be initiated at the disease onset, but
they develop with different dynamics: the peak of inflammatory activity occurs in the early stages of MS, while neurodegeneration with axonal loss is gradually escalating towards more advanced progressive stages (5). Contribution of these processes to MS-related CNS damage corresponds with clinical course of MS, defined as relapsing-remitting, secondary progressive or primary progressive. More recent concept of MS course assumes distinguishing two main phases of the disease: active and inactive/progressive, which may be temporarily overlapping (6). Despite significant progress in diagnostics and therapeutic advances in recent years, there are still some problems which need to be elucidated. Firstly, great individual variability of MS course and response to treatment hinders the prognosis of the disease outcome. Another challenge is associated with managing inactive/progressive phase of the disease, while available treatment options almost exclusively target the active one. Therefore, reliable biomarkers are being sought to allow identification of the disease pathogenic mechanisms and prediction of its clinical course. There is sufficient evidence for relevant indices of inflammatory activity [intrathecal IgG synthesis, level of cytokines and chemokines or adhesion molecules in cerebrospinal fluid (CSF)] as well as neurodegeneration (level of neurofilaments and chitinase in CSF) in CNS. However, none of these markers turned out to be specific for MS, which limits their diagnostic and predictive value. Thus, there is a need to investigate new and more relevant biomarkers potentially useful in MS (7, 8).

Sphingolipids (SLs), as the major component of CNS myelin sheaths, seem to be potential biomarker candidates in MS (9). They participate in numerous inflammatory processes and are responsible for controlling intracellular trafficking and signaling, cell growth, adhesion, vascularization, survival, and apoptosis (10-12). Although SL-specific antibodies and T cells have been identified in MS (13, 14), very little is known about lipid composition in particular stages of MS plaques (acute vs. chronic) (15) as well as the role of bioactive lipids in CNS autoimmunity. SLs also exert pronounced effects on inflammation in the context of autoimmunity, by acting either as targets or regulators of the immune response. In addition, myelin sheath lipids have been reported to induce apoptosis in auto-reactive T cells (16) and ameliorate experimental autoimmune encephalomyelitis (EAE) (17, 18). In particular, ceramide (Cer) and the enzymes linked to its production have been
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It seems that lipids in CNS induce perturbations in balance of anti- and pro-inflammatory that are essentially involved in MS pathology. Recent evidence suggests that alterations in SL pathways may reflect disease activity (23, 24). Due to the activity of some essential hydrolytic enzymes, such as SMases, Cer of different chain lengths may be participated in different cellular processes such as differentiation, proliferation and programmed cell death (12, 25).

Comprehensive profiling of CNS lipids in MS lesions might provide a better insight into their role in the pathogenesis of the disease, including an attempt to define the metabolic pathways leading to autoimmune demyelination and/or neurodegeneration. Such findings might contribute to evaluate the usefulness of SLs as biomarkers of various phases of MS or even as potential targets for therapeutic interventions. Myelin lipids in MS have already been investigated in some studies, but few of them analyzed their distribution in brain MS tissues (17, 26, 27) and in CSF (19, 28-30), which might allow a direct insight into in disease-related CNS damage.

In this study we performed a targeted sphingolipidomic analysis of post mortem brain tissues in patients with MS as well as subjects with CNS affected by other neurological diseases (OND) or with substantially normal CNS (nCNS). The comparative analyses were further conducted for particular types of MS lesions, namely chronic active and chronic inactive. We have observed the distinctive pattern of SL metabolism in chronic inactive MS (In-MS) lesions in comparison to nCNS. Based on our findings we propose ceramide 1-phosphate (C1P) to be a potential new biomarker of MS progressive phase.
MATERIALS AND METHODS

Human autopsy brain tissues

Fresh frozen brain tissues were obtained from the Human Brain and Spinal Resource Center, VA West Los Angeles Healthcare Center, 11301 Wilshire Blvd. Los Angeles, CA 90073 which is sponsored by National Institutes of Health, National MS Society and the US Department of Veterans Affairs. Brain tissue specimens were derived from autopsy of patients with clinically diagnosed and neuropathologically confirmed MS (n=13) and OND (n=15) as well as from 3 controls who had been diagnosed with diseases without CNS involvement, with essentially normal brain confirmed on autopsy findings, nCNS. OND samples were further subdivided into inflammatory (I-OND, n=5) and non-inflammatory (NI-OND, n=10) reference subgroups. Patients’ clinical and autopsy characteristics is provided in Table 1. All procedures performed in this study were in accordance with ethical standards of the institutional ethics committees. An informed consent was obtained at UCLA from the human subjects or their representatives followed by the Declaration of Helsinki. Preservation of anonymity, confidentiality, and masking of samples was maintained throughout all studies.

Histopathology and immunochemistry

Normal appearing and pathological tissues were selected by gross examination and verified by microscopic examination. To assess the presence of myelin and identify areas of demyelinated plaques and NAWM frozen 4 μm thick cryostat tissues sections were stained with Luxol fast blue (LFB) and hematoxylin and eosin (H&E), myelin stains. Neuropathological evaluation comprised also staining for axons (Bielschowsy’s silver impregnation). This allowed identification of lymphocytes, plasma cells as well as foamy macrophages containing myelin degradation products. The stage of lesional development was determined immunochemically as well. Anti-CD68 antibody was used to identify macrophages (data not shown). MS lesions were classified as either chronic active, Ac-MS (profound inflammation with macrophages present) or inactive, In-MS (immunologically silent).

Tissues homogenization and protein determination

Part of each frozen human brain tissue (100 mg) was cut and homogenized with 2 ml of tissue homogenization buffer containing 0.25 M sucrose, 25 mM KCl, 50 mM Tris-HCl and 0.5 mM EDTA,
pH 7.4. Homogenization was performed on ice using a Polytron electric homogenizer until no solid pieces were observed. Next, 100 μl of the tissue homogenate was diluted 1:10 with the homogenization buffer and aliquots of 10 μl were taken for protein determination assay using Pierce BCA protein assay kit. Aliquots corresponding to 1 mg of protein were transferred to 15 ml Falcon tubes and subjected to lipids extraction.

**Lipid extraction**

All solvents were analytical grade from Fisher Scientific (Hampton, NH). SL standards were from MUSC Lipidomics Shared Resource or from a commercially available source (Avanti Polar Lipids, Matreya LLC), with purity of ≥ 98%.

Homogenates of tissues (1 mg per protein) were fortified with 50 μl of the appropriate internal standards (ISs), specifically: sphingosine (Sph)/Cer/dhCer ISs [17C base D-erythro-sphingosine (17C/Sph), 17C base D-erythro-sphingosine 1-phosphate (17C/S1P), 17C base D-erythro-dihydrosphingosine (17C/dhSph), D-erythro-N-palmitoyl-13C-D-erythro-sphingosine (13C/C16-Cer), N-heptadecanoyl-D-erythro-sphingosine (18C/C17-Cer), N-heptadecanoyl-D-erythro-dihydrosphingosine (18C/C17-dhCer), D-erythro-N-palmitoyl-17C-D-erythro-sphingosine (17C/C16-Cer), D-erythro-N-nervonoyl-17C-D-erythro-sphingosine (17C/C24:1-Cer)]; hexosylceramide (HexCer)/lactosylceramide (LacCer) ISs [18C/C8-glucosylceramide (GluCer), 18C/C12-GluCer, 18C/C8-LacCer, 18C/C12-LacCer]; C1P ISs [17C/C16-C1P, 17C/C18:1-C1P, 17C/C24-C1P] and sphingomyelin (SM) ISs [D-erythro-C6-SM (18C/C6-SM), D-erythro-C17-SM (18C/C17-SM)].

Lipids were extracted with 2 ml of one phase solvent system containing ethyl acetate/isopropanol/water (60/30/10%; v/v). The upper organic phase was transferred to a glass tube. To the remaining diluted tissue homogenates an additional 2 ml of extraction solution was added to further facilitate complete extraction. The upper organic phase was then transferred and added to the glass tube containing the initial extract (total 4 ml extract). The lipid extract was divided into two parts. Part A (1 ml) was subjected to base mild alkaline hydrolysis in order to remove glycerolipids interfering with SM analysis as reported previously (31) and used for analysis of SM subspecies. The remaining 3 ml of extract (part B) was used for analysis of Cer, dhCer, sphingoid bases and their 1-
phosphate derivatives as well as HexCer, LacCer and C1P. Both extracts, A and B, after evaporation and reconstitution in 150 µl of acidified with 0.2% formic acid methanol, were stored at 4°C prior to injection on the high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) system.

**Sphingolipidomic analysis by HPLC-MS/MS**

Analyses of SLs were performed by HPLC-MS/MS at MUSC Lipidomics Shared Resource. SLs analyzed included sphingoid bases (C18:1, C18:0) – Sph, dhSph and their derivatives (S1P and dhS1P) as well as dhCer, Cer, SM, HexCer, LacCer and C1P species. HPLC-MS/MS analysis was performed on a ThermoFisher TSQ Quantum or SCIEX Q-Trap triple-stage quadrupole mass spectrometer, operating in a multiple reaction monitoring positive ionization mode, as previously described (32, 33). Chromatographic separations were obtained under a gradient elution using mobile phase A consisting of 2 mM ammonium formate in 0.2% formic acid in water, and mobile phase B consisted of 1 mM ammonium formate in 0.2% formic acid in methanol, on the BDS Hypersil C8, 150 x 3.2 mm, 3 µm particle size column.

Peaks corresponding to the target analytes of SLs and ISs were collected and processed using the Xcalibur™ software system (Fisher Scientific). Quantitative analysis was based on calibration curves using a linear regression model as described previously (20). SLs with unavailable standards were quantified using the calibration curve of its closest counterpart.

**Statistical analysis**

SL levels for each sample were calculated by summing up the total number of all SL subspecies measured expressed in pmoles/mg of protein and then normalizing that total to 100%. Because of the uneven distribution of our data, non-parametric test was used for each two groups comparison. Statistical differences between groups were determined by Mann Whitney test using GraphPad PRISM 7.01, with p<0.05 being considered as statistically significant.
RESULTS

Characteristics of MS lesions studied

Histopathological analysis in clinically diagnosed cases of MS revealed features typical for this disease such as demyelination, oligodendrocyte and axonal loss, inflammation with evidence of monocyte infiltrates present, and some degree of gliosis. MS lesions were classified depending on their activity, examples of which are shown in Figure 1. Of the 13 plaques examined, 4 were classified as chronic active and 9 were categorized as chronic inactive type (Table 1).

Sphingolipid profile in NAWM of the unaffected CNS

Compositional analysis of total SLs indicated that SM was the dominant species of NAWM of nCNS (Figure 2A). The lipid composition of NAWM of nCNS was as follows: SM (72.6 ± 4.4%) followed by Cer (13.5 ± 0.09%), HexCer (11.6 ± 0.54%), LacCer (0.9 ± 0.03%), C1P (0.8 ± 0.04%), sphingoids and their derivatives (0.3 ± 0.01%) as well as dhCer species (0.3 ± 0.02%), respectively (Figure 2B).

The most abundant SM subspecies were identified to be: C18-SM (29.5 ± 2.6%), C24:1-SM (19.6 ± 3.1%), C16-SM (12.9 ± 2.6%) and C24-SM (11.4 ± 1.2%) (Supplemental Figure S1A). The next largest group was comprised of Cer species, which was composed of C18-Cer (52.0 ± 1.9%), C18:1-Cer (16.0 ± 2.7%) and C24:1-Cer (14.2 ± 0.2%) (Supplemental Figure S1B). Contrary to SM precursors of Cer, precursors derived from de novo Cer synthesis, i.e., dhCer species constitutes very low amount of SLs. The major dhCer species were dhC18-Cer (39.6 ± 1.1%), dhC24:1-Cer (26.1 ± 3.0%) and dhC24:0-Cer (6.8 ± 0.9%) (Supplemental Figure S1C). In addition, similar abundance to Cer subspecies constitute HexCer subspecies that included: C18-HexCer (42.0 ± 2.6%), C24:1-HexCer (30.5 ± 0.9%) and C24:0-HexCer (7.4 ± 1.1%) (Supplemental Figure S2A). Comparatively, SLs such as LacCer subspecies: C18-LacCer (53.4 ± 3.2%); C16-LacCer (32.8 ± 4.0%); C24:1-LacCer (9.5 ± 2.4%) (Supplemental Figure S2B) as well as C1P subspecies: C18:0-C1P (40.3 ± 7.0%); C24-C1P (15.8 ± 1.7%) and C24:1-C1P (15.8 ± 1.7%) (Supplemental Figure S3C) made up a very small proportion of the entire sphingolipidome (Figure 2A). Very low abundance SLs including sphingoids and their phosphate derivatives (Figure 2A) were also measured (Supplemental Figure S1D).
detailed molecular distribution of the individual lipid subspecies measured are summarized in Supplemental Figs. S1-S2.

Overall, whereas the lipid profile of Ac-MS lesions (Figure 2C) seemed to be comparable with that of NAWM of nCNS (Figure 2B), the lipid profile of In-MS lesions was significantly different (Figure 2E). The most striking changes in In-MS lesions were observed for C1P (4.2 fold increase), HexCer (4.0 fold increase), Cer (4.4 fold decrease), dhCer (3.6 fold decrease), sphingoids (1.9 fold increase) and SM (1.6 fold decrease) content in comparison to Ac-MS lesions. Another observation was that lipid profile of Ac-MS (Figure 2C) seemed to be similar to the profile from I-OND subgroup (Figure 2D), whereas the profile of In-MS seemed to be comparable with that of NI-OND controls (Figure 2F).

**Cer and its main precursors are dependent on MS activity**

To determine the relationships between active/inactive type of MS-related chronic brain damage and bioactive lipids such as Cer and its main precursors derived from *de novo* Cer synthesis as well as SM hydrolysis, we determined the level of Cer, dhCer and SM subspecies in chronic Ac- and In-MS lesions. Beside NAWM from nCNS, two types of reference groups, I-OND and NI-OND, were included. There was a clear decrease in total Cer level in In-MS plaques in comparison to NAWM from nCNS as well as I-OND reference group (Figure 3F), including major Cer of nCNS subspecies: C16:0- (Figure 3A), C18:0- (Figure 3B) and C18:1- (Figure 3C). Contrary to In-MS plaques, in Ac-MS plaques there was only slight decrease in Cer level in comparison to nCNS (Figure 3F), whereas C16:0- (Figure 3A), C18:0- (Figure 3B), and C18:1-Cer subspecies (Figure 3C) were significantly up-regulated resulting in significantly increased total Cer level compared to NI-OND group (Figure 3F). Most of the major Cer subspecies in Ac-MS lesions were also significantly increased in comparison to In-MS (Figure 3, panels A, B & C) accounting for the significant increase of the total Cer content (Figure 2C and Figure 3F).

The overall Cer level was increased in Ac-MS plaques, mostly due to their major dhCer precursors, i.e. C18:0 (Figure 4B), C24:0 (Figure 4D) and C24:1 (Figure 4E), indicating that *de novo* Cer synthesis was active (Figure 4F). Analyses of dhCer species in In-MS lesions compared to nCNS
and I-OND group elicited no statistically significant differences on major dhCer types except for a significant decrease of C18:0-dhCer (Figure 4B) accounting for the significantly decreased total dhCer level (Figure 4F).

As shown in Figure 5 analyses of SM subspecies in In-MS lesions indicated a significant decrease of C18:0- (Figure 5B), C18:1- (Figure 5C) and C24-SM (Figure 5D) subspecies compared to nCNS suggesting that SM→Cer pathway is active. Contrary to In-MS plaques in Ac-MS lesions no significant differences regarding major SM subspecies were observed (Figure 5).

**Glycosylated Cer derivatives level is involved in MS activity**

It has been recently reported that glycosphingolipids may be participated in CNS chronic inflammation (17) and their levels in CSF may reflect disease progression (28). Therefore, we aimed to determine glycosylated Cer level, i.e., HexCer and LacCer profile in post mortem tissues in chronic MS lesions with or without features of activity (Figure 6 and 7). First, we analyzed whether HexCer species were modified according to disease activity. In Ac-MS plaques there were no significant HexCer level changes compared to nCNS whereas C16-HexCer (Figure 6A), C18-HexCer (Figure 6B), C18:1-HexCer (Figure 6C), C24-HexCer (Figure 6D) and C24:1-HexCer (Figure 6E) were significantly down-regulated compared to NI-OND group. In In-MS lesions all HexCer subspecies: C16-HexCer (Figure 6A), C18-HexCer (Figure 6B), C18:1-HexCer (Figure 6C), C24-HexCer (Figure 6D) and C24:1-HexCer (Figure 6E) were significantly up-regulated in comparison to nCNS (Figure 2B), Ac-MS lesions (Figure 2C) as well as I-OND control (Figure 2D), accounting to 4.0 fold increase of total HexCer (Figure 2E and Figure 6F).

Next, we investigated whether the MS activity dependent on altered HexCer level (Figure 6) in the studied plaques had any influence on the level of LacCer (Figure 7). Less clear discriminative changes in LacCer level between different types of MS lesions were observed. Contrary to our expectation in chronic MS plaques, there was no significant LacCer level changes compared to nCNS as well as OND groups. Indeed, C16-LacCer level in In-MS plaques was significantly decreased in comparison to nCNS (Figure 7A) whereas C24-LacCer level in In-MS plaques was significantly increased in comparison to nCNS, Ac-MS lesions and I-OND (Figure 7D).
Interestingly, some LacCer species, i.e., C24- (Figure 7D) and C24:1-LacCer (Figure 7E) were significantly decreased in Ac-and In-MS lesions compared to NI-OND controls.

**C1P level reflects disease progression**

We next assessed whether C1P subtypes is involved in disease activity (Figure 8). Surprisingly, an enormous up-regulation of C16-C1P (Figure 8A), C18-C1P (Figure 8B), C18:1-C1P (Figure 8C) and C24:1-C1P (Figure 8D) subspecies was observed in In-MS lesions compared to nCNS, Ac-MS lesions as well as I-OND group, as reflected by a striking increase of total C1P (Figure 8F). Contrary to that, Ac-MS plaques indicated significant decrease of C1P subspecies in comparison to NI-OND group (Figure 8, panels A-F).

**Sphingoids alterations in progressive MS**

To further understand the mechanism of MS course, sphingoids and their derivatives: dhSph and Sph as well as dhS1P and S1P, respectively, were quantified in particular phases of the disease (Figure 9). Sph content was increased in Ac-MS lesions in comparison to In-MS (Figure 9B). Elevation in S1P occurred in In-MS plaques compared to nCNS, I-OND and NI-OND group as well as Ac-MS (Figure 9D) while its precursor dhS1P showed significant alteration only compared to I-OND group (Figure 9C).
DISCUSSION

SLs belong to one of the several families of bioactive lipids which activate specific G protein-coupled receptors, thereby acting in both stages involved in MS background: inflammation (34) and neurodegeneration (35). Each phase requires the concerted action of such SL mediators, which are likely to interact and engage in the pathophysiological cross talk. As an important component of CNS, SLs could affect the viability of brain cells (oligodendrocytes, neurons and astrocytes), which is mediated by their signaling. Recent studies indicated that Cer (18-21, 26) and its glycosylated derivatives (17, 28), have attracted the most attention in MS field. Because SL pathway changes have recently emerged as key factors in CNS disorders, including MS, we investigated aberrant SL metabolism in MS post mortem tissues, which might be dependent on the disease progression. The regulation of a vastly intertwined network of bioactive SL molecules with their extensive structural diversity is complex and still not unraveled with respect to MS. Consequently, the full elucidation of their role in the different phases of the disease pathogenic mechanisms, from acute inflammation and its resolution to chronic inflammation, with parallel neurodegeneration, represents conceivably one of the biggest challenges.

Our data suggest different pathological scenarios for an Ac- and In-MS-related damage differentiated mainly by Cer source. Cer is the central hub of the SL pathway, which includes dhCer, SM, HexCer (gluco- and galactosylceramides), LacCer and sphingoid bases (Sph and dhSph) and sphingoid bases 1-phosphates (dhS1P and S1P), and other SLs. In order to test the association between features of chronic MS-related brain damage and SL levels, we applied a sphingolipidomics to quantify bioactive SL mediators in post mortem tissues obtained from subjects with advanced stages of MS. There are two main pathways of Cer production: de novo biosynthesis and endocytic recycling. Which of these pathways dominates for supplying Cer depends on the cell type and specific conditions and remains to be elucidated. Although it was originally thought that SMase is the key enzyme responsible for Cer generation, our ex vivo studies implicate de novo SLs biosynthesis – as indicated by elevated levels of C18:0-dhCer (Figure 4B), C24:0-dhCer (Figure 4D) and C24:1-dhCer (Figure 4E) – in the Ac-MS lesions. De novo pathway for Cer generation via serine palmitoyltransferase (SPT)
activation has been already reported in EAE (26). In line with this, the use of C16-Cer and/or Cer synthase, specifically CerS6 (36) as well as C16-dhCer (37) as biomarkers of MS early activity and/or its progression has been elaborated. In contrary, we have found that, in In-MS lesions Cer may be derived from SM hydrolysis. We did observe a significant decrease of three SM subtypes: C18:0-SM (Figure 5B), C18:1-SM (Figure 5C) and C24:0-SM (Figure 5D) in In-MS plaques in comparison to normal brain as well as Ac-MS lesions. So far, the increased level of acid SMase activity and the increased number of exosomes that carry acid SMase have been reported in the CSF of MS patients (compared to those with other CNS diseases) (38). Interestingly, acid SMase activity did not differ significantly between the sera from patients with relapsing-remitting, secondary progressive and primary progressive MS and no association was found between acid SMase activity and the clinical or radiological signs of the disease activity (39). These data suggest that SLs in CSF might be more relevant as MS biomarkers than in serum. Interestingly, although Cer is generated intracellularly, it can also be found in biological fluids, i.e., plasma (21), where it bounds to microvesicles such as exosomes (20). Also of interest, exogenous Cer was shown to induce acid SMase activity or stimulate the de novo pathway to produce more intracellular Cer, pointing to the existence of a Cer-triggered paracrine amplification loop to increase Cer levels in cells (40).

Cer is the structural backbone of SLs and a precursor of complex SLs. Consequently, further perturbation in its metabolism may have an important implication for disease progression. Firstly, our data indicate that Cer could be metabolized to its glycosylated derivatives, as suggested by significant increase of all HexCer species examined, e.g. C16:0-HexCer (Figure 6A), C18:0-HexCer (Figure 6B), C18:1-HexCer (Figure 6C), C24:0-HexCer (Figure 6D) and C24:1-HexCer (Figure 6E) in In-MS lesions compared to both normal brain as well as Ac-MS plaques. Interestingly, several SL species were already found to be elevated in CSF of MS compared to controls, including C16:0-Cer, C24:0-Cer and C16:0-HexCer, indicating that the SM→Cer→HexCer pathway in MS might be relevant to the effect of damage to neurons (19). It should be emphasized that correlations between C16:0-HexCer and C24:1-HexCer in CSF and degree of disability in Expanded Disability Status Scale (EDSS) were previously observed (28), which further supports the concept of CSF SL components as relevant MS
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biomarkers. The observed increase of all HexCer subspecies (Figure 6) could potentially reflect and implicate further alterations in more complex glycosylated SLs in progressive phase of MS. Contrary to our expectation, we did not observe discriminative differences with respect to LacCer subspecies level (Figure 7). However the increased level of LacCer was already observed in MS brain tissues (17). Glycosylated Cer, specifically C24:1-LacCer besides of C16-GluCer, have also been proposed as lipid based biomarkers for MS (37). The discrepancy between these studies and our findings might result from heterogeneous pathology of MS-affected brain tissues specimens subjected to analysis. Moreover, in the previous studies on CSF findings, histopathological examination was not conducted and/or even MS clinical subtype was not specified.

Secondly, we have found some C1P subspecies, specifically C16:0-C1P (Figure 8A), C18:0-C1P (Figure 8B), C18:1-C1P (Figure 8C), C24:0-C1P (Figure 8D) and C24:1-C1P (Figure 8E) to be increased, resulting in 5.3 fold increase of total C1P (Figure 8E) during progressive MS course. C1P in MS is most likely generated by the ceramide kinase action, and this Ca²⁺ ions dependent enzyme has been reported to be highly active in brain tissue (41) although alternative pathways cannot be completely excluded. For example, the transfer of fatty acyl chain to S1P, or the degradation of SM by the D-type phospholipases would render C1P directly (42). Major sources for C1P are macrophages and leaky damaged cells (43).

The striking increase of C1P, as reported here for the first time in chronic/progressive MS, has undoubtedly biological meaning and diagnostic value. Of note, most of the pro-inflammatory activities of Cer seem to be mediated through C1P besides of its S1P metabolites (44). For example, C1P and S1P could act as chemoattractants for tumor cells and their increased level in several organs after radio-/chemotherapy indicates induction of an unwanted pro-metastatic environment as a side effect of oncologic treatment (45) thus their chemotactic gradient became legitimate targets for anti-metastatic therapies (46). Another activities of C1P include its capability to mediate arachidonic acid release (47) and also to activate group IVA cytosolic phospholipase A₂ (cPLA₂) which is the rate limiting releaser of arachidonic acid used for production of pro-inflammatory eicosanoids (48). Furthermore, it has been proposed that C1P transfer protein prevents excess C1P accumulation after its production by
CERK in the trans Golgi network, thereby regulating cPLA$_2\alpha$ action, diminishing arachidonic acid release and downstream generation by eicosanoid producers such as COX-1 or COX-2 (49). These observation suggested that targeting C1P level at the trans Golgi network potentially targets cPLA$_2\alpha$-mediated eicosanoid biosynthesis and pro-inflammatory pathological process. Interestingly, C1P increases specifically the transport of P-glycoprotein, an ATP-driven efflux pump which regulates the permeability of the blood brain barrier via COX-2/prostaglandin E2 signaling, which offers clinical benefits for drug delivery into CNS to modulate neuroprotection (50).

Although the pro-inflammatory properties of intracellular C1P are well established, as discussed above, increasing experimental evidence indicates that C1P can also exert anti-inflammatory actions in some particular cell types or tissues. Many of the anti-inflammatory effects of C1P include blockade or counteraction of Cer-induced inflammatory responses. In line with this connection, one of the initial anti-inflammatory actions of C1P might be inhibition of stimulated Cer production, which was reported to occur in macrophages through blockade of SPT (51), acid SMase (52) activities; the effects associated to the anti-apoptotic effect of C1P. In addition, C1P was shown to be a potent inhibitor of TNF-α converting enzyme (53), thereby emphasizing the anti-inflammatory action of C1P.

Interestingly, it has also been reported that C1P promotes macrophage chemoattractant protein-1 (MCP-1) release in different types of cells, and this chemokine revealed to be its major mediator of C1P-stimulated cell migration events (54, 55). However C1P-stimulated macrophage migration could be blocked by PA, glycerophospholipid structurally related to C1P (56).

In conclusion, our investigation of chronic MS lesions in brain revealed different SL molecules supposed to differentiate inflammatory and neurodegenerative processes underlying MS pathology. These guardian SL molecules and their corresponding pathologic pathways could be potentially exploited in both: active and inactive MS forms. More neuropathological research is needed in order to define the relationship between the accumulation of these particular SLs and MS activity or progression. All these SL molecules might serve as relevant biomarkers and hopefully also platforms for novel therapies.
DATA AVAILABILITY

The data supporting this study are available in the article, and are available from the corresponding author upon reasonable request.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest with the contents of this article.
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Table 1. Patient clinical characteristics details.

| Sample ID | Brain region | Tissue character | Gender | Age | Post mortem interval (h) | Clinical diagnosis | Group |
|-----------|--------------|------------------|--------|-----|--------------------------|--------------------|-------|
| 4467      | Frontal cortex | Plaque          | Female | 62  | 23.0                      | MS                 | Ac-MS |
| 4218      | Frontal cortex | Plaque          | Female | 63  | 15.0                      | MS                 |       |
| 4546      | Frontal cortex | Plaque          | Male   | 59  | 38.5                      | MS                 |       |
| 4477      | Frontal cortex | Plaque          | Male   | 67  | 19.0                      | MS                 |       |
| 4503      | Frontal cortex | Plaque          | Female | 54  | 24.0                      | MS                 |       |
| 3867      | Frontal cortex | Plaque          | Male   | 75  | 13.0                      | MS                 |       |
| 4959      | Frontal cortex | Plaque          | Female | 47  | 22.1                      | MS                 |       |
| 4934      | Frontal cortex | Plaque          | Female | 51  | 17.5                      | MS                 |       |
| 5056      | Frontal cortex | Plaque          | Female | 59  | 20.1                      | MS                 | In-MS |
| 4832      | Frontal cortex | Plaque          | Male   | 54  | 23.0                      | MS                 |       |
| 4663      | Frontal cortex | Plaque          | Male   | 62  | 16.1                      | MS                 |       |
| 5154      | Frontal cortex | Plaque          | Male   | 63  | 13.0                      | MS                 |       |
| 5268      | Frontal cortex | Plaque          | Male   | 66  | 17.0                      | MS                 |       |
| 4471      | Frontal cortex | NAWM            | Female | 73  | 12.0                      | Chronic encephalitis of Rasmussen |
| 4403      | Frontal cortex | NAWM            | Female | 77  | 18.3                      | Herpes simplex Type I encephalitis |
| 747       | Frontal cortex | NAWM            | Female | 66  | 26.0                      | Subacute-chronic encephalitis (HSV, HE etc.) without inclusion | I-OND |
| 1418      | Frontal cortex | NAWM            | Male   | 69  | 4.5                       | Chronic encephalitis, etiology unknown |
| 924       | Frontal cortex | NAWM            | Male   | 86  | 24.0                      | Herpes zoster encephalitis |
| 4222      | Frontal cortex | NAWM            | Female | 72  | 15.0                      | Parkinson’s disease |
| 3780      | Frontal cortex | NAWM            | Female | 92  | 21.3                      | Parkinson’s disease |
| 3942      | Frontal cortex | NAWM            | Male   | 81  | 15.0                      | Parkinson’s disease |
| 3746      | Frontal cortex | NAWM            | Female | 69  | 24.0                      | Parkinson’s disease |
| 3934      | Frontal cortex | NAWM            | Female | 88  | 19.3                      | Parkinson’s disease |
| 3761      | Frontal cortex | NAWM            | Female | 83  | 12.0                      | Multi-infarct dementia (clinical only) | NI-OND |
| 3769      | Frontal cortex | NAWM            | Male   | 63  | 14.0                      | Parkinson’s disease |
| 3742      | Frontal cortex | NAWM            | Male   | 74  | 23.0                      | Parkinson’s disease |
| 3643      | Frontal cortex | NAWM            | Male   | 81  | 19.0                      | Parkinson’s disease |
| 3779      | Frontal cortex | NAWM            | Male   | 68  | 21.3                      | Dystonia |
| 5072      | Frontal cortex | NAWM            | Male   | 83  | 19.5                      | Chronic obstructive pulmonary disease |
| 5190      | Frontal cortex | NAWM            | Male   | 68  | 20.3                      | Heart Attack |
| 3750      | Frontal cortex | NAWM            | Male   | 77  | 12.3                      | Congestive Heart Failure |

Subcallosal stratum close to lateral ventricle; †Above caudate nucleus close to lateral ventricle; ‡Radiation of corpus callosum; Ac-MS, active multiple sclerosis; NAWM, normal appearing white matter; nCNS, normal CNS; In-MS, inactive multiple sclerosis; I-OND, inflammatory other neurological disease; NI-OND, non-inflammatory other neurological disease.
Figure 1. Plaques morphology in MS cases examined. Tissue sections were stained with Luxol fast blue (LFB; panels A & B) and Haemotoxylin and Eosin (H&E; panels C & D). Chronic active MS (Ac-MS) plaque was characterized by loss of myelin in the left side (panel A) and visible disperse inflammatory lymphocytes infiltration (panel C). Chronic inactive MS (In-MS) lesion indicated demyelination in the bottom of the left corner (panel B) and no evidence of inflammation (panel D). The original magnification: 100x (panels A & C) and 200x (panels B & D); scale bars have been inserted in the micrographs.
Potential sphingolipid biomarkers in progressive MS

A

B

C

D

E

F

compared to nCNS → Cer**: p=0.0045, DihCer**: p=0.0103
SM**: p=0.0045, HexCer**: p=0.0045, LacCer**: p=0.0313
C1P**: p=0.045, Sphingoids**: p=0.0127
compared to I-OND → Cer**: p=0.005, DihCer**: p=0.0025,
SM**: p=0.005, HexCer**: p=0.005, C1P**: p=0.0005
compared to NI-OND → LacCer**: p=0.0137
Figure 2. SL profiles in post mortem human brain tissues. Lipids were extracted from human brain tissues and individual SLs species were quantified by mass spectrometry using sphingolipidomics analysis by reverse-phase HPLC-MS/MS. A) SL level of NAWM from nCNS. SL classes are presented as mean (expressed as pmol/mg of protein) ± SEM (n=3). SL composition in post mortem human brain tissues derived from: B) NAWM from nCNS; C) Ac-MS; D) I-OND; E) In-MS and F) NI-OND. The significant alterations (increase or decrease) are indicated by colored arrows. Data are shown as mean (expressed in % of total SLs) and pie chart was generated with GraphPad PRISM 7.01. Abbreviations used: Ac-MS, active multiple sclerosis; C1P, ceramide-1-phosphate; dhCer, dihydroceramide; HexCer, hexosylceramide; In-MS, inactive multiple sclerosis; I-OND, inflammatory other neurological disease; LacCer, lactosylceramide; NAWM, normal appearing white matter; nCNS, normal CNS; NI-OND-non-inflammatory other neurological disease.
Potential sphingolipid biomarkers in progressive MS

A

B

C

D

E

F

C18:1-Cer (pmol/mg protein)  
nCNS Ac-MS In-MS I-OND Ni-OND

C24:1-Cer (pmol/mg protein)  
nCNS Ac-MS In-MS I-OND Ni-OND

C24:0-Cer (pmol/mg protein)  
nCNS Ac-MS In-MS I-OND Ni-OND

Total Cer (pmol/mg protein)  
nCNS Ac-MS In-MS I-OND Ni-OND

**p = 0.0066
***p = 0.005
***p = 0.0010
**p = 0.045
**p = 0.0014

**p = 0.0045
**p = 0.014

**p = 0.0005

**p = 0.0286
**p = 0.014

**p = 0.0005

**p = 0.0005

**p = 0.0079

**p = 0.0286
**p = 0.0079
Figure 3. XY scatter plots of the major Cer subspecies: A) C16-Cer, B) C18-Cer, C) C18:1-Cer, D) C24-Cer, E) C24:1-Cer as well as F) total Cer in chronic MS plaques (active- Ac-MS and inactive- In-MS) in comparison to normal appearing white matter (NAWM) of the normal CNS (nCNS) and other neurological diseases (inflammatory- I-OND and non-inflammatory-NI-OND). The comparison between Ac-MS and In-MS subgroups was also included. The data are expressed as pmol/mg of protein. Horizontal bars indicate median values. Differences between groups of non-parametric data were determined by the Mann Whitney test using GraphPad PRISM 7.01. nCNS (n=3), Ac-MS (n=4), In-MS (n=9), I-OND (n=5), NI-OND (n=10); Cer, ceramide.
Potential sphingolipid biomarkers in progressive MS

A

B

C

D

E

F

**p=0.0086
**p=0.0091
*p=0.0145
***p=0.0005
*p=0.0079
*p=0.0010
**p=0.0645
*p=0.0286
*p=0.0014
*p=0.0474
***p=0.0010
*p=0.0317
**p=0.0378
*p=0.0286
**p=0.0079
**p=0.0014

C16-dhCer (pmol/mg of protein)

C18-dhCer (pmol/mg of protein)

C18:1-dhCer (pmol/mg of protein)

C24:1-dhCer (pmol/mg of protein)

Total dhCer (pmol/mg of protein)

nCNS, Ac-MS, In-MS, I-OND, Ni-OND
**Figure 4.** XY scatter plots of the major dhCer subspecies: A) C16-dhCer, B) C18-dhCer, C) C18:1-dhCer, D) C24-dhCer, E) C24:1-dhCer as well as F) total dhCer in chronic MS plaques (active- Ac-MS and inactive- In-MS) in comparison to normal appearing white matter (NAWM) of the normal CNS (nCNS) and other neurological diseases (inflammatory- I-OND and non-inflammatory-NI-OND). The comparison between Ac-MS and In-MS subgroups was also included. The data are expressed as pmol/mg of protein. Horizontal bars indicate median values. Differences between groups of non-parametric data were determined by the Mann Whitney test using GraphPad PRISM 7.01. nCNS (n=3), Ac-MS (n=4), In-MS (n=9), I-OND (n=5), NI-OND (n=10); dhCer, dihydroceramide.
Potential sphingolipid biomarkers in progressive MS

(A) C16-SM (pmol/mg protein)
(B) C18-SM (pmol/mg protein)
(C) C18:1-SM (pmol/mg protein)
(D) C24-SM (pmol/mg protein)
(E) C24:1-SM (pmol/mg protein)
(F) Total SM (pmol/mg protein)

*p < 0.05
**p < 0.01
***p < 0.001
Figure 5. XY scatter plots of the major SM subspecies: A) C16-SM, B) C18-SM, C) C18:1-SM, D) C24-SM, E) C24:1-SM as well as F) total SM in chronic MS plaques (active- Ac-MS and inactive- In-MS) in comparison to normal appearing white matter (NAWM) of the normal CNS (nCNS) and other neurological diseases (inflammatory- I-OND and non-inflammatory-NI-OND). The comparison between Ac-MS and In-MS subgroups was also included. The data are expressed as pmol/mg of protein. Horizontal bars indicate median values. Differences between groups of non-parametric data were determined by the Mann Whitney test using GraphPad PRISM 7.01. nCNS (n=3), Ac-MS (n=4), In-MS (n=9), I-OND (n=5), NI-OND (n=10); SM, sphingomyelin.
**Figure 6.** XY scatter plots of the major HexCer subspecies: A) C16-HexCer, B) C18-HexCer, C) C18:1-HexCer, D) C24-HexCer, E) C24:1-HexCer as well as F) total HexCer in chronic MS plaques (active- Ac-MS and inactive- In-MS) in comparison to normal appearing white matter (NAWM) of the normal CNS (nCNS) and other neurological diseases (inflammatory- I-OND and non-inflammatory- NI-OND). The comparison between Ac-MS and In-MS subgroups was also included. The data are expressed as pmol/mg of protein. Horizontal bars indicate median values. Differences between groups of non-parametric data were determined by the Mann Whitney test using GraphPad PRISM 7.01. nCNS (n=3), Ac-MS (n=4), In-MS (n=9), I-OND (n=5), NI-OND (n=10); HexCer, hexosylceramide.
Potential sphingolipid biomarkers in progressive MS

A

B

C

D

E

F

C16-LacCer (pmol of protein)

C18-LacCer (pmol of protein)

C18:1-LacCer (pmol of protein)

C24:1-LacCer (pmol of protein)

C24-LacCer (pmol of protein)

Total LacCer (pmol of protein)

nCNS Ac-MS In-MS I-OND NI-OND

nCNS Ac-MS In-MS I-OND NI-OND

nCNS Ac-MS In-MS I-OND NI-OND

nCNS Ac-MS In-MS I-OND NI-OND

nCNS Ac-MS In-MS I-OND NI-OND

nCNS Ac-MS In-MS I-OND NI-OND

* p<0.05
** p<0.01
*** p<0.001
Figure 7. XY scatter plots of the major LacCer subspecies: A) C16-LacCer, B) C18-LacCer, C) C18:1-LacCer, D) C24-LacCer, E) C24:1-LacCer as well as F) total LacCer in chronic MS plaques (active- Ac-MS and inactive- In-MS) in comparison to normal appearing white matter (NAWM) of the normal CNS (nCNS) and other neurological diseases (inflammatory- I-OND and non-inflammatory-NI-OND). The comparison between Ac-MS and In-MS subgroups was also included. The data are expressed as pmol/mg of protein. Horizontal bars indicate median values. Differences between groups of non-parametric data were determined by the Mann Whitney test using GraphPad PRISM 7.01. nCNS (n=3), Ac-MS (n=4), In-MS (n=9), I-OND (n=5), NI-OND (n=10); LacCer, lactosylceramide.
Potential sphingolipid biomarkers in progressive MS

A

B

C

D

E

F
Figure 8. XY scatter plots of the major C1P subspecies: A) C16-C1P, B) C18-C1P, C) C18:1-C1P, D) C24-C1P, E) C24:1-C1P as well as F) total C1P in chronic MS plaques (active- Ac-MS and inactive- In-MS) in comparison to normal appearing white matter (NAWM) of the normal CNS (nCNS) and other neurological diseases (inflammatory- I-OND and non-inflammatory-NI-OND). The comparison between Ac-MS and In-MS subgroups was also included. The data are expressed as pmol/mg of protein. Horizontal bars indicate median values. Differences between groups of non-parametric data were determined by the Mann Whitney test using GraphPad PRISM 7.01. nCNS (n=3), Ac-MS (n=4), In-MS (n=9), I-OND (n=5), NI-OND (n=10); C1P, ceramide-1-phosphate.
**Figure 9.** XY scatter plots of the major sphingoids subspecies: A) dhSph, B) Sph, C) dhS1P, D) S1P as well as E) total sphingoids in chronic MS plaques (active- Ac-MS and inactive- In-MS) in comparison to normal appearing white matter (NAWM) of the normal CNS (nCNS) and other neurological diseases (inflammatory- I-OND and non-inflammatory-NI-OND). The comparison between Ac-MS and In-MS subgroups was also included. The data are expressed as pmol/mg of protein. Horizontal bars indicate median values. Differences between groups of non-parametric data were determined by the Mann Whitney test using GraphPad PRISM 7.01. nCNS (n=3), Ac-MS (n=4), In-MS (n=9), I-OND (n=5), NI-OND (n=10); dhSph, dihydrosphingosine; dihydrosphingosine-1-phosphate; S1P, sphingosine-1-phosphate Sph, sphingosine.