



Published in final edited form as:

Mol Genet Metab. 2018 July ; 124(3): 204–209. doi:10.1016/j.ymgme.2018.05.001.

Deoxysphingolipid precursors indicate abnormal sphingolipid metabolism in individuals with primary and secondary disturbances of serine availability

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Abstract

Patients with primary serine biosynthetic defects manifest with intellectual disability, microcephaly, ichthyosis, seizures and peripheral neuropathy. The underlying pathogenesis of peripheral neuropathy in these patients has not been elucidated, but could be related to a decrease in the availability of certain classical sphingolipids, or to an increase in atypical sphingolipids. Here, we show that patients with primary serine deficiency have a statistically significant elevation in specific atypical sphingolipids, namely deoxydihydroceramides of 18-22 carbons in length. We also show that patients with aberrant plasma serine and alanine levels secondary to mitochondrial disorders also display peripheral neuropathy along with similar elevations atypical sphingolipids. We hypothesize that the etiology of peripheral neuropathy in patients with primary mitochondrial disorders is related to this elevation of deoxysphingolipids, in turn caused by increased availability of alanine and decreased availability of serine. These findings could have important therapeutic implications for the management of these patients.

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Declarations of interest
none

Keywords

Serine Biosynthesis Defect; Serine deficiency; Deoxysphingolipids; Deoxydihydroceramide

1. Introduction

Serine is a non-essential amino acid that can be synthesized *de novo* from 3-phosphoglycerate, a glycolytic and gluconeogenic intermediate. Among the major roles of serine in the cell are to participate in the early biosynthetic steps of sphingolipid metabolism. Herein, we describe the presence of pathological sphingolipid biosynthesis in primary and secondary causes of serine deficiency.

In the first biosynthetic step of serine biosynthesis, the NAD⁺-dependent enzyme 3-phosphoglycerate dehydrogenase (PHGDH) converts 3-phosphoglycerate into 3-phosphohydroxypyruvate, which in turn is converted to 3-phosphoserine by phosphoserine aminotransferase (PSAT). Then the phosphoserine phosphatase (PSPH) removes the phosphate group from phosphoserine to form L-serine (Figure 1). Primary defects in all three enzymatic reactions of serine biosynthesis have been described. The most severely affected patients present with Neu-Laxova syndrome, a fetal or neonatal lethal condition characterized by severe growth retardation, arthrogyrosis, microcephaly, ichthyosis, and dysmorphic facial features including ectropion and eclabion [1,2]. Patients with milder forms of the disease present with acquired microcephaly, developmental delay and intellectual disability of variable severity, ichthyosis, and progressive peripheral neuropathy.

The pathogenesis of the aforementioned neurological manifestations is not fully understood. One possibility is limited serine substrate for the rate-limiting enzyme of sphingolipid biosynthesis, serine palmitoyltransferase (SPT) leading to decreased total sphingolipids. Indeed, *Phgdh* knockout mice show decreased brain levels of serine-derived lipids, such as phosphatidylserine (PS), sphingomyelin, and ganglioside GD3 [3,4]. Moreover, Glinton et al. recently described reduced levels of various phospholipid species including glycerophosphoethanolamine, glycerophosphocholine, and sphingomyelin in individuals with untreated primary serine deficiency [5]. They found that these deficiencies normalized with serine and glycine supplementation.

A second possibility is that elevation of atypical sphingolipids, in addition to deficiency of classical sphingolipids, contributes to the neurological manifestations of serine synthetic disorders. Atypical sphingolipids are synthesized when SPT uses a non-canonical substrate—such as alanine or glycine—instead of serine in the key step of sphingolipid synthesis (Figure 1). SPT is an enzymatic complex composed of subunits encoded by the ubiquitously expressed *SPTLC1* and *SPTLC2*, and in some tissues—but not the brain—also *SPTLC3*. Mutations in *SPTLC1* and *SPTLC2* lead to hereditary sensory and autonomic neuropathy types IA and IC (HSAN1A and HSAN1C), respectively [6,7].

In support of this mechanism, it has been shown that the neuropathy in *SPTLC1* mutant mice is caused by accumulation of neurotoxic 1-deoxysphingolipids, and that supplementation with oral L-serine reduces this toxic accumulation [8]. Moreover, the

deprivation of exogenous L-serine in mouse embryonic fibroblasts lacking PHGDH leads to the accumulation of cytotoxic 1-deoxysphingolipids, the most abundant of which is 1-deoxydihydroceramide (DoxDHCer) [9]. This accumulation was normalized after the addition of L-serine to the medium.

Variable availability of substrates for SPT, including serine and alanine, is not limited only to primary disorders of serine biosynthesis, and can be seen in primary mitochondrial disease (PMD). This is particularly relevant because peripheral neuropathy is an important feature in many affected patients [10]. In addition to elevation of alanine, a common amino acid finding in PMD caused by increased transamination from pyruvate [11], we have observed persistently low plasma serine levels in a cohort of individuals with primary mitochondrial disease with peripheral neuropathy. We considered that this low serine and elevated alanine could lead to aberrant SPT substrate utilization, and production of atypical sphingolipids, with resultant peripheral neuropathic disturbances.

In this study we compare serine, sphingolipids, and 1-deoxysphingolipids in plasma from patients with primary serine deficiency (PSD) and PMD with peripheral neuropathy to control subjects. We show, for the first time, that patients with both primary and secondary forms of serine biosynthetic abnormalities exhibit elevation of atypical sphingolipids in the form of DoxDHCer. We also show a highly significant correlation between the plasma alanine/serine ratio and levels of atypical sphingolipids in PMD. These atypical sphingolipids likely contribute to the pathogenesis of neuropathy in the setting of functional serine deficiency.

2. Materials and methods

2.1 Patients

Two patients with primary serine synthetic defects were evaluated at the National Institutes of Health Clinical Center after enrollment under protocol #76-HG-0238, “Diagnosis and Treatment of Patients with Inborn Errors of Metabolism or Other Genetic Disorders,” approved by the National Human Genome Research Institute (NHGRI) Institutional Review Board (IRB). Patients or their guardians gave written, informed consent. PSD patient 1, enrolled in the NIH Undiagnosed Diseases Program [12–14], had PSAT deficiency, while PSD patient 2 (previously reported [15]) has PSPH deficiency. Plasma samples were obtained when the patients were not receiving serine or glycine supplementation.

Plasma samples from individuals with molecularly confirmed PMD and from normal controls (individuals without a known inborn error of metabolism or other genetic condition) were obtained from the Kennedy Krieger Biochemical Genetics Laboratory under IRB study NA_00069372, Metabolic Analysis of Archived Biofluids (PI. H. Vernon). All samples were drawn at 4-5 hours fasting.

We identified plasma samples from 6 individuals with molecularly confirmed primary mitochondrial disease (3 with MERRF, 1 with autosomal dominant POLG related mtDNA deletion disorder, 1 with LHON, and 1 with MELAS), and for 4 of these individuals we were able to obtain two independently drawn plasma samples from different dates (Table 1).

The 3 MERRF patients and the POLG patient had significant sensory neuropathy of their lower extremities diagnosed by physical exam and/or consistent nerve conduction studies. The patients with LHON and MELAS had no features of peripheral neuropathy based on history or physical exam, though nerve conduction studies were not performed.

Ages of controls ranged from 12-76 years (mean 35.7 years \pm 24 SD), with 4 males and 7 females. Ages of patients ranged from 12-72 years (mean 36.5 years \pm 25 SD), with 2 males and 4 females. Patients' specific mutations and characteristics can be found in supplementary table 1.

2.2 Plasma amino acid analysis

Amino acids were quantified via ion exchange chromatography. Plasma was de-proteinized, centrifuged, and the supernatant used for further analysis. Quantification of amino acids was conducted via ion-exchange liquid chromatography with ninhydrin detection on a Biochrom 30 Amino Acid Analyzer.

2.3 Sphingolipid/ceramide analysis

For the 12 controls and the patients with PSD, lipids were extracted with protein precipitation with methanol in the presence of internal standards. The d31-SM(16:0) was used as internal standard for SM. Cer(17:0) was the internal standard for Cer, DHCer, DoxCer, and DoxDHCer; PS(28:0) were internal standards for PS; d5-GluC(18:0) was internal standard for GluC; d3-LC(16:0) was internal standard for LC; C17 sphingosine-1-phosphate as internal standard for sphingosine, sphinganine, SIP, 1-deoxysphingosine, and 1-deoxysphinganine. The lipids were separated by column-switching high-performance liquid chromatography (HPLC) and monitored by multiple-reaction monitoring (MRM) detection on an Applied Biosystems Sciex 4000QTRAP tandem mass spectrometer (MS/MS) equipped with an electrospray ion source. A quality control (QC) sample was prepared from pooled study plasma samples and injected every five study samples to monitor the LC-MS/MS assay performance.

The results of the QC samples provided the basis for accepting or rejecting the data. Only the lipid species in QC samples with coefficient of variation of less than 20% are reported. Since at this stage this was a pilot project screening for potential biomarkers, no absolute quantification method was developed for these lipids, but we used instead a relative quantification method. The DoxDHCer(m18:0), DoxDHCer(m20:0), and DoxDHCer(m22:0) data are expressed as peak area ratios of these deoxydihydroceramides to their internal standard (Cer(17:0)). Unpaired Student's t-test was performed with Prism version 6.0c (Graphpad Software Inc, La Jolla, CA). Statistical significance was assigned to a two-tailed p-value <0.05 .

For the patients with PMD and peripheral neuropathy, ceramides and sphingoid bases were extracted from plasma according to the method described by Bligh and Dyer [16]. The lower phase, containing both ceramides and sphingoid bases, was dried and the residue was dissolved in sodium hydroxide in methanol. Samples were either deacylated for analysis of ceramides or not for analysis of sphingoid bases. Next, internal standards were added to each sample (d7-sphingosine, d7-sphinganine and d3-deoxysphinganine) and samples were dried

and dissolved in butanol and water. Samples were analyzed by LC-MS/MS. Levels of ceramides and sphingoid bases were calculated using calibration lines, according to the internal standard ratio method. Sphingoid bases were separated by RP-UPLC using an Acquity I-Class UPLC with BEH C18 column, 2.1 × 50 mm with 1.7-μm particle size (Waters Corporation, Milford, MA, USA) and detected by electron spray ionization in positive ionization mode (ESI+) using a MS/MS-instrument (Xevo TQ MS, Waters Corporation) in multiple reaction monitoring (MRM) mode. Levels of the biomarkers were calculated using calibration curves within the appropriate concentration range, according to the internal standard ratio method.

It is worth noting that the ceramides and sphingoids in PMD and PSD patients were detected using different sample preparation and extraction methodologies. In the PSD patients, the ceramide and sphingoid base species were measured intact, thus allowing for measurement of individual ceramide species. In the PMD patients, the DoxDHCer(m18:0) and Cer(d18:1) species of different acyl chain lengths were deacylated to Dox-Sphinganine(m18:0) and Sphingosine(d18:1) respectively, and measured as a sum total of the bases (DoxSO(m18:1) was measured in the non-de-acylated sample). However, here we have shown the validity of both methodologies in detecting relevant variant ceramides in these patient groups.

3. Results

3.1 Sphingolipids in PSD

The values of DoxDHCer (m18:0), (m20:0) and (m22:0) were significantly elevated compared to controls (Figure 2). For DoxDHCer(m18:0), the mean peak ratio for controls was 0.0984 ± 0.018 vs 0.5997 ± 0.220 for patients (P-value <0.0001); for DoxDHCer(m20:0), the control mean was 0.1632 ± 0.016 vs 0.5249 ± 0.202 in patients (P-value 0.0004); for DoxDHCer(m22:0), the control mean was 0.3674 ± 0.026 vs 0.9394 ± 0.293 for affected patients (P-value 0.0003) (figure 3). The specific peak area ratios of the different DoxDHCer are provided in table 1.

3.2 Plasma amino acids in PMDs

Plasma amino acids were compared between the PMD cohort and the control cohort (supplementary table 1). For patients having two independent samples measured, the results were averaged prior to cohort comparisons. The results from the LHON patient were removed from the comparison analysis, since the serine level was a significant outlier for the PMD the population. Full plasma amino acid profiles can be found in Supplementary table 2.

The mean plasma serine level was significantly different between the PMD and control cohort ($71.5 \text{ umol/L} \pm 22 \text{ SD}$ vs $106.9 \text{ umol/L} \pm 18 \text{ SD}$, respectively), with a P-value of 0.017. Alanine was significantly different between the PMD and control cohort ($444.2 \text{ umol/L} \pm 55 \text{ SD}$ vs $361.2 \text{ umol/L} \pm 59 \text{ SD}$, respectively), with a P-value of 0.025. Serine/Alanine ratios were significantly increased in the PMD population compared to controls (6.12 ± 2.8 and for the control cohort was 3.41 ± 0.5 , respectively), with a P-value of 0.038).

3.3 Sphingolipids in PMDs

We compared DoxDHCer(m18:0), deoxysphingosine (DoxSO)(m18:1), and the ratios of these metabolite classes to ceramide(d18:1) (DoxDHCer(m18:0)/Cer(d18:1), DoxSO(m18:1)/Cer(d18:1)) between PMD patients and controls (Table 2). DoxDHCer(m18:0) was significantly elevated in PMD patients compared to controls (49.7nM \pm 17 SD vs. 21.2nM \pm 10 SD, (P-value 0.00043)) (Figure 3). DoxSO(m18:1) was also significantly elevated in PMD patients compared to controls (8.1uM \pm 4 SD vs. 2.3nM \pm 0.6 SD (P-value 0.0011)) (Figure 3). Both ratios were also elevated in PMD patients compared to controls (DoxDHCer(m18:0)/Cer(d18:1): 0.012 \pm 0.004 vs. 0.003 \pm 0.002, P-value 0.00001, and DoxSO(m18:1)/Cer(d18:1): 0.002 \pm 0.001 vs. 0.00035 \pm 0.0001 (P-value 0.00092)).

When comparing the plasma serine/alanine ratio to the plasma DoxDHCer (m.18:0) in the PMD patients, there is a strong correlation with DoxDHCer (m.18:0) ($R^2 = 0.74$, figure 4). When comparing the plasma serine/alanine ratio with DoxSO, there is not a significant direct correlation between the two values ($R^2 = 0.29$); however, there is a clear clustering of PMD patients versus controls (Figure 4).

4. Discussion

Dysregulation in both sphingolipid biosynthesis and breakdown have been implicated in the etiology of various peripheral neuropathies. This includes the biosynthetic sphingolipid defects of SPT in HSN1 leading to accumulation of deoxysphingolipids. More recently, defects in sphingosine 1-phosphate lyase (SPL), which catalyzes the final step of sphingolipid catabolism, have been shown to underlie an autosomal recessive form of Charcot-Marie-Tooth neuropathy [17]. This enzymatic defect leads to elevations in sphingosine 1-phosphate and in the sphingosine/sphinganine ratio.

We have shown, for the first time, that abnormal deoxysphingolipids are elevated in the plasma of individuals with both primary and secondary causes of abnormal serine availability. We hypothesize that this is due to a mechanism involving alternative sphingolipid metabolism similar to that seen in the SPTLC abnormalities, HSN1A and HSN1C. Specifically, that mechanism consists of alternative amino acid substrate utilization (alanine vs. serine) by serine palmitoyltransferase in sphingolipid synthesis. In the situations presented here, however, this alternative substrate utilization is due to substrate availability rather than abnormal enzyme function.

For PSD patients, abnormal deoxysphingolipids levels are probably due to straightforward serine availability for SPT, whereas the etiology of these abnormal deoxysphingolipids in PMD may be more complex. The strong association between the serine/alanine ratio and DoxDHCer identified in this study suggests that availability of both amino acid substrates is relevant. There are several possible factors contributing to the abnormal amino acid availability for SPT and the consequent production of DoxSO in PMD. Firstly, elevated alanine, resulting from increased transamination from elevated pyruvate, is commonly seen in PMD [11]. The potential reasons for decreased serine availability are less straightforward. One possible explanation for serine deficiency in PMD could be a functional deficiency of

the NAD⁺-dependent first enzyme of serine biosynthesis, 3-phosphoglycerate dehydrogenase, due to deficiency of NAD⁺ [18,19]. Another possible explanation could be potential shunting of serine away from sphingolipid metabolism, and towards stress response pathways of glutathione synthesis and one-carbon metabolism; these pathways are upregulated in multiple models of primary mitochondrial disease including the Deletor mouse model of mtDNA deletions [20,21].

It is not yet clear if all PMD patients are at risk for functional serine deficiency, or if this occurs in only a subset of PMD patients. Of the cases presented here, most PMD patients had abnormally low plasma serine, with some in the extreme low range seen in PSD. Retrospective studies of PMD cohorts suggest that peripheral neuropathy is common, and potentially undiagnosed [10]. While further functional studies are necessary to determine the precise cause of the serine deficiency in individuals with mitochondrial disease, the presence of these abnormal sphingolipids has the potential to serve not only as a biomarker for mitochondrial disease, but also potentially as a biomarker for those with PMD who are at risk for peripheral neuropathy. Further studies may also reveal whether serine supplementation could lead to prevention of peripheral neuropathy in PMD or improvement in clinical status of those affected by peripheral neuropathy, as it has been shown to do in PSD and HSAN1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors wish to thank the patients and their families for their cooperation and support. This study is supported in part by the National Institutes of Health Intramural Research Program of the National Human Genome Research Institute and the Common Fund, Office of the Director; the NIH grants 5RO1-N5021328-030 and RO1OD010944-05.

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Highlights

- Atypical sphingolipids are elevated primary serine deficiency disorders
- Atypical sphingolipids are elevated in some cases of primary mitochondrial disease
- This may be due to aberrant amino acid substrates for serine palmitoyltransferase

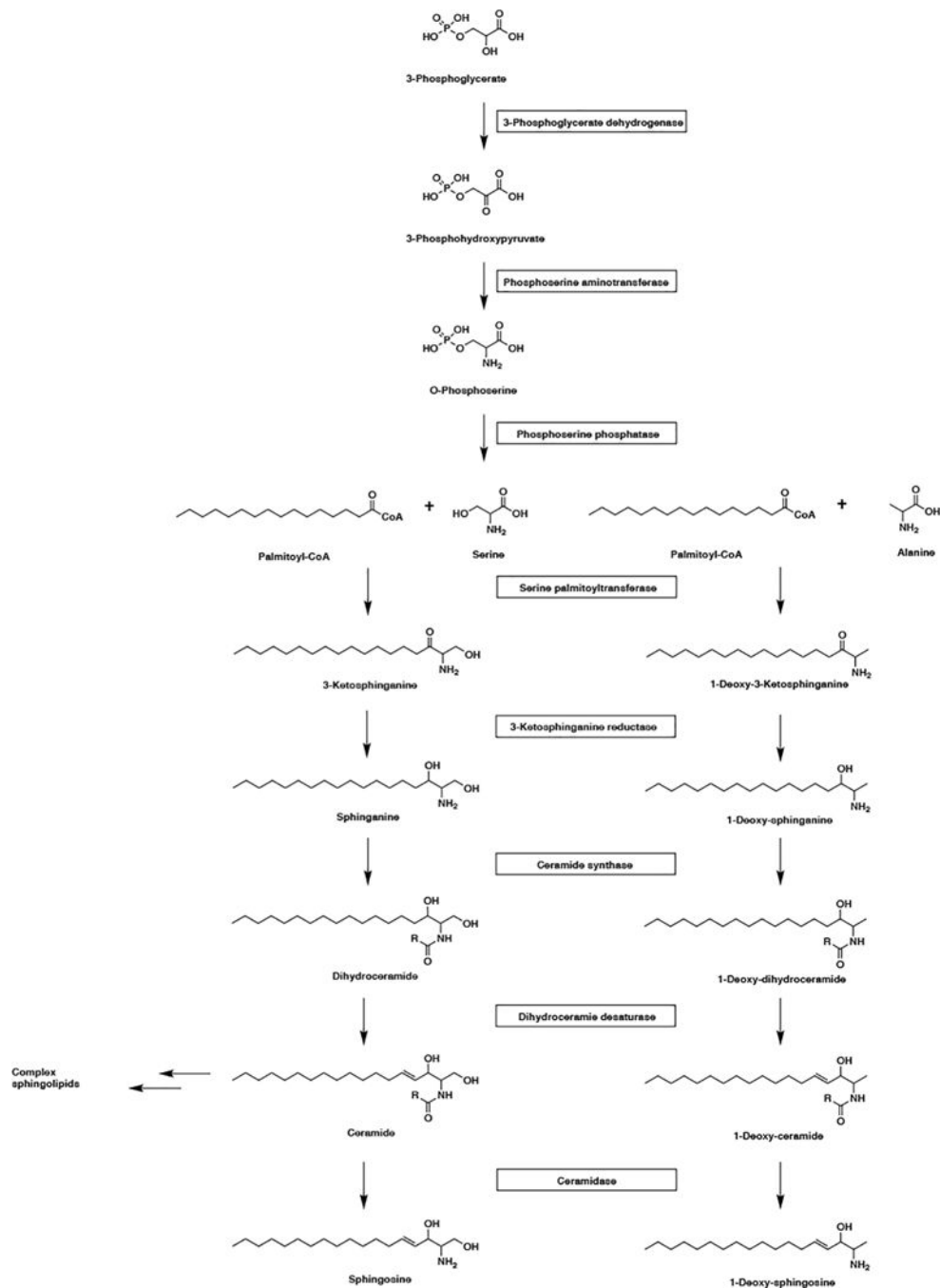


Figure 1. Biosynthesis of serine, sphingolipids and 1-deoxy-sphingolipids

The NAD^+ -dependent enzyme 3-phosphoglycerate dehydrogenase converts 3-phosphoglycerate into 3-phosphohydroxypyruvate, which in turn is converted to 3-phosphoserine by phosphoserine aminotransferase. The phosphoserine phosphatase (PSPH) dephosphorylates phosphoserine to form L-serine. In classical sphingolipid de novo synthesis, serine palmitoyl transferase (SPT) condenses serine and palmitoyl CoA to synthesize 3-ketosphinganine. Next, 3-keto-dihydro-sphingosine is reduced to form dihydro-sphingosine by 3-ketosphinganine reductase. Dihydro-sphingosine is acylated by

ceramide synthase to form dihydroceramide, which is desaturated to give ceramide. Deacylation of ceramide by ceramidase generates sphingosine. The ceramide can be also converted into complex sphingolipids such as sphingomyelin and glycosphingolipids. Atypical sphingolipids are synthesized when SPT uses a non-canonical substrate such as alanine in the first step of sphingolipid synthesis. Following same sequence as classical sphingolipid de novo synthesis, 1-deoxy-ceramide is produced and converted into 1-deoxy-sphingosine. Due to lack of 1-hydroxyl group, no analogs of complex sphingolipids can be synthesized from 1-deoxy-ceramide.

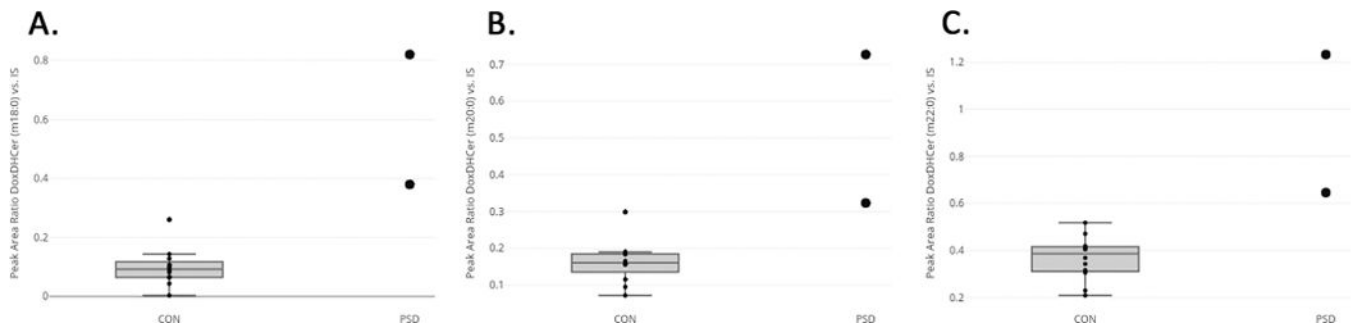


Figure 2. Deoxysphingolipids in patients with PSDs

DoxDHCer species as measured by peak area ratio compared to internal standard were significantly elevated in individuals with PSD (n=2) compared to controls (n=12). a)

DoxDHCer (m18:0), b) DoxDHCer (m20:0) c) DoxDHCer (m22:0). DoxDhCer, deoxydihydroceramide.

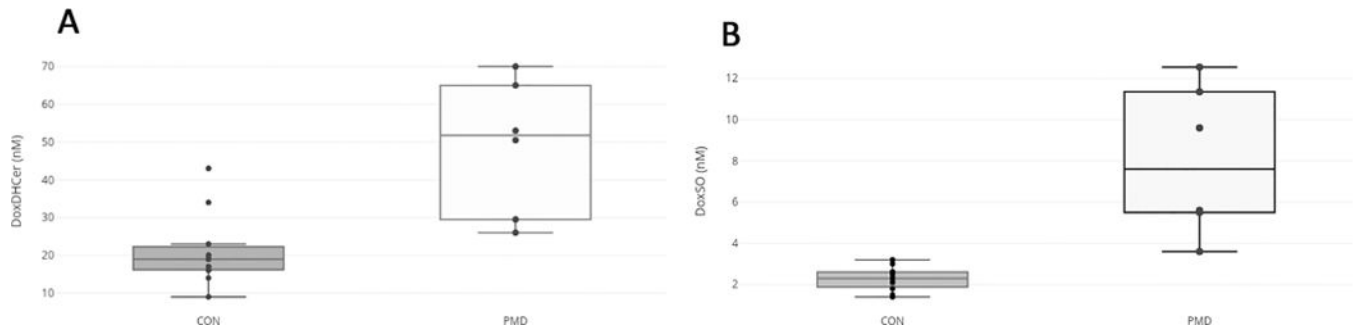


Figure 3. Deoxysphingolipids in patients with PMDs

Total DoxDhCer (a) and Total DoxSO (b) were significantly elevated in individuals with PMD (n=6) compared to controls (n=11). DoxDhCer, deoxydihydroceramide; DoxSO, deoxysphingoids.

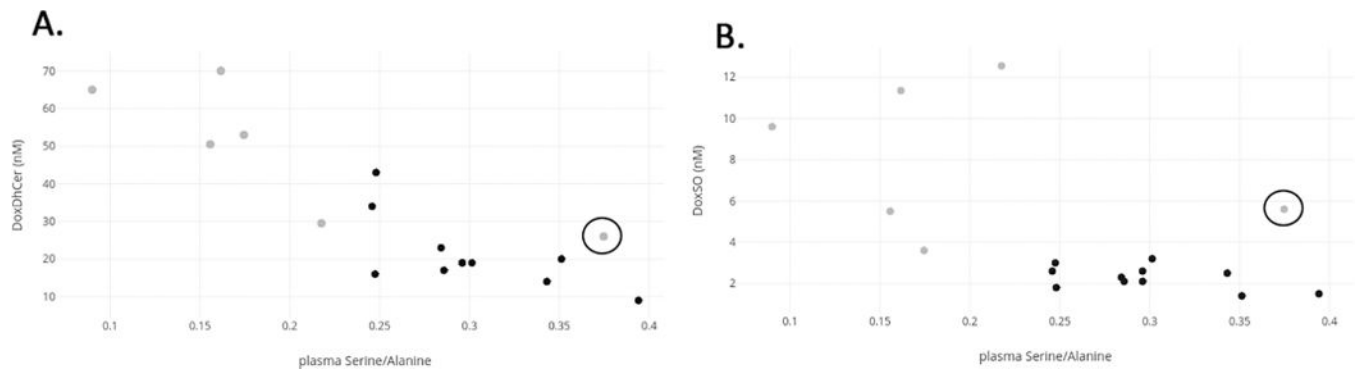


Figure 4. Correlation between plasma amino acids and deoxysphingolipids in patients with PMDs

a) Strong correlation between plasma serine/alanine ratio and DoxDhCer in PMD patients (light gray) vs controls (dark gray) ($R^2 = 0.74$). b) No significant direct correlation between plasma serine/alanine ratio and DoxSO ($R^2 = 0.29$), although there is clear clustering of PMD patients (light gray) versus controls (dark gray). DoxDhCer, deoxydihydroceramide; DoxSO, deoxysphingoids. The PMD data points differentiated by a circle around represent the patient with LHON plus (patient 6) who does not manifest peripheral neuropathy and who has an above average plasma serine level of 142 μ M.

Table 1

Deoxydihydroceramides in patients with PSDs and controls.

Patient		
DoxDHCer(m18:0)	DoxDHCer(m20:0)	DoxDHCer(m22:0)
0.37962	0.32331	0.64632
0.81978	0.72654	1.23255
Controls		
DoxDHCer(m18:0)	DoxDHCer(m20:0)	DoxDHCer(m22:0)
0.12822	0.19015	0.40674
0.10687	0.18399	0.40536
0.06363	0.09508	0.20939
0.04315	0.11518	0.30621
0.06606	0.15612	0.41883
0.09674	0.16519	0.36892
0.00338	0.07192	0.23018
0.09916	0.18439	0.51814
0.14335	0.15616	0.31697
0.08866	0.15725	0.34316
0.08177	0.18487	0.41326
0.26016	0.29861	0.47123

Table 2

Deoxysphingolipids in patients with PMDs and controls.

	PMD Patients			
	DoxDHCer (nM)	DoxSO (nM)	DoxDHCer/Cer	DoxSO/Cer
Patient 1, Sample 1	68	9.1	0.0122	0.0016
Patient 1, Sample 2	72	13.6	0.0136	0.0026
Patient 2	65	9.6	0.0133	0.002
Patient 3, Sample 1	40	4.4	0.01	0.0011
Patient 3, Sample 2	61	6.6	0.0178	0.0019
Patient 4, Sample 1	56	4.4	0.0157	0.0012
Patient 4, Sample 2	50	2.8	0.0144	0.0008
Patient 5, Sample 1	28	11.4	0.0085	0.0035
Patient 5, Sample 2	31	13.7	0.0094	0.0042
Patient 6	26	5.6	0.0065	0.0014
	Controls			
	DoxDHCer (nM)	DoxSO (nM)	DoxDHCer/Cer	DoxSO/Cer
	14	2.5	0.002	0.0004
	17	2.1	0.0019	0.0002
	19	3.2	0.0033	0.0006
	16	3	0.0021	0.0004
	9	1.5	0.0011	0.0002
	43	1.8	0.0083	0.0003
	34	2.6	0.0048	0.0004
	20	1.4	0.0043	0.0003
	19	2.6	0.0029	0.0004
	19	2.1	0.0027	0.0003
	23	2.3	0.0038	0.0004