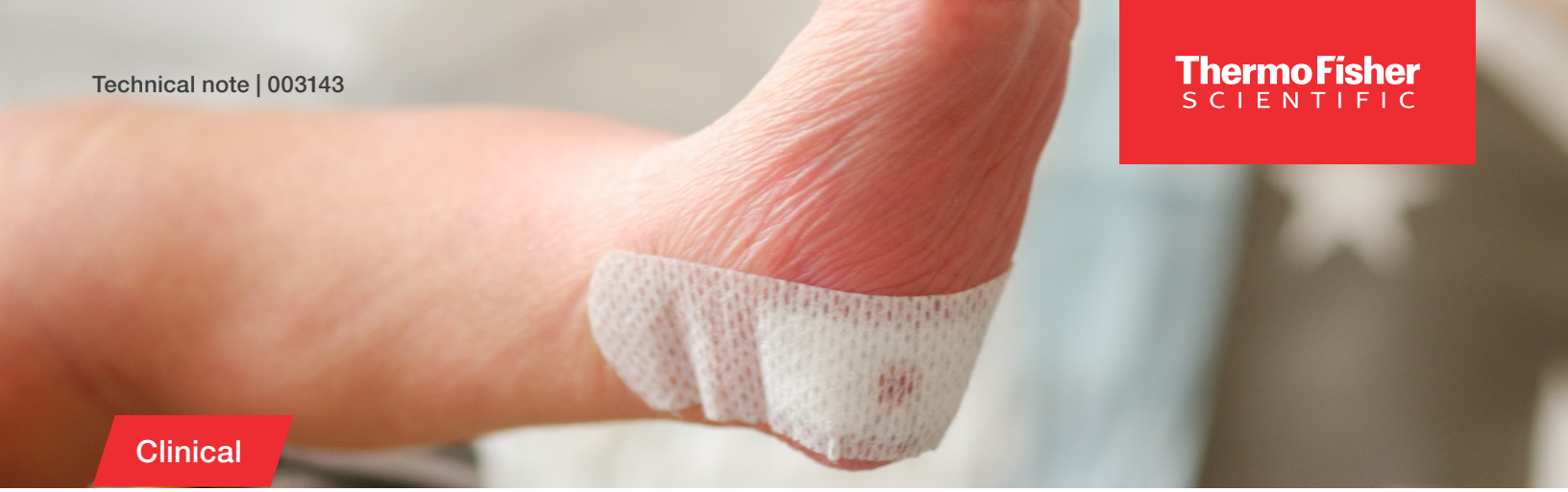

Technical note | 003143

Clinical

Quantitation of lysosphingolipids in dried blood spots and plasma using high-resolution mass spectrometry for sphingolipidoses

Authors

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Keywords

Sphingolipidoses, Fabry disease, Gaucher disease, GM1 Gangliosidosis, GM2 Gangliosidosis, Niemann-Pick A/B, C disease, lysosphingolipids quantification, dried blood spots, inborn errors of metabolism, plasma, high-resolution mass spectrometry, Orbitrap Exploris mass spectrometer, TraceFinder software

Goal

To simultaneously quantify specific endogenous lysosphingolipid markers in both plasma and dried blood spots for six lysosomal diseases monitored for inborn errors of metabolism using a validated multiple analyte UHPLC-Thermo Scientific™ Orbitrap™ mass analyzer-MS/MS method.

Introduction

Sphingolipidoses are a group of inherited metabolic disorders characterized by the accumulation of sphingolipids within cells, particularly within lysosomes. These disorders result from deficiencies in enzymes involved in the breakdown of sphingolipids, which play important roles in various cellular functions such as signaling and structural integrity. Sphingolipids are a class of complex lipids composed of a sphingosine backbone, a long-chain fatty acid, and various head groups. Due to the diversity of sphingolipid species and their involvement in cellular functions, sphingolipidoses manifest with overlapping phenotypes and varying symptom severity, rendering them difficult to diagnose. Lysosphingolipids, derived from the deacylation of glycosphingolipids, have emerged as potential biomarkers of several sphingolipidoses, including Gaucher disease (GD), Fabry disease (FD), Niemann-Pick type A/B (NPA/B) and type C (NPC) diseases, and GM1 and GM2 gangliosidoses (Figure 1).[1] Early detection of sphingolipidoses through screening for inborn errors of metabolism (IEM) allows for timely intervention and treatment, thus helping improve outcomes and quality of life for the affected infants.

Screening for sphingolipidoses typically involves measuring specific biomarkers or enzyme activities in dried blood spots (DBS) obtained from a heel prick blood sample collected shortly after birth. DBS is an alternative sampling method where capillary blood is collected and dried on a paper card. DBS has been used in the screening

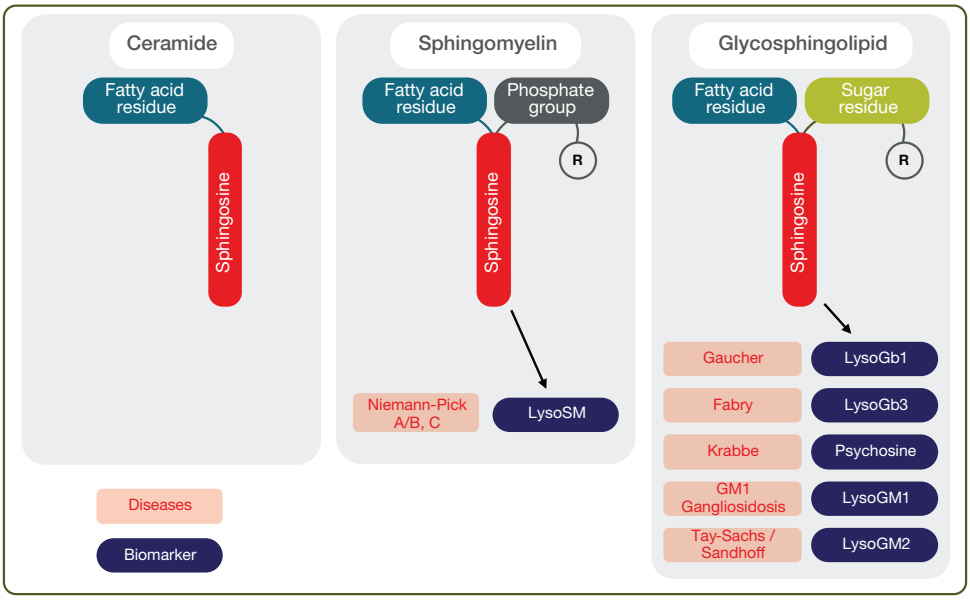

Figure 1. Overview of the biochemical components of sphingolipids, their deacylated lysosphingolipid biomarkers (highlighted in blue), and their respective diseases (red)

of IEM in newborns since the early 1960s. Compared to the traditional venous liquid blood collection method, the DBS technique is advantageous for its minimal invasiveness, smaller sample volume, improved analyte stability, and ease of storage and transportation. These properties have also prompted its increasing usage in therapeutic drug monitoring (TDM), clinical toxicology, and sports anti-doping.[2,3] Recently, advanced technologies such as high-resolution mass spectrometry have shown promising results in IEM for the improved detection accuracy for otherwise indistinguishable isobars and isomers compared to nominal mass-resolution triple quadrupole mass spectrometry.[4]

In this work, we implemented a simple and reliable ultra-high performance liquid chromatography (UHPLC) – high-resolution accurate-mass (HRAM) Orbitrap mass spectrometry (MS) method to quantify multiple lysosphingolipid markers for the detection of six sphingolipidoses simultaneously in plasma or DBS:

- Glucosylsphingosine or lysoglucosylceramide (LysoGb1) for GD
- Lysoglobotriaosylceramide (Lyso-Gb3) for FD
- Lysosphingomyeline (LysoSM) for NPA/B
- LysoSM509, which is a LysoSM carboxylated analog known as N-palmitoyl-O-phosphocholine-serine, for NPA/B and NPC
- Lysomonosialoganglioside GM1 and GM2 (LysoGM1 and LysoGM2) for GM1 and GM2 gangliosidoses, respectively

The method was validated with good precision and stability and was used to measure the lysosphingolipid levels in a group of 30 control subjects and 202 subjects suspected of having sphingolipidoses (total of 59 DBS and 143 plasma). This work highlighted the high-standard analytical performances provided by the high-resolution mass spectrometry-based methods. The results indicated that each sphingolipidosis had a distinct pattern of lysosphingolipids, and the lysosphingolipid levels showed consistent patterns between DBS and plasma samples.

Experimental

Sample preparation

The lysosphingolipids, LysoGb1, LysoGb3, LysoSM, LysoGM1, and LysoGM2, were purchased from BioValley (Paris, France). Internal standards; [$^{2}H_{5}$]-LysoGb1 (for LysoGb1, LysoGb3, LysoGM1, LysoGM2) and LysoSM(d17:1) (for LysoSM) were purchased from Sigma (St Louis, MO, USA). LysoSM509 was not commercially available and was semi-quantified using the calibration curve of LysoSM. Individual analyte stock solutions were prepared at 10 µg/mL and diluted in acetonitrile to six levels of non-zero calibration points: LysoGb1 (0.8–500 nmol/L), LysoGb3 (4–100 nmol/L), LysoSM and LysoSM509 (2–500 nmol/L), LysoGM1 and LysoGM2 (2–250 nmol/L), and two quality control (QC) samples, low and high, in amber glass vials. The internal standard (IS) solutions were prepared at 10 µg/mL.

In brief, analytes were extracted from three 3.2 mm ID DBS discs or 9.6 µL of EDTA plasma in the presence of methanol/acetonitrile/water (45:45:10) containing the IS with a final concentration of 3 nmol/L. After incubation in the well plate at 45 °C for 20 min with agitation, the mixture was centrifuged, and the supernatant was diluted with water before LC-MS analysis.

Liquid chromatography

The LC separation was performed on a Thermo Scientific™ Vanquish™ Flex UHPLC system with a Thermo Scientific™ Syncronis™ C18 column (50 x 2.1 mm, 1.7 µm) maintained at 30 °C. Mobile phase A was 0.2% formic acid in water, and mobile phase B was 0.2% formic acid in methanol. The gradient is specified in Table 1, and the injection volume was 15 µL.

Table 1. LC gradient for the lysosphingolipid separation

Time (min)	Flow rate (mL/min)	%B
0.0	0.3	50
1.0	0.3	100
1.5	0.3	100
4.5	0.3	50
5.0	0.3	50
6.0	0.3	50

Mass spectrometry

The analyte quantitation was achieved using the Thermo Scientific™ Orbitrap Exploris™ 120 mass spectrometer coupled to a Thermo Scientific™ OptaMax™ NG ion source with a heated electrospray ionization (HESI) probe in the positive mode. The MS source parameters and scan event properties for the data-dependent MS^2 mode are listed in Table 2. The precursor and fragment ions *m/z* values of the analytes and their IS solutions are shown in Table 3.

Data analysis

Data were acquired and processed using Thermo Scientific™ TraceFinder™ software (ver. 5.1 SP3 Clinical). The mass tolerance for the analyte quantitation using the fragment ions was set to 5 ppm. Other plots were generated in R (version 4.0.0) using data exported from TraceFinder software.

Table 2. Orbitrap Exploris 120 MS settings

Ion source properties	
Ion source type	H-ESI
HESI probe position	Center - 1.0 - L/M (x - y - z)
Global parameters	
Spray voltage (V)	+3,500
Sheath gas (Arb)	35
Aux gas (Arb)	10
Sweep gas (Arb)	1
Ion transfer tube temp. (°C)	325
Vaporizer temp. (°C)	300
Expected LC peak width (s)	6
Lock mass correction	EASY-IC™, Run Start
tSIM scan properties	
Multiplex	5
Isolation window (*m/z*)	2
Resolution	30,000
AGC	Standard
Max injection time (ms)	Auto
Microscans	1
Data type	Profile
Targeted mass list	See Table 3
Data-dependent properties	
Intensity threshold	5.0 e3
Dynamic exclusion	Auto
Targeted mass list	See Table 3
Mass tolerance (ppm)	10
Ignore charge state	True
Apex detection (%)	30
Number of dependent scans	3
ddMS² scan properties	
Resolution	30,000
Isolation window (*m/z*)	1
Collision energy type	Absolute
HCD collision energies (V)	20, 35, 45
AGC	Standard
Max injection time (ms)	Auto
Microscans	1
Data type	Profile

Table 3. The retention time and *m/z* of the analytes and their IS solutions

Analyte	Retention time (min)	Precursor ions (*m/z*)	Fragment ions (*m/z*)	IS	IS precursor ions (*m/z*)	IS fragment ions (*m/z*)
LysoGb1	2.25	462.3425	282.2792	$[^2H_5]$-LysoGb1	467.3739	237.6727
LysoGb3	2.24	786.4482	282.2792	$[^2H_5]$-LysoGb1	467.3739	237.6727
LysoSM	2.25	465.3452	184.0733	LysoSM(d17:1)	451.3295	184.0733
LysoSM509	2.50	510.3428	184.0733	-	-	-
LysoGM1	2.40	1280.6230	282.2792	$[^2H_5]$-LysoGb1	467.3739	237.6727
LysoGM2	2.36	1118.5701	282.2792	$[^2H_5]$-LysoGb1	467.3739	237.6727

Method validation

Linear calibration curves were generated using a weighing factor of 1/x from the limit of detection (LOD) to the upper limit of quantification (ULOQ). Thirty blank samples were injected to compute the standard deviation (SD) of the background, and the LOD for each analyte was defined as 3 x SD, whereas 10 x SD was accepted as the lower limit of quantification (LLOQ). The two levels of DBS and plasma QC samples were used to evaluate intra-day (n = 20) and inter-day (n = 9) accuracy and precision. For carryover analysis, high QC samples (H1, H2, H3) were analyzed in triplicate followed by three low QC samples (L1, L2, L3). The final blank carryover was calculated by taking the percent ratio of the peak areas, 100*(mean L1 – mean L3)/(mean H – mean L3). Accepted carryover values were <1%. Stability has been assessed using pools of plasma and DBS samples aliquoted and analyzed after 24 h, 48 h, 7 days (both in the autosampler at 4 °C and at 20 °C), 15 days, and 21 days (20 °C).

Results and discussion

Quantifying lysosphingolipids in plasma or DBS is a valuable screening method for detecting sphingolipidoses in suspected populations. For the first time, specific lysosphingolipid markers for Fabry, Gaucher, GM1 and GM2 gangliosidoses, Niemann-Pick type A/B, and C diseases were quantified simultaneously in both plasma and DBS using a multiple analyte UHPLC-HRAM-MS/MS method.

Representative extracted ion chromatograms of the five analytes in a control sample and a donor sample are shown in Figure 2. Good linearity was achieved for the calibration curves of lysoGb1, lysoGb3, lysoSM, lysoGM1, and lysoGM2 with the mean regression coefficients (R^2) ranging from 0.9940 to 0.9999 (Figure 3). Due to lack of commercially available synthetic standard, LysoSM509 was semi-quantified using the LysoSM calibration curve. The LOD and LOQ values of each analyte, and the precision of low and high QC samples for both plasma and DBS samples are listed in Table 4. All inter- and intra-day %CV were below 20%, indicating high reproducibility of the reported assay.

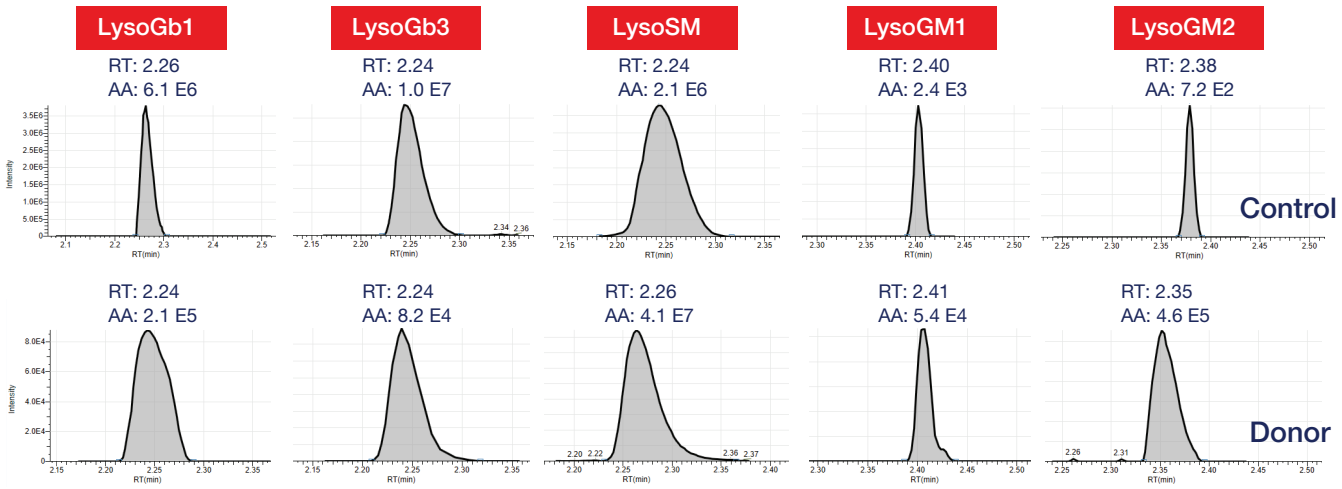

Figure 2. Representative extracted ion chromatograms of the five lysosphingolipids in control samples (top row) and in the donor samples (bottom row)

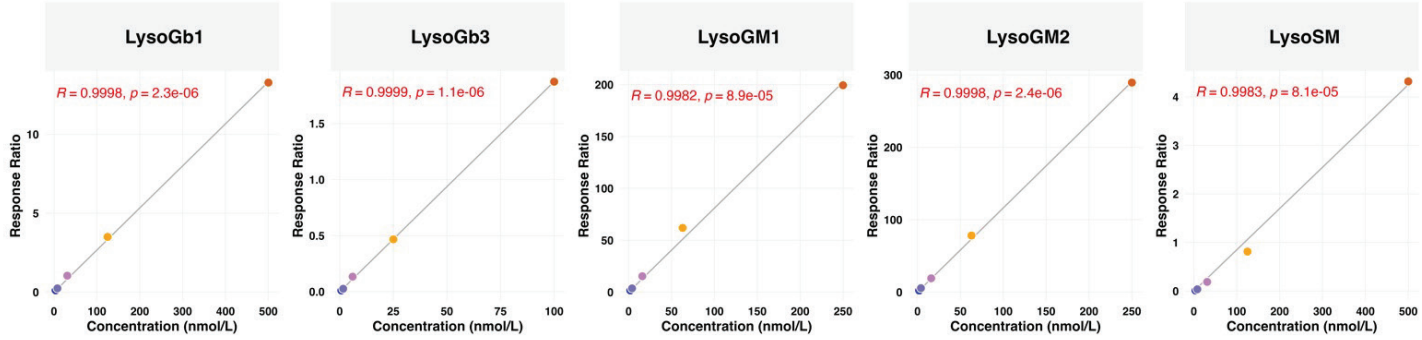

Figure 3. Calibration curves of the five analytes in methanol/acetonitrile/water 45:45:10

Table 4. The detection limits and precisions of lysosphingolipids from DBS or plasma samples (intra-day, n = 20, and inter-day, n = 9)

Analytes	LOD (nM)	LOQ (nM)	QC1, DBS		QC1, plasma		QC2, DBS		QC2, plasma	
			Interday %CV	Intraday %CV	Interday %CV	Intraday %CV	Interday %CV	Intraday %CV	Interday %CV	Intraday %CV
LysoGb1	0.28	0.96	12.20	5.60	8.30	3.60	6.50	2.50	6.20	3.30
LysoGb3	0.16	0.56	15.60	10.60	12.60	5.40	7.80	3.90	6.90	3.50
LysoSM	0.30	1.00	5.40	6.00	10.00	5.00	11.60	2.20	9.30	4.50
LysoGM1	0.09	0.30	4.10	12.10	8.40	12.80	5.10	12.30	7.00	4.50
LysoGM2	0.02	0.08	8.80	11.20	9.90	9.80	15.10	13.00	5.80	2.90

Analyte stabilities in the pooled plasma and DBS samples were assessed for up to 7 days in the autosampler at 4 °C, and up to 21 days at 20 °C. The stability results expressed as a percentage of variation compared to Day 0 are summarized in Table 5. Except for LysoGM1, the change of concentrations for all other four analytes from DBS or plasma in the 4 °C autosampler are within ± 30% up to 7 days. LysoSM and LysoGM1 concentrations changed more significantly at 20 °C than at 4 °C. The instability of these analytes is unknown and requires further investigation.

The reference cut-off was defined as the 99th percentile[5] and was set as follows:
LysoGb1: (DBS = 34.36 nM, Plasma = 0.94 nM),
LysoGb3: (DBS = 3.26 nM, Plasma = 0.41 nM),
LysoGM1: (DBS = 3.72 nM, Plasma = 3.15 nM),
LysoGM2: (DBS = 0.97 nM, Plasma = 1.95 nM),
LysoSM: (DBS = 93.93 nM, Plasma = 10.28 nM),
LysoSM509: (DBS = 3.75 nM, Plasma = 1.06 nM).

The method measured the levels of lysosphingolipids in a group of 30 control subjects and 202 subjects with sphingolipidoses: 53 Gaucher, 79 Fabry, 7 NPA/B, 15 NPC, and 3 GM2 (total of 59 DBS and 143 plasma). Lysosphingolipid levels in DBS showed similar patterns as those from plasma, and distinguished controls from positive subjects. All the results are presented in Figure 4.

Table 5. Analyte stabilities at 4 °C (up to 7 days) and 20 °C (up to 21 days). Stability is expressed as a percentage of variation compared to Day 0.

		DBS					Plasma				
		Day1	Day2	Day7	Day15	Day21	Day1	Day2	Day7	Day15	Day21
+ 20 °C	LysoGb1	9.84	-2.19	19.36	16.34	8.97	22.05	18.85	43.97	48.96	48.20
+ 20 °C	LysoGb3	2.81	-21.50	-0.39	-11.78	-15.43	11.47	6.29	35.00	22.05	23.41
+ 20 °C	LysoSM	-69.83	-48.70	-68.55	-75.87	-74.86	19.39	24.26	46.70	37.39	23.29
+ 20 °C	LysoGM1	44.82	548.51	522.74	340.09	352.81	-19.47	3.64	-2.99	26.85	44.92
+ 20 °C	LysoGM2	-4.83	17.50	15.15	17.94	9.34	-7.84	-33.41	-1.55	44.68	38.16
4 °C Autosampler	LysoGb1	-0.74	-1.52	-2.10			-1.39	-0.52	-2.48		
4 °C Autosampler	LysoGb3	1.35	15.88	12.34			3.34	4.37	2.25		
4 °C Autosampler	LysoSM	3.27	1.23	0.30			0.81	5.37	-17.36		
4 °C Autosampler	LysoGM1	-14.46	70.49	63.10			-17.64	-33.31	-34.18		
4 °C Autosampler	LysoGM2	-10.30	-23.50	23.82			-10.08	-5.35	-13.70		

Biomarkers	LysoGb1	LysoGb3	LysoSM, LysoGM509	LysoGM1	LysoGM2
Disorders	Gaucher	Fabry	Niemann-Pick A/B, C	GM1 gangliosidosis	GM2 gangliosidosis

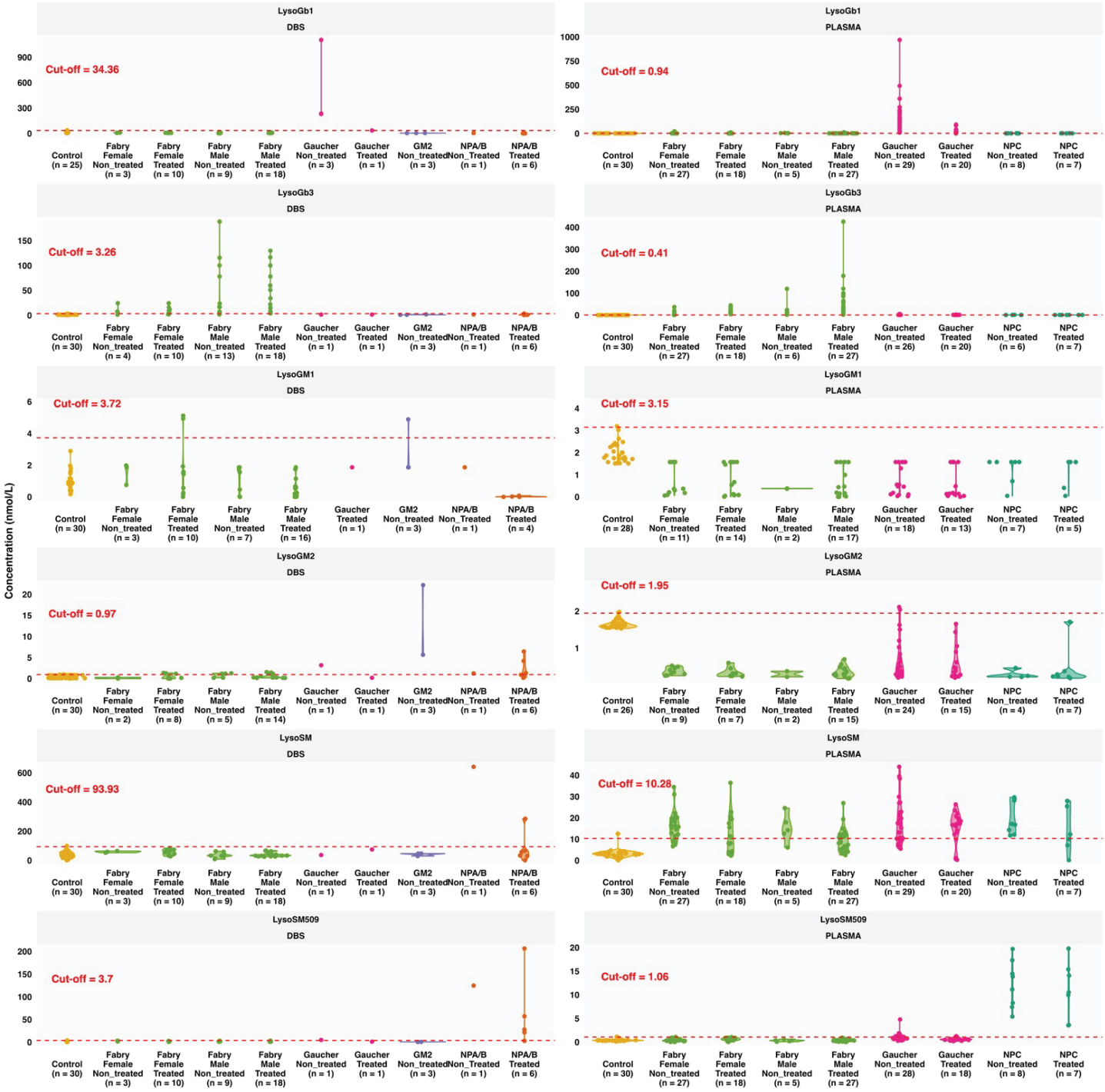

Figure 4. Overview of donor lysosphingolipid levels in plasma and DBS samples

Conclusions

A multiple analyte UHPLC-HRAM-MS/MS method was developed on an Orbitrap Exploris 120 mass spectrometer to measure various lysosphingolipids in plasma and DBS to detect Fabry, Gaucher, GM1 and GM2 gangliosidoses, Niemann-Pick type A/B, and C diseases simultaneously. In both DBS and plasma samples, quantified analytes were able to distinguish between controls and positive donors in a similar manner, reinforcing the usage of DBS to perform these measurements in a multiple analyte fashion. This method can be used to complement the first-tier enzymatic assays for sphingolipidoses to improve the detection performance.

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