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Urinary Globotriaosylsphingosine-Related Biomarkers for Fabry Disease Targeted by Metabolomics

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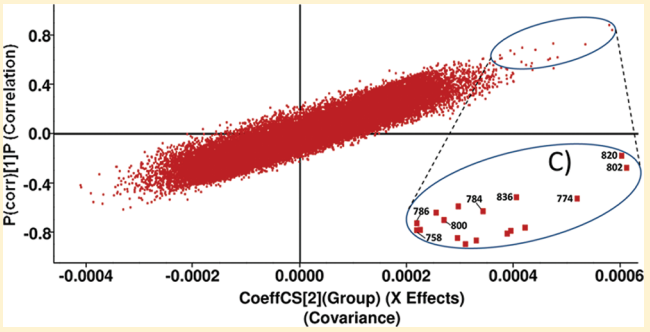
Supporting Information

ABSTRACT: Fabry disease is a lysosomal storage disorder caused by deficiency of α-galactosidase A, resulting in glycosphingolipid accumulation in organs and tissues, including plasma and urine. Two disease-specific Fabry biomarkers have been identified and quantified in plasma and urine: globotriaosylceramide (Gb_3) and globotriaosylsphingosine (lyso-Gb_3). The search continues for biomarkers that might be reliable indicators of disease severity and response to treatment. The main objective of this study was to target other urinary biomarkers using a time-of-flight mass spectrometry metabolomic approach. Urinary metabolites of 63 untreated Fabry patients and 59 controls were analyzed. A multivariate statistical analysis performed on a subset of male samples revealed seven novel Fabry biomarkers in urine, all lyso-Gb_3 analogues having modified sphingosine moieties. The empirical formulas of the sphingosine modifications were determined by exact mass measurements ($-C_2H_4$, $-C_2H_4+O$, $-H_2$, $-H_2+O$, $+O$, $+H_2O_2$, $+H_2O_3$). We evaluated the relative concentration of lyso-Gb_3 and its seven analogues by measuring area counts for each analogue in all Fabry patients. All samples were normalized to creatinine. We found higher concentrations for males with Fabry disease compared to females. None of these biomarkers were detected in controls. To our knowledge, this is the first time that lyso-Gb_3-related Fabry disease biomarkers are detected in urine.



Fabry disease (OMIM no. 301500) is a lysosomal storage disorder (LSD) caused by deficiency of α-galactosidase A (α-GAL or GLA; EC 3.2.1.22), resulting in the accumulation of glycosphingolipids, predominantly globotriaosylceramide (Gb_3) (Figure 1A), in biological fluids and in multiple organs and tissues, such as the walls of small blood vessels, unmyelinated nerves, the heart, and the kidneys.[1] Although it is an X-linked disorder which presents earlier and is clinically more severe in affected males, a large proportion of heterozygous females are also affected.[2−4] Fabry disease presents a heterogeneous clinical phenotype with manifestations such as painful acroparesthesia, angiokeratomas, and hypohidrosis in early childhood or adolescence,[5] progressing to renal insufficiency, cardiomyopathy, and cerebrovascular accidents in adulthood.[1] Life expectancy of both affected males and females is significantly reduced.[4] Treatment with enzyme replacement therapy (ERT) has been shown to stabilize the progression of the disease.[4]

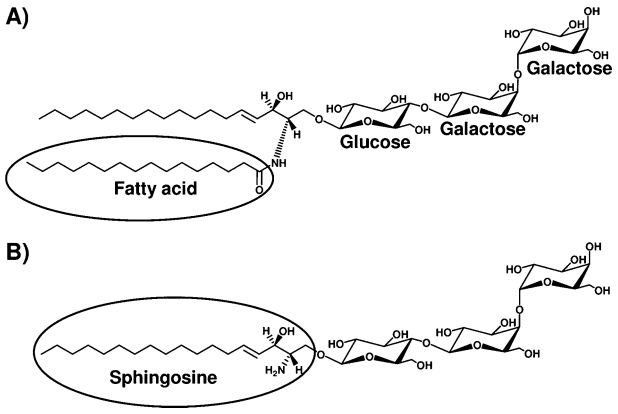


Figure 1. (A) Structure of globotriaosylceramide (Gb_3) with palmitic acid (C_{16}) fatty acid as an example. (B) Structure of globotriaosylsphingosine (lyso-Gb_3).

Recently, another biomarker has been detected in plasma[6] and urine[7] of Fabry patients: globotriaosylsphingosine (lyso-Gb_3). It is similar to the Gb_3 molecule lacking the fatty acid chain (deacylation of the Gb_3 molecule) (Figure 1B).

Recent publications have emphasized that lyso-Gb_3 might be a potential diagnostic biomarker for Fabry disease.[8] Plasma lyso-Gb_3 concentrations have also been shown to be of diagnostic value, and exposure to lyso-Gb_3 in plasma correlates

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Table 1. UPLC and ESI-TOF-MS Methods for the Analysis of Urinary Metabolites and Urinary Creatinine

UPLC methods			ESI-TOF-MS methods		
parameter	urinary metabolites	urinary creatinine	parameter	urinary metabolites	urinary creatinine
column	Acquity UPLC HSS T3	Acquity UPLC BEH Hilic	scan mode	MS-TOF	MS-TOF
company	Waters Corp. (Milford, MA)	Waters Corp. (Milford, MA)	ionization mode	ESI	ESI
length	50 mm	50 mm	polarity	positive	positive
i.d.	2.1 mm	2.1 mm	analyzer mode	V	V
particle diameter	1.8 μm	1.7 μm	dynamic range	extended	extended
temp	30 °C	30 °C	capillary voltage	3.2 kV	2.0 kV
			sampling cone voltage	35 V	25 V
flow rate	0.50 mL/min	0.50 mL/min	extraction cone voltage	5.0 V	5.0 V
			source temperature	120 °C	120 °C
mobile phase A	ACN 60%/IPr 40%/FA 0.1%	ACN	desolvatation temperature	450 °C	450 °C
mobile phase B	H_2O 95%/MeOH 5%/FA 0.1%	NH_4HCO_2 50 mM/FA 0.5%	cone gas flow	30 L/h	30 L/h
			desolvatation gas flow	700 L/h	700 L/h
gradient	0−1 min → 100% B	0−1 min → 5% B	trap. collision energy	6.0 V	6.0 V
	1−2 min → 100−90% B	1−3 min → 5−15% B	transfer collision energy	4.0 V	4.0 V
	2−9 min → 90−65% B	3−5 min → 15−50% B	data format	centroid	centroid
	9−14 min → 65−35% B	5−6 min → 50% B	mass range	50−1000 Da	50−1000 Da
	14−15 min → 35−10% B	6−7 min → 50−5% B	scan time	0.1 s	0.1 s
	15−17 min → 10% B	7−10 min → 5% B			
	17−20 min → 10−100% B		**Lock Mass**		
			compd	terfenadine (500 nM)	na
weak wash	H_2O 50%/ACN 50%/FA 0.1%	ACN 100%	exact mass	472.3215 Da	na
strong wash	MeOH 100%/FA 0.1%	water 100%	solvent	H_2O/ACN 5%/FA 0.5%	na
			scan time	0.5 s	na
injection volume	5 μL	2 μL	interval	5.0 s	na
injection mode	partial loop overfill	partial loop overfill	sampling cone voltage	30 V	na
			trap. collision energy	6.0 V	na
autosampler temp	15 °C	15 °C	mass window	±0.2 Da	na
			scan average	3	na
solvent delay	0−1 min and 17−20 min	0−1 min	flow rate	∼4 μL/min	na

with severity of Fabry disease manifestations.[9] On the basis of the effect on human podocytes, a possible role for lyso-Gb_3 in glomerular injury has also been postulated.[10] Reduction of plasma lyso-Gb_3 concentrations in response to enzyme replacement therapy has also been demonstrated.[11] Administration of the recombinant GLA enzyme to Fabry mice was reported to result in a greater decrease of tissue lyso-Gb_3 compared to Gb_3 levels, particularly in the kidney.[12]

We recently undertook a metabolomic study of plasma using a mass spectrometry approach and detected novel lyso-Gb_3-related analogues in Fabry patients (submitted for publication). This prompted us to evaluate urine specimens of Fabry disease patients. The main objective of this study focused on the analysis of urine specimens of males and females affected with Fabry disease to target novel biomarkers which might reflect disease severity and response to therapy. To attain this goal, we opted for a metabolomic approach employing time-of-flight mass spectrometry. The study of the metabolome has gained interest over the years for different diseases because of its ultimate potential to be helpful clinically for early diagnosis of a disease and as a measure of response to treatment.[13] Moreover, metabolomics is an emerging aspect of biomarker research focused on generating important information about biochemical pathways that are modified in disease states and restored to normal by treatment of affected patients, such as in Fabry disease.[14] A metabolomic approach allows the metabolic profiling of large numbers of small molecules (or metabolites) of up to 1500 Da in different biological fluids.[14,15] It is an unbiased targeting approach to classify samples based on metabolic patterns that are modified in response to disease, treatment, or other genetic perturbations. No single biomarker will likely be the lone determinant of the prognosis and the outcome of treatment of a specific disease.[14] However, the evaluation of patterns of metabolites that are unique to a group of patients compared rigorously with healthy reference control values could be of diagnostic and prognostic value. Metabolomics performed in Fabry patients compared with healthy age-matched controls could favor the discovery of biomarkers which might specifically be related to disease severity and progression and also contribute to a deeper understanding of the pathophysiology of the disease. We present the results of a metabolomic study based on ultraperformance liquid chromatography coupled to a time-of-flight mass spectrometry investigation of Fabry disease.

EXPERIMENTAL SECTION

Ethics Approval. This project was approved by the Research Ethics board (REB) of the Faculty of Medicine and Health Sciences and the Centre hospitalier universitaire de Sherbrooke (CHUS) and the institutional REBs of all the collaborators.

Sample Collection. After informed consent was obtained, random urine samples were collected from Fabry patients in whom the diagnosis had been confirmed by demonstrating marked enzyme deficiency in leucocytes or by mutation analysis. None of the patients had ever undergone enzyme

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replacement therapy (ERT) at the time of the sample collection. Random urine samples were neither filtered nor centrifuged. Sixty-three untreated Fabry patients were evaluated: 18 males (age range from 1 to 60 years, mean age: 22 years) and 45 females (age range from 0.5 to 78 years, mean age: 34 years). Control urine samples from 59 healthy subjects, 23 males (age range from 3 to 57 years, mean age: 20 years) and 36 females (age range from 2 to 59 years, mean age: 33 years), were analyzed for comparison. Urine samples from a male Fabry patient having the p.Arg342 (R342X) mutation were analyzed before and after ERT. This 35 year-old patient presented a number of Fabry disease-related clinical manifestations: hypohydrosis, angiokeratomas, tinnitus, proteinuria, abdominal pain, cornea verticillata, and ischemic cerebrovascular disease. He received agalsidase-β 1.0 mg/kg/2 weeks (Fabrazyme, Genzyme Corporation, Cambridge MA). Samples were collected at intervals over a period of 2 years: two samples were collected before and three samples after initiation of ERT. Samples were stored in multiple aliquots at −20 °C until analysis. A Supporting Information table (Table S-1) presents the age, gender, and mutations of Fabry patients and controls.

Reagents. HPLC grade methanol (MeOH), acetonitrile (ACN), and 2-propanol (iPr) were from EMD Chemicals Inc. (Darmstadt, Germany). Formic acid (FA) (99+%) was from Acros Organics (New Jersey, U.S.A.). ACS grade ammonium hydroxide (NH_4OH) (29%) and ammonium formate were from Fisher Scientific (Fair Lawn, NJ). ACS grade hydrochloric acid (HCl) (37%) was from Anachemia Canada (Montreal, Canada). Creatinine and terfenadine were from Sigma-Aldrich (St. Louis, MO). The creatinine-d_3 (methyl-d_3) (99.8 atom % D) standard was purchased from CDN Isotopes (Pointe-Claire, Canada). Laboratory water was purified to ultrapure grade with the use of a Nanopure Infinity water purification system (Ultrapure, 18.3 MΩ, Barnstead, Dubuque, IA).

Instrumentation and Parameters. The ultraperformance liquid chromatography−electrospray ionization time-of-flight mass spectrometry (UPLC−ESI-TOF-MS) analyses were performed on a Synapt ESI-QTOF-MS system hyphenated with an Acquity UPLC, both from Waters Corporation (Milford, MA). Between these two systems, a Rheodyne injection valve model MXP9900-000 from IDEX Health & Science (Oak Harbor, WA) was installed to direct the UPLC eluent toward the mass spectrometer or to divert it in the waste. The UPLC and MS methods programmed to analyze the metabolites in the samples under study are detailed in Table 1.

To increase the mass accuracy during the analysis of urinary metabolites, the "lock mass" option was activated for real-time recalibration. For this purpose, an HPLC isocratic pump model 515 from Waters Corp (Milford, MA) was used to inject the calibrating solution (terfenadine 500 nM) through the reference ionization probe during the sample analysis. The flow rate of the isocratic pump was set to 0.500 mL/min, and that flow rate was decreased to approximately 4 μL/min before its entrance in the mass spectrometer, using a "T" splitter.

Sample Preparation. *Metabolite Analysis in Urine.* Urine samples were processed by solid-phase extraction (SPE) as described earlier by Auray-Blais et al.[7] Briefly, the SPE cartridges (Oasis, MCX, 30 mg, 60 μm, Waters Corp., Milford, MA) were conditioned successively with 1 mL of MeOH and 1 mL of HCl 1 N. Simultaneously, 500 μL aliquots of well-mixed urine samples from untreated Fabry patients and healthy controls were transferred to glass tubes along with 500 μL of MeOH, to prevent adsorption of hydrophobic compounds to the wall of the tubes, and acidified with 100 μL of HCl 1 N. Each urine sample was loaded on a conditioned SPE cartridge, which was successively washed with 1 mL of 2% FA in water and 1 mL of 0.2% FA in MeOH. Samples were eluted into glass tubes with 600 μL of 2% NH_4OH in MeOH and evaporated to dryness under a stream of nitrogen. Residues were reconstituted with 200 μL of 50% ACN/water/0.1% formic acid for further UPLC−ESI-TOF-MS analysis. Urine samples from the aforementioned four groups of individuals were randomly prepared and analyzed to prevent composition discriminations between the groups due to the sample preparation order and to the analysis sequence (instead of samples composition).

For the structural elucidation of Fabry disease biomarkers by UPLC−ESI-TOF-MS, a 5 mL urine aliquot from an untreated Fabry male (patient 9 in Supporting Information Table S-1) was processed to obtain a sample 10 times more concentrated than that obtained by the procedure mentioned above.

Creatinine Analysis in Urine. The creatinine from all urine samples was analyzed using a method developed by our group by means of isotope dilution mass spectrometry on the QTOF system. Twenty-five microliters of urine and 25 μL of internal standard (creatinine-d_3 2 mM) were added to an Eppendorf tube containing 950 μL of 65% ACN/35% ammonium formate 50 mM/0.5% FA. After mixing, 2 μL of the solution was further diluted with 200 μL of 65% ACN/35% ammonium formate 50 mM/0.5% FA prior to UPLC−ESI-TOF-MS analysis. For the calibration curve, the 25 μL urine sample was replaced by 25 μL of the standard solutions (0, 1, 2, 5, 7.5, 10, 15, 20, and 30 mM of creatinine in water). Since no urine standard exempt of creatinine is available, urine samples from two healthy control males with high (15.868 mM) and low (2.371 mM) levels of creatinine were used to measure intraday (n = 5) and interday (n = 5) precisions. The limit of detection (LOD) for creatinine was defined using a signal-to-noise ratio of 3.

Data Mining and Multivariate Data Analysis. The UPLC−ESI-TOF-MS results generated during the metabolomic study were processed by MarkerLynx XS (Waters Corp.). This data mining software aligned the metabolites detected in different samples according to the exact mass and retention time and also measured their peak areas. For that process, the mass range chosen was between 100 and 1000 Da, the intensity threshold was 5 counts, the mass window was 0.05 Da, and the retention time window was 0.20 min. Only the mass spectra acquired between 1 and 15 min during the UPLC run were processed in order to discard contaminants not retained on the UPLC column or eluted during the washing of the column. The results treated with MarkerLynx XS were downloaded in EZInfo, a software of Umetrics (Umeå, Sweden) integrated to the extended statistics tools box of MassLynx V4.1 (Waters Corp.), for multivariate analysis. Using EZInfo, unsupervised principal component analysis (PCA) followed by the supervised orthogonal partial least-squares-discriminant analysis (OPLS-DA) was performed to discriminate the 18 untreated Fabry males from the 23 healthy male controls. For PCA and OPLS-DA, the unit variance (UV) scaling (eq 1) was chosen to amplify the statistical contribution of the metabolites close to the noise level.

$$\mathrm{UV} = \frac{x - y}{\mathrm{SD}} \tag{1}$$

x = area of the metabolite; y = mean area of the metabolite; SD = standard deviation of the areas of the metabolite.

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An S-plot was later generated to highlight the metabolites contributing the most to the discrimination of the two groups of samples during OPLS-DA and considered to be potential Fabry disease biomarkers. The more abundant a biomarker is in patient samples and absent in control samples, the more it obviously will be revealed by the S-plot. For that reason, only the male samples were used for the multivariate analysis (PCA, OPLS-DA, S-plot) because, in general, the clinical, physiological, and biochemical manifestations of Fabry disease are more severe for male patients compared to females. In order to highlight even more Fabry disease biomarkers in the S-plot, a second iteration of the multivariate analysis was performed with 11 Fabry male patients specifically presenting the highest level of the biomarker with the mass to charge (m/z) ratio 802 revealed at the first iteration. These samples were expected to be more characteristic of Fabry disease because they already showed high levels of urinary excretion of Gb_3 and lyso-Gb_3 biomarkers.

System Stability Evaluation. To evaluate the system stability over the metabolomic run, a quality control urine sample was analyzed at the beginning of the analytical run, at every 18 samples, and at the end of the run for a total of nine replicates. Data mining was performed, as described in the previous section, for all the urine samples and all the replicates of the QC analyzed. The MarkerLynx threshold was then increased to 25 counts. Thereafter, a PCA analysis was performed on all urine samples and replicates of the QC using the UV scaling to visualize the similarity and dissimilarity of the samples.

Structural Characterization of Fabry Disease Biomarkers. The biomarkers pointed out during the multivariate statistical analysis were structurally characterized by tandem mass spectrometry. The UPLC and MS methods were the same as the ones presented in Table 1 for the analysis of urinary metabolites except that the MS mode was QTOF-MS (quadrupole time-of-flight-mass spectrometry) instead of TOF-MS. The precursor ions were thus isolated in the quadrupole (Q), fragmented in the collision trap, and the resulting fragments were analyzed in the TOF system. The following parameters were selected for the fragmentation: collision energy ramps were 20−30 V for the metabolites with m/z lower than 810 and 25−35 V for the ones with m/z higher than 810.

Absolute Quantification of Creatinine in Urine. In each urine sample, the creatinine concentration was calculated using the QuanLynx 4.1 software (Waters). The calibration curve was quadratic with a $1/X$ weighting function, and the origin was excluded. The mass window was 0.04 Da and the retention time window was 0.12 min. For the smoothing, four iterations of the "mean" method were used with a smoothing width of 3.

Relative Quantification of Fabry Disease Biomarkers. Fabry disease biomarkers targeted during the multivariate analysis performed on 11 male urine samples were integrated in all the analyzed samples (males and female patients) using QuanLynx. The Apex track integration algorithm was selected with a mass window of 0.1 Da and a retention time window of 0.2 min. For the smoothing, three iterations of the "mean" method were used with a smoothing width of 2.

RESULTS AND DISCUSSION

UPLC−ESI-TOF-MS Analysis of Metabolites and Creatinine in Urine. Figure 2A shows an example of a UPLC−ESI-TOF-MS base peak chromatogram obtained during the metabolomic analysis of an untreated Fabry male urine sample purified by SPE and analyzed on a HSS T3 column (Waters). Figure 2B shows the extracted ion chromatograms of creatinine and creatinine-d_3 (2 mM) analyzed by UPLC−ESI-TOF-MS from a urine sample of the same untreated Fabry male patient. A UPLC normal phase column (BEH Hilic, Waters) was used. Figure 2C presents the creatinine at the LOD (0.2 mM).

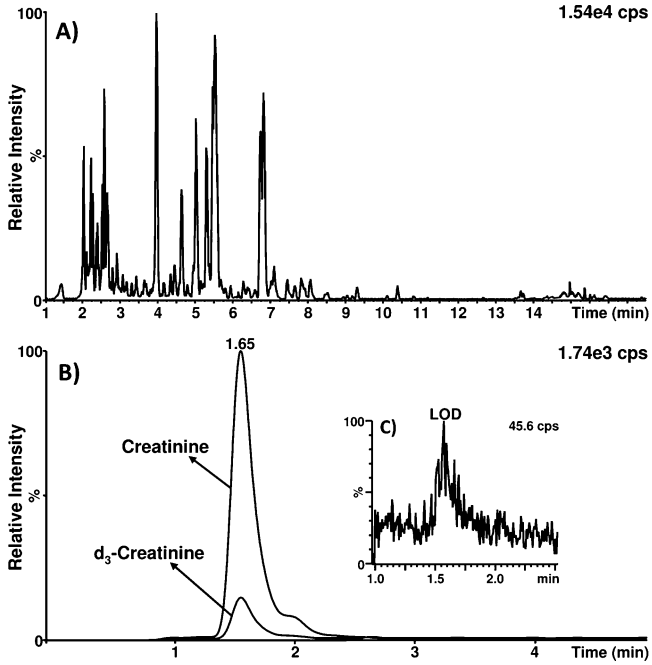

Figure 2. UPLC−ESI-TOF-MS analysis of a urine sample from an untreated Fabry male patient (creatinine = 18.542 mM). (A) Base peak ion chromatogram. The sample was purified by solid-phase extraction (MCX, Oasis) and analyzed on a UPLC C_{18} column (HSS T3, Waters). (B) Extracted ion chromatograms of creatinine and creatinine-d_3 (2 mM). The sample was diluted and analyzed on a UPLC normal phase column (BEH Hilic, Waters). (C) Creatinine at the limit of detection (LOD) (0.2 mM).

Data Mining and Multivariate Data Analysis. The compilation with MarkerLynx of the UPLC−ESI-TOF-MS metabolomic results obtained for 11 untreated Fabry males and 23 healthy control males provided a list of 47 263 metabolites aligned according to their exact mass and to their retention time. The multivariate statistical analysis (PCA, OPLS-DA, and S-plot) of these results was performed using EZInfo as described previously. The OPLS-DA score plot (Figure 3A) shows a complete differentiation between the two sample groups. Each symbol in the graph corresponds to one sample. The x-axis represents the intergroup variation, and the y-axis represents the intragroup variation. The ellipse corresponds to the Hotelling T^2 range with a significance level of 95% (p = 0.05). The control sample 4 (see Supporting Information Table S-1) was outside the Hotelling T^2 ellipse. This sample was thus considered an outlier and not included in the multivariate statistical analysis. In the S-plot (Figure 3B), each point corresponds to a metabolite defined by MarkerLynx. The S-plot presents for each metabolite the reliability (y-axis) in function of the covariance (x-axis). The more a metabolite is located at the top right or at the bottom left of the graph, the greater its influence on the discrimination of the sample groups in the OPLS-DA score plot. Metabolites located at the top right corner of the S-plot were found only in Fabry samples

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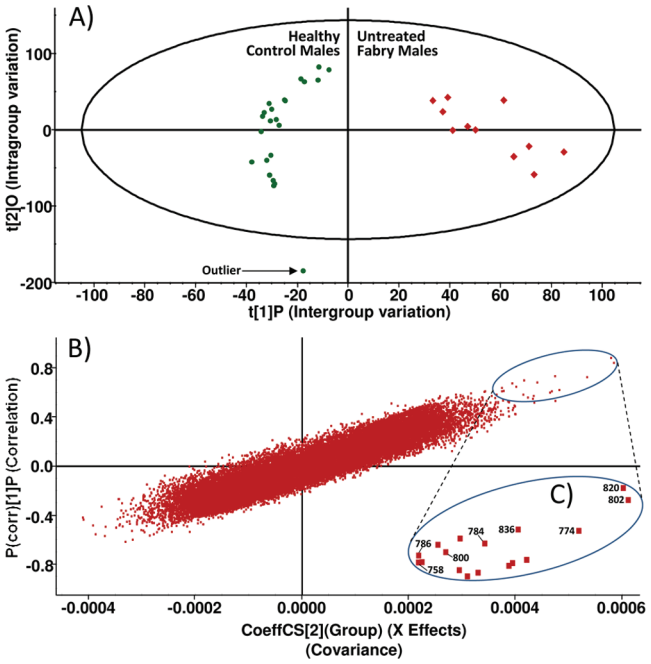

Figure 3. (A) OPLS-DA score plot derived from the UPLC–ESI-TOF-MS metabolomic analysis of urine samples from untreated Fabry males (n = 11) and healthy control males (n = 23). The ellipse corresponds to the Hotelling T^2 range with a significance level of p = 0.05. (B) S-plot showing the correlation in function of the covariance for the metabolites used to discriminate the two sample groups in the OPLS-DA score plot. (C) Zoom of the section of the S-plot where the Fabry disease biomarkers (lyso-Gb_3 (m/z 786) and its analogues) are annotated.

compared to control samples, and the metabolites at the bottom left were not detected in Fabry samples compared to control samples. Fabry disease metabolites are considered as potential biomarkers if they are present in Fabry patient samples, but not in normal control samples. Therefore, among the metabolites located in the top right of the figure, only the ones present in at least six Fabry patients and in none of the control samples were retained as potential biomarkers. We present eight metabolites respecting these criteria (m/z 758, 774, 784, 786, 800, 802, 820, and 836), which are annotated in Figure 3C. Owing to the very low threshold (5 counts/s) set in MarkerLynx to detect very low abundance biomarkers, other metabolites at the top right corner of the S-plot were found to be background noise. The m/z 786 metabolite corresponds to lyso-Gb_3, a known urinary biomarker of Fabry disease.[7]

System Stability Evaluation. A Supporting Information figure (Figure S-1) shows the PCA score plot derived from the UPLC–ESI-TOF-MS metabolomic analysis of all the urine samples (63 Fabry patients, 59 healthy controls, and 1 QC (nine replicates)). In the PCA score plot, each point corresponds to a different sample or QC. The more two points are close together, the more the composition of these two samples (or QCs) is similar. In Supporting Information Figure S-1, the nine replicates of the QC are tightly clustered indicating a good instrumental stability over the whole metabolomic run.

Structural Characterization of Fabry Disease Biomarkers. To obtain structural information about these seven new potential Fabry disease biomarkers, tandem mass spectrometry analyses (ESI-QTOF-MS: electrospray-quadrupole time-of-flight-mass spectrometry) were performed on a concentrated urine sample from an untreated Fabry male as described in the Sample Preparation section. Figure 4 shows the fragmentation patterns of lyso-Gb_3 (Figure 4A) and the novel Fabry disease biomarkers detected (Figure 4B–H). All these Fabry disease biomarkers have fragmentation profiles similar to lyso-Gb_3 and are therefore considered to be closely related structurally to lyso-Gb_3. Like lyso-Gb_3, the new biomarkers present three successive fragments separated by 162 amu, corresponding to the consecutive losses of three sugar units [two galactose units (Gal) and one glucose (Glc) unit]. The fragment matching the sphingosine (m/z 282) moiety in lyso-Gb_3 is also present in the mass spectra of the new biomarkers but with mass shifts of −28, −12, −2, +14, +16, +34, and +50. These results suggest that the seven targeted novel biomarkers are analogues of lyso-Gb_3, with modified sphingosine moieties. The structures of the sphingosine moieties of the glycosphingolipids vary somewhat according to the tissue in which they occur.[16] Further investigation of the amounts of the different lyso-Gb_3 species accumulating in the plasma and urine of patients with Fabry disease may therefore shed some light on the tissue of origin of the Gb_3 from which they are derived.

In Table 2, the exact mass measured during the metabolomic analysis for these seven new biomarkers were used to determine the empirical formulas of the sphingosine modifications. The differences between the exact mass measured for lyso-Gb_3 analogues and the theoretical exact mass of lyso-Gb_3 were compared to the theoretical exact mass of all the possible chemical modifications corresponding to the addition or subtraction of carbon (C), hydrogen (H), nitrogen (N), and oxygen (O) atoms. In Table 2, the "Proposed modification" corresponds to the empirical formula of the modifications matching the lowest delta (Δ) mass (or mass error) between lyso-Gb_3 and its analogues. Table 2 also presents the UPLC retention times of lyso-Gb_3 and its analogues. As expected, the addition of polar atoms (O) or the removal of nonpolar atoms (C and H) on the sphingosine moiety decreased the retention times of the lyso-Gb_3 analogues compared to lyso-Gb_3.

Absolute Quantification of Creatinine in Urine. Urinary creatinine concentrations were selected to normalize the concentrations of the various compounds identified in urine samples as already presented by our group.[17] However, because urinary creatinine excretion varies with age and sex, metabolomic studies in which concentrations of compounds normalized to creatinine levels should always include studies on age- and gender-matched controls.[17]

A UPLC–ESI-TOF-MS method was developed and validated to analyze the creatinine on the same mass spectrometer that was used for the metabolomic analyses. A creatinine deuterated internal standard (creatinine-d_3) was spiked in the samples to compensate for the losses during the sample preparation and for the possible mass spectrometer variability. Figure 2B presents the extracted ion chromatograms of creatinine and creatinine-d_3 analyzed in the urine sample of an untreated Fabry male (patient 9). The LOD of creatinine, with the proposed dilutions preceding the analysis, is 0.2 mM. Figure 2C shows the extracted ion chromatogram of creatinine at LOD. The correlation coefficient (R^2) for the quadratic calibrations curves was greater than 0.999. The intraday precisions (CV%) (n = 5) were 3.4% for the low creatinine level sample (2.371 mM) and 0.8% for the high creatinine level sample (15.868 mM). The interday precisions (CV%) (n = 5)

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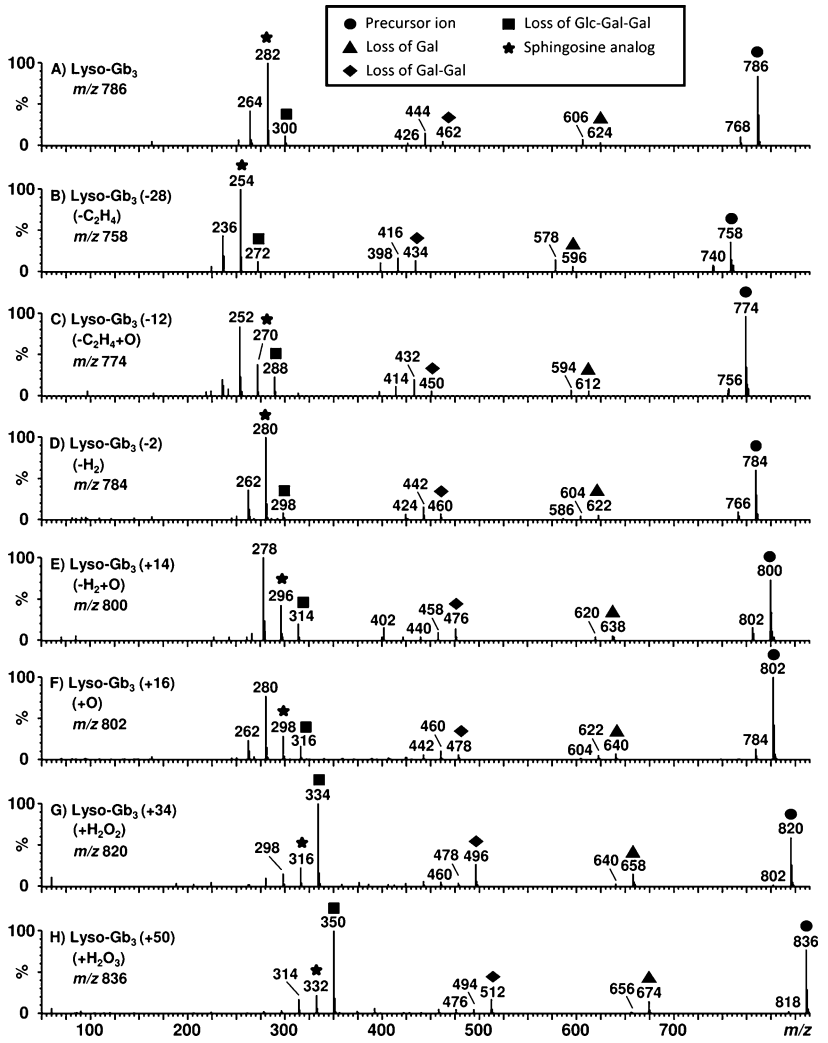

Figure 4. ESI-QTOF-MS fragmentation spectra of lyso-Gb_3 and its analogues. Collision energy ramps: (A–F) 20–30 V; (G and H) 25–35 V. Urine sample from an untreated male Fabry patient.

Table 2. Exact Mass and Retention Time of Lyso-Gb_3 and Analogues

lyso-Gb_3 and analogues	measured mass (amu)	Δ with lyso-Gb_3(amu)[a]	proposed modification[b]	theor mass (amu)[c]	Δ mass (ppm)[d]	retention time (min)
lyso-Gb_3	786.4493	n.a.	n.a.	786.4487	0.8	11.84
lyso-Gb_3 (−28)	758.4121	−28.0366	− C_2H_4	758.4174	−7.0	10.57
lyso-Gb_3 (−12)	774.4067	−12.0420	− C_2H_4 + O	774.4123	−7.2	6.23
lyso-Gb_3 (−2)	784.4290	−2.0197	− H_2	784.4331	−5.2	11.01
lyso-Gb_3 (+14)	800.4275	13.9788	− H_2 + O	800.4280	−0.6	7.99
lyso-Gb_3 (+16)	802.4447	15.9960	+ O	802.4436	1.4	8.32
lyso-Gb_3 (+34)	820.4516	34.0029	+ H_2O_2	820.4542	−3.1	8.22
lyso-Gb_3 (+50)	836.4486	49.9999	+ H_2O_3	836.4491	−0.6	7.12

[a]Difference between the measured exact mass and the theoretical exact mass of lyso-Gb_3. [b]Empirical formula of the modification corresponding to the mass difference between lyso-Gb_3 and its analogue. [c]Theoretical mass of lyso-Gb_3 with the proposed modification. [d]Difference, in parts per million (ppm), between the measured and theoretical masses.

were, respectively, 1.6% and 1.9% for the low- and high-level samples.

Relative Quantification of Fabry Disease Lyso-Gb_3 Analogues. Figure 5 shows the extracted ion chromatograms of lyso-Gb_3 and its analogues for an untreated male Fabry patient (Supporting Information Table S-1, patient 9). The peaks of lyso-Gb_3 −12, +16, and +50 analogues are split suggesting the presence of structural isomers for these analogues.

The results of the analyses of peak areas of lyso-Gb_3 and its analogues were measured with QuanLynx for all the samples (Fabry and controls) and normalized to creatinine for each patient. These results are presented in Supporting Information Table S-1 and summarized in Figure 6. The abundances of lyso-Gb_3 and its analogues are presented according to the four urine sample groups: untreated Fabry males (n = 18), control males (n = 20), untreated Fabry females (n = 45), and control females (n = 36). The ranges for each analogue are in brackets. Lyso-

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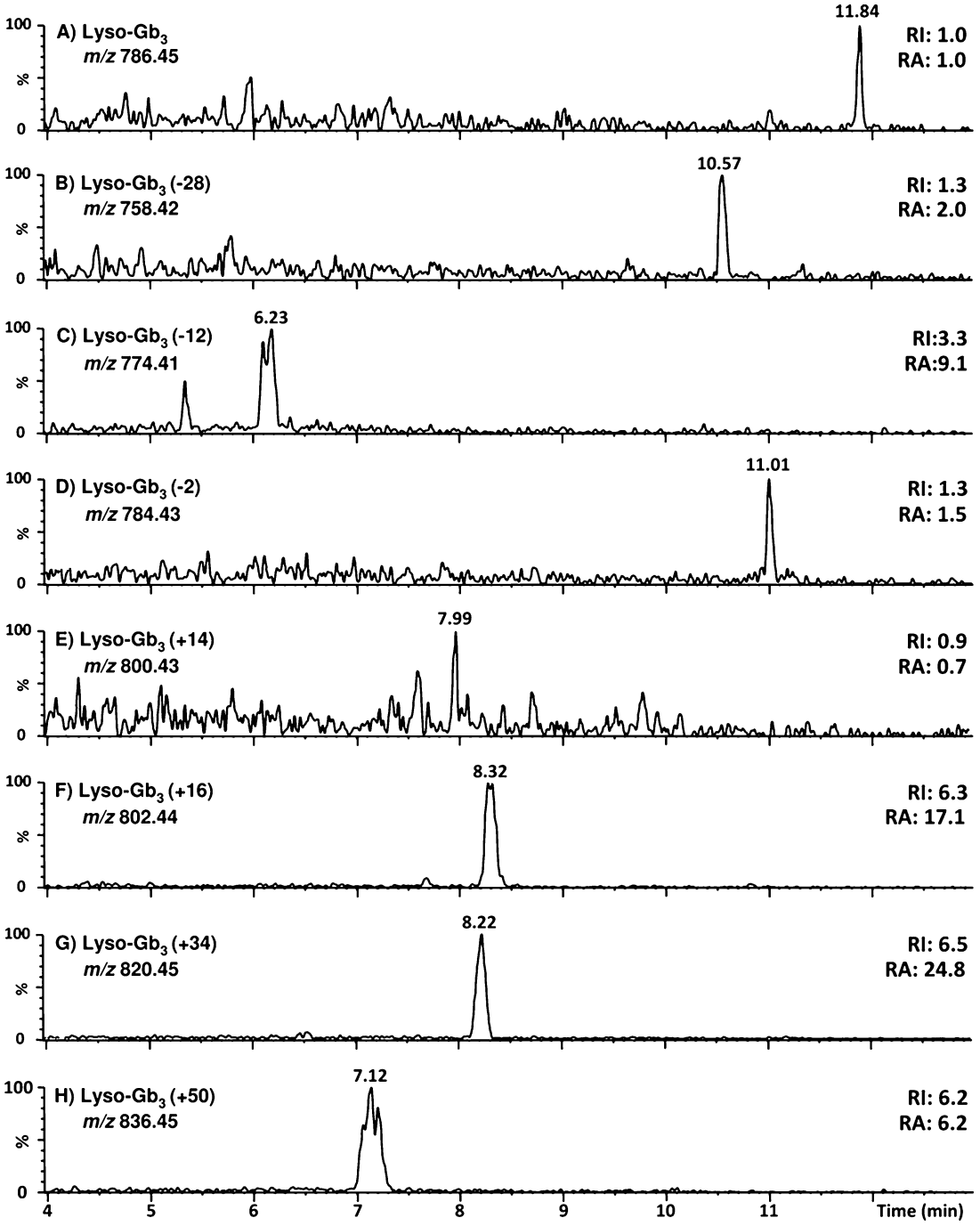

Figure 5. Extracted ion chromatograms of lyso-Gb_3 and its analogues in a urine sample from an untreated male Fabry patient (creatinine = 18.542 mM). Mass window = 0.2 Da. RI = relative intensity. RA = relative area.

Gb_3 and its analogues were not detected in any of the control samples, making them strong candidates as Fabry disease biomarkers. The levels of these eight biomarkers were higher in untreated Fabry males compared to untreated Fabry females. Because disease manifestations are generally less severe in affected females than affected males, these results suggest that the urinary excretion of these specific biomarkers correlates with clinical severity.[7,18] Since the structures of lyso-Gb_3 and its analogues are closely related, similar response factors are expected for these compounds. Except for the lyso-Gb_3 (+14) analogue, all the lyso-Gb_3 analogues have relative concentrations superior to lyso-Gb_3 based on area counts. For example, the relative concentration of the lyso-Gb_3 (+16) in untreated Fabry males is approximately 17 times higher than that of lyso-Gb_3. Some of the new biomarkers may thus be more reliable than lyso-Gb_3 for the diagnosis and follow-up of Fabry disease patients. Lyso-Gb_3-related analogues at m/z −12, +16, +50 are present in higher relative levels of excretion than lyso-Gb_3 (m/z 786) itself in male Fabry patients. In the female Fabry cohort, two lyso-Gb_3-related analogues at m/z −2 and +16 were predominantly excreted. A study with a larger cohort would allow the confirmation of this tendency. Nevertheless, the urinary levels of excretion in females are approximately 10 times less elevated than those found in male Fabry patients. Lyso-Gb_3 and its analogues were not detected in four male and eight female Fabry patient urine samples studied. This may be

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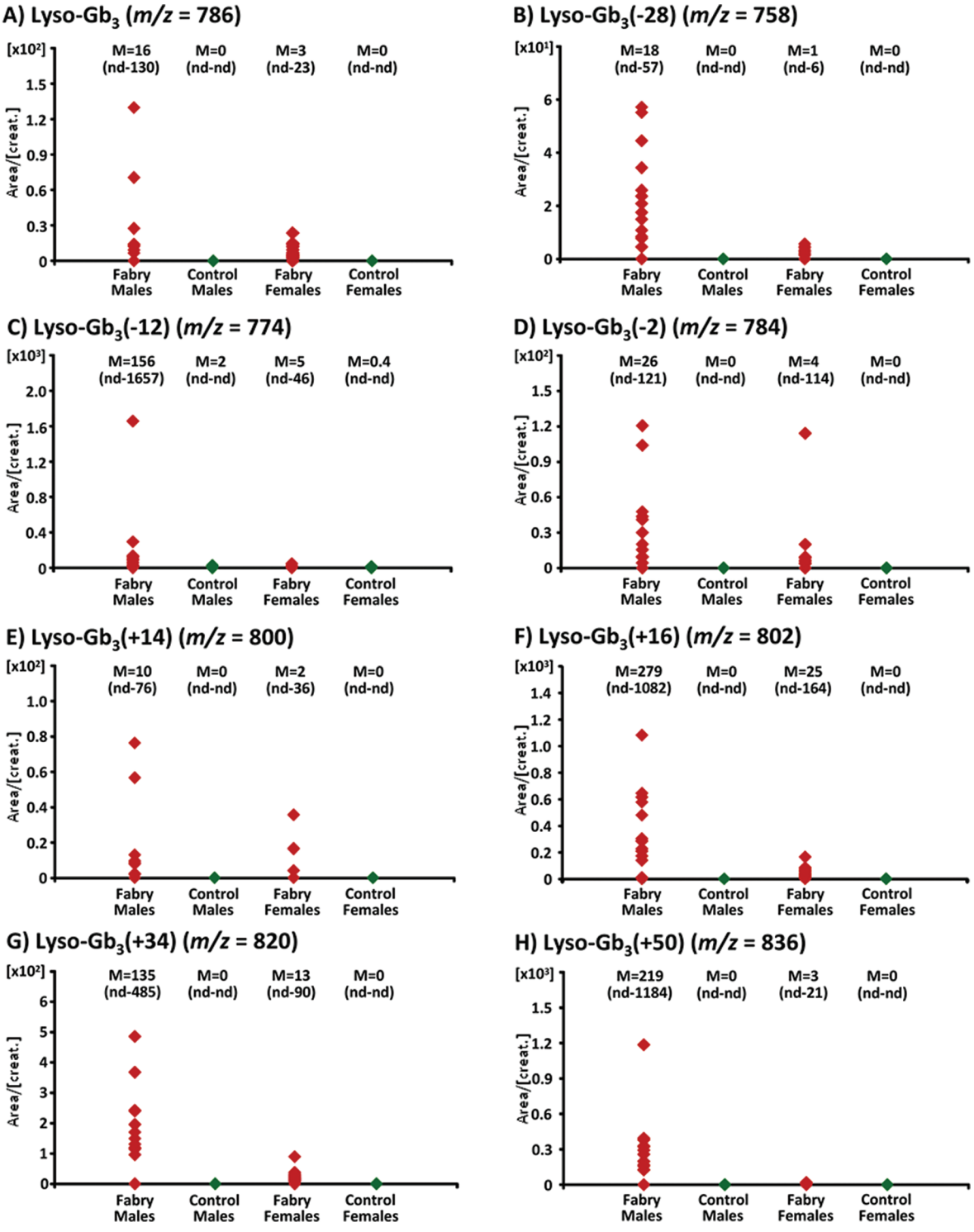

Figure 6. Abundance of lyso-Gb_3 and its analogues in four urine sample groups: (1) untreated Fabry males (n = 18), (2) control males (n = 20), (3) untreated Fabry females (n = 45), and (4) control females (n = 36). UPLC−QTOF-MS analyses: [creat] = urinary creatinine concentration in mol/L; M = mean; minimum and maximum values in brackets; nd = not detected.

due to the low concentration of some urine samples having particularly reduced urinary creatinine excretion levels. It may also result from the low sensitivity of the full scan mass spectrometry method employed for the metabolomic study compared to a multiple reaction monitoring (MRM) method performed on a triple-quadrupole mass spectrometer.

Figure 7 shows the results of the analysis of urine samples of a male Fabry patient obtained at intervals before and after the initiation of ERT. All lyso-Gb_3 analogues were normalized to creatinine and are significantly decreased after starting ERT.

CONCLUSIONS

The metabolomic study reported here, with the multivariate statistical data analysis performed (PCA, OPLS-DA, and S-plot), has identified novel urinary Fabry disease biomarkers. Among these biomarkers, lyso-Gb_3, a well-known Fabry disease

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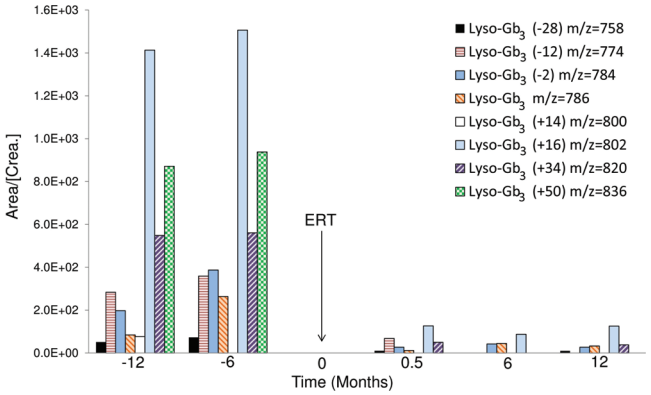

Figure 7. Urinary excretion of lyso-Gb_3 and seven related analogues normalized to creatinine in a male Fabry patient before (12 months) and after ERT (12 months).

biomarker, and lyso-Gb_3 (−2 Da, m/z 784), previously reported as lyso-ene-Gb_3,[7] were also detected. Six other biomarkers were identified and characterized as analogues of lyso-Gb_3 by tandem mass spectrometry, having modified sphingosine moieties. The empirical formula for the sphingosine moieties were determined by exact mass measurements. However, further characterization of these analogues, using techniques such as nuclear magnetic resonance (NMR), will be needed for complete structural elucidation. These eight biomarkers were present in most of the Fabry cohorts, and none was found in controls. In general, these biomarkers were more abundant for Fabry males compared to Fabry females. A male Fabry patient showed decreased excretion of all lyso-Gb_3-related analogues after receiving ERT, revealing a potential applicability for the pretreatment follow-up and the monitoring of the specific primary treatments of Fabry patients. Future work perspectives will involve the development of a sensitive multiplex methodology for the quantification of all these lyso-Gb_3-related analogues on a triple-quadrupole mass spectrometer. The excretion levels of all lyso-Gb_3 analogues will be studied in a larger cohort of Fabry patients to evaluate correlations with clinical severity and progression of disease.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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