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Mass spectrometry analysis of metals, other elements and lipids in urine samples of Fabry disease patients

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ABSTRACT

Fabry disease is an X-linked lysosomal storage disorder caused by deficiency of alpha-galactosidase A leading to accumulation of globotriaosylceramide in tissues and biological fluids of affected patients. Mass spectrometry is a powerful tool to quantify components of interest in biological fluids. Our study had four objectives: (1) to devise an ICP-MS methodology for quantitative determination of metal and other element concentrations in urine specimens of Fabry patients; (2) to analyze urinary Gb_3/creatinine and lyso-Gb_3/creatinine in these patients; (3) to evaluate correlations between urinary lipid concentrations *versus* metals and other elements in Fabry patients and healthy controls; (4) to evaluate which metals and other elements discriminate groups of patients and controls according to gender and treatment. We found that the excretion of barium was elevated in Fabry females and calcium and strontium levels were lower in Fabry males compared to controls. Preliminary results for treated and untreated Fabry disease patients indicate that ERT seems to have little effect on urine elements analyzed. Statistically significant correlations were established between urinary lyso-Gb_3/creatinine, Gb_3/creatinine and levels of magnesium, copper, mercury, nickel, lead, barium and calcium, whereas no significant correlations were found for the other 15 elements examined. Our results indicate that further studies are warranted in larger cohorts of Fabry disease patients for the investigation of possible roles of metals and other elements.

1. Introduction

Trace metals (e.g., zinc, copper and iron) are involved in cellular processes (e.g., proliferation, myelinization and signalling) and are essential for growth and functioning of the brain [1]. Approximately one-third of all proteins are believed to require metal cations as cofactors with catalytical functions [1]. The determination of essential and beneficial trace metals (such as copper, zinc, iron, manganese, molybdenum, magnesium, and others), metalloids (such as selenium), or non-metals (like sulfur, phosphorus, iodine, and chlorine), which are of vital importance in biological systems, is a main issue in modern bioanalytics. Certain trace metals protect against many diseases and from reactive oxygen species (ROS) [2].

The investigation of body fluids with respect to nutrient (essential) elements and toxic elements – which are challenging topics for analytical chemistry – requires the determination of concentrations at the trace and ultratrace level [3]. Urine is the most frequently investigated medical matrix [4–11]. Samples are collected easily in a non invasive manner from patients and controls. ICP-MS (inductively coupled plasma mass spectrometry), as a universal and sensitive analytical mass spectrometry technique, possesses very low limits of detection, as demonstrated with the analysis of physiological fluids, such as urine [4,5]. The analysis of biological fluids by this technique may be useful in the study of the pathophysiology of lysosomal storage disorders (LSDs).

LSDs are a group of nearly 45 disorders presenting variable phenotypes. LSDs caused by the same enzyme deficiency may present clinically in the newborn period or as late as adult life, owing to the variable effects of different mutations [12]. Most LSDs are associated with accumulation of substrates in biological fluids and secondary biochemical changes resulting from lysosomal storage. While the extent and severity of LSDs depend on the type and amount of accumulated substrate, almost all disorders are progressive [13]. Our interest has developed in the identification of disease-specific biological changes in blood or urine that are reli-

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Nomenclature

CHUS	Centre hospitalier universitaire de Sherbrooke
ERT	Enzyme replacement therapy
α-gal A	Alpha-galactosidase A
Gb_3	Globotriaosylceramide
GSG	Glucosylsphingosine
HPLC	High performance liquid chromatography
ICP-MS	Inductively coupled plasma mass spectrometry
ICP-QMS	Quadrupole-based inductively coupled plasma mass spectrometry
IS	Internal standard
ISE	Internal standard element
Isotopes	^{7}Li (lithium), ^{11}B (boron), ^{23}Na (sodium), ^{24}Mg (magnesium), ^{31}P (phosphorus), ^{39}K (potassium), ^{40}Ca (calcium), ^{47}Ti (titanium), ^{52}Cr (chromium), ^{56}Fe (iron), ^{60}Ni (nickel), ^{63}Cu (copper), ^{64}Zn (zinc), ^{75}As (arsenic), ^{82}Se (selenium), ^{85}Rb (rubidium), ^{88}Sr (strontium), ^{98}Mo (molybdenum), ^{103}Rh (rhodium), ^{118}Sn (tin), ^{137}Ba (barium), ^{202}Hg (mercury), ^{208}Pb (lead)
LA-ICP-MS	Laser ablation-inductively coupled plasma mass spectrometry
LC–MS/MS	liquid chromatography–tandem mass spectrometry
LOD	Limit of detection
LSDs	Lysosomal storage disorders
Lyso-Gb_3	Globotriaosylsphingosine
REB	Research Ethics Board
TOF-MS	Time-of-flight mass spectrometry
QTOF-MS	Quadrupole time-of-flight mass spectrometry

ably predictive of clinical severity and which play an important role in monitoring the effectiveness of the treatment of patients.

Fabry disease (OMIM 301500) is an X-linked lysosomal storage disorder affecting both males and females to various degrees. It is caused by deficiency of alpha-galactosidase A (α-gal A, EC 3.2.1.22) leading to the accumulation of a neutral glycosphingolipid, globotriaosylceramide (Gb_3), in the walls of small blood vessels, nerves, dorsal root ganglia, renal tubular and epithelial cells and cardiomyocytes [14]. Advancements in technology have made analysis of Gb_3 levels in urine feasible and reliable for mass and high-risk screening (aimed at a specific population at increased risk for a given disorder) [15–20], as well as for monitoring patients on treatment by enzyme replacement therapy (ERT) [21,22]. We recently developed and validated a tandem mass spectrometry (MS/MS) methodology for quantitative analysis of another biomarker, lyso-Gb_3 (globotriaosylsphingosine) a deacylated Gb3, in urine samples of Fabry patients [23]. We established correlations between Gb_3/creatinine, lyso-Gb_3/creatinine excretion and measurements of kidney function in Fabry disease patients [23]. Considering the likelihood of complex secondary metabolic abnormalities in patients with Fabry disease, we undertook an investigation of the levels of certain metals and other elements in the urine of patients with the disease. Our study had four objectives: (1) to devise an ICP-MS methodology for quantitative determination of metal and other element concentrations in urine specimens of Fabry patients; (2) to analyze urinary Gb_3/creatinine and lyso-Gb_3/creatinine in the patients; (3) to evaluate correlations between urinary lipid concentrations *versus* metals and other elements in Fabry patients and healthy controls; (4) to evaluate which metals and other elements discriminate groups of patients and controls according to gender and treatment.

Table 1
ICP-MS operating conditions and measurement parameters.

Rf power (W)	1480
Sample uptake rate (mL/min)	0.3
Gas flow rate (L/min)	
Coolant gas flow	14
Auxiliary gas flow	1.4
Nebulizer gas flow	1.1
Ion sampling depth (mm)	5
Ion lens setting	Adjusted to obtain maximum ion intensity
Nebulizer	Concentric nebulizer
Spray chamber	Scott-type double pass spray chamber
Sample/skimmer diameter orifice	Nickel 1.0 mm/0.4 mm
Scanning mode	Peak-hopping
Dwell time (ms)	50
Integration mode	Peak area
Points per spectral peak	1
Isotopes measured	^{7}Li, ^{11}B, ^{23}Na, ^{24}Mg, ^{31}P, ^{39}K, ^{40}Ca, ^{47}Ti, ^{52}Cr, ^{56}Fe, ^{60}Ni, ^{63}Cu, ^{64}Zn, ^{75}As, ^{82}Se, ^{85}Rb, ^{88}Sr, ^{98}Mo, ^{103}Rh, ^{118}Sn, ^{137}Ba, ^{202}Hg, ^{208}Pb

2. Materials and methods

2.1. 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 other REBs from collaborators.

2.2. Urine specimen collection from Fabry patients and controls

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. All samples were received in a coded manner, with the key code kept at each collaborating centre. Control urine samples from 8 healthy subjects (4 males and 4 females; aged 18–59 years) were analyzed to establish normal reference values. We analyzed urine samples from 27 Fabry disease patients: 13 males (aged 17–59 years) and 14 females (aged 23–66 years). Eight of the males and 5 of the females were receiving enzyme replacement therapy (ERT) with either agalsidase-alpha 0.2 mg/kg/2 weeks (Replagal™, Shire Human Genetic Therapies Inc., Lexington, MA) or agalsidase-beta 1.0 mg/kg/2 weeks (Fabrazyme®, Genzyme Corporation, Cambridge, MA).

2.3. Chemicals and reagents

All chemicals used were analytical reagent grade. Sub-boiling distillation of nitric acid from Merck was used. High purity deionized water (18.2 MΩ cm) obtained from a Milli-Q system was used for all dilution of mixed metal standard solution and urine samples. The working standard solutions were prepared in 1% HNO_3 by serial dilution of ICP multi-element standard stock solution IV (Merck) immediately prior to use. Rh single element standard solution (Merck CertiPrep) was used as the internal standard element (ISE).

A standard mixture of total globotriaosylceramide isoforms, HPLC reagents and solvents were purchased and prepared as previously described [15]. $C_{17:0}$-Gb_3 isoform internal standard (IS) was purchased from Matreya (Pleasant Gap, PA). Creatinine standards were purchased from Sigma (St. Louis, MO, USA). A stock solution of creatinine, 10 mmol/L in water, was prepared and the following reference standards were used: 0.1, 1, 4 and 10 μmol/4 mL. Deuterated creatinine methyl-[2H]creatinine (d_3-creat) (CDN Iso-

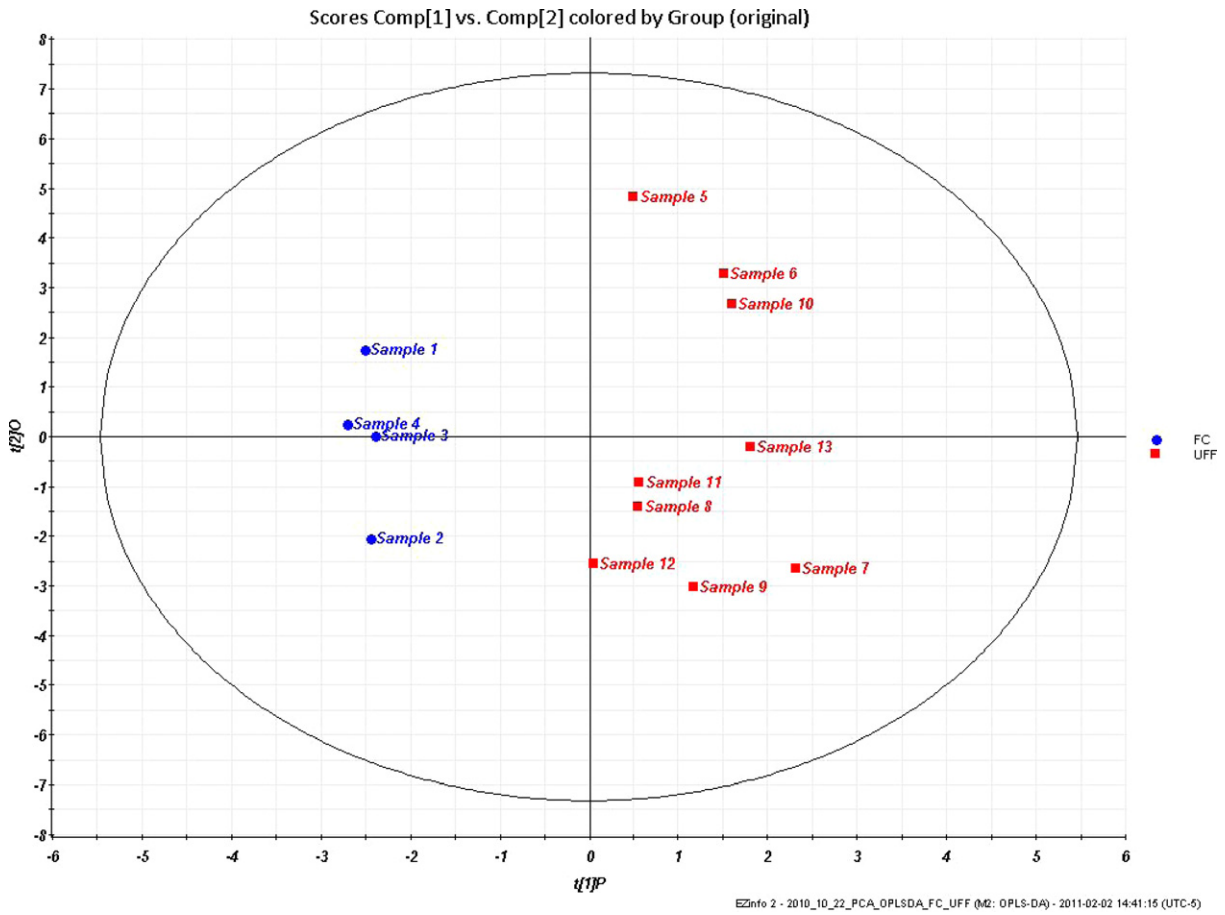

Fig. 1. Scoring plot of untreated female Fabry patients (UFF, $n = 9$, samples 5–13) *versus* female controls (FC, $n = 4$, samples 1–4).

topes, Pointe-Claire, Que., Canada) was the internal standard used for the creatinine quantification at 40 μg/20 μL in water and was stored at 4 °C. Filter papers were Whatman no. 903.

For lyso-Gb_3 analysis, acetonitrile LC–MS grade was purchased from Fisher Scientific (Fair Lawn, NJ). Laboratory water was purified to ultra pure grade with the use of a Nanopure Infinity water purification system (Ultrapure, 18.3 MΩ, Barnstead, Dubuque, IA). lyso-Globotriaosylsphingosine (Lyso-Gb_3 from pig) and 1-β-D-glucosylsphingosine (GSG from plant) were obtained from Matreya.

2.4. Processing and analysis of urinary specimens

Prior to ICP-MS determination, frozen urine samples from controls and Fabry patients were gradually thawed, first at 4 °C in a refrigerator for several hours and finally at room temperature. Urine samples were diluted, acidified (to 1% HNO_3 final concentration) and spiked with the internal standard (Rh, 10 ng/mL final concentration). The diluted urine samples were analyzed for each element after external calibration [8–11].

Urine samples for Gb_3 analysis were deposited on a Whatman 903 filter paper, left to dry at room temperature overnight. Samples were processed as previously described [15,18]. Briefly, a 5-cm diameter filter paper disc was punched from each sample and 1 μg of $C_{17:0}$-Gb_3 and 20 μg of d_3-creat were added as internal standards. Elution was performed by rotary shaking filter papers with 4 ml of methanol in glass vials for 60 min. Ten microliters were injected into the LC–MS/MS system.

For lyso-Gb_3, aliquots (500 μl) of well-mixed urine samples from Fabry patients and controls were transferred to screw glass tubes with phenolic caps along with 500 μl of glucosylsphingosine (GSG) internal standard at 4 nmol/L in methanol. Samples were processed using solid phase extraction according to a previously described methodology [23].

Waters (Waters Corp., Milford, MA, USA) QuanLynx software was used to quantify Gb_3/creatinine and lyso-Gb_3/creatinine data.

2.5. Mass spectrometry instrumentation

A quadrupole based inductively coupled plasma mass spectrometer (ICP-QMS, Agilent 7500, Tokyo, Japan) operating at standard mode was used for the measurement of the urinary concentrations of metals and other elements. The ICP mass spectrometer was equipped with a Cetac ASX-510 Autosampler (Cetac Technologies, Omaha, USA) for automation of the analyses. The experimental conditions were optimized to obtain the highest signal/background ratio for $^{103}Rh^+$ (concentration: 10 μg/L), the oxide ion ratios (MO^+) less than 3% and minimum double charge (M^{2+}) ions. Optimized experimental parameters of ICP-MS are summarized in Table 1.

Analysis of Gb_3 [18], lyso-Gb_3 and creatinine [23] was performed on a Quattro micro tandem quadrupole instrument (Waters Corp.) coupled to an Alliance 2795XE system (Waters Corp.) (LC–MS/MS) with the electrospray ionization operating in positive-ion mode (ES+). Mass spectrometry and high performance liquid chromatography parameters were previously reported for Gb_3 [18] and lyso-Gb_3 [23].

2.6. Statistical analysis

Correlation analysis was performed on urinary concentrations of elements and lipids using the CORR Procedure with the SAS soft-

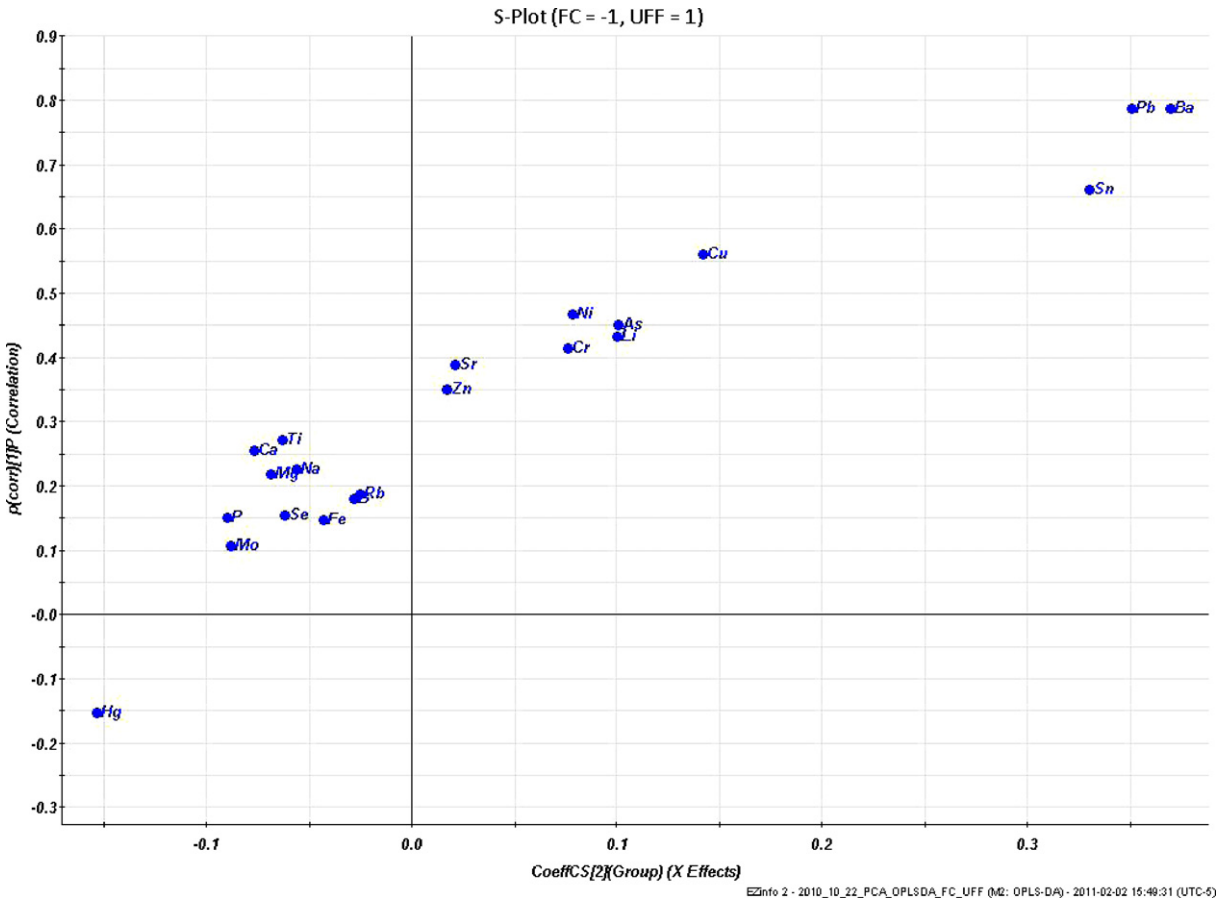

Fig. 2. S-plot statistical analysis to target specific isotopes of interest from 21 different isotopes studied in untreated female Fabry patients ($n = 9$) and controls ($n = 4$).

ware (Statistical Analysis System, version 9.1.3, SAS Institute Inc., Cary, NC, USA). Multivariate analysis using logarithm concentrations of Gb_3/creatinine or lyso-Gb_3/creatinine as the dependent variable *versus* element concentrations, gender and treatment as independent variables was performed by Mixed Procedure (SAS software) in Fabry patients and controls. Additional multivariate data analysis was performed with EZinfo software (Umetrics) using orthogonal partial least squares discriminant analysis (OPLS-DA). This analysis allowed us to define which element discriminates groups of interest. Potassium levels were not used for the multivariate analysis with EZInfo.

3. Results and discussion

Urine was investigated with respect to elemental and molecular composition by ICP-MS combined to biomolecular mass spectrometry. ICP-MS with multi-element capability allows the measurements of metals, metalloids and other selected elements over a wide dynamic range of over seven orders of magnitude. We found that the concentrations measured in urine samples varied from 0.4 ng/mL for Hg and up to 4 mg/mL for sodium. In general, the alkali metals, Na and K, the earth alkali metal, Ca, and the non-metal, phosphorus, are detected with higher concentrations at μg/mL up to mg/mL level. The concentration of the lowest atomic weight metal, Li, measured by ICP-MS varied between 30 and 800 ng/mL, and the heavy toxic metal, Hg, was found within the 0.4 and 4 ng/mL range. The limits of detection (LODs) were 0.02 ng/mL, 0.09 ng/mL and 0.01 ng/mL for Hg, Se and As, respectively.

Results of quantitative determination of urinary boron, lithium, sodium, magnesium, phosphorus, potassium, calcium, titanium, chromium, iron, nickel, copper, zinc, arsenic, selenium, rubidium, strontium, molybdenum, tin, barium and mercury by ICP-MS, as well as liquid chromatography coupled to tandem mass spectrometry analysis of lyso-Gb_3 and Gb_3 urinary excretion values, are presented in Table 2.

Results of multivariate data analysis with EZinfo software using OPLS-DA, which enables the separation of predictive from non-predictive (orthogonal) variation, are presented in Figs. 1 and 2. Scoring plots allowed us to see which observations are similar (so near each other) and which are dissimilar (so far away of each other). Fig. 1 presents the scoring plot of untreated female Fabry patients ($n = 9$) *versus* controls ($n = 4$). These heterozygous patients were clearly discriminated from controls. An S-plot of this statistical analysis allowed us to target two specific isotopes, barium and lead which were different from the 19 other isotopes under study (Fig. 2).

We used a graphical representation, a trend plot, which reveals the general pattern of level change in patients and controls for a specific element under study. The trend plot for barium is shown in Fig. 3 for untreated female Fabry patients ($n = 9$) *versus* female controls ($n = 4$). Urinary barium levels were found to be lower in all controls.

Fig. 4 presents the scoring plot results for untreated male Fabry patients ($n = 5$) *versus* controls ($n = 4$). Similar to females, the scoring plot provides a clear discrimination between untreated male Fabry patients and controls. Fig. 5 presents the S-plot for male controls ($n = 4$) and untreated male Fabry patients ($n = 5$) for all 21 isotopes. We found that calcium and strontium were clearly discriminated

Table 2
ICP-MS analysis of metals and other elements and liquid chromatography coupled to tandem mass spectrometry of Gb_3 and lyso-Gb_3 in urine samples of male and female Fabry patients and controls. Li, B and all others are expressed as ng/mL. Only Na, Mg, P, K, Ca are expressed as μg/mL. All values at 0 are below the limits of detection (LOD). Status: Female controls (FC); Male controls (MC) Untreated female Fabry patients (UFF); Treated female Fabry patients (TFF); Untreated male Fabry patients (UMF); Treated male Fabry patients (TMF).

Sample ID	Age	Status	LGb_3	Gb_3	Li	B	Na	Mg	P	K	Ca	Ti	Cr
Sample 1	30	FC	0	n/a	164.5	338.1	2373.1	71.5	343.0	2653.5	44.5	517.2	33.2
Sample 2	42	FC	0	n/a	30.8	LOD	253.7	5.0	16.1	349.9	4.2	51.0	7.7
Sample 3	48	FC	0	n/a	110.7	235.5	1240.3	50.4	222.3	1466.4	33.3	381.4	21.8
Sample 4	59	FC	0	n/a	152.3	1129.7	2277.5	40.1	134.5	3056.9	21.2	240.0	20.4
Sample 5	26	UFF	30	68	804.2	1685.0	4277.6	100.5	369.2	Very high	75.8	976.0	78.9
Sample 6	27	UFF	50	127	396.1	1966.5	2535.2	133.5	735.7	4440.9	66.6	935.1	71.4
Sample 7	51	UFF	77	725	66.3	LOD	835.4	21.7	123.2	1186.9	14.0	173.4	22.9
Sample 8	42	UFF	30	3	189.4	463.6	1459.7	55.5	171.4	803.1	15.5	218.9	36.9
Sample 9	62	UFF	0	20	324.0	LOD	358.6	9.7	41.2	488.3	4.0	49.7	17.5
Sample 10	39	UFF	0	2	603.6	471.4	4548.9	145.7	252.1	1955.4	67.1	858.1	54.6
Sample 11	47	UFF	31	11	117.8	591.3	1399.5	20.8	130.5	1403.5	25.9	260.3	27.1
Sample 12	52	UFF	16	304	49.3	118.7	706.9	17.2	74.4	344.5	3.6	60.5	17.6
Sample 13	38	UFF	44	58	418.3	1074.3	3052.3	32.3	130.2	2753.7	56.2	568.0	35.8
Sample 14	38	TFF	0	20	93.8	27.8	2001.4	32.1	87.1	403.4	21.6	247.2	15.9
Sample 15	66	TFF	13	2	430.4	658.0	4290.0	82.4	464.9	2608.3	35.4	527.8	52.5
Sample 16	60	TFF	0	2	207.2	660.1	2369.4	46.6	518.3	3226.9	10.4	260.1	42.1
Sample 17	23	TFF	11	3	159.8	128.8	2298.3	46.2	425.8	760.2	26.4	348.6	25.1
Sample 18	48	TFF	93	0	90.2	369.1	1108.9	10.5	51.9	1084.1	17.0	161.8	14.2
Sample 19	18	MC	0	n/a	550.1	2326.7	903.5	141.6	1175.8	1816.9	83.4	1186.6	73.9
Sample 20	42	MC	0	n/a	660.2	2356.5	2732.3	29.5	123.3	2279.2	55.4	608.9	39.9
Sample 21	44	MC	0	n/a	173.3	466.7	2093.1	34.2	48.8	1763.5	55.7	553.4	25.0
Sample 22	57	MC	0	n/a	643.4	1939.5	6336.8	69.1	613.8	3809.9	69.3	829.7	64.8
Sample 23	34	UMF	159	327	130.9	266.9	2229.3	18.0	465.7	903.3	9.2	168.3	33.1
Sample 24	36	UMF	220	4059	137.5	84.7	1152.3	26.8	216.5	2081.2	2.8	69.9	19.6
Sample 25	17	UMF	0	1	557.0	208.6	4229.2	13.0	333.2	3361.8	42.6	596.9	50.9
Sample 26	20	UMF	149	14	120.1	456.2	3540.9	21.0	246.7	4109.0	13.7	224.0	45.6
Sample 27	25	UMF	353	150	54.2	148.0	760.1	18.0	79.3	369.2	3.4	61.6	18.5
Sample 28	50	TMF	187	1944	178.7	329.9	1267.2	41.3	202.9	931.8	5.8	109.3	20.3
Sample 29	58	TMF	111	3565	114.0	373.1	2086.7	16.7	220.6	1636.0	26.7	339.0	30.2
Sample 30	38	TMF	27	26	100.6	52.1	934.1	26.8	60.1	385.6	14.1	168.6	13.8
Sample 31	33	TMF	92	144	373.8	595.4	3861.2	75.7	411.4	2964.3	32.4	470.0	39.3
Sample 32	52	TMF	28	14	304.6	341.0	1423.1	30.2	295.1	877.6	9.7	159.0	27.6
Sample 33	59	TMF	107	351	154.7	LOD	1411.3	25.5	256.7	501.1	1.7	83.9	32.0
Sample 34	41	TMF	16	11	134.5	329.6	1015.5	35.6	74.5	1040.3	13.7	194.4	24.9
Sample 35	21	TMF	59	660	397.1	430.7	2260.7	20.6	25.7	939.1	35.6	365.0	28.9

Sample ID	*Fe*	Ni	Cu	Zn	*As*	Se	Rb	*Sr*	Mo	Sn	Ba	*Hg*	Pb
Sample 1	1351.4	8.9	33.5	456.2	4.3	76.3	1115.8	74.5	77.4	2.3	1.4	6.8	3.1
Sample 2	678.6	−0.2	3.2	57.2	*LOD*	LOD	196.1	*LOD*	4.1	0.9	0.7	4.3	2.9
Sample 3	1027.1	5.5	17.5	364.7	2.1	13.4	754.1	37.5	39.0	1.8	1.3	4.0	2.9
Sample 4	1528.2	4.0	28.8	89.8	4.8	24.2	1389.5	5.0	10.8	0.8	0.7	4.0	2.9
Sample 5	1627.5	21.5	75.4	560.7	20.7	165.4	4737.6	3.8	157.1	6.9	2.7	14.3	4.3
Sample 6	1303.2	19.3	40.4	559.4	16.7	132.4	2565.7	146.9	126.2	8.6	5.1	6.7	4.2
Sample 7	707.1	4.5	48.7	500.0	1.4	11.8	642.7	19.5	13.8	9.2	3.9	4.7	4.0
Sample 8	822.1	9.7	28.3	300.2	5.3	19.1	840.9	55.6	4.8	2.2	5.2	*LOD*	3.1
Sample 9	1013.8	5.4	15.0	122.9	*LOD*	0.3	413.8	*LOD*	10.7	2.0	4.4	*LOD*	3.8
Sample 10	2403.6	26.9	69.6	693.0	20.0	52.9	1343.9	233.9	49.2	1.5	5.2	*LOD*	3.8
Sample 11	1638.2	8.7	26.7	200.1	13.9	26.3	843.0	*LOD*	27.8	8.1	2.1	8.6	3.8
Sample 12	896.4	LOD	24.6	224.0	*LOD*	7.2	280.1	*LOD*	13.8	6.5	1.6	0.7	3.4
Sample 13	1304.1	11.9	62.9	124.4	15.3	27.2	1454.5	150.7	18.4	10.2	2.7	0.4	3.5
Sample 14	33.7	LOD	25.5	200.7	*LOD*	LOD	217.8	23.8	9.7	3.7	4.8	0.9	3.7
Sample 15	2125.0	18.0	67.3	393.1	33.5	62.2	1425.5	87.2	42.8	9.1	6.8	7.9	3.9
Sample 16	*LOD*	6.4	46.7	655.9	12.6	33.7	1960.3	0.0	53.8	1.5	4.2	*LOD*	3.3
Sample 17	*LOD*	6.9	44.6	511.8	5.7	20.7	455.3	42.2	34.3	0.5	4.8	*LOD*	2.9
Sample 18	563.5	1.8	145.3	118.6	1.9	0.0	659.9	*LOD*	5.9	19.9	2.7	*LOD*	3.6
Sample 19	*LOD*	14.4	31.6	1183.3	18.8	186.3	1617.0	268.1	64.7	50.6	4.4	*LOD*	3.6
Sample 20	129.7	7.7	46.9	487.3	11.9	38.6	1420.3	115.6	79.2	3.6	1.5	*LOD*	3.5
Sample 21	317.8	4.9	38.8	598.0	5.6	17.0	924.1	121.8	41.2	3.1	3.2	*LOD*	3.3
Sample 22	2446.2	14.9	82.8	822.3	21.1	91.2	1629.3	151.8	126.3	2.9	4.4	13.3	4.4
Sample 23	263.8	LOD	44.9	607.9	2.1	22.8	764.2	28.8	17.1	6.2	3.3	3.0	3.6
Sample 24	87.5	LOD	22.4	405.1	*LOD*	8.5	1090.2	*LOD*	19.4	8.0	3.3	2.9	3.8
Sample 25	753.8	19.2	66.2	996.3	14.3	47.1	1519.8	*LOD*	67.2	1.5	4.0	*LOD*	2.8
Sample 26	2419.1	6.6	59.6	244.3	11.1	49.3	1636.1	*LOD*	18.0	5.6	2.5	4.4	3.7
Sample 27	1047.2	−0.1	26.1	267.3	*LOD*	9.4	300.3	*LOD*	14.9	8.4	2.0	1.5	3.5
Sample 28	142.8	LOD	33.1	417.4	1.7	5.7	418.3	9.5	27.7	40.4	3.0	2.0	3.5
Sample 29	114.9	2.5	42.0	632.6	*LOD*	9.6	831.3	54.5	20.7	11.5	2.6	1.6	3.6
Sample 30	95.3	LOD	13.7	273.9	*LOD*	16.8	206.1	*LOD*	35.2	4.6	2.5	1.0	3.6
Sample 31	107.0	LOD	51.7	446.0	23.1	43.8	1611.8	95.2	39.1	9.5	4.7	0.8	3.8
Sample 32	1236.8	10.3	39.1	345.6	4.5	16.5	686.1	34.5	21.5	3.6	5.2	1.7	3.0
Sample 33	756.8	7.9	49.3	552.6	3.0	12.8	339.3	*LOD*	13.4	8.5	4.3	0.9	3.0
Sample 34	*LOD*	4.1	25.4	289.4	3.4	51.7	665.5	*LOD*	41.8	0.8	4.2	*LOD*	2.9
Sample 35	1731.1	6.8	42.2	260.6	17.4	24.0	574.3	1.4	58.5	6.0	2.0	2.0	3.4

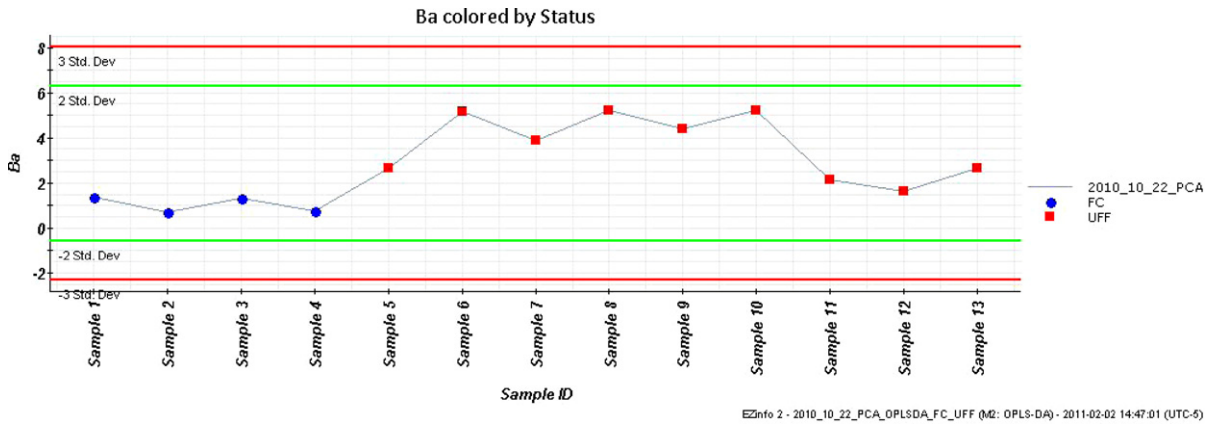

Fig. 3. Trend plot for barium for untreated female Fabry patients (UFF, $n = 9$, samples 5–13) *versus* urine of female controls (FC, $n = 4$, samples 1–4).

compared to other metals studied. Boron was discriminated in only 4 out of 5 patients (data not shown). In Fig. 6, a trend plot for strontium in untreated Fabry patients ($n = 5$) *versus* urine of male controls ($n = 4$) is presented. Urinary strontium values were found to be lower in all male Fabry patients under study.

Fig. 7 presents scoring plot results from OPLS-DA of female Fabry patients ($n = 14$) *versus* female controls ($n = 4$), independent of treatment. Both groups are well separated. Fig. 8 shows OPLS-DA scoring plot results of male Fabry patients (MF, $n = 13$) *versus* male controls (MC, $n = 4$), independent of treatment where Fabry samples were not as well discriminated. Fig. 9 presents the S-plot for female controls ($n = 4$) and female Fabry patients ($n = 14$) independent of treatment, for all 21 isotopes. Fig. 10 shows a S-plot for male controls ($n = 4$) and male Fabry patients ($n = 13$) independent of treatment, for all 21 isotopes. Barium was definitely discriminated in both male and female cohorts (only female results shown in Fig. 11), whereas calcium and strontium were only discriminated in male Fabry patients (see Figs. 12 and 13). Preliminary results presented for treated and untreated Fabry disease patients (Fig. 2 *versus* Fig. 9, and Fig. 5 *versus* Fig. 10) indicate that ERT seems to have little effect on urine elements analyzed. Nevertheless, studies on larger cohorts of patients are needed (considering that two ERT products and different doses were used) to draw definite conclusions.

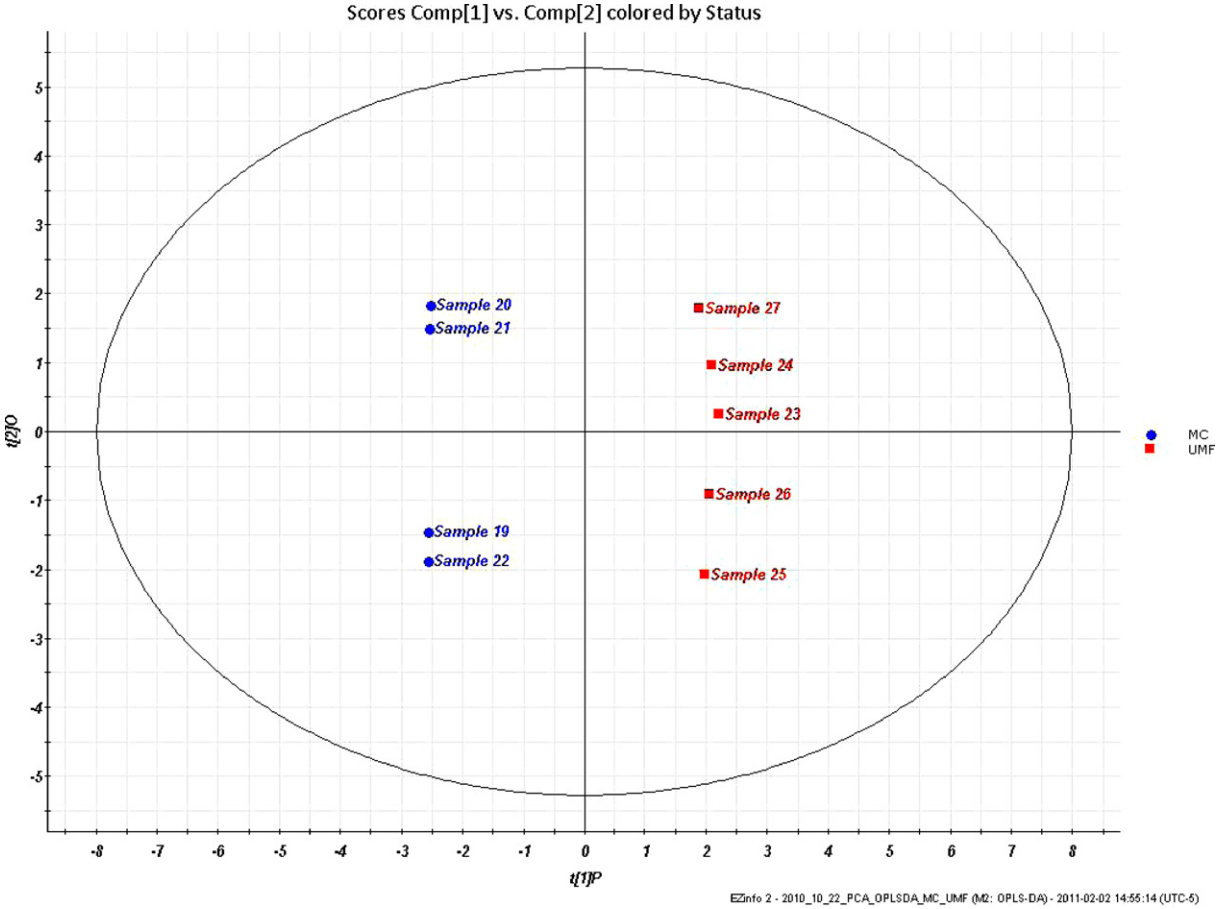

Fig. 4. Scoring plot results of untreated male Fabry patients (UMF, $n = 5$, samples 23–27) *versus* male controls (MC, $n = 4$, samples 19–22).

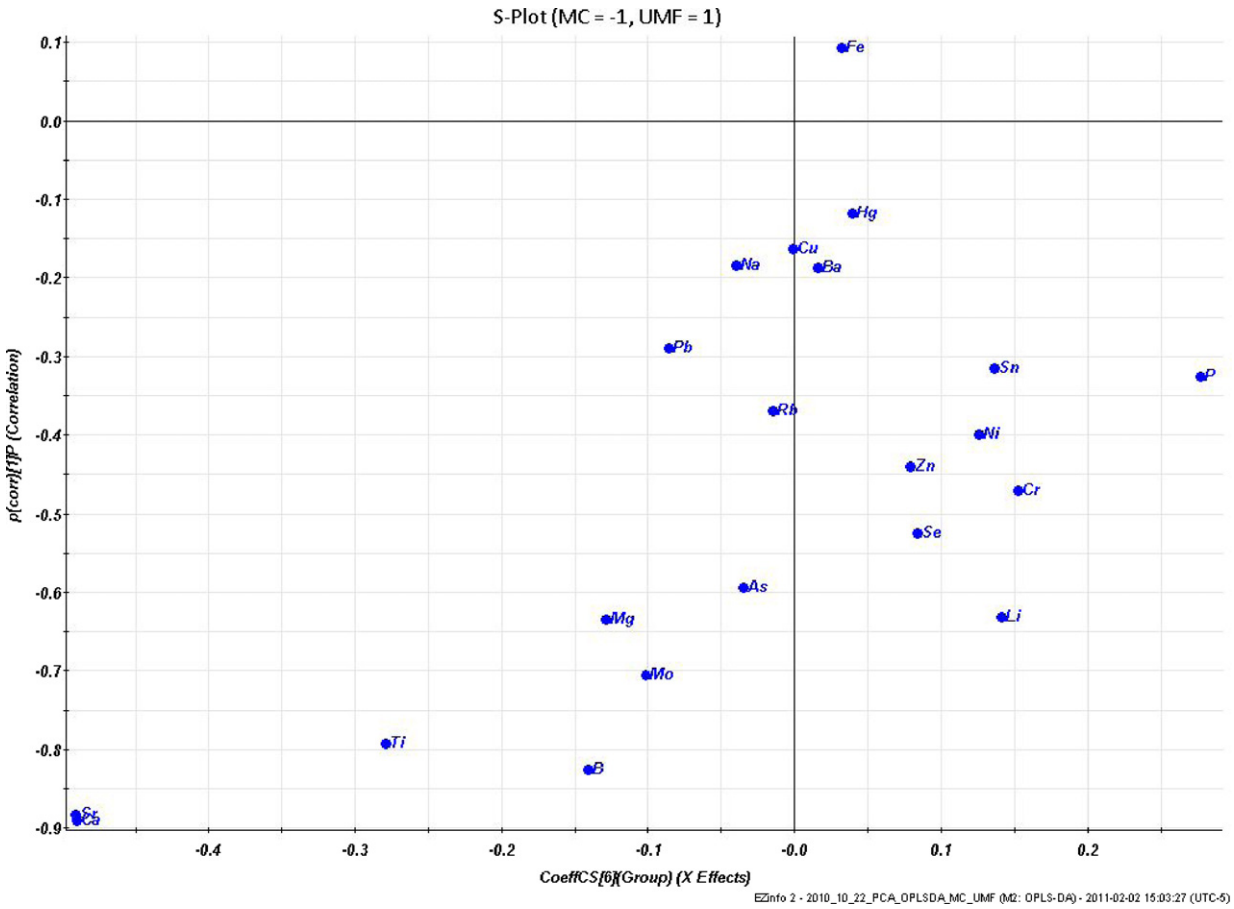

Fig. 5. S-plot statistical analysis to target specific isotopes of interest from 21 different isotopes studied in untreated male Fabry patients ($n = 5$) and controls ($n = 4$). Calcium and strontium isotopes gave a positive discrimination, whereas boron was not discriminant in all male patients studied.

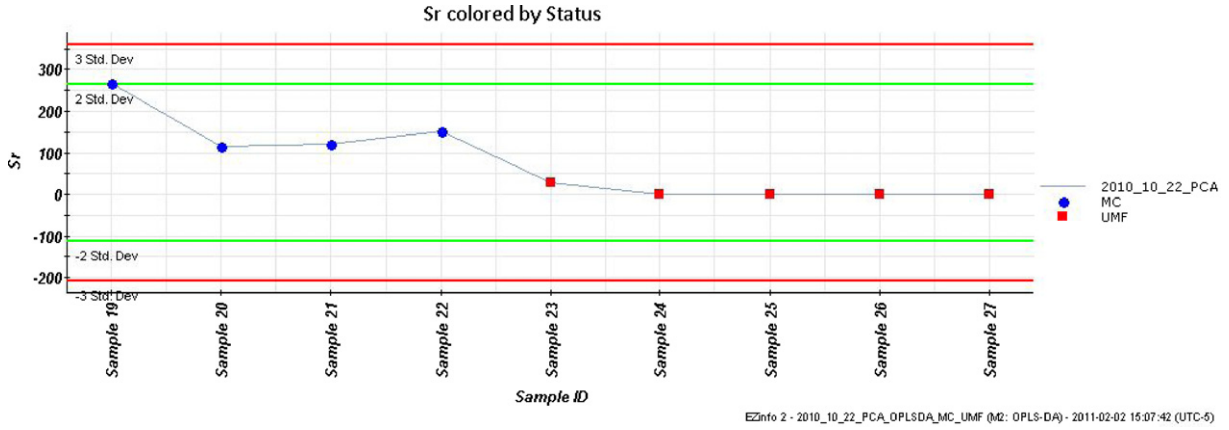

Fig. 6. Trend plot for strontium in untreated male Fabry patients (UMF, $n = 5$, samples 23–27) *versus* urine of male controls (MC, $n = 4$, samples 19–22).

Table 3
Absolute values of significant correlations between urine lyso-Gb_3/creatinine and Gb_3/creatinine with different metals: calcium, titanium, tin, nickel, and mercury.

Globotriaosylsphingosine (lyso-Gb_3)	Ca	Ti	Sn
Spearman correlation coefficients	0.41980	0.38674	0.64667
Prob > |r| under H0: Rho = 0	0.0121	0.0217	<0.0001
Number of observations	35	35	35
Globotriaosylceramide (Gb_3)	Ni	Sn	Hg
Spearman correlation coefficients	0.46553	0.48596	0.45025
Prob > |r| under H0: Rho = 0	0.0144	0.0102	0.0184
Number of observations	27	27	27

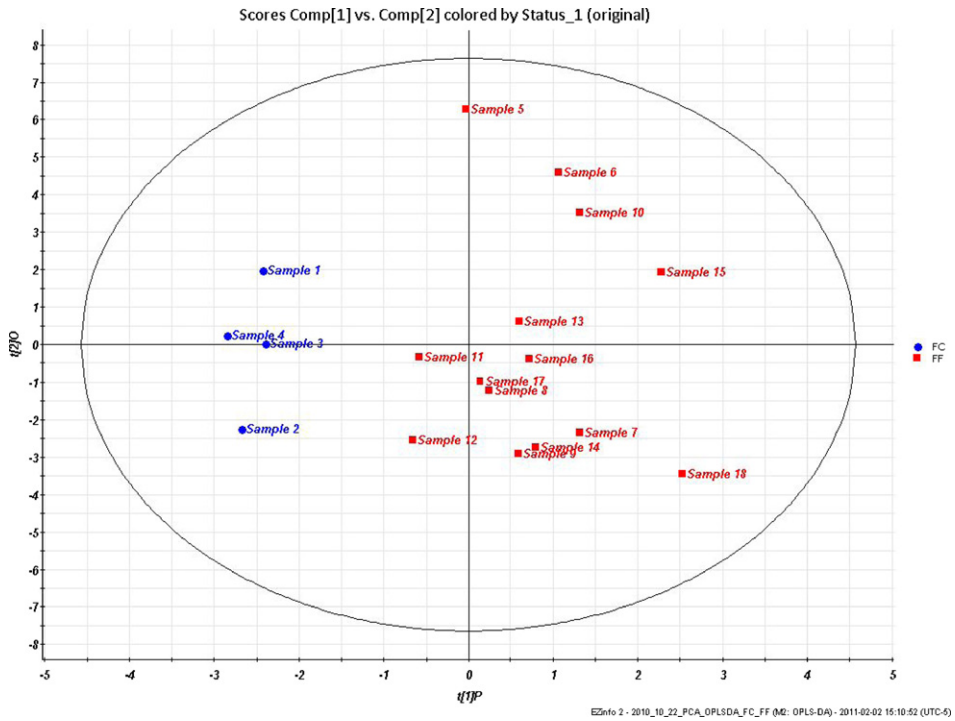

Fig. 7. Scoring plot results of all female Fabry patients (FF, $n = 14$, samples 5–18) *versus* female controls (FC, $n = 4$, samples 1–4).

Data in Table 3 show that significant statistical correlations were obtained for several elements *versus* Gb_3/creatinine and lyso-Gb_3/creatinine as demonstrated by relatively high Spearman correlation coefficients. Table 4 presents statistically significant slopes between the dependent variables lyso-Gb_3/creatinine and Gb_3/creatinine compared to different element levels Mg, Cu, Hg, Ni, Pb, Ba, Ca. We found in all Fabry patients (males and females, treated or untreated): (1) high urinary levels of lyso-Gb_3/creatinine

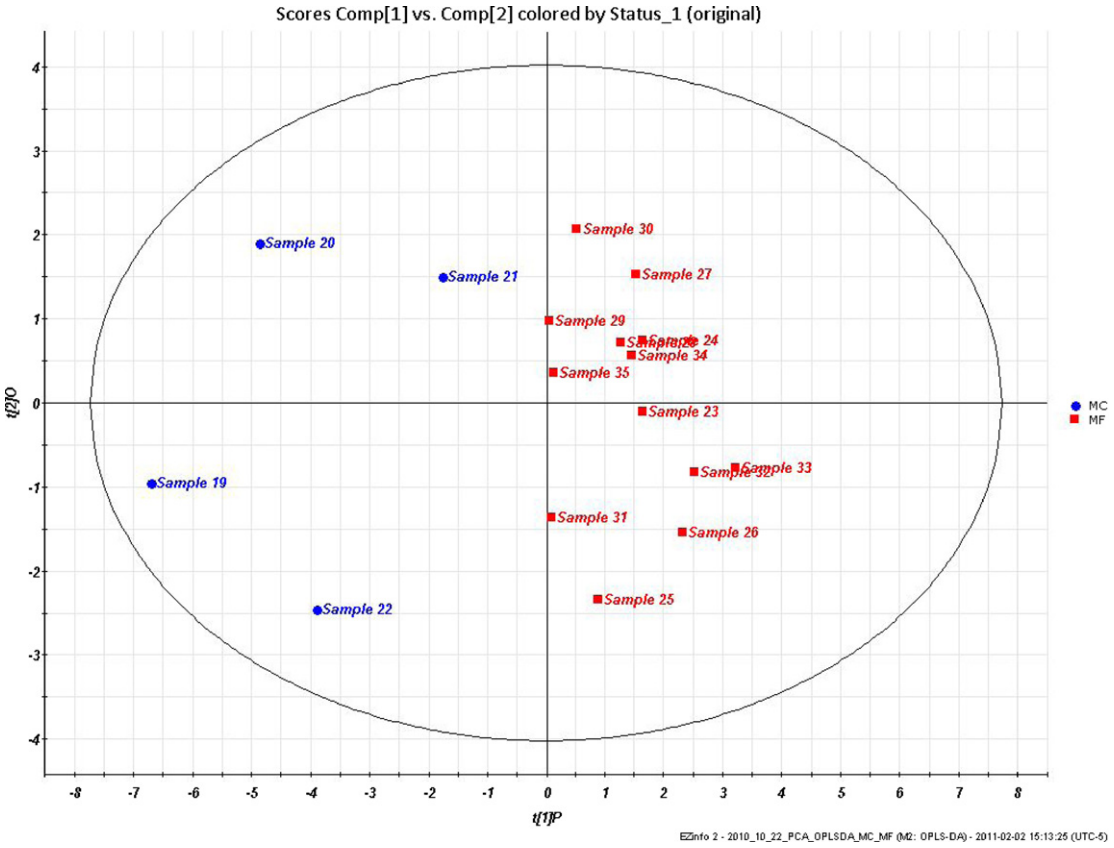

Fig. 8. Scoring plot results of male Fabry patients (MF, $n = 13$, samples 23–35) *versus* male controls (MC, $n = 4$, samples 19–22).

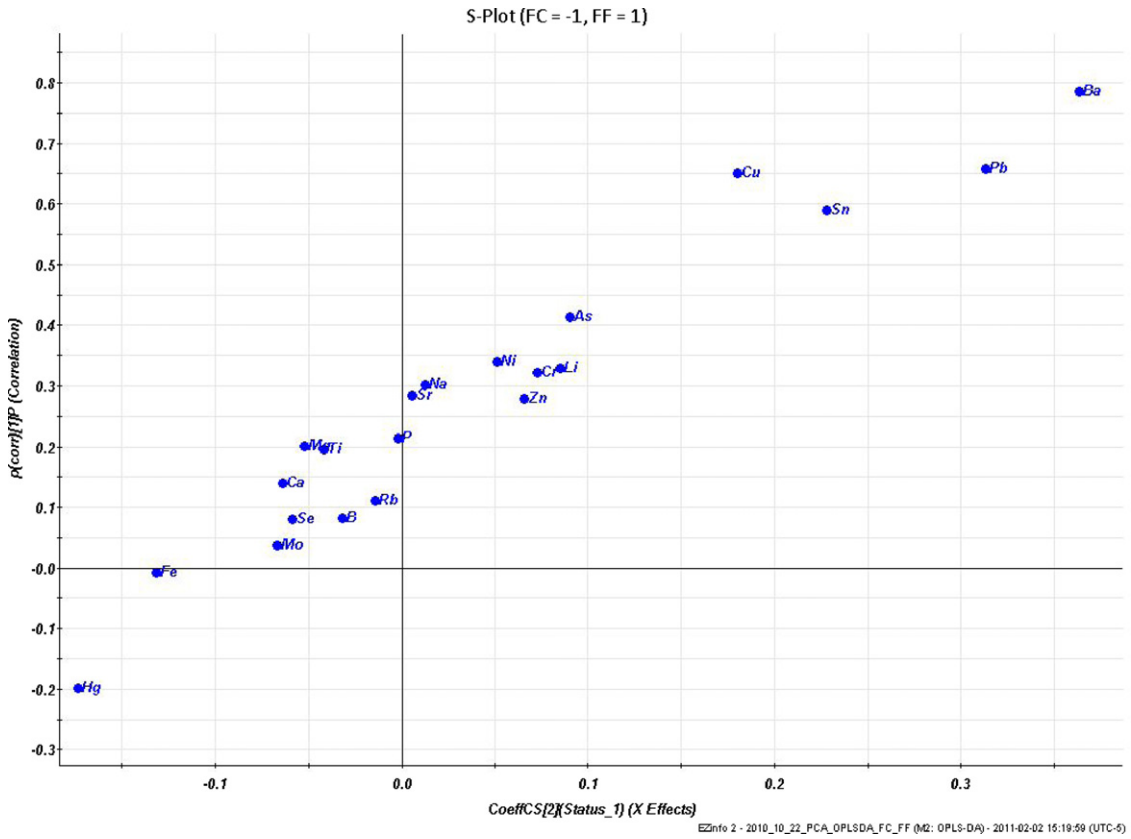

Fig. 9. S-plot for female controls ($n = 4$) and female Fabry patients ($n = 14$) independent of treatment, for all 21 isotopes.

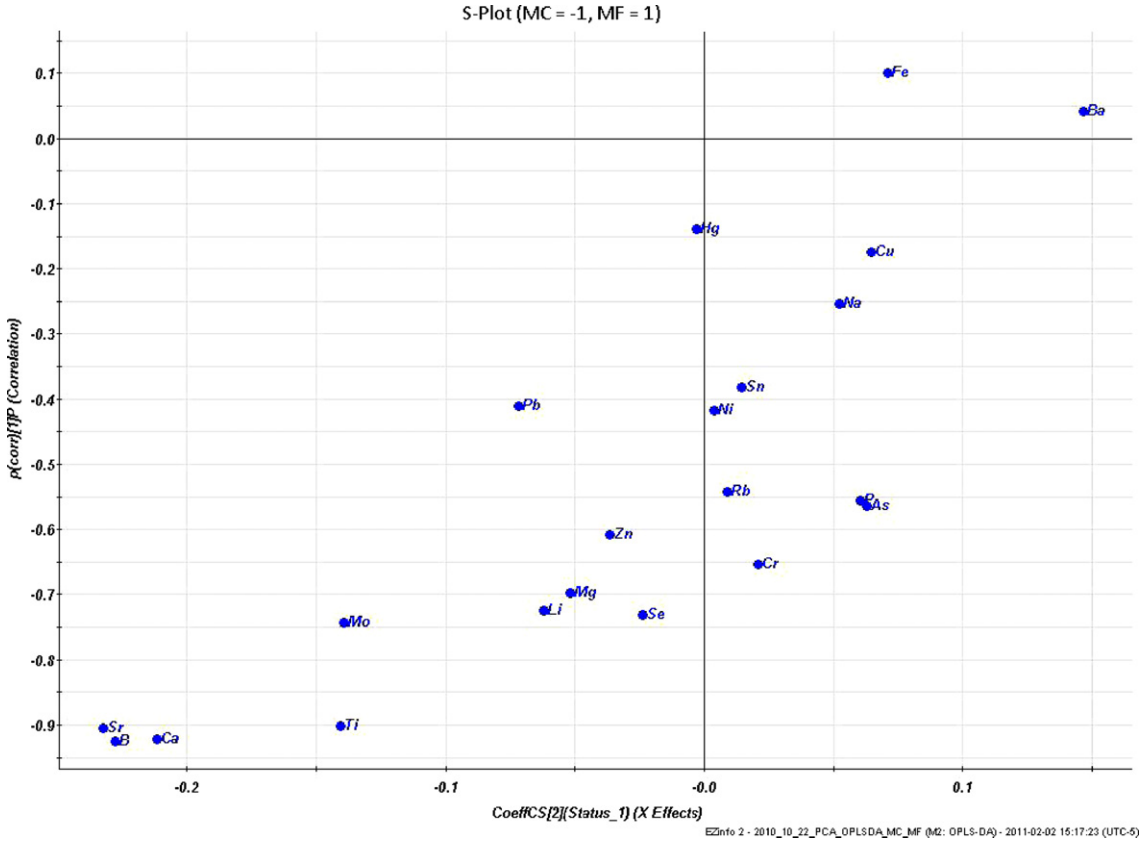

Fig. 10. S-plot for male controls ($n = 4$) and all male Fabry patients ($n = 13$) independent of treatment, for all 21 isotopes.

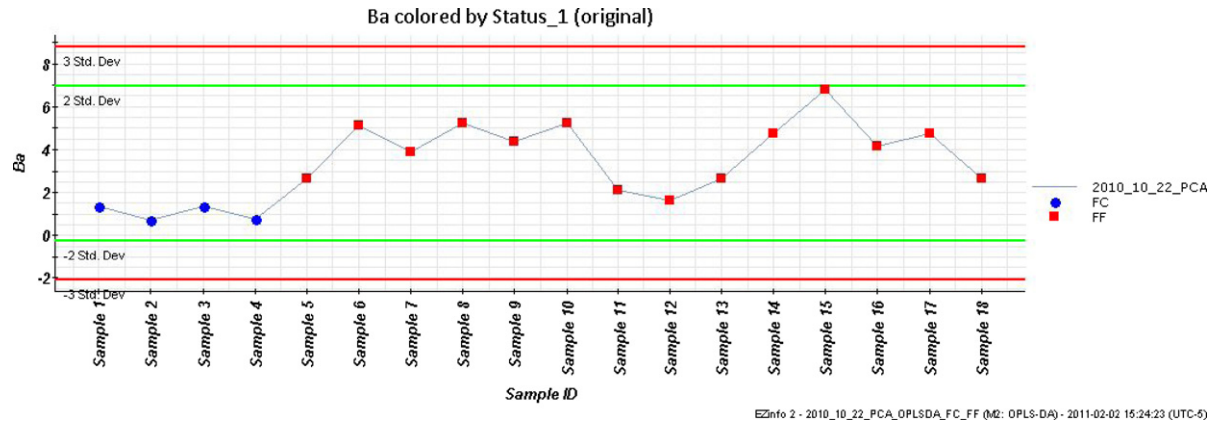

Fig. 11. Trend plot for barium from all female Fabry patients (FF, $n = 14$, samples 5–18) *versus* female controls (FC, $n = 4$, samples 1–4).

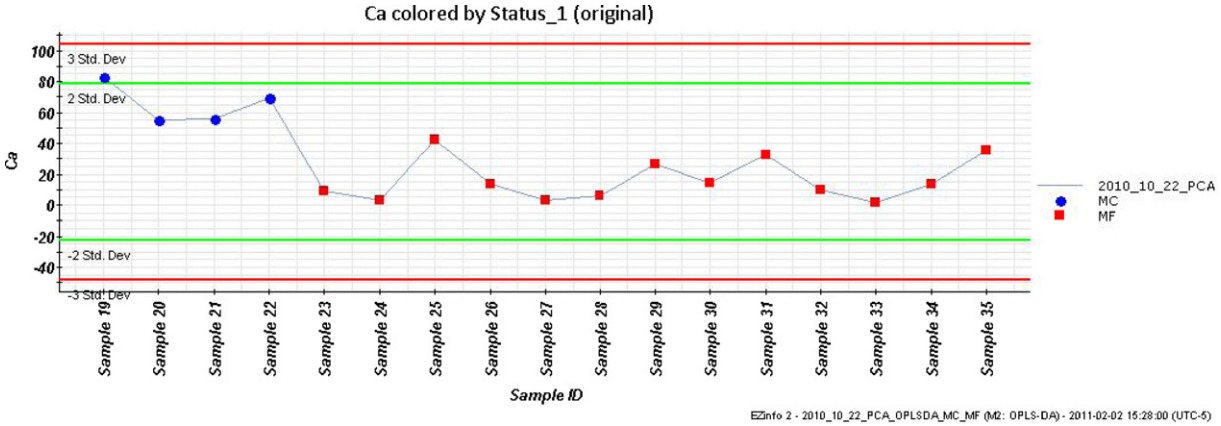

Fig. 12. Trend plot for calcium from all male Fabry patients (MF, $n = 13$, samples 23–35) *versus* male controls (MC, $n = 4$, samples 19–22).

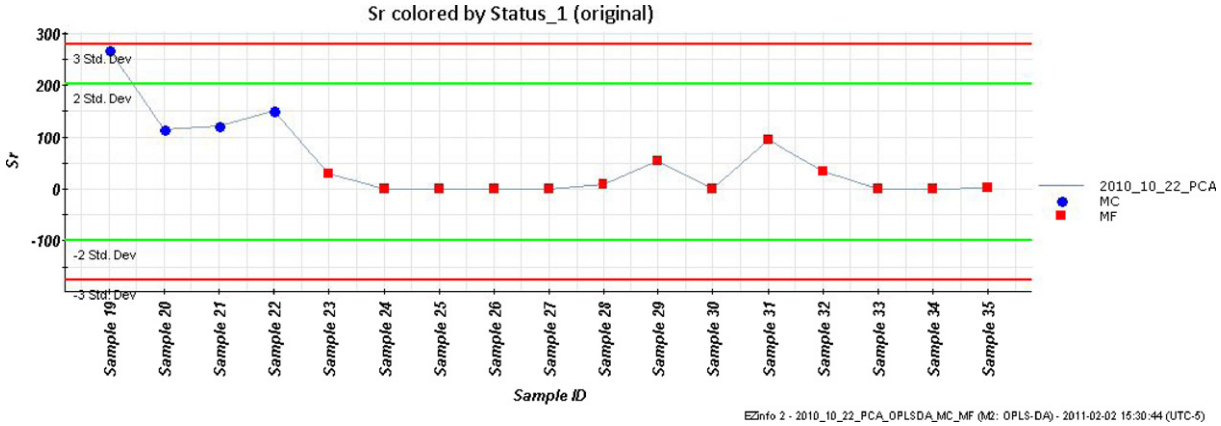

Fig. 13. Trend plot for strontium from all male Fabry patients (MF, $n = 13$, samples 23–35) *versus* male controls (MC, $n = 4$, samples 19–22).

Table 4
Results obtained from a multivariate analysis between the dependent variables lyso-Gb_3 and Gb_3 compared to different significant element levels such as Mg, Ni, Cu, Hg, Pb, Ba, and Ca.

Globotriaosylsphingosine (lyso-Gb_3)	Mg	Ni	Cu	Hg	Pb
Slope estimate	0.02432	−0.2336	0.02527	0.2579	−1.4601
Pr > |t|	0.0171	0.0001	0.0175	0.0024	0.0720
Number of observations	26	26	26	26	26
Globotriaosylceramide (Gb_3)	Ba	Ca	Pb	Hg	Ni
Slope estimate	5.142	1.278	−6.38200	−1.191	−1.087
Pr > |t|	0.0012	0.0011	0.0101	0.0168	0.0009
Number of observations	26	26	26	26	26

corresponding to high urinary levels of excretion of magnesium (Mg), copper (Cu) and mercury (Hg) and low levels of nickel (Ni) and lead (Pb); and (2) high urinary levels of Gb_3/creatinine corresponding to high urinary levels of excretion of barium (Ba) and calcium (Ca) but low levels of lead (Pb), mercury (Hg) and nickel (Ni). However, no significant slope correlations were found between urine lyso-Gb_3/creatinine or Gb_3/creatinine levels and the rest of the 15 elements under study.

Further investigations will focus on the development and applications of a sensitive analytical technique to analyze metal, metalloid and other element concentrations in urine samples dried on filter paper using laser ablation (LA) ICP-MS.

4. Conclusion

In this study, we devised an ICP-MS methodology for metals and other elements for quantification analysis in urine specimens of treated and untreated Fabry hemizygotes (males) and heterozygotes (females). We used LC–MS/MS methodologies developed previously to analyze lipids such Gb_3 and lyso-Gb_3 in these same patients [15,17,18,23], in order to evaluate correlations between metals, and other elements *versus* lipids in urine specimens of Fabry patients *versus* controls. To our knowledge, this is the first documented quantitative analysis of urinary metals and other elements in the urine of Fabry patients compared with specific biomarkers related to Fabry disease.

We found that the excretion of barium was elevated in Fabry females and calcium and strontium levels were lower in Fabry males compared to controls. Our preliminary results show that the enzyme replacement therapy treatment had a minor effect upon the urinary excretion of barium, calcium, and strontium elements in both male and female Fabry disease patients. Studies on larger cohorts would make it possible to evaluate the influence of other factors such as diet, medication, and kidney function parameters on urinary excretion of elements.

Statistically significant correlations were established between urinary lyso-Gb_3/creatinine, Gb_3/creatinine and levels of magnesium, copper, mercury, nickel, lead, barium and calcium. However, no significant correlations were found between urine lyso-Gb_3/creatinine or Gb_3/creatinine levels and the rest of the 15 elements under study. No statistical analysis is shown comparing controls *versus* Fabry disease patients because of the small numbers in each cohort.

Our results indicate that further studies are warranted in larger cohorts of Fabry disease patients for the investigation of possible roles of metals and other elements. Because heavy metals disturb enzyme functions and cellular signalling processes and generate oxidative stress which can lead to the programmed cell death of the cell (apoptosis), they could also play either a more basic or a modulating role [24] in the quite heterogeneous phenotype related to Fabry disease.

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References

[1] J.S. Becker, D. Salber, New mass spectrometric tools in brain research, Trends Anal. Chem. 29 (9) (2010) 966–979.
[2] J.S. Becker, M. Zoriy, A. Matusch, B. Wu, D. Salber, C. Palm, S. Becker, Bioimaging of metals by laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS), Mass Spectrom. Rev. 29 (1) (2010) 156–175.
[3] J.S. Becker, Inorganic Mass Spectrometry: Principles and Applications, John Wiley and Sons, Chichester, 2007.
[4] J.S. Becker, M. Burow, S.F. Boulyga, C. Pickhardt, R. Hille, P. Ostapczuk, ICP-MS determination of uranium and thorium concentrations and $^{235}U/^{238}U$ isotope ratios at trace and ultratrace levels in urine, Atomic Spec. 23 (2002) 177–182.
[5] M.V. Zoriy, C. Pickhardt, P. Ostapczuk, R. Hille, J.S. Becker, Determination of Pu in urine at ultratrace level by sector field inductively coupled plasma mass spectrometry, Int. J. Mass Spectrom. 232 (2004) 217–224.
[6] B.L. Batista, J.L. Rodrigues, L. Tormen, A.J. Curtius, F. Barbosa, Reference concentrations for trace elements in urine for the Brazilian population based on q-ICP-MS with a simple dilute-and-shoot procedure, J. Braz. Chem. Soc. 20 (2009) 1406–1413.
[7] Ö. Yorbik, I. Kurt, A. Haşimi, Ö. Öztürk, Chromium, cadmium, and lead levels in urine of children with autism and typically developing controls, Biol. Trace Elem. Res. 135 (2009) 10–16.
[8] P. Heitland, H.D. Koster, Biomonitoring of 30 trace elements in urine of children and adults by ICP-MS, Clin. Chim. Acta 365 (1–2) (2006) 310–318.
[9] P. Heitland, H.D. Koster, Fast, simple and reliable routine determination of 23 elements in urine by ICP-MS, J. Anal. Atom. Spec. 19 (12) (2004) 1552–1558.
[10] I. Rodushkin, F. Ödman, Application of inductively coupled plasma sector field mass spectrometry for elemental analysis of urine, J. Trace Elem. Med. Biol. 14 (4) (2001) 241–247.
[11] A.T. Townsend, A.K.A. Miller, S. McLean, S. Aldous, The determination of copper, zinc, cadmium and lead in urine by high resolution ICP-MS, J. Anal. Atom. Spec. 13 (11) (1998) 1213–1219.
[12] F. Platt, S.U. Walkley, Lysosomal Disorders of the Brain, Recent Advances in Molecular and Cellular Pathogenesis and Treatment, Oxford University Press, New York, 2004.
[13] W.R. Wilcox, Lysosomal storage disorders: the need for better pediatric recognition and comprehensive care, J. Pediatr. 144 (5 Suppl.) (2004) S3–S14.
[14] J.T.R. Clarke, Narrative review: Fabry disease, Ann. Int. Med. 146 (2007) 425–433.
[15] C. Auray-Blais, D. Cyr, K. Mills, R. Giguère, R. Drouin, Development of a filter paper method potentially applicable to mass and high-risk urinary screenings for Fabry disease, J. Inherit. Metab. Dis. 30 (1) (2007) 106.
[16] C. Auray-Blais, D. Cyr, R. Drouin, Quebec Neonatal Mass Urinary Screening Programme: from micromolecules to macromolecules, J. Inherit. Metab. Dis. 30 (4) (2007) 515–521.
[17] C. Auray-Blais, D. Cyr, R. Drouin, J.T.R. Clarke, D.S. Millington, Use of urinary globotriaosylceramide for Fabry screening in Canada, Clin. Therap. 30 (Suppl. C) (2008) S79–S80.
[18] C. Auray-Blais, D. Cyr, A. Ntwari, M.L. West, J. Cox-Brinkman, D.G. Bichet, D.P. Germain, R. Laframboise, S.B. Melançon, T. Stockley, J.T.R. Clarke, R. Drouin, Urinary globotriaosylceramide excretion correlates with the genotype in children and adults with Fabry disease, Mol. Genet. Metab. 93 (2008) 331–340.
[19] C. Auray-Blais, J.T.R. Clarke, S.P. Young, D.S. Millington, R. Schiffmann, Proposed high-risk screening protocol for Fabry disease in patients with renal and vascular disease, J. Inherit. Metab. Dis. 32 (2) (2009) 303–308.
[20] R. Schiffmann, S. Waldek, A. Benigni, C. Auray-Blais, Biomarkers of Fabry disease nephropathy, Clin. J. Am. Soc. Nephrol. 5 (2) (2010) 360–364.
[21] R. Schiffmann, J.B. Kopp, H.A. Austin, S. Sabnis, D.F. Moore, T. Weibel, J.E. Balow, R.O. Brady, Enzyme replacement therapy in Fabry disease: a randomized controlled trial, JAMA 285 (2001) 2743–2749.
[22] D.P. Germain, S. Waldek, M. Banikazemi, D.A. Bushinsky, J. Charrow, R.J. Desnick, P. Lee, T. Loew, A.C. Vedder, R. Abichandani, W.R. Wilcox, N. Guffon, Sustained, long-term renal stabilization after 54 months of Agalsidase β therapy in patients with Fabry disease, Am. Soc. Nephrol. 18 (2007) 1547–1557.
[23] C. Auray-Blais, A. Ntwari, J.T.R. Clarke, D. Warnock, J.P.F.S. Oliveira, S.P. Young, D.S. Millington, D.G. Bichet, S. Sirrs, M.L. West, R. Casey, W.L. Wu, J. Keutzer, K. Zhang, R. Gagnon, How well does urinary lyso-Gb_3 function as a biomarker in Fabry disease? Clin. Chim. Acta 411 (2010) 1906–1914.
[24] Ö. Yorbik, I. Kurt, A. Haşimi, Ö. Öztürk, Chromium, cadmium, and lead levels in urine of children with autism and typically developing controls, Biol. Trace Elem. Res. 135 (2010) 10–15.