Genotype, Phenotype and Disease Severity Reflected by Serum LysoGb3 Levels in Patients with Fabry Disease

Albina Nowak [a], Thomas P. Mechtler [b], Thorsten Hornemann [c], Joanna Gawinecka [c], Eva Theswet [a], Max J. Hilz [d], David C. Kasper [b]

[a] *Department of Internal Medicine, University Hospital Zurich and University of Zurich, Rämistrasse 100, 8091 Zürich, Switzerland; albina.nowak@usz.ch; eva.theswet@uzh.ch*

[b] *ARCHIMED Life Science, Leberstrasse 20, 1110 Vienna, Austria; t.mechtler@archimedlife.com; d.kasper@archimedlife.com*

[c] *Institute for Clinical Chemistry, University Hospital Zurich and University of Zurich, Rämistrasse 100, 8091 Zürich, Switzerland; thorsten.hornemann@usz.ch; joanna.gawinecka@usz.ch*

[d] *University College London, Institute of Neurology, Queen Square, London, WC1N 3BG, United Kingdom; m.hilz@ucl.ac.uk*

Correspondence:

Dr. Albina Nowak

Department of Internal Medicine

University Hospital Zurich

Rämistrasse 100

CH-8091 Zürich

Phone: 0041-44-255 10 54, Fax: 0041-44-255 45 67

Email: albina.nowak@usz.ch

Abstract

Background

Fabry disease (FD) is a rare X-linked lysosomal storage disease caused by mutations in the α-galactosidase A (*GLA*) gene causing deficiency of α-galactosidase A which results in progressive glycosphingolipid accumulation, especially globotria**o**sylceramide (Gb3), in body liquids and lysosomes. In a large cohort of FD patients, we aimed to establish genotype/phenotype relations as indicated by serum LysoGb3 (deacylated Gb3).

Methods

In 69 consecutive adult FD patients (males: n=28 (41%)) with a *GLA*-mutation confirmed diagnosis, we conducted a multidisciplinary clinical characterization during their routine annual examinations, and measured serum LysoGb3 levels by high-sensitive electrospray ionization liquid chromatography tandem mass spectrometry.

Results

Serum levels of LysoGb3 were significantly higher in Classic compared with Later-Onset phenotype and higher in the latter compared with controls, both in males (52 [40-83] vs 9.5 [4.5-20] vs 0.47 [0.41-0.61]ng/ml, P<0.001) and in females (9.9 [7.9-14] vs 4.9 [1.6-4.9] vs 0.41 [0.33-0.48]ng/ml, P<0.001), respectively. Multivariate linear regression analysis showed that LysoGb3 levels were independently associated with, serum creatinine (β=0.09, 95%CI 0.04-0.13, P<0.001) and the presence of cardiomyopathy (β=25, 95%CI 9.8-41, P=0.002). LysoGb3 levels were higher in males with frame-shift and nonsense mutations than in males with missense mutations (84 [72-109] vs 41 [37-52]ng/ml, P=0.002).

Conclusion

LysoGb3 relates to disease severity, enzyme replacement response, and to the genotype severity in males. LysoGb3 supports identifying patients at risk who require intensive monitoring and treatment. LysoGb3 appears to be one marker of metabolic phenotyping of FD.

Keywords: Fabry disease; *GLA*-mutation; LysoGb3; biomarker; genotype phenotype relation; disease severity.

1. Introduction

Fabry disease (FD) (OMIM#301500) is an X-linked disease, resulting from the deficient activity of the lysosomal enzyme α-galactosidase A (α-Gal A) [1, 2]. The enzymatic defect causes the progressive accumulation of globotriaosylceramide (Gb3) and related glycosphingolipids in the plasma and in tissue lysosomes throughout the body [1].

There are two major phenotypes, Classic and Later-Onset [1, 3-6]. The Classic phenotype is more severe due to very low or absent α-Gal A activity, with the typical early symptoms such as acroparesthesias, angiokeratoma, corneal opacities and hypohidrosis, particularly in males. With advancing age, the progressive deposition of glycosphingolipids lead to cardiomyopathy, deterioration of kidney function, and premature strokes [7, 8]. The Later-Onset phenotype is typically less severe with a significant residual α-Gal A activity in males, who usually lack the early symptoms but present with a cardiomyopathy or chronic kidney disease in the adult age [3, 9-11]. The phenotypic heterogeneity can delay the correct diagnosis. In females, α-Gal A activity can be normal due to random X-chromosomal inactivation [12]. Even genetic testing can result in novel *GLA* variants with unknown clinical significance [13]. This raises questions with regard to disease onset and progression, particularly in asymptomatic patients identified in family screening.

Recently, enhanced deposits of globotriaosylsphingosine (LysoGb3) have been shown to be a characteristic feature of FD [14]. The deacylated Gb3, LysoGb3 (also called LysoGl3), also known as globotriaosylsphingosine, has been reported as a potential diagnostic tool in both classic and uncertain cases [15]. The utility of LysoGb3 is still controversial, as discussed in previous studies [15, 16], due to concerns that LysoGb3 levels may not be strongly associated with disease phenotype [17]. To answer these concerns, we evaluated whether a

genotype/phenotype association can be established using serum LysoGb3 levels. To this end, we analyzed a clinical, biochemical and genetic characterization of a large FD patient's cohort that was regularly monitored at a single FD center. This is the first study to associate LysoGb3 levels with the FD-related comorbidities and the genotype severity.

2. Methods

2.1. Study participants and clinical work-up

The study was conducted in accordance with the principles of the Helsinki Declaration. Informed consent for collecting clinical data and blood samples for biobanking was obtained from all patients.

We recruited 69 consecutive adult patients (males: n=28 (41%)) at the University Hospital Zurich, Switzerland, between January 2014 and December 2016. All patients had a confirmed *GLA*-mutation diagnosis and presented for routine annual examinations at our FD center. The cohort was established in 2001 when ERT was approved and offered to FD patients. ERT was prescribed at the licensed dose of either 0.2 mg/kg body weight of recombinant agalsidase-α (Replagal) or 1 mg/kg body weight agalsidase-β (Fabrazyme) and given intravenously every 14 days.

All patients had a comprehensive workup, including medical history, cardiac, renal, and neurological evaluations. The occurrence of stroke or TIA (transient ischemic attack) was evaluated during annual examinations by asking the patient and/or using the medical records. Standard transthoracic 2D-echocardiography was routinely performed in all patients. LVMMI was calculated using the Devereux formula [18]. Cardiomyopathy was defined as the presence of diastolic dysfunction and/or left ventricular hypertrophy on echocardiography or heart MRI.

For the present analyses, all clinical and routine laboratory results were obtained from the patients` medical records.

The healthy group consisted of 13 females and 13 males aged between 17 and 69 years.

2.2. Phenotyping

The phenotyping was performed blinded to the LysoGb3 levels and as reported previously [5, 19]. The phenotype was classified based on the genotype. Nonsense, frameshift, consensus splice site and certain missense mutations encode for 0 to 1% residual α-Gal activity and cause Classic phenotype in males. Alternative splicing mutations and certain other missense mutations encode for more than 1% of normal α-Gal activity and cause Later-Onset phenotype in males. The phenotype was confirmed based on the age of symptoms onset for each mutation. For novel missense mutations, the phenotype was classified based on clinical symptoms and signs in males and by in vitro expression assays [4, 20].

2.3. LysoGb3 measurement

For serum LysoGb3 levels, blood samples were centrifuged and serum was immediately frozen at −80°C for a later batch analysis. The samples were measured by high-sensitive electrospray ionization liquid chromatography tandem mass spectrometry (ESI LC-MS/MS) using an adapted method from Gold [21]. A 7-point serum calibrator and an internal standard for LysoGb3 quantification (covering the analytic range from 0-120 ng/mL; lower limit of quantification: 0.3 ng/mL), and three level controls (3, 30 and 100 ng/mL) for quality control were used (ARCHIMED Life Science GmbH, Vienna, Austria; www.archimedlife.com). Further experimental details on mass spectrometric conditions and sample work-up will be available upon request.

2.4. Statistical analysis

We used descriptive statistics for the baseline characteristics and laboratory parameters. Categorical variables were expressed as proportions, continuous

variables as means with standard deviations and medians with interquartile ranges (IQR). Normal distribution was assessed by Kolmogorov-Smirnov-Test. Comparisons between the study groups were performed using the t test, Mann–Whitney U test, the Chi-square or one-way analysis of variance (ANOVA) test as appropriate. Correlations were determined according to the method of Spearman.

Receiver operating characteristics (ROC) procedure was used to predict the Classic phenotype by serum LysoGb3 levels in all FD males and females. ROC was also used to predict FD among FD patients and controls.

Univariate linear regression analysis was applied to assess the association between serum levels of LysoGb3 and sex, phenotype as well as disease activity as reflected by the FD-related clinical work-up parameters. Multivariate linear regression model was used to evaluate which of these disease activity parameters are independently associated with the LysoGb3 levels after adjustment for sex and phenotype.

Statistical analyses were performed using SPSS/PC (version 22.0; SPSS Inc., Chicago, IL, USA) software package. A statistical significance level of 0.05 was used. All hypothesis testing was two-tailed.

3. Results

3.1. Baseline characteristics

The baseline characteristics and sex of all patients are presented in Table 1. In male patients, serum creatinine levels were higher and cardiomyopathy more frequent than in female patients. All patients on renal replacement therapy were male.

Table 1. Baseline characteristics

	All patients N=69	Males N=28	Females N=41	P-value
Age, years	40 [31-53]	43 [35-51]	37 [30-56]	0.62
Phenotype n (%)				0.31
Classic	61 (88)	23 (82)	38 (93)	
Later-Onset	9 (12)	5 (18)	4 (7)	
On ERT n (%)	52 (75)	25 (89)	27 (64)	0.02
Serum creatinine, µmol/L	123 ± 175	198 ± 258	72 ± 29	0.003
Urine protein/creatinine ratio mmol/L **	0.06 ± 0.19	0.05 ± 0.07	0.06 ± 0.23	0.94
On dialysis n (%)	3 (3)	3 (11)	0 (0)	0.03
Kidney transplant n (%)	5 (7)	5 (18)	0 (0)	0.005
Cardiomyopathy n (%)	33 (48)	18 (64)	15 (37)	0.02
LVMMI, g/m^2	103 ± 55	125 ± 62	88 ± 44	0.007
Stroke/TIA n (%)	10 (14)	4 (14)	6 (15)	1.00

* estimated according to the CKD-Epi formula

** Patients on renal replacement excluded

Plus-minus values are means ± SD. Numbers with ranges in square brackets are medians and interquartile ranges.

Abbreviations: ERT, Enzyme Replacement Therapy; MSSI, Mainz Severity Score Index, LVMMI, Left Ventricular Mass Index; TIA, Transient Ischemic Attack.

3.2. LysoGb3 in relation to sex and phenotype

In males and females, serum levels of LysoGb3 were significantly higher in Classic than in Later-Onset phenotype patients. In healthy controls, LysoGb3 levels were lower than in FD patients (Figure 1A and 1B).

Figure 1. Serum levels of LysoGb3 in males (A) and females (B) depending of phenotype and in comparison to healthy controls*.

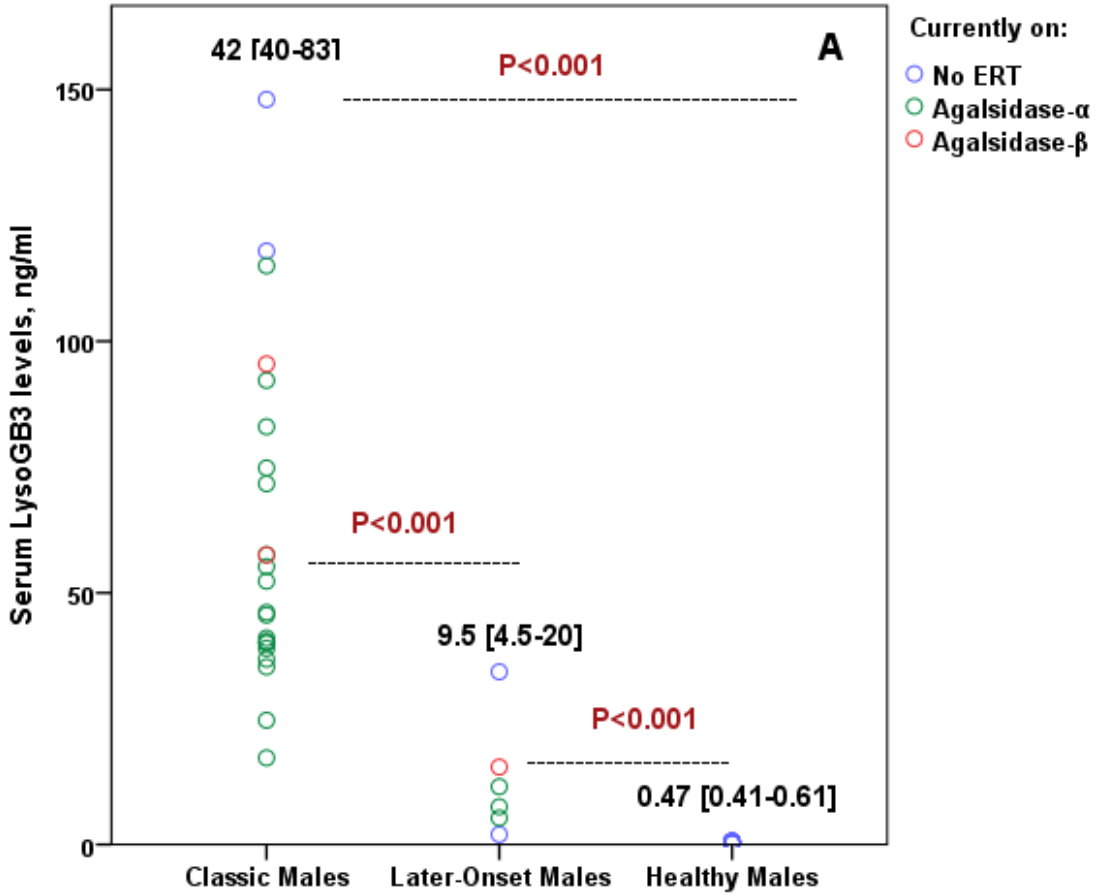


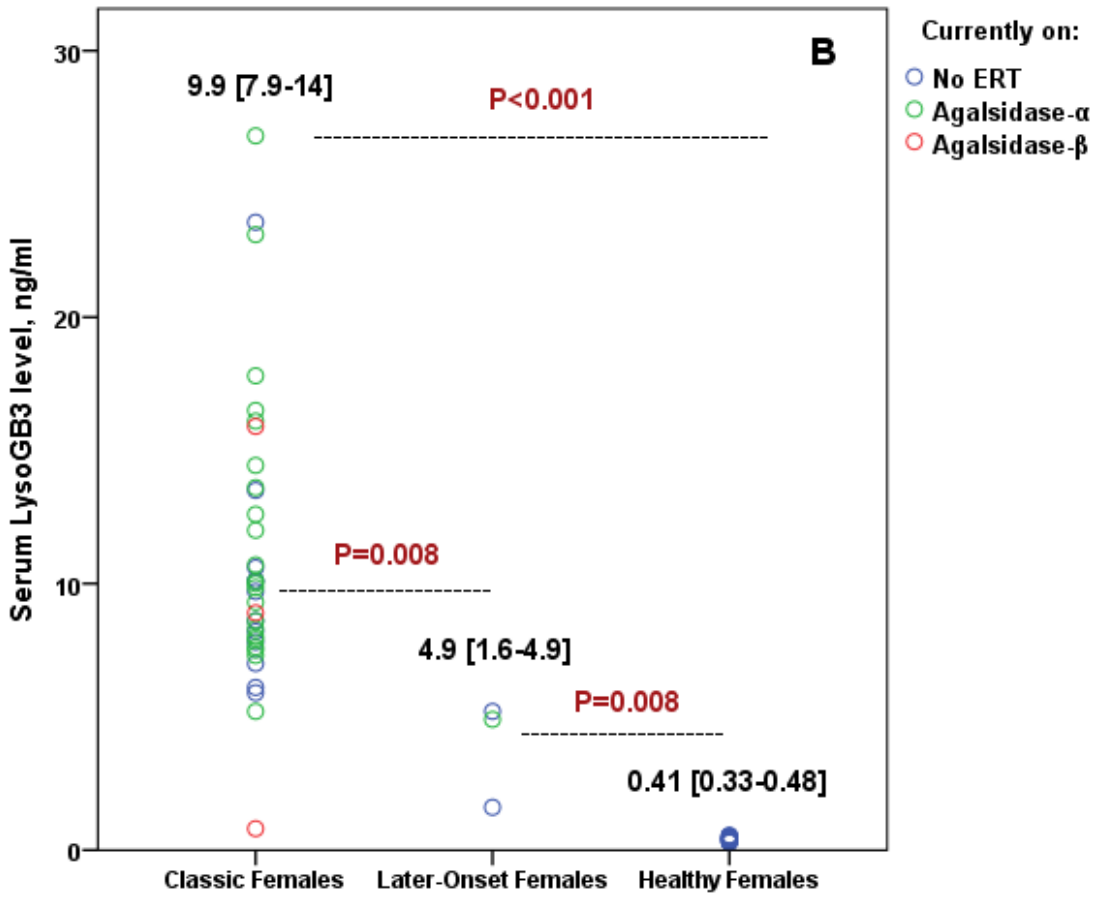
Table legend to Figure 1A.

Classic Males

Age	GLA Mutation	Predicted Enzyme Protein Change	Cumulative Dose Agalsidase-α, mg	Cumulative Dose Agalsidase-β, mg	Serum Lyso-Gb3 level (ng/ml)
27	c.559_560delAT	p.M187Vfs*6			148.02
29	c.1147_1149del	p.F383del			117.95
50	Deletion exon 2		3591.20		115.00
18	c.744_745delTA	p.F248LfsX7		6580.00	95.50
44	c.744_745delTA	p.F248LfsX8	1935.20	2124.00	92.20
55	c.899T>A	p.L300H	1988.00	16614.00	83.00
49	c.744_745delTA	p.F248LfsX8	2407.20	408.00	74.80
31	c.1055_1057dupCTA	p.A352_M353insT	963.60		71.70
44	c.370-2A>G	Cons. Splice Site	3539.20		57.60
30	c.679C>T	p.R220X	1240.00	17236.00	57.52
59	c.1033T>C	p.S345P	5088.00		55.20
61	c.1033T>C	p.S345P	4089.60		52.31
67	c.581C>T	p.T194I	4316.80		46.10
39	c.827G>A	p.S276N	3312.00		45.60
44	c.581C>T	p.T194I	3164.00		41.00
36	c.581C>T	p.T194I	4121.60		40.40
59	c.899T>A	p.L300H	864.00	13536.00	40.10
51	c.581C>T	p.T194I	3778.80		40.00
23	c.125T>C	p.M42T	1776.00		39.00
40	c.370-2A>G	Cons. Splice Site	3175.20		36.80
35	c.125T>C	p.M42T	3192.00		35.30
39	c.1033T>C	p.S345P	2148.80	10472.00	24.70
47	c.613C>T	p.Pro205Ser	3608.00		17.20

Later-Onset Males

Age	GLA Mutation	Predicted Enzyme Protein Change	Cumulative Dose Agalsidase-α, mg	Cumulative Dose Agalsidase-β, mg	Serum Lyso-Gb3 level (ng/ml)
39	c.902G>A	p.R301Q			34.30
41	c.337T>C	p.F113L		1456.00	15.40
65	c.902G>A	p.R301Q	2275.20	17064.00	11.50
44	c.902G>A	p.R301Q	1776.00	8288.00	7.50
63	c.644A>G	p.N215S	1008.00		5.30
41	c.1196G>C	p.W399S			2.00



Numbers with ranges in square brackets are medians and interquartile ranges

11 Table legend to Figure 1B.

Classic Heterozygotes

Age	GLA Mutation	Predicted Enzyme Protein Change	Cumulative Dose Agalsidase-α, mg	Cumulative Dose Agalsidase-β, mg	Serum Lyso-Gb3 level (ng/ml)
68	c.581C>T	p.T194I	3328.00		26.81
35	c.1167dupT	p.V390CfsX9			23.56
48	c.365delA	p.N122IfsX8	2049.60		
65	c.581C>T	p.T194I	3476.00		17.80
51	c.1033T>C	p.S345P	1364.00		16.50
68	c.1033T>C	p.S345P	1531.20		16.10
55	c.796G>T	p.D266T		2100	
65	c.640-3C>G	Cons. Splice Site	5149.6		14.44
28	c.1033T>C	p.S345P	907.20		13.60
48	c.72G>A	p.Y24X			13.50
36	c.1235_1236delCT	p.N122IfsX8	194.40		12.60
63	c.581C>T	p.T194I	3113.60		12.00
35	c.901C>T	p.R301X	79.20		10.70
31	c.1055_1057dupCTA	p.A352_M353insT			10.60
25	c.1147_1149del	p.F383del			10.10
39	c.581C>T	p.T194I			10.10
17	c.1167dupT	p.V390CfsX9	1356.80		10.10
41	c.640-3C>G	Cons. Splice Site	4238.00		10.00
51	c.1167dupT	p.V390CfsX9	1298.00		9.85
37	c.581C>T	p.T194I			9.70
63	c.72G>A	p.Y24X	3245.20		9.30
56	c.796G>T	p.D266T		2024.00	8.90
62	c.744_745delTA	p.F248LfsX7			8.60
23	c.125T>C	p.M42T	2360.00		8.60

34	c.581C>T	p.T194I	3366.00		8.30
29	c.704C>A	p.Ser235Tyr			8.2
31	c.125T>C	p.M42T	2419.20		8.00
33	c.744_745delTA	p.F248LfsX7	379.60		7.90
42	c.1033T>C	p.S345P			7.80
24	c.1167dupT	p.V390CfsX9	1560.00		7.61
29	c.125T>C	p.M42T	1786.00		7.5
26	c.125T>C	p.M42T	2195.20		7.30
23	c.1033T>C	p.S345P			7.00
32	c.581C>T	p.T194I			6.10
36	c.154T>C	p.C52R			5.90
39	c.744_745delTA	p.F248LfsX7	775.20	5928.00	5.20
48	c.870G>C	p.M290I			.80

Later-Onset Heterozygotes

Age	GLA Mutation	Predicted Enzyme Protein Change	Cumulative Dose Agalsidase-α, mg	Cumulative Dose Agalsidase-β, mg	Serum Lyso-Gb3 level (ng/ml)
37	c.902G>A	p.R301Q			5.20
73	c.902G>A	p.R301Q	2128.00		4.90
33	c.337T>C	p.F113L			1.6

12

There was one overlap in LysoGb3 levels between males with the Classic and Later-Onset phenotypes: the male with the highest LysoGb3 level within the Later-Onset phenotype group was newly diagnosed having FD and not yet on ERT. Among the females, one Classic and three Later-Onset had similar LysoGb3 values. There was no overlap in LysoGb3 levels between FD patients and controls.

The ROC curve indicated a high predictive value for LysoGb3 to identify FD patients among patients and controls: AUC=1 for each sex, with the best calculated cutoff for sensitivity and specificity at 34.8 ng/ml for males and 8.1 ng/ml for females.

For prediction of the Classic versus Later-Onset phenotype among FD patients, LysoGb3 levels nearly ideally predicted the Classic phenotype in males: AUC=0.98 (best calculated cutoff 43.3 ng/ml); in females, the predictive accuracy of LysoGb3 levels was high: AUC=0.81 (best calculated cutoff 9.9 ng/ml).

3.3. LysoGb3 in relation to disease severity

In an univariate linear regression analysis, LysoGb3 levels were associated with sex, phenotype, serum creatinine, renal replacement, LVMMI, presence of cardiomyopathy and stroke/TIA. In a multivariate linear regression analysis, if adjusted for sex and phenotype, LysoGb3 levels remained independently associated with the same parameters (Table 2).

Table 2. Linear regression for serum LysoGb3 as the dependent variable.

	Univariate		**Multivariate ***	
Characteristics	β (95% CI)	P Value	β (95% CI)	P Value
Sex, male	52.3 (44.2-62.4)	<0.001	n.a.	
Classic phenotype	30.6 (22.7-38.5)	<0.001	n.a.	
Age, year	0.58 (0.40-0.75)	<0.001	1.01 (0.64-1.38)	<0.001
Serum creatinine, µmol/L	0.11 (0.07-0.15)	<0.001	0.09 (0.04-0.13)	<0.001
Urin protein/creatinine ratio, mmol/L	46.8 (-4.3 to 97.8)	0.07		
Renal replacement	45.4 (21.7-69.0)	<0.001	42.2 (18.9-65.5)	0.001
Cardiomyopathy	32.1 (19.8-44.5)	<0.001	25.5 (9.81-41.1)	0.002
LVMMI, g/m^2	0.24 (0.18-0.30)	<0.001	0.29 (0.20-0.39)	<0.001
Stroke/TIA	27.6 (5.53-49.6)	0.02	23.0 (0.93-45.1)	0.04

*Adjusted for sex and phenotype

Abbreviations: LVMMI, left-ventricular myocardial index; TIA, trans ischemic attack.

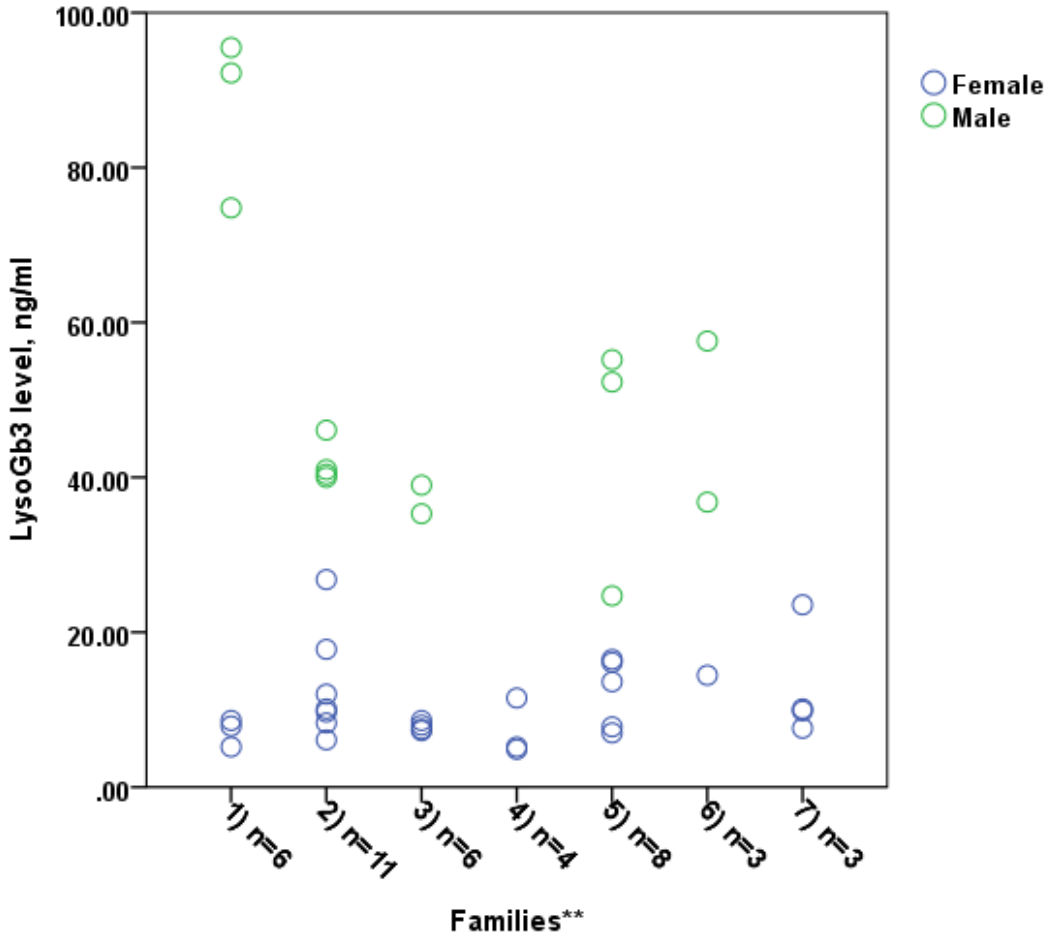
LysoGb3 levels significantly correlated with the serum creatinine (R=0.28, P=0.02), LVMMI (R=0.27, P=0.03) and protein/creatinine ratio (R=0.33, P=0.007). LysoGb3 levels weekly correlated with age in females (R=0.34, R=0.03) but not in males (R=-0.22, P=0.26).

3.4. LysoGb3 in relation to genotype and between family members

If LysoGb3 measured during the routine annual examination was available in at least 3 family members, the levels were grouped by the family, as shown in Figure 2. Within one family, the LysoGb3 levels were mostly similar among males and females respectively and were always higher in males than in females. If LysoGb3 showed greater differences between the family members with the same sex, it could be partly associated with differences in the disease burden and whether the patient was treated with enzyme replacement. In detail, in Family 1, the 44-year-old male with LysoGb3 of 92.2 ng/ml much earlier developed end-stage renal disease and required kidney transplantation than did his 49-year-old brother who had LysoGb3 of 74.8 ng/ml. The relatively high LysoGb3 level of 95.5 ng/ml of their 18-year-old oligosymptomatic nephew remains difficult to interpret; the phenomenon of high LysoGb3 levels in children and younger adults is known from the literature [22] and may represent an early plateau during the pre-symptomatic period, this storage already begins during the fetal phase [23]. In Family 2, the 68-year-old female with the highest LysoGb3 level of 26.8 ng/ml has the most severe phenotype among the females within the same family being hemiplegic due to recurrent strokes despite ERT. In Family 4, the 35-year-old female had a higher LysoGb3 level (23.6 ng/ml) than had her aunt and cousin which might be explained by the fact that she was not yet on ERT due to compliance reasons. Previous studies showed that LysoGb3 decreases following ERT initiation [24]. In Family 5, the 39-year-old male with the

lowest LysoGb3 level of 24.7 ng/ml has a less severe phenotype with normal kidney function and a lower MSSI than his 59 and 61-year-old uncles who had LysoGb3 values of 55.2 and 52.3 ng/ml and are both on renal replacement therapy.

Figure 2. Plasma LysoGb3 levels per family*.



* Families with at least three family members were plotted

** Table legend to Figure 2.

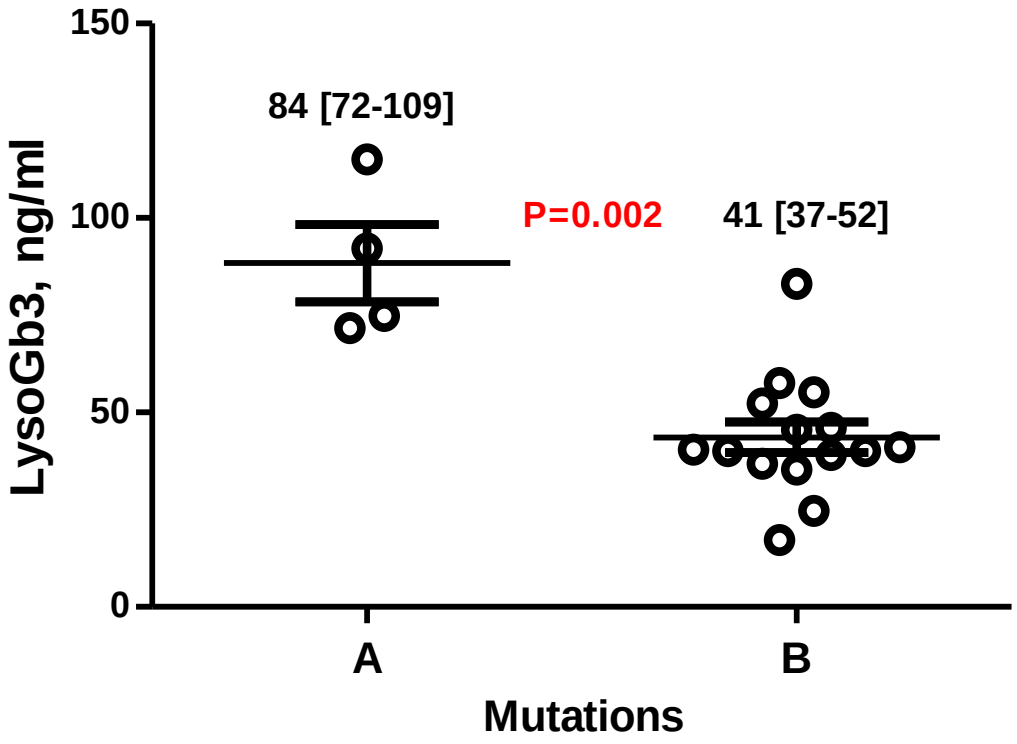
Nr	*GLA* Mutation / Predicted Enzyme Protein Change	Phenotype	LysoGb3	Sex	Age	ERT
1	c.559_560delAT / p.M187Vfs*6 (6 family members)	Classic	95.5	m	18	+
			92.2	m	44	+
			74.8	m	49	+
			8.6	f	62	-
			7.9	f	20	+
			5.2	f	39	+
2	c.581C>T / p.T194I (11 family members)	Classic	46.1	m	67	+
			41.0	m	44	+
			40.4	m	36	+
			40.0	m	51	+
			26.8	f	68	+
			17.8	f	65	+
			12.0	f	63	+
			10.1	f	39	-
			9.7	f	37	-
			8.3	f	34	+
			6.1	f	32	-
3	c.125T>C / p.M42T (6 family members)	Classic	39.0	m	23	+
			35.3	m	35	+
			8.6	f	25	+
			7.5	f	29	+
			7.3	f	26	+
			8.0	f	31	+
4	c.1167dupT / p.V390CfsX9 (4 family members)	Classic	23.6	f	35	-
			10.1	f	17	+
			9.9	f	51	+
			7.6	f	24	+
5	c.1033T>C / p.S345P (8 family members)	Classic	55.2	m	59	+
			52.3	m	61	+
			24.7	m	39	+
			16.5	f	51	+
			16.1	f	68	+
			13.6	f	28	+
			7.8	f	42	-
			7.0	f	23	-
6	c.370-2A>G / Cons. Splice	Classic	57.6	m	44	+

	Site		36.8	m	40	+
	(3 family members)		14.4	f	65	+
7	c.902G>A / p.R301Q	Later-Onset	11.5	m	65	+
	(3 family members)		5.2	f	37	-
			4.9	f	73	+

Abbreviations: ERT, Enzyme Replacement Therapy.

The mutations within the Classic phenotype patients were subdivided into two groups according to their structure and function. Group A was defined by frame-shift or nonsense mutations that lead to a major change in the gene products which can be caused by deletions, insertions, duplications and some point mutations [1]. Group B included missense mutations caused by individual point mutations that lead to single amino acid changes. Only patients with the same ERT preparation (α-agalsidase) with treatment duration of at least 5 years at stable dose were included into this analysis in order to balance the ERT effect on the LysoGb3 levels [24]. In males, LysoGb3 levels were higher in group A than in group B (Figure 3). In contrast, in females, LysoGb3 levels did not differ significantly between group A (n= 6) and group B (n=13) (10.0 [8.3-15.2] vs 12.0 [8.2-16.3]; P=0.77).

Figure 3. Serum Lysob3 levels in affected males* according to mutation severity by structure and function: **A** frame-shift and nonsense-mutation versus **B** missense mutations.



*Included only males with the same ERT preparation (α-agalsidase) at a stable dose of at least 5 years.

Table legend to Figure 3.

Mutations A

GLA Mutation	Predicted Aminoacid	Serum Lyso-Gb3 (ng/ml)
Deletion exon 2		115.0
c.744_745delTA	p.F248LfsX7	92.2
c.744_745delTA	p.F248LfsX7	74.8
c.1055_1057dupCTA	p.A352_M353insT	71.7

Mutations B

GLA Mutation	Predicted Aminoacid	Serum Lyso-Gb3 (ng/ml)
c.125T>C	p.M42T	39.0
c.125T>C	p.M42T	35.3
c.581C>T	p.T194I	41.0
c.581C>T	p.T194I	46.1
c.581C>T	p.T194I	40.0
c.581C>T	p.T194I	40.4
c.899T>A	p.L300H	40.1
c.899T>A	p.L300H	83.0
c.370-2A>G	Cons. Splice Site	36.8
c.370-2A>G	Cons. Splice Site	57.6
c.827G>A	p.S276N	45.6
c.1033T>C	p.S345P	52.3
c.1033T>C	p.S345P	24.7
c.1033T>C	p.S345P	55.2
c.613C>T	p.P205S	17.2

Discussion

In this relatively large and well characterized cohort of genetically proven FD patients, serum levels of LysoGb3 were significantly higher than in the healthy controls. More importantly, LysoGb3 levels were significantly higher in the Classic than in the Later-Onset phenotype in male and female patients. After adjustment for sex and phenotype, LysoGb3 was independently associated with the relevant co-morbidities such as kidney function, renal replacement therapy, cardiomyopathy, stroke and TIA. Within families, LysoGb3 levels were generally similar within one sex but always higher in males than in females. However, higher LysoGb3 levels in family members of the same sex were found in patients with higher disease activity, not on ERT, or at young age.

Interestingly, the analysis of just the Classic phenotype, showed that serum LysoGb3 levels were higher among the males with severe mutations, such as frame-shift or nonsense mutations, that are known to lead to grossly altered gene products. These high LysoGb3 levels may be partly interpreted as a result of particularly low or absent α-Gal A activities in males with such mutations resulting in accumulation of LysoGb3 [25]. Additionally, LysoGb3 could be de-novo synthesized by sequential glycosylation of sphingoid bases by the action of a specific enzyme, more accelerated in males with “severe” mutations.

In contrast to LysoGb3 levels in the male patients, LysoGb3 levels of female patients did not depend on the mutation severity which might be ascribed to the random X-chromosomal inactivation in the heterozygous [12].

In FD, the correct risk stratification based on an understanding of the genotype and phenotype relationship is an urgent though unmet clinical need. Since FD has become treatable with ERT [2, 26] and more recently with further treatment strategies [27-30], there is increasing awareness of FD among primary care physicians and

different specialists, and systematic screening among high-risk populations [31-33] and newborns [34] has become more frequent. This has resulted in increased detection of mutations with unknown clinical relevance [35, 36].

The diagnosis is further complicated in females; at least 40% of the *GLA*-mutation confirmed females have normal or slightly decreased α-Gal A activities and require *GLA* sequencing to confirm heterozygosity [12, 37]. In males, the diagnosis of FD requires demonstrating of decreased α-Gal A activity in leucocytes; the diagnosis then can be confirmed by additional *GLA* mutation analysis. However, males with the Later-Onset phenotype may still have a significant residual enzyme activity. Consequently, male Later-Onset phenotype FD patients often lack the typical early-onset classical manifestations, but they do show later disease manifestation and a predominance of single organ disease, particularly of the heart and kidney [3, 4, 9-11]. In these patients, FD might not be recognized as the cause of heart, kidney, or cerebrovascular diseases. These might be misdiagnosed and ascribed to more common pathology including aging processes or cardiovascular risk factors [38].

A highly sensitive and specific biomarker could fill the diagnostic gap and help avoid invasive biopsies and assure a swift diagnosis in patients with suspected FD. Such a biomarker may also improve disease staging and risk stratification as well as support the decision whether a patient should be started on ERT or should only be closely monitored.

Previous studies identified LysoGb3 as a helpful diagnostic tool in classic and uncertain cases [14], and particularly in females [5]. Rombach and colleagues found LysoGb3 levels to be associated with white matter lesions in males, and with MSSI and left- ventricular mass in females [39]. Lenders and colleagues reported associations of LysoGb3 levels with the serum-mediated ERT inhibition [40].

In Later-Onset (so-called Non-classical) phenotype FD patients, Smid observed that LysoGb3 levels are similar in patients of the same sex and with the same *GLA* variant [16]. Our findings confirm the relation between LysoGb3 levels and Classic and Later-Onset phenotypes [16]. Moreover, our results show an independent association of LysoGb3 with the most important clinical manifestations such as renal, cardiac and cerebrovascular disease, and treatment response expressed by serum-mediated ERT inhibition. Notably, our results did not show an influence of the long-term cumulative dose of ERT on the LysoGb3 levels. This finding is in accordance with previous studies showing that LysoGb3 levels decrease after the ERT initiation and reach a plateau already after 2-3 months [24, 41].

Our study is the first to have analyzed LysoGb3 levels per family. It is valuable because family members usually have similar modifying genes and live under similar environmental conditions. Our data are also novel in showing a strong relation between LysoGb3 and mutation severity in males. The additional measurement of LysoGb3 may therefore augment the functional characterization of *GLA* mutations. However, previous studies [15, 16] show some overlap between LysoGb3 values of FD females and healthy individuals. While augmenting functional characterization of *GLA* mutations, LysoGb3 cannot replace a detailed clinical characterization using a multidisciplinary approach, family history, genetic testing and the phenotypic descriptions of the family mutation. Thus, more sensitive biomarkers will be needed to distinguish between the Later-Onset phenotype FD patients, particularly women, and healthy persons.

LysoGb3 is not only a biomarker. It might also be involved in the FD pathology. LysoGb3 has been shown to promote Notch1-mediated inflammatory and fibrogenic response in podocytes potentially contributing to Fabry nephropathy [42]. LysoGb3 treatment inhibited the proliferation and differentiation of fibroblasts into

myofibroblasts, reducing collagen synthesis and herewith compromising vascular remodeling [43]. It directly inhibited the α-Gal A activity and induced smooth muscle cell proliferation [14]. Administration of LysoGb3 stimulated the up-regulation of voltage-dependent Ca^{2+} channels in nociceptive neurons, suggesting that it may induce pain through direct actions on sensory neurons [44].

Several limitations merit consideration. First, the number of our Later-Onset group patients was low. Second, the study was not designed to evaluate the ability of LysoGb3 to predict the development of major clinical events. Third, the study did not assess epigenetic phenomena, such as the influence of the degree of the random X-chromosomal inactivation on LysoGb3 levels in females. Finally, for the use as a clinical biomarker, standardization of the technical methods and inter-laboratory testing is needed in order to compare LysoGb3 measurements among laboratories.

In conclusion, the use of LysoGb3 as an additional laboratory biomarker appears to improve the detection and management of clinically relevant FD. LysoGb3 levels are associated with the important clinical sequelae of FD such as nephro-, cardiomyopathy and cerebrovascular disease, thus, LysoGb3 may represent the new concept of metabolic phenotype. LysoGb3 helps to stratify persons at risk and may provide guidance towards a more individualized treatment of patients.

Role of the funding source

The LysoGb3 measurements were determined by *ARCHIMED Life Science, Vienna, Austria.* The *ARCHIMED Life Science* laboratory members (TPM and DCK) participated in writing and approving the manuscript. The laboratory members were blinded to patients' names and all clinical and biochemical information and had no

role in the collection of samples, interpretation of data and the decision to submit the article for publication.

Conflict of interest

AN is a consultant to Shire, received lecturing honoraria and research support from Sanofi Genzyme and Shire and received financial publication support of this paper from Sanofi Genzyme.

References

[1] R. Desnick, Y. Ioannou, C. Eng, α-galactosidase A deficiency: Fabry disease. The metabolic and molecular bases of inherited disease. (eds Scriver CR, Beaudet AL, Sly WS, Valle D.) p3733-3774, McGraw-Hill, New York, 2001

[2] R.J. Desnick, R. Brady, J. Barranger, A.J. Collins, D.P. Germain, M. Goldman, G. Grabowski, S. Packman, W.R. Wilcox, Fabry disease, an under-recognized multisystemic disorder: expert recommendations for diagnosis, management, and enzyme replacement therapy Ann Intern Med 138 (2003) 338-346

[3] W. von Scheidt, C.M. Eng, T.F. Fitzmaurice, E. Erdmann, G. Hubner, E.G. Olsen, H. Christomanou, R. Kandolf, D.F. Bishop, R.J. Desnick, An atypical variant of Fabry's disease with manifestations confined to the myocardium N Engl J Med 324 (1991) 395-39910.1056/NEJM199102073240607.

[4] M. Spada, S. Pagliardini, M. Yasuda, T. Tukel, G. Thiagarajan, H. Sakuraba, A. Ponzone, R.J. Desnick, High incidence of later-onset fabry disease revealed by newborn screening Am J Hum Genet 79 (2006) 31-4010.1086/504601.

[5] A. Nowak, T.P. Mechtler, R.J. Desnick, D.C. Kasper, Plasma LysoGb3: A useful biomarker for the diagnosis and treatment of Fabry disease heterozygotes Molecular genetics and metabolism (2016) 10.1016/j.ymgme.2016.10.006.

[6] M. Arends, C. Wanner, D. Hughes, A. Mehta, D. Oder, O.T. Watkinson, P.M. Elliott, G.E. Linthorst, F.A. Wijburg, M. Biegstraaten, C.E. Hollak, Characterization of Classical and Nonclassical Fabry Disease: A Multicenter Study J Am Soc Nephrol (2016) 10.1681/ASN.2016090964.

[7] R. Schiffmann, D.G. Warnock, M. Banikazemi, J. Bultas, G.E. Linthorst, S. Packman, S.A. Sorensen, W.R. Wilcox, R.J. Desnick, Fabry disease: progression of nephropathy, and prevalence of cardiac and cerebrovascular events before enzyme replacement therapy Nephrol Dial Transplant 24 (2009) 2102-211110.1093/ndt/gfp031.

[8] K.D. MacDermot, A. Holmes, A.H. Miners, Anderson-Fabry disease: clinical manifestations and impact of disease in a cohort of 60 obligate carrier females J Med Genet 38 (2001) 769-775

[9] S. Nakao, T. Takenaka, M. Maeda, C. Kodama, A. Tanaka, M. Tahara, A. Yoshida, M. Kuriyama, H. Hayashibe, H. Sakuraba, et al., An atypical variant of Fabry's disease in men with left ventricular hypertrophy N Engl J Med 333 (1995) 288-29310.1056/NEJM199508033330504.

[10] S. Nakao, C. Kodama, T. Takenaka, A. Tanaka, Y. Yasumoto, A. Yoshida, T. Kanzaki, A.L. Enriquez, C.M. Eng, H. Tanaka, C. Tei, R.J. Desnick, Fabry disease: detection of undiagnosed hemodialysis patients and identification of a "renal variant" phenotype Kidney Int 64 (2003) 801-80710.1046/j.1523-1755.2003.00160.x.

[11] J. Shabbeer, M. Yasuda, S.D. Benson, R.J. Desnick, Fabry disease: identification of 50 novel alpha-galactosidase A mutations causing the classic phenotype and three-dimensional structural analysis of 29 missense mutations Hum Genomics 2 (2006) 297-309

[12] L. Echevarria, K. Benistan, A. Toussaint, O. Dubourg, A.A. Hagege, D. Eladari, F. Jabbour, C. Beldjord, P. De Mazancourt, D.P. Germain, X-chromosome inactivation in female patients with Fabry disease Clin Genet 89 (2016) 44-5410.1111/cge.12613.

[13] J. Lukas, S. Scalia, S. Eichler, A.M. Pockrandt, N. Dehn, C. Cozma, A.K. Giese, A. Rolfs, Functional and Clinical Consequences of Novel alpha-Galactosidase A Mutations in Fabry Disease Hum Mutat 37 (2016) 43-5110.1002/humu.22910.

[14] J.M. Aerts, J.E. Groener, S. Kuiper, W.E. Donker-Koopman, A. Strijland, R. Ottenhoff, C. van Roomen, M. Mirzaian, F.A. Wijburg, G.E. Linthorst, A.C. Vedder, S.M. Rombach, J. Cox-Brinkman, P. Somerharju, R.G. Boot, C.E. Hollak, R.O. Brady, B.J. Poorthuis, Elevated globotriaosylsphingosine is a hallmark of Fabry disease Proc Natl Acad Sci U S A 105 (2008) 2812-281710.1073/pnas.0712309105.

[15] M. Niemann, A. Rolfs, S. Stork, B. Bijnens, F. Breunig, M. Beer, G. Ertl, C. Wanner, F. Weidemann, Gene mutations versus clinically relevant phenotypes: lyso-Gb3 defines Fabry disease Circ Cardiovasc Genet 7 (2014) 8-1610.1161/CIRCGENETICS.113.000249.

[16] B.E. Smid, L. van der Tol, M. Biegstraaten, G.E. Linthorst, C.E. Hollak, B.J. Poorthuis, Plasma globotriaosylsphingosine in relation to phenotypes of Fabry disease J Med Genet 52 (2015) 262-26810.1136/jmedgenet-2014-102872.

[17] J. Lukas, A.K. Giese, A. Markoff, U. Grittner, E. Kolodny, H. Mascher, K.J. Lackner, W. Meyer, P. Wree, V. Saviouk, A. Rolfs, Functional characterisation of alpha-galactosidase a mutations as a basis for a new classification system in fabry disease PLoS Genet 9 (2013) e100363210.1371/journal.pgen.1003632.

[18] R.B. Devereux, N. Reichek, Echocardiographic determination of left ventricular mass in man. Anatomic validation of the method Circulation 55 (1977) 613-618

[19] A. Nowak, G. Koch, U. Huynh-Do, M. Siegenthaler, H.P. Marti, M. Pfister, Disease Progression Modeling to Evaluate the Effects of Enzyme Replacement Therapy on Kidney Function in Adult Patients with the Classic Phenotype of Fabry Disease Kidney Blood Press Res 42 (2017) 1-1510.1159/000464312.

[20] M. Yasuda, J. Shabbeer, S.D. Benson, I. Maire, R.M. Burnett, R.J. Desnick, Fabry disease: characterization of alpha-galactosidase A double mutations and the D313Y plasma enzyme pseudodeficiency allele Hum Mutat 22 (2003) 486-49210.1002/humu.10275.

[21] H. Gold, M. Mirzaian, N. Dekker, M. Joao Ferraz, J. Lugtenburg, J.D. Codee, G.A. van der Marel, H.S. Overkleeft, G.E. Linthorst, J.E. Groener, J.M. Aerts, B.J. Poorthuis, Quantification of globotriaosylsphingosine in plasma and urine of fabry patients by stable isotope ultraperformance liquid chromatography-tandem mass spectrometry Clin Chem 59 (2013) 547-55610.1373/clinchem.2012.192138.

[22] F.A. Wijburg, B. Benichou, D.G. Bichet, L.A. Clarke, G. Dostalova, A. Fainboim, A. Fellgiebel, C. Forcelini, K. An Haack, R.J. Hopkin, M. Mauer, B. Najafian, C.R. Scott, S.P. Shankar, B.L. Thurberg, C. Tondel, A. Tylki-Szymanska, U. Ramaswami, Characterization of early disease status in treatment-naive male paediatric patients with Fabry disease enrolled in a randomized clinical trial PLoS One 10 (2015) e012498710.1371/journal.pone.0124987.

[23] A.C. Vedder, A. Strijland, M.A. vd Bergh Weerman, S. Florquin, J.M. Aerts, C.E. Hollak, Manifestations of Fabry disease in placental tissue Journal of inherited metabolic disease 29 (2006) 106-11110.1007/s10545-006-0196-0.

[24] M.J. van Breemen, S.M. Rombach, N. Dekker, B.J. Poorthuis, G.E. Linthorst, A.H. Zwinderman, F. Breunig, C. Wanner, J.M. Aerts, C.E. Hollak, Reduction of elevated plasma globotriaosylsphingosine in patients with classic Fabry disease following enzyme replacement therapy Biochim Biophys Acta 1812 (2011) 70-7610.1016/j.bbadis.2010.09.007.

[25] T. Togawa, T. Kodama, T. Suzuki, K. Sugawara, T. Tsukimura, T. Ohashi, N. Ishige, K. Suzuki, T. Kitagawa, H. Sakuraba, Plasma globotriaosylsphingosine as a biomarker of Fabry disease Molecular genetics and metabolism 100 (2010) 257-26110.1016/j.ymgme.2010.03.020.

[26] R. Schiffmann, J.B. Kopp, H.A. Austin, 3rd, 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

[27] D.G. Warnock, D.G. Bichet, M. Holida, O. Goker-Alpan, K. Nicholls, M. Thomas, F. Eyskens, S. Shankar, M. Adera, S. Sitaraman, R. Khanna, J.J. Flanagan, B.A. Wustman, J. Barth, C. Barlow, K.J. Valenzano, D.J. Lockhart, P. Boudes, F.K. Johnson, Oral Migalastat HCl Leads to Greater Systemic Exposure and Tissue Levels of Active alpha-Galactosidase A in Fabry Patients when Co-Administered with Infused Agalsidase PLoS One 10 (2015) e013434110.1371/journal.pone.0134341.

[28] K.M. Ashe, E. Budman, D.S. Bangari, C.S. Siegel, J.B. Nietupski, B. Wang, R.J. Desnick, R.K. Scheule, J.P. Leonard, S.H. Cheng, J. Marshall, Efficacy of Enzyme and Substrate Reduction Therapy with a Novel Antagonist of Glucosylceramide Synthase for Fabry Disease Mol Med 21 (2015) 389-39910.2119/molmed.2015.00088.

[29] D.P. Germain, R. Giugliani, D.A. Hughes, A. Mehta, K. Nicholls, L. Barisoni, C.J. Jennette, A. Bragat, J. Castelli, S. Sitaraman, D.J. Lockhart, P.F. Boudes, Safety and pharmacodynamic effects of a pharmacological chaperone on alpha-galactosidase A activity and globotriaosylceramide clearance in Fabry disease: report from two phase 2 clinical studies Orphanet journal of rare diseases 7 (2012) 9110.1186/1750-1172-7-91.

[30] D.P. Germain, D.A. Hughes, K. Nicholls, D.G. Bichet, R. Giugliani, W.R. Wilcox, C. Feliciani, S.P. Shankar, F. Ezgu, H. Amartino, D. Bratkovic, U. Feldt-Rasmussen, K. Nedd, U. Sharaf El Din, C.M. Lourenco, M. Banikazemi, J. Charrow, M. Dasouki, D. Finegold, P. Giraldo, O. Goker-Alpan, N. Longo, C.R. Scott, R. Torra, A. Tuffaha, A. Jovanovic, S. Waldek, S. Packman, E. Ludington, C. Viereck, J. Kirk, J. Yu, E.R. Benjamin, F. Johnson, D.J. Lockhart, N. Skuban, J. Castelli, J. Barth, C. Barlow, R. Schiffmann, Treatment of Fabry's Disease with the Pharmacologic Chaperone Migalastat N Engl J Med 375 (2016) 545-55510.1056/NEJMoa1510198.

[31] G. Serebrinsky, M. Calvo, S. Fernandez, S. Saito, K. Ohno, E. Wallace, D. Warnock, H. Sakuraba, J. Politei, Late onset variants in Fabry disease: Results in high risk population screenings in Argentina Mol Genet Metab Rep 4 (2015) 19-2410.1016/j.ymgmr.2015.05.006.

[32] O. Saito, E. Kusano, T. Akimoto, Y. Asano, T. Kitagawa, K. Suzuki, N. Ishige, T. Akiba, A. Saito, E. Ishimura, M. Hattori, A. Hishida, C. Guili, H. Maruyama, M. Kobayashi, T. Ohashi, I. Matsuda, Y. Eto, Prevalence of Fabry disease in dialysis patients: Japan Fabry disease screening study (J-FAST) Clin Exp Nephrol 20 (2016) 284-29310.1007/s10157-015-1146-7.

[33] B. Goeggel Simonetti, M.L. Mono, U. Huynh-Do, P. Michel, C. Odier, R. Sztajzel, P. Lyrer, S.T. Engelter, L. Bonati, H. Gensicke, C. Traenka, B. Tettenborn, B. Weder, U. Fischer, A. Galimanis, S. Jung, R. Luedi, G.M. De Marchis, A. Weck, C.W. Cereda, R. Baumgartner, C.L. Bassetti, H.P. Mattle,

K. Nedeltchev, M. Arnold, Risk factors, aetiology and outcome of ischaemic stroke in young adults: the Swiss Young Stroke Study (SYSS) J Neurol 262 (2015) 2025-203210.1007/s00415-015-7805-5.

[34] S. Elliott, N. Buroker, J.J. Cournoyer, A.M. Potier, J.D. Trometer, C. Elbin, M.J. Schermer, J. Kantola, A. Boyce, F. Turecek, M.H. Gelb, C.R. Scott, Pilot study of newborn screening for six lysosomal storage diseases using Tandem Mass Spectrometry Molecular genetics and metabolism (2016) 10.1016/j.ymgme.2016.05.015.

[35] B.E. Smid, C.E. Hollak, B.J. Poorthuis, M.A. van den Bergh Weerman, S. Florquin, W.E. Kok, R.H. Lekanne Deprez, J. Timmermans, G.E. Linthorst, Diagnostic dilemmas in Fabry disease: a case series study on GLA mutations of unknown clinical significance Clin Genet 88 (2015) 161-16610.1111/cge.12449.

[36] B.E. Smid, L. van der Tol, F. Cecchi, P.M. Elliott, D.A. Hughes, G.E. Linthorst, J. Timmermans, F. Weidemann, M.L. West, M. Biegstraaten, R.H. Lekanne Deprez, S. Florquin, P.G. Postema, B. Tomberli, A.C. van der Wal, M.A. van den Bergh Weerman, C.E. Hollak, Uncertain diagnosis of Fabry disease: consensus recommendation on diagnosis in adults with left ventricular hypertrophy and genetic variants of unknown significance Int J Cardiol 177 (2014) 400-40810.1016/j.ijcard.2014.09.001.

[37] G.E. Linthorst, B.J. Poorthuis, C.E. Hollak, Enzyme activity for determination of presence of Fabry disease in women results in 40% false-negative results J Am Coll Cardiol 51 (2008) 2082; author reply 2082-208310.1016/j.jacc.2008.02.050.

[38] L. van der Tol, E. Svarstad, A. Ortiz, C. Tondel, J.P. Oliveira, L. Vogt, S. Waldek, D.A. Hughes, R.H. Lachmann, W. Terryn, C.E. Hollak, S. Florquin, M.A. van den Bergh Weerman, C. Wanner, M.L. West, M. Biegstraaten, G.E. Linthorst, Chronic kidney disease and an uncertain diagnosis of Fabry disease: approach to a correct diagnosis Molecular genetics and metabolism 114 (2015) 242-24710.1016/j.ymgme.2014.08.007.

[39] S.M. Rombach, N. Dekker, M.G. Bouwman, G.E. Linthorst, A.H. Zwinderman, F.A. Wijburg, S. Kuiper, M.A. Vd Bergh Weerman, J.E. Groener, B.J. Poorthuis, C.E. Hollak, J.M. Aerts, Plasma globotriaosylsphingosine: diagnostic value and relation to clinical manifestations of Fabry disease Biochim Biophys Acta 1802 (2010) 741-74810.1016/j.bbadis.2010.05.003.

[40] M. Lenders, J. Stypmann, T. Duning, B. Schmitz, S.M. Brand, E. Brand, Serum-Mediated Inhibition of Enzyme Replacement Therapy in Fabry Disease J Am Soc Nephrol 27 (2016) 256-26410.1681/ASN.2014121226.

[41] O. Goker-Alpan, M.J. Gambello, G.H. Maegawa, K.J. Nedd, D.J. Gruskin, L. Blankstein, N.J. Weinreb, Reduction of Plasma Globotriaosylsphingosine Levels After Switching from Agalsidase Alfa to Agalsidase Beta as Enzyme Replacement Therapy for Fabry Disease JIMD reports 25 (2016) 95-10610.1007/8904_2015_483.

[42] M.D. Sanchez-Nino, D. Carpio, A.B. Sanz, M. Ruiz-Ortega, S. Mezzano, A. Ortiz, Lyso-Gb3 activates Notch1 in human podocytes Hum Mol Genet 24 (2015) 5720-573210.1093/hmg/ddv291.

[43] J.Y. Choi, M.Y. Shin, S.H. Suh, S. Park, Lyso-globotriaosylceramide downregulates KCa3.1 channel expression to inhibit collagen synthesis in fibroblasts Biochem Biophys Res Commun 468 (2015) 883-88810.1016/j.bbrc.2015.11.050.

[44] L. Choi, J. Vernon, O. Kopach, M.S. Minett, K. Mills, P.T. Clayton, T. Meert, J.N. Wood, The Fabry disease-associated lipid Lyso-Gb3 enhances voltage-gated calcium currents in sensory neurons and causes pain Neurosci Lett 594 (2015) 163-16810.1016/j.neulet.2015.01.084.