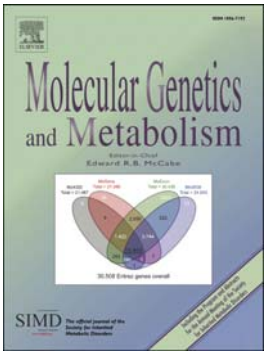

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Plasma LysoGb3: A Useful Biomarker for the Diagnosis and Treatment of Fabry Disease Heterozygotes

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Abstract

Background

Fabry disease (FD) is a rare X-linked lysosomal storage disorder due to mutations in the α-galactosidase A gene (*GLA*) that result in absent or markedly reduce α-galactosidase A (α-GalA) enzymatic activity. As a result, the major glycosphingolipid substrates, globotriaosylceramide (Gb3) and globotriaosylsphingosine (LysoGb3) accumulate in plasma, urine and tissue lysosomes. In females, the diagnosis can be complicated by the fact that 40-50% of *GLA*-mutation confirmed heterozygotes have normal or only slightly decreased leukocyte α-GalA activities. Recently, LysoGb3 has been appreciated as a novel FD biomarker, especially for therapeutic monitoring.

Methods

Among our *GLA*-mutation proven FD patients, we screened 18 heterozygotes whose leukocyte α-GalA activity was determined at initial diagnosis. For these females, we measured their serum LysoGb3 levels using highly-sensitive electrospray ionization liquid chromatography tandem mass spectrometry.

Results

We identified three unrelated females in whom the accumulating LysoGb3 was increased, whereas their leukocyte α-GalA activities were in the normal range.

Conclusion

LysoGb3 serves as an useful biomarker to improve the diagnosis of FD heterozygotes and for therapeutic evaluation **and monitoring**.

Abstract Word Count 171

Keywords: Fabry disease, heterozygotes, LysoGB3, biomarker, α-Galactosidase deficiency.

1. Introduction

Fabry disease (FD) is a rare X-linked lysosomal storage disorder caused by mutations in the α-galactosidase A (*GLA*) gene leading to markedly decreased or absent α-GalA enzyme activity [1, 2]. The enzymatic defect leads to glycosphingolipid accumulation in plasma, urine and tissue lysosomes. The resulting clinical manifestations include kidney and heart failure, cerebrovascular disease and premature demise. Males are more severely affected than female heterozygotes, whose manifestations can range from asymptomatic to rarely as severely affected as males [1, 2].

Currently, the diagnosis of affected males involves the demonstration of markedly decreased α-GalA activity in plasma, leucocytes or dried blood spots (DBS), which can be confirmed by *GLA* mutation analysis [1]. However, due to random X-chromosomal inactivation, at least 40% of *GLA*-mutation confirmed heterozygotes have normal or slightly decreased α-GalA activity requiring *GLA* sequencing to confirm heterozygosity [2-4] .

The phenotypic heterogeneity also complicates the clinical diagnosis of FD in females. There are two major subtypes, the Type 1 “Classic” and the Type 2 “Later-Onset” phenotypes [4]. Affected males with the Type 2 phenotype have residual α-GalA activity, later-onset cardiac and/or renal disease, and lack the major early-onset classical manifestations, including angiokeratoma, acroparesthesias, hypohidrosis, and the ocular abnormalities [5-7]. Even genetic testing of patients at-risk or suspicious for FD has been compromised by the recently recognized occurrence of various benign missense mutations [8-10].

Previously, increased plasma or urine levels of globotriaosylceramide (Gb3) were used to help identify heterozygosity, but its analysis is laborious and multi-procedural

[11]. More recently, the easier measurement of its deacylated form, globotriaosylsphingosine (LysoGb3) has become appreciated as a novel diagnostic tool that can classify Classic and Later-Onset males [12, 13].

Here, we report three unrelated heterozygotes among the FD patients treated or followed at the University Hospital Zurich with increased LysoGb3, whereas their leukocyte α-GalA activities were in the normal range. In these women, the serum LysoGb3 levels were useful diagnostic markers to identify heterozygotes who should be evaluated for treatment.

2. Methods

2.1. Patients and clinical work-up

This study was conducted in accordance with the principles of the Helsinki Declaration. All patients signed a written informed consent. All authors have read and approved the manuscript.

Between January 2014 and December 2016, blood for biobanking was drawn from 61 consecutive FD patients (36 females, 59%) at the University Hospital Zurich, Zurich, Switzerland, all with a *GLA*-mutation confirmed diagnosis, who presented for routine annual examinations at our FD center. In 11 of these patients (5 females), kidney biopsies had been performed, all of which had the typical FD pathology **including the prominent Gb3 accumulation in vascular endothelial, interstitial and mesangial cells, and in particular, in podocytes (Fig. 1) [14].** For serum LysoGb3 levels, blood samples were centrifuged and serum was immediately frozen at −80°C. All patients had a comprehensive work-up, including medical history, cardiac, renal, and neurological evaluations. In this retrospective cohort study, all clinical and routine laboratory results including α-GalA activities were obtained from the patients` medical records. α-GalA activities were originally determined at the time of diagnosis in the males and mutation analyses were performed in all females. For this study, we screened the 18 heterozygotes who previously had a documented α-GalA activity assay at the original diagnosis. For these heterozygotes, we determined the serum LysoGb3 levels.

2.2. Phenotyping

Phenotyping was based on *GLA* mutation type: frame-shift, nonsense, consensus splice-site mutations, large insertions and deletions, and some missense mutations,

which result in no or <2% of mean normal enzymatic activity were classified as having the Type 1 Classic phenotype. In contrast, some missense and some alternative splicing mutations result in enzymes with residual enzymatic activity and the Type 2 Later-Onset phenotype. To determine if the missense mutations caused the Classic or Later-Onset phenotype, clinical data, substrate levels, and *in vitro* expression assays were evaluated to determine the mutation's phenotype [7, 8].

2.3. LysoGb3 measurement

Serum LysoGb3 levels were measured by highly-sensitive electrospray ionization liquid chromatography tandem mass spectrometry (ESI LC-MS/MS) **on a Shimadzu 8050 class I medical device using an adapted method from Gold et al [15].** 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 calibrator levels (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 are available on request.**

The reference range was defined as the prediction interval that included 95% of values of a reference group: cut-off ≤1.1 ng/mL.

3. Results

3.1. Fabry disease diagnosis in three female patients with normal α-GalA activity

In our study cohort of 61 patients, there were 36 *GLA*-mutation confirmed heterozygotes. Of these, 18 previously had α-GalA activity determinations, and of these, three had normal α-GalA activities and increased serum LysoGb3 levels, as described in detail below:

Female 1 was 55 years old when she was admitted to the hospital with her third cryptogenic stroke, the first having occurred when she was 50 years old. At initial examination, the patient also described burning sensations in her hands and feet and an inability to sweat. Her son died at 28 years old from recurrent epileptic seizures. He also complained of burning paresthesias in his hands and feet. Her mother died at 78 years; her father died of a myocardial infarction at the age of 65 years.
Her clinical examination revealed a few angiokeratomas on her trunk, and a slit-lamp examination revealed the characteristic cornea verticillata. Laboratory studies demonstrated normal kidney function and microalbuminuria (urinary albumin/creatinine 4.2 mg/mmol). *GLA* mutation analysis identified *GLA* pathologic missense mutation c.796G>T (p.Asp266Tyr) which made her heterozygote diagnosis. **This previously reported mutation causes the Classic phenotype** [16].
Female 2 was diagnosed with FD at 39 years of age by family mutation screening. Her asymptomatic daughter was incidentally found to have proteinuria at 11 years. The daughter`s kidney biopsy revealed myelin-figures in podocyte lysosomes by electron microscopy. Female 2's father died of kidney failure at 43 years of age. Her paternal aunt had left ventricular hypertrophy and a pacemaker. She died at 67 years of kidney failure following a hip fracture operation. The clinical examination of Female

2 revealed no angiokeratoma, but the slit-lamp examination showed cornea verticillata. Laboratory studies demonstrated normal kidney function and microalbuminuria (urinary albumin/creatinine 3.6 mg/mmol). Genetic testing confirmed the pathogenic family missense mutation c.1033T>C (p.Ser345Pro). **This previously reported missense mutation causes the Classic phenotype** [16].

Female 3 suffered from chronic acroparesthesias and was diagnosed with FD at 12 years old by family screening. Her 41 year old maternal uncle had acroparesthesias, myalgias, anhidrosis, proteinuria and renal insufficiency, which led to a kidney biopsy. Electron microscopy revealed numerous lamellar inclusions in the lysosomes of podocytes, mesangial, vascular endothelial and tubular cells and genetic testing was undertaken. Another maternal uncle committed suicide at 28 years of age due to recurrent excruciating acroparesthesias shortly before the family was diagnosed as having FD. The 31 year old mother of Female 3, a 38 year old maternal uncle, and a 36 year old maternal aunt also had the family mutation. Female 3 did not have angiokeratomas, however, the slit-lamp examination showed cornea verticillata. Laboratory studies demonstrated normal kidney function and proteinuria of less than 0.5 gram per 24 hours. However, the electron microscopy of her kidney biopsy revealed numerous lamellar inclusions in podocyte lysosomes, typical of the Fabry nephropathy. Genetic testing confirmed the presence of the pathogenic family mutation c.744_745delTA (p.Phe248LeufsX7). **This previously reported frameshift mutation causes the Classic phenotype** [16].

All three heterozygotes **had Classic mutations** with normal leukocyte α-GalA activities and markedly elevated serum LysoGb3. Their clinical and biochemical

characteristics are summarized in the Table 1. **All symptoms described in the text and Tables were diagnosed prior to enzyme replacement therapy (ERT).**

Table 1. Current clinical and biochemical characteristics of the three females with the α-galactosidase A activity within the normal range at the time of diagnosis*.

	Female 1	Female 2	Female 3
α-gal A activity, % of lower range	108	288	120
LysoGb3, ng/mL; cut-off<1.1 ng/mL	8.6	7.8	7.9
Current age, years	57	42	20
Age at diagnosis, years	55	39	12
Diagnosed as	index patient	family screening	family screening
Mutation	c.796G>T	c.1033T>C	c.744_745delTA
Predicted amino acid change	p.Asp266Tyr	p.Ser345Pro	p.Phe248LeufsX7
Category	missense	missense	deletion
Phenotype	classic	classic	classic
Receiving ERT	+	+	+
Hypohidrosis	+	-	-
Acroparesthesia	+	-	+
Angiokeratoma	+	-	-
Cornea verticillata	+	+	+
Cardiomyopathy	-	-	-
eGFR**, ml/min/1.73m^2	94	86	128
Urine prot/creatinine, mg/mmol	normal	normal	900
Stroke history †	5	0	0

* Plus (+) or minus (-) indicates presence or absence of the clinical finding
** According to CKD-EPI equation
† Number of strokes

3.2. Clinical and biochemical parameters of two female patients with normal α-galactosidase activity and normal LysoGb3 levels.

Two of the 18 females who had α-GalA activity determinations, had normal α-GalA activities and normal serum LysoGb3 levels. Their clinical and biochemical findings are summarized in the Supplementary Table A1 (Females **4** and **5**). These females were diagnosed by family screening.

3.3. Clinical and biochemical findings of 13 female patients with reduced α-GalA activities and increased LysoGb3 levels.

Thirteen of the 18 females, who had α-GalA activity determinations, had reduced α-GalA activities and increased serum LysoGb3 levels. Their clinical and biochemical findings are summarized in Table A1 (Females **6-18**). Two of these females were diagnosed with FD as index patients (Females **6** and **12**), the other 11 by family screening. The LysoGb3 levels were increased in all these patients with reduced α-GalA activities.

4. Discussion

We identified three of 18 females with *GLA*-mutation confirmed FD in whom their α-GalA activities were normal, however, their serum LysoGb3 levels were markedly increased (Table 1). In FD heterozygotes, the α-GalA activities are often borderline normal or normal due to random X-chromosomal inactivation [3]. Our findings suggest that this biomarker may improve initial diagnose of clinically relevant FD, particularly in female patients. These findings relate to which females should be evaluated for treatment.

Importantly, the initiation of treatment is based on a confirmed pathogenic mutation, low levels of enzymatic activity and evidence of early clinical manifestations. The LysoGb3 provides another indicator of disease activity as it may reflect the overall total body substrate accumulation. In heterozygotes with normal levels of α-GalA activity that are asymptomatic, tissue biopsies have been used to determine which women had marked glycosphingolipid accumulation. However, invasive biopsies may not be feasible or acceptable in many heterozygotes [17]. **In heterozygotes from classic families,** the serum LysoGb3 may serve as a marker of tissue involvement to evaluate the need of treatment. **Although, normal LysoGb3 does not necessarily mean that there is no need for treatment, as the clinical manifestations indicate therapeutic intervention.**

Different methods have been used to measure LysoGb3 including LC-MS/MS. Quality control materials and inter-laboratory testing are required to standardize LysoGb3 measurements among laboratories. This will permit composition of results among laboratories in order to further evaluate the potential use of this marker particularly in patients with the Later-Onset phenotype to improve its diagnostic utility, need for treatment, and therapeutic monitoring.

Certainly, **therapeutic decisions are not made by biomarker levels only** and extensive clinical characterization using a multidisciplinary approach, a detailed family history and knowledge of previously reported phenotypic descriptions of the specific *GLA* mutation are of utmost importance to identify the phenotype and disease severity. Among our females, two had both normal α-GalA activities and normal LysoGb3 levels, which emphasizes the need for further evaluation beyond the use of biomarkers. As previously reported, many heterozygotes from families with the Later-Onset phenotype will have no FD manifestations and no serum LysoGb3 accumulation [12]. **For example, the female, who had a mutation resulting in the Later-Onset phenotype, had normal LysoGb3 and would have been misdiagnosed by the biomarker (Supplemental Table, female 5). It should be noted that the severity of both phenotypes can vary among affected males. Especially "Type 2" mutations, where there is more variability in the onset and severity of the cardiac and/or renal manifestations [18, 19]. These are based on the amount of residual α-GalA activity expressed by the given Later-Onset mutation. In the Later-Onset heterozygotes, the LysoGb3 levels may be very low or normal due to random X-chromosomal inactivation. Thus, the LysoGb3 levels may not diagnose the mildly-affected or asymptomatic Later-Onset heterozygotes. Clearly, *GLA* mutation analysis remains the diagnostic method of choice for Fabry heterozygotes, and the LysoGb3 levels serve as biomarkers to evaluate the disease activity and are helpful to determine when to intervene therapeutically.**

Three recent reports have determined the plasma LysoGb3 levels in males and females with the Classic, Later-Onset and benign *GLA* mutations: Lukas et al [20], Niemann et al [13] and Smid et al [12]. Lukas et al from the Centogene laboratory, reported a variety of mutations and their LysoGb3 levels (typically 1 or 2 females). Niemann et al classified patients clinically by

new criteria and found that LysoGb3 levels varied in 11 women with pathogenic mutations. Smid et al determined the LysoGb3 levels in 14 non-classical patients and found an overlap with normal levels regardless of α-GalA activities. Importantly, Smid et al recognized the need to further expand experience with LysoGb3 particularly in Later-onset (or so called Non-classical) heterozygotes. Therefore, we present our experience with 18 heterozygotes, with Classic, Later-Onset, and a benign mutation, in whom the enzymatic activities and LysoGb3 levels were determined. These studies further expand experience with LysoGb3 and demonstrate that even classic female patients with normal enzyme levels have elevated LysoGb3 and further confirm that heterozygotes with Later-Onset mutations can have elevated or normal LysoGb3 levels.

Higher LysoGB3 serum levels and lower leukocyte α-GalA activities are not directly linked in heterozygotes presumably due to random X-chromosomal inactivation. The leukocyte α-GalA activities reflect "Lyonization" in bone marrow myeloid cells. Whereas the serum LysoGb3 levels likely represent the level of Gb3 and LysoGb3 in multiple cell types in heterozygotes, the leukocyte α-GalA activity can be in the normal range but reduced in other cell types such as myocytes and podocytes so that the accumulation of Gb3 will be leaked into the plasma and urine. Thus, the LysoGb3 levels can be a better indicator of overall disease severity.

These studies are limited since the sample size is relatively small and larger studies are needed to confirm our findings. In addition, *the degree of random X-chromosomal inactivation is the most prominent epigenetic factor that determines if a Type 1 Classic or Type 2 Later-Onset heterozygote will be symptomatic* [3]. *It would be useful to correlate the degree of "Lyonization" with the LysoGb3 levels. Such studies should be undertaken in the future.*

Conclusion

LysoGb3 appears a useful biomarker in addition to the leukocyte enzyme activity in females. The measurement of LysoGb3 can improve the initial diagnosis of FD, particularly in females with normal and/or borderline α-GalA activities, and the presentation of non-specific symptoms such as heat intolerance, pain, and gastrointestinal symptoms. **As illustrated by our study, LysoGb3 was a useful marker along with the leukocyte α-GalA activity for the diagnosis of heterozygotes with FD,** and may be useful to assess which heterozygotes should be considered for treatment, even though they have normal α-GalA activities.

Role of the funding source

The LysoGb3 measurements were provided by *ARCHIMED Life Science, Vienna, Austria.* 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. The laboratory members (TPM and DCK) participated in writing and approving the manuscript.

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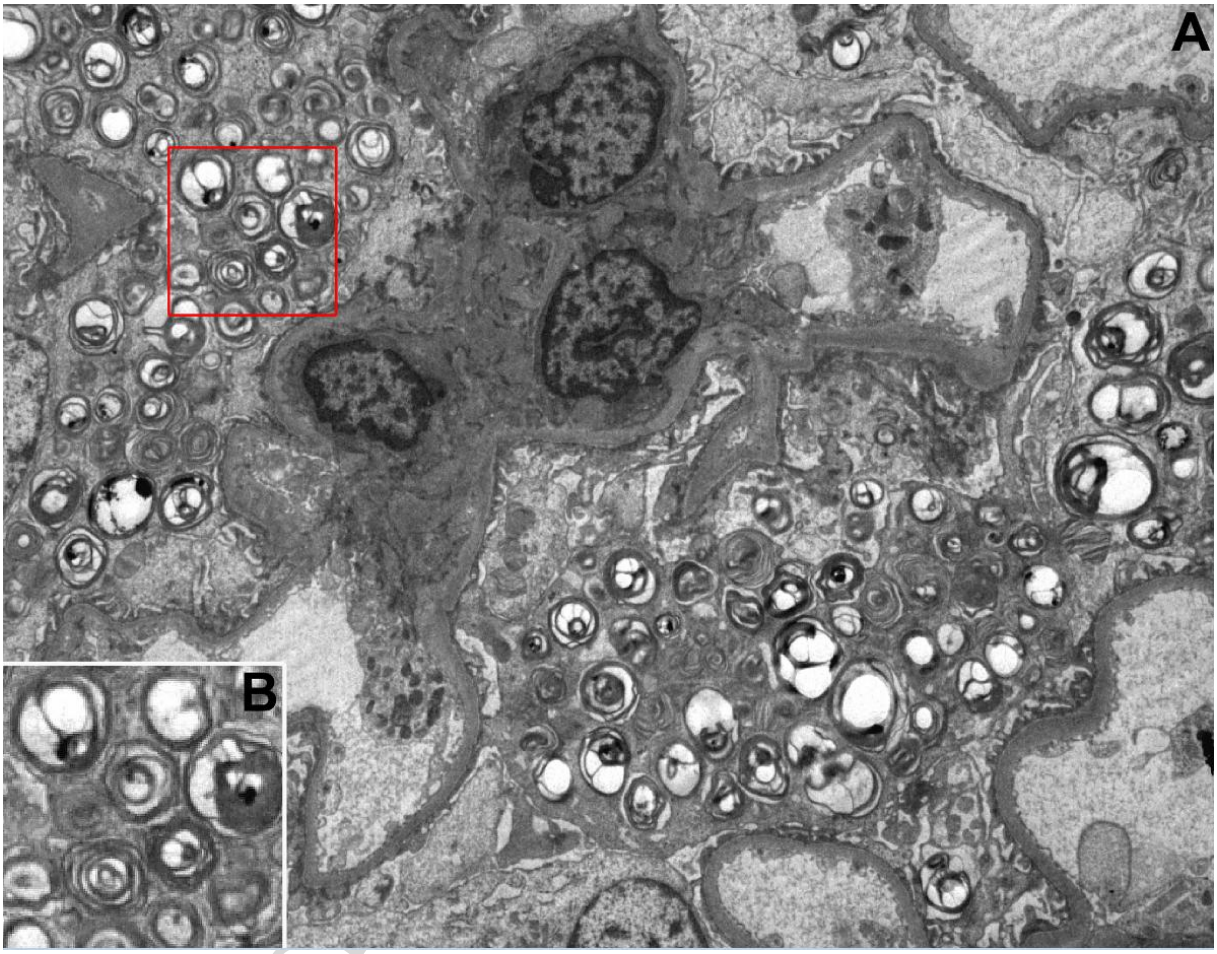
Figure 1. Histological findings of Fabry Disease in a kidney biopsy. A: Lysosomal inclusions with lamellated structures – myeloid bodies in podocytes (TEM, ×2800). B: Higher magnification of the podocyte shows lysosomes packed with storage material.



Courtesy Dr. Ariana Gaspert, Pathology Department, University Hospital Zurich, Switzerland