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Plasma globotriaosylsphingosine: Diagnostic value and relation to clinical manifestations of Fabry disease

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ABSTRACT

Fabry disease is an X-linked lysosomal storage disorder due to deficiency of alpha-Galactosidase A, causing accumulation of globotriaosylceramide and elevated plasma globotriaosylsphingosine (lysoGb3). The diagnostic value and clinical relevance of plasma lysoGb3 concentration was investigated. All male and adult female patients with classical Fabry disease could be discerned by an elevated plasma lysoGb3. In young pre-symptomatic Fabry heterozygotes, lysoGb3 levels can be normal. Individuals carrying the R112H and P60L mutations, without classical Fabry symptoms, showed no elevated plasma lysoGb3. Multiple regression analysis showed that there is no correlation of plasma lysoGb3 concentration with total disease severity score in Fabry males. However, plasma lysoGb3 concentration did correlate with white matter lesions (odds ratio: 6.1 per 100 nM lysoGb3 increase (95% CI: 1.4–25.9, $p=0.015$). In females, plasma lysoGb3 concentration correlated with overall disease severity. Furthermore, plasma lysoGb3 level was related to left ventricular mass (19.5 ± 5.5 g increase per 10 nM lysoGb3 increase; $p=0.001$). In addition, it was assessed whether lifetime exposure to lysoGb3 correlates with disease manifestations. Male Fabry patients with a high lysoGb3 exposure (>10,000 U), were moderately or severely affected, only one mildly. Female patients with a low exposure (<1000 U) were asymptomatic or mildly affected. A large proportion of the females with an exposure >1000 U showed disease complications. Plasma lysoGb3 is useful for the diagnosis of Fabry disease. LysoGb3 is an independent risk factor for development of cerebrovascular white matter lesions in male patients and left ventricular hypertrophy in females. Disease severity correlates with exposure to plasma lysoGb3.

1. Introduction

Fabry disease (OMIM 301500) is caused by deficient activity of the lysosomal enzyme α-Galactosidase A (E.C. 3.2.1.22) [1]. The deficiency leads to lysosomal accumulation of glycosphingolipids with a terminal α-galactosyl moiety, in particular globotriaosylceramide (Gb3), also known as ceramide trihexoside (CTH) [2]. Primarily affected cells are smooth muscle and endothelial cells, contributing to the predominance of vascular complications in Fabry disease [3,4]. Although its inheritance is X-linked, both males and females can be affected by Fabry disease [5–10]. Male patients typically present with acroparesthesias as well as angiokeratoma and hypo- or anhydrosis already at a young age. In the third and fourth decade of life, renal, cardiac, cerebral complications, and hearing loss may develop [4,5]. Atypical manifestations have been described for male patients with high residual enzyme activity. In such individuals the disease may be limited to one organ only, such as the heart, or may show a milder course [4–7]. Intriguingly, despite significant amounts of residual α-Galactosidase A activity, female carriers display a vast spectrum of disease severity, ranging from total absence of clinical disease to severe organ damage [8–12]. The observed residual enzyme activity in plasma or blood cells from female carriers varies considerably due to random X-inactivation, ranging from normal to nearly completely absent, and is a poor predictor of the clinical course [13]. Also in other aspects, the relationships between residual enzyme activity, Gb3 accumulation and disease manifestations in Fabry patients are still puzzling. For example, it has been documented that in male hemizygotes prominent storage of Gb3 already occurs *in utero* whilst clear clinical symptoms develop much later in life [14,15]. Furthermore, it has become apparent that no strict correlation exists between clinical manifestation of Fabry disease and plasma Gb3 levels [12,15,17]. In hemizygotes with classical manifestations of Fabry

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disease, the plasma Gb3 level is already abnormally high at very young age prior to prominent clinical symptoms, whereas in heterozygotes, even symptomatic females, Gb3 levels are generally within the normal range. Based on these findings, monitoring of the clinical course in Fabry patients by analysis of their plasma Gb3 is presently considered to be of little value [12,16,17]. In urine of symptomatic Fabry patients, both heterozygotes and hemizygotes, Gb3 levels are generally abnormally high [18,19]. However, again no correlation of this parameter with disease manifestation has been observed [12,16,17].

The enigmatic relationships between residual α-Galactosidase A capacity, Gb3 levels and disease manifestations has prompted us to consider an additional factor in the pathogenesis of Fabry disease. In analogy to Krabbe disease, the occurrence of a deacylated storage lipid was hypothesized. Indeed, globotriaosylsphingosine (lysoGb3) was found to be dramatically elevated in plasma of all male patients with classical Fabry disease [20]. Plasma lysoGb3 was also increased in plasma of symptomatic female patients, although to a lesser extent in male patients. Comparable findings were made in Fabry mice lacking α-Galactosidase A activity [20].

In our initial investigation, a relatively small number of Fabry patients was studied, in particular few females [20]. In the follow-up investigation presented here, the entire cohort of Dutch patients with classic manifestation of Fabry disease was analyzed with respect to plasma lysoGb3 content. The aim of this study was two-fold. Firstly, the value of plasma lysoGb3 in confirming the diagnosis of Fabry disease was determined. This is relevant for individuals carrying an abnormality in the α-Galactosidase A gene with unknown consequence, or pre-symptomatic female carriers for which demonstration of true Fabry disease is problematic with present laboratory methods (e.g. those with normal plasma or urinary Gb3). The second aim of the investigation was to determine the relationship between plasma lysoGb3 and clinical manifestations of Fabry disease. Our earlier observation that lysoGb3 is capable of promoting proliferation of smooth muscle cells *in vitro* [20] suggested that lysoGb3 could contribute to the noted intima media thickening and vascular pathology, and thus may constitute a risk for disease manifestations [5,21–23]. The results of our investigation are reported here.

2. Methods

2.1. Patients

All 92 Fabry patients, including 69 adult and 23 paediatric patients from 28 families, with classical Fabry disease seen at the outpatient clinic of the Academic Medical Center from 1999 to 2008 were included in the study. Classical Fabry disease was defined as a mutation in the α-Galactosidase A gene and the occurrence of typical manifestations of the disease, such as acroparesthesias, angiokeratoma, hypo- or anhydrosis in at least one male family member. Patients with the R112H and P60L mutation did not meet the criteria of classical Fabry disease and were analyzed separately. Healthy controls, comparable for age were recruited for analysis of plasma lysoGb3. None of the patients received enzyme therapy at the time of plasma lipid analysis. The diagnosis was confirmed by demonstration of deficient leukocyte α-Galactosidase A activity and genotyping [24]. All patients gave informed consent. For all patients, a full medical history was obtained as well as a routine physical examination, including height, weight and blood pressure measurements. Body mass index was determined and hypertension was diagnosed in case of a systolic BP≥140 mm Hg and/ or a diastolic BP≥90 mm Hg in three consecutive measurements performed on the same arm. Dyslipidemia was diagnosed according to the Dutch guidelines for dyslipidemia and cardiovascular risk management. Smoking status was scored as current, smoking in the past, or never smoking. Renal function (eGFR) was estimated using the abbreviated MDRD equation in adults and the Schwartz formula in children [25,26]. Glomerular filtration rate (GFR) measured by simultaneous infusion of iothalamate and hippuran (mGFR) was performed in adults and in most children ($n=50$) and Cr^{51} EDTA in 2 adult patients. Renal failure was defined as an mGFR<60 ml/min. Microalbuminuria was defined as albumin exceeding 30 mg in a 24-h urine portion in two consecutive portions taken a least 1 week apart. Proteinuria was diagnosed when total protein count was more than 300 mg in these samples. A standard brain magnetic resonance imaging (MRI) evaluation for presence of white matter lesions and (lacunar) infarctions was available from 83 patients. An electrocardiogram (ECG) and cardiac ultrasound investigation was available from 87 and 76 patients, respectively. Left ventricular hypertrophy (LVH) was defined as a left ventricular mass (LVmass) of >259 g in males and >166 g in females [27]. Hearing tests were performed by using conventional audiometry and were available from 79 patients. Hearing loss was defined as a PTA (the average hearing loss in dB at 0.5, 1.0 and 2.0 kHz)>25 dB of the most affected ear [28]. The presence of acroparesthesia was obtained by medical history of pain in hands and feet. The presence of angiokeratoma was scored when this was confirmed by physical examination of the skin by a medical professional specialized in Fabry disease. Overall severity of disease was assessed by the MSSI scoring system, a composite score of clinical symptoms [29].

2.2. Plasma Gb3 and lysoGb3 measurements

Quantitation of Gb3 in plasma samples was performed as described previously [30]. Quantitative measurements of plasma lysoGb3 were performed as described with minor modifications [20]. In brief, 100 µl of plasma was extracted with 900 µl of chloroform/ methanol 1/2 (vol/vol). The extract was centrifuged for 10 min. at 14,000*g* and the pellet was discarded. To the supernatant, 300 µl chloroform and 450 µl MQ-H_2O was added, mixed, and centrifuged for 2 min at 14,000*g* to separate the phases. The upper phase was collected, and the lower chloroform phase was re-extracted with 1.2 ml of methanol/ MQ-H_2O 1/1 (vol/vol) to quantitatively extract lysoGb3. The combined upper phases were dried under nitrogen flow, taken up in 1 ml MQ-H_2O, and extracted twice with 1 ml of water-saturated 1-butanol. LysoGb3 was recovered from the butanol phase with an overall recovery of >90%. The butanol phase was dried, dissolved in 120 µl of freshly prepared 0.1 M NaOH in methanol and incubated at 37 °C for 1 h. Of this solution, 50 µl was derivatized with 25 µl of o-phtaldialdehyde (OPA) reagent (5 mg of OPA, 0.1 ml of ethanol, 5 µl of 2-mercaptoethanol, and 10 ml of 3% boric acid, pH 9.0). The OPA-derivatized lysoGb3 was separated by HPLC and identified by fluorescence detection as described previously [30]. All plasma samples were extracted in duplicate. Quantification was performed by addition of lysoGb3 (Sigma-Aldrich) to normal plasma at concentrations ranging from 0 to 1 mM. The limit of detection of plasma lysoGb3 was 3 nM. Quantification of lysoGb3 was based on a commercially available standard (Sigma-Aldrich) as described earlier [20]. We plan to synthesize a large batch of lysoGb3 ourselves and use this standard in the future, given the observation that commercial lysoGb3 batches show a contamination with N-acetylated lysoGb3.

2.3. Electronmicroscopy

After fixation, the material was post fixed in 1% osmiumtetroxide, block-staining with 1% uranyl acetate, one-step dehydration in dimethoxypropane and embedding in epoxyresin LX-112. LM sections were stained with toluidine blue. EM sections were stained with tannic acid, uranyl acetate and lead citrate and examined in an Philips CM10 (FEI).

Photographs were taken with a Morada digital camera, (S.I.S.).

2.4. Statistical analysis

Statistical analyses were performed using SPSS 17.0 (IBM, Chicago). Results are expressed as median (range). Differences between groups were assessed by the Mann–Whitney *U*-test. Correlations between variables were described by Spearman's rank correlation coefficients. Multiple regression logistic and linear regression was used to determine the association between lysoGb3 and disease parameters adjusted for age as a continuous variable, gender, smoking status, body-mass index and the presence or absence of a history of hypertension, and the presence of other cardiovascular disease including diabetes, hemochromatosis and dyslipidemia [31].

Linear regression was applied to quantify the association between continuous outcome variables and lysoGb3, while logistic regression was used for binary outcome variables. In the former case, B values represent the slope of the linear regression line ($y = a + B\,x$) while keeping all other variables constant and reflects the change of the outcome variable when lysoGb3 changes 1 unit (i.e. 10 nM or 100 nM). With logistic regression the odds ratio was determined which reflects the change of the odds of the binary outcome when lysoGb3 changes 1 unit while keeping all other variables constant.

3. Results

3.1. Diagnostic value of plasma lysoGb3

The average age of the male ($n = 37$) and female Fabry patients ($n = 55$) did not differ significantly, the median being 33 and 35 years, respectively. As has been previously described, lysoGb3 values were around 15 times higher in males as compared to females [20], ranging from 51 to 489 (median 286) in males to 0–99 (median 18) in females (Fig. 1). Also, the disease severity was significantly higher in the male Fabry patients than in female patients, reflected in an average higher MSSI score of 21 and 7, respectively (Table 1).

To determine the usefulness of plasma lysoGb3 measurement for confirmation of the diagnosis of Fabry disease, the concentrations of Gb3 and lysoGb3 were determined in plasma specimens of the 92 subjects as well as healthy controls (Fig. 1). In male Fabry patients, a striking increase in plasma Gb3 concentration was observed. Almost all of the male Fabry patients could also be recognized by their abnormally high plasma Gb3 concentration, although in some cases the Gb3 levels were close to the upper normal range (Fig. 1A). In the case of female Fabry patients, plasma Gb3 concentration did not allow a clear discrimination from normal subjects: only few individuals showed values slightly above the upper normal range (Fig. 1A). In sharp contrast to the findings with plasma Gb3, almost all female Fabry patients showed plasma lysoGb3 concentrations clearly exceeding normal levels. Only in two heterozygotes aged 5 years and 7 years respectively, lysoGb3 was within the normal range. The latter individual showed a plasma lysoGb3 of 5.3 nM at the age of 11 years, slightly above the upper normal range. These findings indicate that demonstration of high plasma lysoGb3, (> 3 nM), may be used for confirming the diagnosis of Fabry disease. A (near) normal level in very young female individuals however, does however not exclude the status of a carrier of Fabry disease.

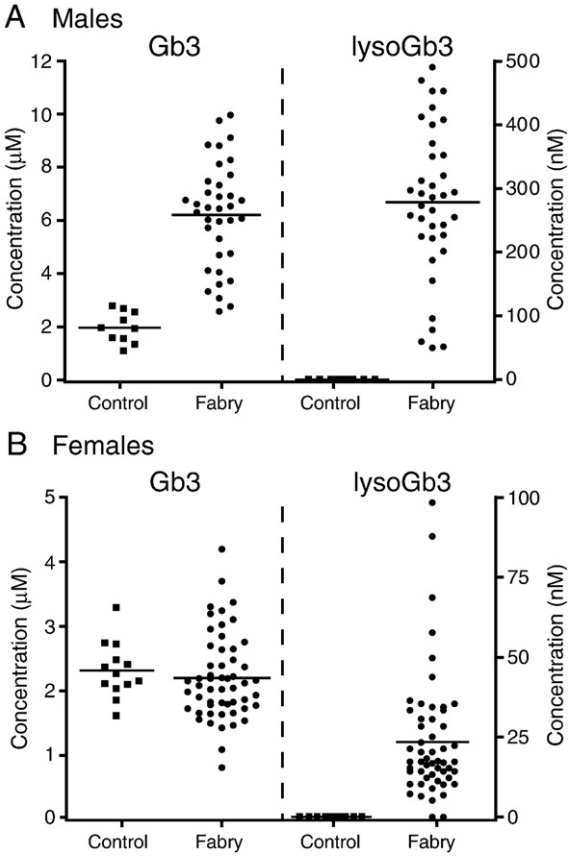

Fig. 1. Diagnostic value of lysoGb3 in Fabry disease. Plasma Gb3 and lysoGb3 concentrations in males (A) and females (B). Males: Gb3: controls $n = 10$, patients $n = 36$; lysoGb3: controls $n = 9$, patients $n = 37$). Females: Gb3: controls $n = 13$, patients $n = 54$; lysoGb3: controls $n = 9$, patients $n = 55$).

3.2. Age, overall disease severity and plasma lysoGb3 in male and female Fabry patients

The effect of age on lysoGb3 levels is depicted in Fig. 2. In case of male Fabry patients, no significant correlation was found between lysoGb3 levels and the age of the investigated individuals. This is consistent with a previous observation of a high lysoGb3 level in cord blood of an affected male [20]. In female Fabry patients, lysoGb3 levels showed a trend to be higher in older individuals ($\rho = 0.25$; $p = 0.06$). Similar findings were made when plasma lysoGb3 levels were related to MSSI scores of Fabry patients reflecting overall disease severity (Fig. 3). Male Fabry patients did not show a correlation between lysoGb3 level and MSSI score ($\rho = 0.08$; $p = 0.63$). In contrast, in female Fabry patients there was a significant, albeit not strict, correlation between plasma lysoGb3 and MSSI score ($\rho = 0.53$; $p < 0.0001$).

3.3. Plasma lysoGb3 and clinical manifestations

Given the impact of age and gender on disease severity in Fabry patients, multiple regression analysis was performed to assess whether plasma lysoGb3 is an independent contributor to disease manifestations. For this purpose, male and female patients were analyzed separately. Multiple regression logistic and linear regression was used to determine the association between lysoGb3 and disease parameters adjusted for age as a continuous variable, and general

Table 1
Baseline characteristics: lysoGb3 and MSSI score.

	Males	Females	Males vs. females (*p*-value)
Number of patients, *N*	37	55	
Age in years Median (range)	33 (3.6–64)	35 (2.0–71)	0.9
MSSI Median (range)	21 (5–59)	7 (0–32)	<0.0001
LysoGb3 (nM) Median (range)	286 (51–489)	18 (0–99)	<0.0001

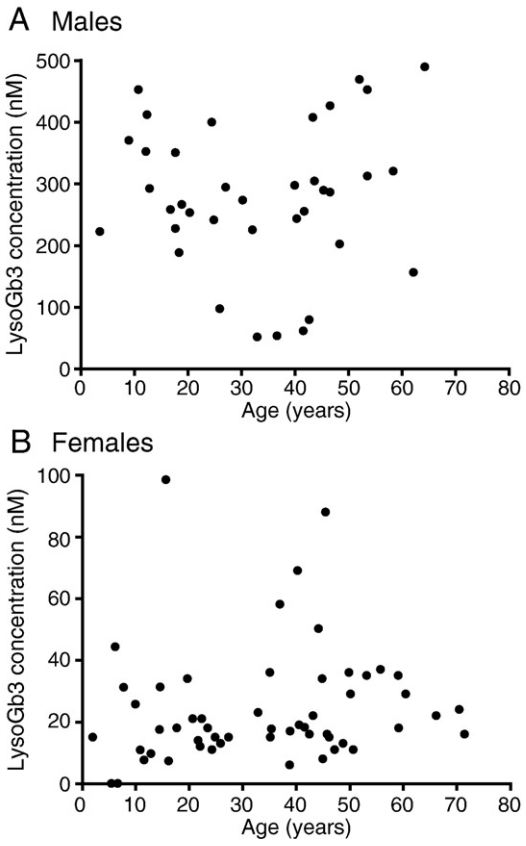

Fig. 2. Relation of plasma lysoGb3 levels and age. Plasma lysoGb3 levels related to age for 37 male (A) and 55 female patients (B) with Fabry disease.

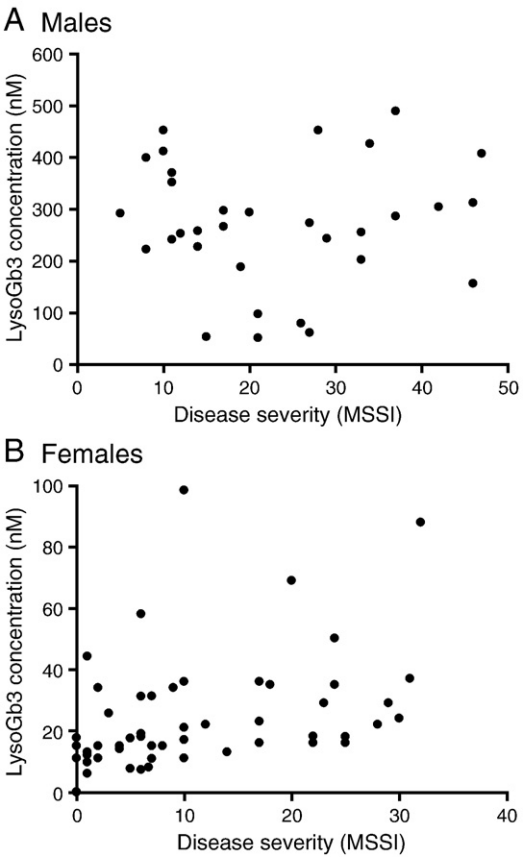

Fig. 3. Relation of plasma lysoGb3 with clinical severity of Fabry disease. Plasma lysoGb3 levels in 37 male (A) and 55 female patients (B) related to overall disease severity (Mainz Severity Score Index (MSSI)).

cardiovascular risk factors (Table 2). For Fabry males parameters are related to an increase in lysoGb3 of 100 nM; for Fabry females we used a smaller increment of 10 nM since the range of lipid abnormality in females is much lower (see Fig. 1). Table 2 shows that based upon linear regression analysis plasma lysoGb3 is not associated with the MSSI score in males (1.3 ± 1.0 increase of MSSI score per 100 nM increase of lysoGb3 ($p = 0.23$)). In contrast, for female Fabry patients a significant association was noted between plasma lysoGb3 and MSSI score (1.6 ± 0.4 increase of MSSI per 10 nM increase of plasma lysoGb3 ($p < 0.001$)).

Next, specific disease manifestations were analysed. When adjusted for age, plasma lysoGb3 concentration does not appear to correlate with LVmass in males ($p = 0.53$). However, a significant influence was noted for the effect on LVmass in female Fabry patients (24.8 ± 5.6 g increase per 10 nM increase of lysoGb3 ($p < 0.0001$) and 19.5 ± 5.5 ($p = 0.001$) after adjustment for hypertension) (Table 2). There was no apparent correlation of plasma lysoGb3 concentration with hearing loss, renal failure (both mGFR and eGFR), microalbuminuria, proteinuria, or presence of angiokeratoma (Table 2).

The association of plasma lysoGb3 with the development of white matter lesions and cerebrovascular accidents formed a clear exception. Plasma lysoGb3 concentration contributes independently to the development of these lesions in the brain in male Fabry patients. The odds ratio was 6.1 (95% CI: 1.4–25.9) per 100 nM lysoGb3 increase ($p = 0.015$). In females the odds ratio was 1.2 (95% CI: 0.9–1.9) per 10 nM lysoGb3 increase, which was not significant ($p = 0.18$). Fig. 4 illustrates the occurrence of cerebrovascular white matter lesions for male Fabry patients. Most male patients around 35 years develop brain lesions [32]. The ones without such white matter abnormalities all have comparatively low plasma lysoGb3 levels (see Fig. 4). In females (not shown), the patient with the highest plasma lysoGb3 had a white matter lesion already at the age of 13.

3.4. Exposure to plasma lysoGb3 and clinical manifestations

Next we examined whether the lifetime exposure to lysoGb3 contributes to the likelihood of presentation of symptoms. Maximum lifetime exposure was estimated by calculating the product of plasma lysoGb3 concentration and the age of an individual. This is very likely an overestimation in the case of female Fabry patients. The calculated cumulative lysoGb3 exposure of individual Fabry patients was compared to the disease severity (see Table 3). For this purpose, patients were stratified as asymptomatic or mildly affected (MSSI 0–19), moderately affected (MSSI 20–39), or severely affected (MSSI >40) [29]. Four ranges of exposure values were distinguished: 0–1000, 1000–5000, 5000–10,000 and >10,000 U (nM lysoGb3 x year). In the female patients with a low lysoGb3 exposure of 0–1000 U ($n = 38$), 36 were asymptomatic or mildly affected. The two patients with a low lysoGb3 exposure who had a higher MSSI score suffered from hypertension and obesity. These risk factors may have contributed to the higher MSSI score. Of the female patients with a higher lysoGb3 exposure of 1000–5000 ($n = 17$), 11 were moderately affected (MSSI 20–39), but 6 had a MSSI below 20. These 6 cases were examined in more detail. The first case (35 y, MSSI 10) had proteinuria, but no other complications. A recent follow-up MRI examination, at the age of 40, revealed a lacunar infarction. The second case (44 y, MSSI 9) had microalbuminuria, modest LVH, white matter lesions and hearing loss. The third case (15 y, MSSI 10) had acroparesthesias and several white matter lesions. The fourth case (49 y, MSSI 17) showed angiokeratoma, LVH, white matter lesions, and microalbuminuria. The fifth case (53 y, MSSI 18) had LVH, white matter lesions and proteinuria. The sixth case (37 y, MSSI 6) had LVH and angiokeratoma. In other words, 5 out of the 6 female patients with relatively high lysoGb3 had significant disease symptoms at the time of the lysoGb3 assessment, not reflected in a high MSSI score

Table 2
Multiple regression analysis of contribution of plasma lysoGb3 to disease manifestations.

Linear regression	Males		Females	
	B-value per 100 nM lysoGb3	*p*	*B*-value per 10 nM lysoGb3	*p*
MSSI	1.3 ± 1.0	0.23	1.6 ± 0.4	<**0.001**
LVmass	−5.1 ± 8.0	0.53	24.8 ± 5.6*	<**0.001**
Renal function (mGFR)	−7.1 ± 6.8	0.32	0.3 ± 1.6	0.84
Logistic regression	**Males**		**Females**	
	Odds ratio (95% CI) per 100 nM lysoGb3	*p*	Odds ratio (95% CI) per 10 nM lysoGb3	*p*
MSSI (>20)	0.8 (0.2–2.4)	0.68	1.9 (1.1–3.5)	**0.03**
LVH	0.6 (0.2–2.0)	0.41	1.6 (0.9–2.7)	0.11
WML	6.1 (1.4–25.9)	**0.015**	1.2 (0.9–1.9)	0.18
Hearing loss	3.0 (0.9–10.1)	0.07	1.0 (0.4–2.4)	0.99
Renal failure (mGFR)**	1.5 (0.5–4.5)	0.49	1.3 (0.05–34.6)	0.87
Microalbuminuria	0.9 (0.5–1.8)	0.80	1.2 (0.9–1.6)	0.32
Proteinuria	0.9 (0.4–1.7)	0.66	1.0 (0.6–1.5)	0.93
Angiokeratoma	1.0 (0.6–1.9)	0.92	1.4 (1.0–1.9)	0.07
Acroparesthesia	2.3 (0.8–7.0)	0.14	1.0 (0.7–1.4)	0.94

B-value = 19.5 ± 5.5, $p = 0.001$ when adjusted for hypertension.

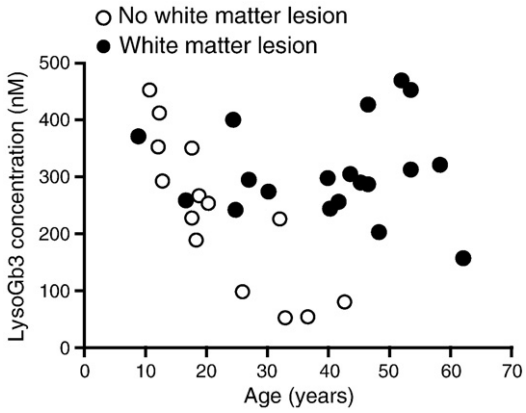

Fig. 4. Correlation between plasma lysoGb3 concentration and incidence of white matter lesions in male Fabry patients. Plasma lysoGb3 levels were correlated with occurrence of white matter lesions in male Fabry patients ($n = 33$).

exceeding 19. Of the male patients ($n = 37$), only a single, 3-year-old individual, had a low lysoGb3 exposure (802 U) together with a low MSSI of 8. Table 3 shows that the proportion of male patients with severe disease is clearly increasing with higher lysoGb3 exposure. Of the 12 male patients with a modestly higher lysoGb3 exposure of 1000–5000 U, 8 were mildly affected. The remaining 4 patients were moderately affected. Of the 12 patients with a lysoGb3 exposure of 5000–10,000, 6 were mildly affected, 5 moderately affected and a single individual severely affected. The latter case was a smoker with hypertension, which may have contributed to his relatively high MSSI. Finally, in the 12 male patients with a very high lysoGb3 exposure exceeding 10,000 U, 6 were moderately affected and 5 were severely affected. Only one individual was mildly affected with a MSSI of 17. The percentage of moderately and severely affected patients increased significantly with increasing lysoGb3 exposure in both females ($p < 0.001$) and males ($p = 0.008$). Finally, the relationship between plasma lysoGb3 exposure and the incidence of white matter lesions was examined more closely. It was found that in male Fabry patients the plasma lysoGb3 exposure (Fig. 5B) correlates better than age (Fig. 5A) with existence of a white matter lesion. In female patients this difference was less pronounced.

3.5. R112H and P60L mutation

A subset of patients did not classify for classical Fabry disease. This concerned 11 patients with the R112H mutation (5 males, 7 females) and 4 patients with the P60L mutation (2 males, 2 females). Their lysoGb3 ranged from 0 to 11 nM in females and 0–20 nM in males. Although, the α-Galactosidase A activity in plasma and leukocytes was reduced in most of these individuals, they did not express any of the classical Fabry symptoms. A single 61-year-old male from this group developed renal insufficiency at the age of 43. A kidney biopsy performed at that time led to the diagnosis of Fabry disease. Recent reanalysis of that biopsy showed some storage in the epithelial cells, but no storage in the endothelium as is usually detected in Fabry patients (Fig. 6). This case was considered an atypical variant of Fabry disease by the pathologist. Furthermore this patient suffered from hypertension that could have attributed to development of renal

Table 3
LysoGb3 exposure and disease severity.

LysoGb3 exposure	<1000 U	1000-5000 U	5000–10,000 U	>10,000 U
Females ($n = 55$)	Total $n = 38$ Asymp-mild $n = 36$ Moderate $n = 2$# Severe none	Total $n = 17$ Mild $n = 6$* Moderate $n = 11$ Severe none	None	None
Males ($n = 37$)	Total $n = 1$ Asymp-mild $n = 1$ Moderate none Severe none	Total $n = 12$ Mild $n = 8$ Moderate $n = 4$ Severe none	Total $n = 12$ Mild $n = 6$ Moderate $n = 5$ Severe $n = 1$##	Total $n = 12$ Mild $n = 1$ Moderate $n = 5$ Severe $n = 6$

LysoGb3 exposure = plasma lysoGb3 concentration x age; expressed in Units (nM x year).
Asymptomatic-mild = MSSI 0–19; moderate = MSSI 20–39; severe = MSSI >40.
* 5/6 patients with MSSI 0–19 had significant symptoms; # hypertension, overweight; ##: hypertension, smoker.

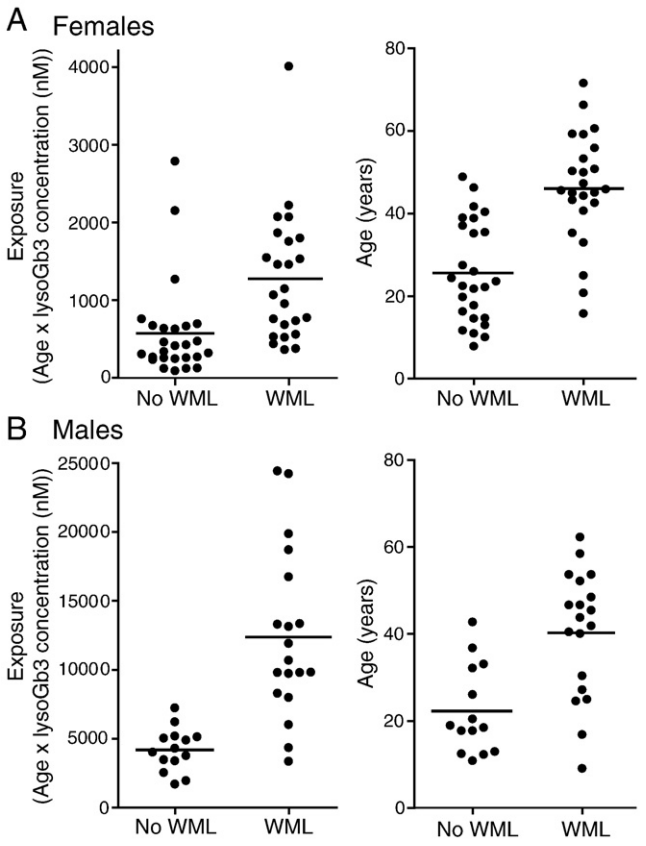

Fig. 5. White matter lesions in females (A) and males (B) relation to lysoGb3 exposure and to age. Plasma lysoGb3 exposure expressed in nM × year. WML: white matter lesion detected by MRI.

insufficiency. These observations suggest that in case of a low plasma lysoGb3 values in males a diagnosis of Fabry disease may need reconsideration in individuals with an α-Galactosidase A mutation with unknown consequence.

4. Discussion

Our investigation of 92 hemizygotes and heterozygotes for Fabry disease, seen at the Academic Medical Centre prior to therapy, has revealed that measurement of plasma lysoGb3 concentration is useful for confirmation of the diagnosis of Fabry disease. In all hemizygous male and adult heterozygous female Fabry patients the plasma lysoGb3 concentration was found to be abnormally high and clearly distinguishable from normal. In very young female Fabry heterozygotes a lack of plasma lysoGb3 elevation may however be uninformative. For example, we were unable to detect elevated plasma lysoGb3 in a 7-year-old Fabry patient. However, at the age of 11 years the plasma lysoGb3 was clearly elevated. The findings for plasma lysoGb3 levels sharply contrast with those for plasma Gb3 concentrations. Confirmation of the diagnosis of Fabry disease by demonstration of elevated plasma Gb3 concentration is only possible in male Fabry patients. Most female Fabry patients show plasma Gb3 levels within the normal range.

Our study further revealed that plasma lysoGb3 is not elevated in a number of individuals carrying an α-Galactosidase A gene with a nucleotide change which is not unequivocally linked to Fabry disease. Examples of this are the R112H substitution and the P60L substitution. Although, the α-Galactosidase A activity in plasma and leukocytes is reduced in these individuals, they do not develop marked elevated plasma lysoGb3. Since their urinary Gb3 is not increased, it seems questionable whether these individuals should be considered as Fabry patients at all. These observations suggest that plasma lysoGb3 measurement may prove to be a useful additional assessment for confirmation of Fabry disease in individuals with an α-Galactosidase A mutation with unknown consequence.

A pathophysiological role of elevated plasma lysoGb3 in Fabry disease may be considered. Barbey and co-workers [22] were the first to report an increased carotid intima-media thickness in the absence of atherosclerotic plaques in adult Fabry patients, a finding which was soon confirmed by others [21]. Barbey and colleagues [22] proposed that a circulating factor contributes to the noted vessel remodelling in Fabry patients and subsequent vasculopathy. We earlier demonstrated that exposure of smooth cells in culture to lysoGb3 concentrations as

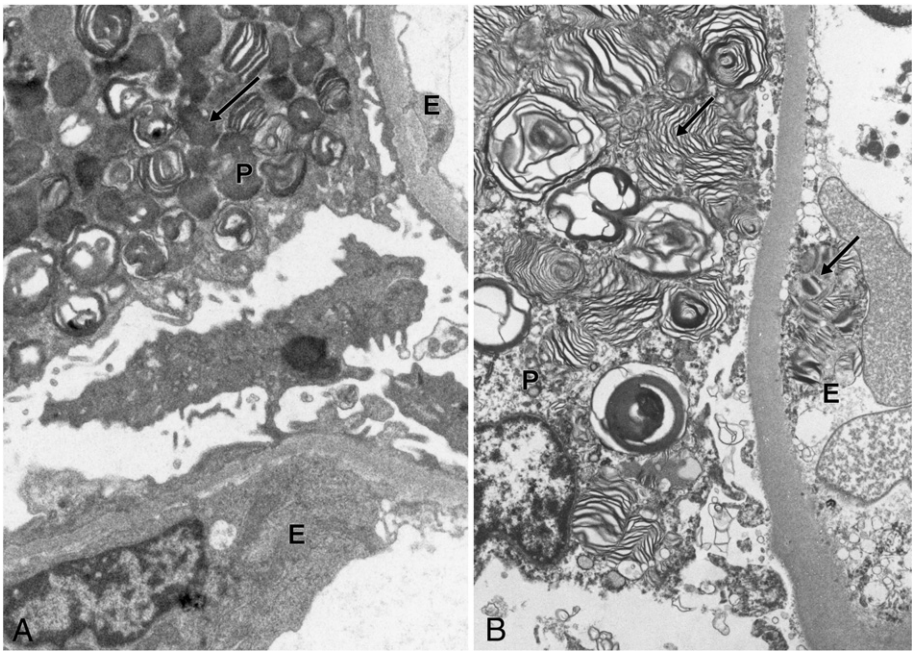

Fig. 6. (A) Kidney biopsy from the patient with the R112H mutation. Epithelial cells of the podocytes show zebra bodies (arrow). In the endothelial cells (E) these zebra bodies are absent (original magnification 20,000×). (B) Kidney biopsy from a patient with a classical Fabry mutation and phenotype. Zebrabodies (arrows) are present in both epithelial cells and endothelial cells (E) (original magnification 30,000×).

occurs in male Fabry patients prominently stimulates the proliferation of these cells [20]. Our present investigation using multiple logistic and linear regression with adjustments for age and cardiovascular risk factors, revealed some significant relationships between plasma lysoGb3 concentration and clinical manifestations. This is remarkable since relatively low numbers of male ($n=37$) and female ($n=55$) Fabry patients were studied. The relationships become more pronounced when data of both genders are combined with appropriate adjustment for gender. For example, the linear correlation between lysoGb3 and MSSI is 1.4 ± 0.5 ($p=0.007$) per 10 nM increase of lysoGb3 and 24.3 ± 5.3 g increase of LVmass ($p<0.0001$) per 10 nM increase of lysoGb3 for the patients combined. The logistic correlation between lysoGb3 and white matter lesions shows an odds ratio (95% CI) of 1.2 (1.0–1.3) ($p=0.004$) for both males and females per 10 nM increase.

We investigated whether lifetime exposure to plasma lysoGb3 influences disease severity. The individual lifetime exposure was crudely estimated by calculating the product of plasma lysoGb3 level and age of the investigated subject. A correlation indeed was noted between plasma lysoGb3 exposure and disease classification, using the Mainz severity scoring index (MSSI) [29], both for male and female Fabry patients. This finding is striking since white matter abnormalities and micro-albuminuria hardly contribute to the Mainz severity score.

It should be mentioned that age by itself is a major factor that determines the clinical outcome in Fabry patients. The contribution of lysoGb3 on top of age is most apparent with respect to white matter lesions: in patients with a high exposure to lysoGb3, white matter lesions were clearly more frequently present. The inverse was true as well: in the older male patients, only the ones with a relatively low lysoGb3 level had not yet developed white matter lesions. For females, the most important conclusion is that levels are much lower than in males, but when relatively high lysoGb3 values are identified at a young age, this almost invariably relates to the presence of disease signs and/or symptoms.

Unfortunately, no IMT data had been collected prior to therapeutic intervention for most of the Fabry patients included in this study. We therefore are unable to correlate IMT values with plasma lysoGb3 concentration or estimated exposure. Very recently Barbey and colleagues reported a slight elevation in sphingosine-1-phosphate in plasma of Fabry patients [33]. In their report it is suggested that this lipid abnormality may underlie the carotid intima media thickening in Fabry patients. Spingosine-1-phosphate is known to be an important regulator of the vessel wall [34]. Since lysoGb3 and sphingosine-1-phosphate show structural resemblance it is of interest to investigate the effect of lysoGb3 on sphingosine-1-phosphate signaling in the vessel wall.

It is surprising that our data suggest that plasma lysoGb3 concentration is an independent risk factor for different disease manifestations in males and in females (white matter lesions in males and left ventricular hypertrophy in females). It will be of interest to study a larger cohort of FD patients with complete clinical assessment, particularly mildly affected male patients, to establish whether the noted gender-difference truly exists or is caused by the limited number of individuals investigated in our study.

Since almost a decade enzyme replacement therapy, based on chronic intravenous administration of recombinant α-Galactosidase A, has become available for the treatment of Fabry patients [35–37]. Two enzyme preparations are currently registered. One enzyme is produced by Chinese hamster ovary (CHO) cells with classic recombinant technology (agalasidase β, Fabrazyme) and the other enzyme is produced by cultured human skin fibroblasts with an activated promoter of the α-Gal A gene (agalasidase α, Replagal). Both recombinant enzymes are quite comparable in properties and differ only slightly in glycan composition [24]. We earlier reported on responses in plasma Gb3 levels in Fabry patients treated with the two enzyme preparations at several dosing regimens [37]. The impact of enzyme replacement therapy on plasma lysoGb3 concentration is subject of a separate ongoing investigation. Preliminary findings were reported in our previous study [20], indicating that treatment of Fabry patients with either enzyme preparations gives rise to a reduction, but not complete correction of plasma lysoGb3 [20]. Multiple regression analyses revealed that, when adjusted for age and cardiovascular risk factors, the plasma lysoGb3 concentration in male Fabry patients is an independent risk for cerebral white matter lesions and in female Fabry patients for left ventricular hypertrophy. Our observation that lysoGb3 concentration decreases upon enzyme replacement therapy is therefore encouraging.

In conclusion, the measurement of plasma lysoGb3 is valuable for confirmation of the diagnosis of Fabry disease, particularly in female heterozygotes. Our investigation indicates that exposure to plasma lysoGb3 correlates with severity of disease manifestations. Plasma lysoGb3 concentration is an independent risk factor for development of cerebrovascular white matter lesions in male patients and left ventricular mass in females. Further investigations in patients and mice with an α-Galactosidase A deficiency will give more insight into the role of circulating lysoGb3 in the vasculopathy of Fabry disease.

Disclosure

This is to certify that Prof. Dr. J.M.F.G. Aerts, Prof. Dr. C.E.M. Hollak and Dr. G.E. Linthorst received reimbursement of expenses and small honoraria for lectures on the management of lysosomal storage diseases, including Fabry disease, from Genzyme Corp, Boston, MA, USA and Shire HGT, Boston, MA, USA. All honoraria are donated to the Gaucher Stichting, a national foundation that supports research in the field of lysosomal storage disorders. Prof. Dr. J.M. Aerts received a joint unrestricted study grant from Genzyme and Shire HGT to conduct studies on pathogenic mechanisms in Fabry disease. Prof. Dr. F.A. Wijburg also received reimbursement of expenses, honoraria for lectures on the management of lysosomal storage diseases, including Fabry disease, from Genzyme and Shire HGT and research grants from Genzyme and Shire HGT.

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