Central hemodynamics and arterial function
van den Bogaard, B.

Citation for published version (APA):
van den Bogaard, B. (2012). Central hemodynamics and arterial function

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Chapter 5

Vascular aspects of Fabry disease in relation to clinical manifestations and elevations in plasma lysoGb3

Saskia M Rombach
Bas van den Bogaard
Eric de Groot
Johanna EM Groener
Ben J Poorthuis
Gabor E Linthorst
Bert-Jan H van den Born
Carla EM Hollak
Johannes MFG Aerts

Manuscript in preparation
ABSTRACT

Background Fabry disease is an X-linked hereditary lysosomal storage disorder due to a deficiency of α-galactosidase A causing intralysosomal accumulation of globotriaosylceramide (Gb3) in various cell types. Apart from accumulation of Gb3, increased plasma levels of globotriaosylsphingosine (lysoGb3) have been detected. Clinically, the disease presents as a vascular disease, with cerebral, cardiac and renal complications. The aim of this study was to compare vascular parameters in patients with Fabry disease and study the relationship with plasma lysoGb3.

Methods Carotid intima media thickness (IMT), brachial flow-mediated dilation (FMD), pulse wave velocity (PWV), and advanced glycation end products (AGES) were measured in 57 classically affected patients (22 males, 35 females), and 55 healthy matched controls (20 males, 35 females). In addition, 10 atypical Fabry patients (5 males, 5 females) without elevated plasma lysoGb3 levels were investigated. Most patients received enzyme replacement therapy (ERT).

Results Comparing classical Fabry males to matched unaffected controls, brachial FMD was decreased, (4.7 (0.8-17.4) % versus 5.8 (1.9-13.6) %, \( p = 0.016 \)) and carotid IMT was increased (0.65 (0.43-0.96) mm vs 0.60 (0.39-1.09) mm, \( p = 0.022 \)). PWV and the AGES did not differ. In females with classical disease and atypical patients IMT, FMD and PWV were not different compared to controls, although the AGES were slightly increased in atypical patients. Clinically, IMT thickness was associated with cerebral white matter lesions and stroke (OR 4.0 (95% CI: 1.8-9.1) per 0.1 mm increase in IMT, \( p < 0.001 \)). In classically affected females, a small increase in lysoGb3 was associated with an increase in IMT independent of age. In the classically affected males, all with increased IMT and high levels of plasma lysoGb3 (median 24 (range 7-87) nM), lysoGb3 levels did not add to a higher IMT, suggestive for a ceiling effect. Similarly for FMD, in both classically affected males and females, elevated lysoGb3 levels (> 7 nM) contributed to a 2.9% lower FMD independent of age and gender (\( p = 0.02 \)).

Conclusion Increased carotid IMT and decreased brachial FMD occur in patients with classic Fabry disease, particularly in males, indicating increased cardiovascular risk. Increased plasma lysoGb3 is associated with increased IMT and decreased FMD independent of age and gender. These observations still exist despite ERT.
INTRODUCTION

Fabry disease is an X-linked hereditary lysosomal storage disorder due to a deficiency of α-galactosidase A. Its distribution is panethnic, with an estimated birth prevalence of 1:40,000-117,000,\(^1,2\) although recent screening studies suggest higher prevalence rates.\(^3,4\) The primary cause of disease is a deficient activity of the lysosomal alpha-Galactosidase A (alfa-galA, EC 3.2.1.22). Due to this, globotriaosylceramide (Gb3, also named GL-3 or CTH) is stored in various cell types, especially endothelial cells. Clinically, the disease presents as a vascular disease, with gradual development of white matter lesions in the brain, cardiac hypertrophy, and progressive kidney disease. Premature coronary artery and cerebral artery disease were found in the NIH cohort,\(^5\) but this has not been confirmed in other studies.\(^6,7\) Interestingly, a proportion of female carriers with alpha-Galactosidase A deficiency also develop disease, albeit with a more protracted course. The chronic presence of a large amount of endogenous circulating alpha-Galactosidase A enzyme is apparently unable to correct the lack of enzyme in those cells that only express the mutated allele. Even more intriguing, atypical variants of Fabry disease can be discerned, consisting of individuals with more subtle alpha-Galactosidase A abnormalities resulting in an atypical course with limited manifestations such as primarily cardiomyopathy or renal insufficiency.\(^8-11\) The latter group of individuals constitutes a major problem in diagnosis, which is solely based on the demonstration of a predicted amino acid substitution as revealed by the gene analysis and/or detection of reduced enzyme activity in assays using artificial substrates. In these individuals, there may be an absence of elevated levels of Gb3 in urine and Gb3 and globotriaosylsphingosine (lysoGlobotriaosylceramide – lysoGb3) in plasma, which questions the relationship between storage and symptomatology in these patients.

Since 2001 enzyme replacement therapy (ERT) has become available. Two commercial products, agalsidase alfa and agalsidase beta, have shown to reduce cardiac mass, reduce pain, improve quality of life and stabilise kidney function in some patients with preserved renal function.\(^12-14\) However, despite reduction in endothelial Gb3 storage as a result of therapy, disease progression does occur,\(^15-17\) suggesting another mechanism causing vasculopathy.\(^18\) The pathogenesis of the vasculopathy in Fabry disease is, however, poorly understood.\(^19\) Abnormal functional control of the vessel, reflected by an altered cerebral blood flow velocity, the presence of endothelial dysfunction as well as an increased prothrombotic state with the formation of reactive oxygen species have all been suggested to underlie the vasculopathy.\(^20,21\)

Fabry patients show an increased IMT of the common carotid, brachial and radial artery compared to controls.\(^6,22-24\) Flow mediated dilation (FMD), a measure of endothelial dysfunction, is impaired in Fabry disease.\(^23\) Furthermore, Fabry patients prior to ERT treatment were found to have an increased pulse wave velocity (PWV), a measure of aortic stiffness.\(^24\) Recently accumulation of advanced glycation end products (AGES) have been reported as marker of cardiovascular disease in individuals with an increased IMT and patients at risk for cardiovascular...
complications but not yet in Fabry disease. None of these vascular measures have been studied in relation to other clinical symptoms beside left ventricular hypertrophy.

A potential pathogenic factor in the vasculopathy may be lysoGb3, a water-soluble lipid which was recently found to be highly elevated in plasma of classically affected Fabry patients. This lipid was shown to stimulate smooth muscle cell proliferation in vitro. Therefore lysoGb3 might contribute to the reported increased IMT in Fabry patients.

The primary aim of the study was to compare carotid IMT, FMD, PWV, and AGES in patients with Fabry disease to healthy matched controls. We evaluated how these parameters of vascular damage relate to disease severity and clinical parameters, including, gender, age, treatment status, the extent of renal dysfunction and the presence of brain lesions and left ventricular hypertrophy. The second aim of the study was to investigate the hypothesis that an increase in plasma lysoGb3 levels is associated with increase in IMT.

METHODS

Study design and participants

All patients with a confirmed diagnosis of Fabry disease that were scheduled for a regular follow-up visit at the outpatient clinic were invited to participate in the study from March 2009 through July 2010. The diagnosis was confirmed by decreased enzyme-activity in males and genotyping in both males and females. As has been reported previously, patients with the R112H and P60L substitutions are considered to be atypical Fabry patients based on their atypical course of disease and apparent lack of lipid abnormalities. Exclusion criteria were diabetes mellitus, primary dyslipidemia, or other relevant comorbidity. The participating patients were matched with healthy controls for age, gender, and smoking status. Healthy controls were recruited by asking the patients to bring a relative with an excluded diagnosis of Fabry disease or friend. Additional controls were recruited by advertisement. Participants were instructed to fast overnight and refrain from smoking. In all participants a full medical and family history was obtained including questions regarding cardiovascular disease through a standardized questionnaire. Blood pressure was measured using a validated automatic oscillometric device (Omron 705it). For the pulse wave velocity measurement blood pressure was measured three times on the right arm. The first measurement was discarded, and the average of the latter two measurements was used for analyses. A history of hypertension was defined as a previous diagnosis of hypertension (a systolic blood pressure (SBP) ≥140 mmHg and/or a diastolic blood pressure (DBP) ≥90 mmHg in three consecutive measurements) with an indication for antihypertensive medication. The estimated glomerular filtration (eGFR) rate was calculated by the abbreviated MDRD equation. In the Fabry patients, data on left ventricular mass (LVM), cerebral white matter lesions (WMLs), and stroke were available as part of ongoing data collection. Left ventricular hypertrophy in males was defined as LVM >51 g/m² in males.
and >48 g/m² in females. A WML was diagnosed by a neuroradiologist on T2 weighted MRI images. All patients gave written informed consent according to the protocol approved by the institutional review board.

**Laboratory assessments**

Creatinine, glucose, and total cholesterol profile (TC, HDL-c, LDL-c and triglycerides) were assessed. In Fabry patients, lysoGb3 levels in plasma were measured with a newly developed method based on tandem mass spectrometry with isotope labeled lysoGb3 as internal standard.

**Measurements**

**Intima-media thickness.** For cIMT measurements, bilaterally non-invasive ultrasound images of predefined arterial wall segments of the right and left common carotid artery, the carotid bulb, and the internal carotid artery were acquired. cIMT was defined as the average of the six cIMT measurements. M-mode images were acquired to provide data on arterial wall stiffness and lumen in the distal common carotid arteries. The image analyst was blinded for the clinical and genetic status of the subject. The assessments were performed by the echo core lab of the AMC Vascular Imaging.

**Flow mediated dilatation.** Assessment of brachial FMD was performed as described previously. A blood pressure cuff was placed around the right forearm. The scan followed a standard protocol: Brachial artery diameter at the level of the antecubital crease was measured for a period of 1 minute, followed by 5 minutes of occlusion. To minimize intrasession variance, the measurement arm was stabilized using a custom-built ultrasound probe holder/ arm rest. FMD was expressed at each examination as (lumen diameter after ischemia -diameter at baseline)/diameter at baseline.

**Pulse wave velocity.** To determine the pulse wave velocity (PWV), pressure waveforms were recorded at the carotid and femoral artery sequentially. Wave transit time was calculated by the SphygmoCor (version 8, AtCor) system software, using the R wave of a simultaneously recorded ECG as a reference frame. Surface distance between the two recording sites was measured, thus allowing PWV to be determined. The measurements were done twice on the right side of the subject and the average of the two measurements was used for analyses.

**Advanced glycation end products.** Skin auto-fluorescence (AF) was measured with the AGE Reader (DiagnOptics Technologies BV, Groningen, The Netherlands). Measurements were performed at the volar site of the forearm, in a semi dark environment, preventing surrounding light from interfering with the measurement.

**Statistical analysis**

Values are expressed as median (range) or mean±standard deviation (SD). Comparison between groups was made by using the Mann-Whitney-test or the Kruskal-Wallis test where applicable.
Univariate, multiple linear regression, and logistic regression were applied to determine the association between the vascular outcome parameter, lysoGb3, and clinical disease manifestations. These analyses were adjusted for other cardiovascular risk factors, including age, gender, BMI, smoking status, a history of hypertension, SBP and DBP, TC/HDL-c ratio, and LDL-c. The predictors and their interaction terms were tested and kept in the model if significant at $p=0.05$. Model fit was assessed by the coefficient of determination ($R^2$). Previously, the impact of lifetime exposure to elevated plasma lysoGb3 on clinical manifestations in Fabry patients has been investigated.\textsuperscript{31} For this purpose, lifetime exposure to plasma lysoGb3 was assessed as follows: age at baseline $\times$ lysoGb3 at baseline + years of ERT treatment $\times$ lysoGb3 at the time of the evaluation. As part of a sensitivity analysis, multiple imputation for missing values for FMD and PWV data was performed, using age, gender and subgroup as well as blood pressure for PWV as predictor variables, which led to the same conclusions (data not shown). A $p$-value <0.05 was considered statistically significant. Statistical analyses were performed with SPSS 17.0.

**Patients and measurements**

In total, 57 adult Fabry patients (22 males, 35 females) with a classical mutation in the alpha-galactosidase A gene were investigated. In addition, 10 individuals with the R112H (3 males, 3 females) and the P60L substitution (2 males, 2 females) were included. The Fabry cohort was matched with a healthy control group of 20 males and 35 females. At the time the measurements were performed, 42 out of 57 classically affected patients and 2 atypical patients received ERT; agalsidase alfa 0.2 mg/kg/2 weeks (n=17) or agalsidase beta (n=27). Of the patients receiving agalsidase beta, 11 patients received a dosage of 1.0 mg/kg/2 weeks, 3 patients received a dosage of 0.2 mg/kg/2 weeks as prescribed as part of a previous trial\textsuperscript{15} and 13 patients temporarily received a dosage ranging from 0.25 up to 0.50 mg/kg/2 weeks due to the global shortage.\textsuperscript{36} Baseline characteristics are reported in table 1. PWV data were available for 76% of the participants (40 classical Fabry patients, 8 atypical patients and 45 controls). The main reason for unsuccessful PWV measurements was a cardiac rhythm disturbance. FMD data were available for 78% of the participants, including 46 classical Fabry patients, 8 atypical patients and 41 controls. Twenty-three FMD measurements were rejected by the analyst because of inferiority of the images. AGES were measured in 96% of the participants.

**RESULTS**

**Vascular parameters in Fabry patients**

The findings for FMD, IMT, PWV, and AGES in Fabry patients and controls are shown in table 2.  

**Classically affected Fabry males and females.** Comparing the total cohort of classically affected Fabry patients with matched controls, FMD was significantly lower (4.7% vs 5.8%, $p=0.02$). Carotid IMT was increased (0.65 vs 0.60 mm in controls $p=0.02$). PWV and AGES were
Arterial stiffness in premature atherosclerosis

not significantly different between classical Fabry patients and controls. A further analysis by gender revealed that in males (age 38.4±14.3 years) IMT was increased and FMD decreased if compared to male controls. This in contrast with the results for the Fabry females (age 45.7±13.3 years) that were comparable to the matched female controls (table 2). Figure 1 shows the IMT and FMD depicted by age for classically affected patients and controls.

Atypical patients. Comparing the atypical patients (age 52.0±13.3 years) with controls revealed that none of the vascular parameters was significantly abnormal with the exception of AGES in males (Table 2). The atypical cases showed a trend towards a higher TC/HDL-c profile compared to the controls (p=0.05) as well as a trend towards a higher BMI (p=0.06).

<table>
<thead>
<tr>
<th>Table 1 Baseline clinical characteristics of the study population.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n</strong></td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td>**Males, n (%) / **</td>
</tr>
<tr>
<td>57</td>
</tr>
<tr>
<td><strong>Females, n(%)</strong></td>
</tr>
<tr>
<td>22 (39%)</td>
</tr>
<tr>
<td>35 (61%)</td>
</tr>
<tr>
<td><strong>Age: mean±SD median, (range)</strong></td>
</tr>
<tr>
<td>42.9±18.8</td>
</tr>
<tr>
<td>44.2 (19-76)</td>
</tr>
<tr>
<td><strong>Ethnicity (caucasian; mediterranean; asian)</strong></td>
</tr>
<tr>
<td>55;1:1</td>
</tr>
<tr>
<td><strong>Agalsidase alfa;beta;no ERT (n)</strong></td>
</tr>
<tr>
<td>17;25:15</td>
</tr>
<tr>
<td><strong>History of hypertension,n(%)</strong></td>
</tr>
<tr>
<td>11 (19%)</td>
</tr>
<tr>
<td><strong>SBP, mmHg</strong></td>
</tr>
<tr>
<td>121±19</td>
</tr>
<tr>
<td><strong>DBP, mmHg</strong></td>
</tr>
<tr>
<td>73±9</td>
</tr>
<tr>
<td><strong>BP-lowering medication, n (%)</strong></td>
</tr>
<tr>
<td>30 (53%)</td>
</tr>
<tr>
<td><strong>Glucose</strong></td>
</tr>
<tr>
<td>4.9±0.6</td>
</tr>
<tr>
<td><strong>TC/HDL-c ratio</strong></td>
</tr>
<tr>
<td>3.2±0.8</td>
</tr>
<tr>
<td><strong>LDL-c, mmol/l</strong></td>
</tr>
<tr>
<td>2.9±0.9</td>
</tr>
<tr>
<td><strong>Triglycerides, mmol/l</strong></td>
</tr>
<tr>
<td>0.87±0.40</td>
</tr>
<tr>
<td><strong>Statin use, n (%)</strong></td>
</tr>
<tr>
<td>3 (5.3%)</td>
</tr>
<tr>
<td><strong>BMI, kg/m²</strong></td>
</tr>
<tr>
<td>24.4±4.1</td>
</tr>
<tr>
<td><strong>Current smoking, n (%)</strong></td>
</tr>
<tr>
<td>11 (19%)</td>
</tr>
<tr>
<td><strong>eGFR (median, range)</strong></td>
</tr>
<tr>
<td>88 (23-148)</td>
</tr>
<tr>
<td><strong>LVH, n (%)</strong></td>
</tr>
<tr>
<td>35 (61%)</td>
</tr>
<tr>
<td><strong>WML, n (%)</strong></td>
</tr>
<tr>
<td>39 (68%)</td>
</tr>
<tr>
<td><strong>LysoGb3 pretreatment</strong></td>
</tr>
<tr>
<td>10.7 (2.3-124.3)</td>
</tr>
<tr>
<td><strong>LysoGb3</strong></td>
</tr>
<tr>
<td>8.4 (2.1-86.9)</td>
</tr>
</tbody>
</table>

Data on left ventricular hypertrophy (LVH) and WML (white matter lesions) were not available in the control subjects. BP-lowering medication: including angiotensin-converting-enzyme inhibitors, angiotensin receptor blockers prescribed for microalbuminurie/proteinuria. p-values are calculated for the differences between the three groups (classic, atypical and control group). NA=not applicable, ND=not determined, eGFR=estimated glomerular filtration rate. *Reference interval for lysoGb3 in healthy controls: 0.3-0.6 nM.
The design of the study did not allow us to assess the effect of ERT on vascular parameters. Almost all male patients (21 of 22) and female patients (21 of 35) received ERT. The median ERT duration was 5.7 (0.5-9.9) years. The 21 females with ERT were older than the 14 females without treatment (50.5±13.3 vs 38.7± years, \(p < 0.01\)), and more severely affected which may explain the higher IMT in the ERT treated females compared to controls (0.67 (0.43-0.88) mm vs 0.60 (0.39-1.09) mm, \(p = 0.06\)).

Vascular parameters and clinical parameters

### Classical patients
In univariate analysis IMT was associated with renal function, LVH and WMLs including previous strokes (table 3). However, in the multivariate analysis including age and gender, the relationship of IMT with the clinical manifestations lost significance. Comparing the univariate analyses, the presence of WML and a history of stroke was predicted most accurately by IMT as apposed to age and gender (\(R^2\)\_WML and IMT =0.38, \(R^2\)\_WML and age =0.33, \(R^2\)\_WML and gender =0.02).

In univariate analysis, FMD and PWV were related to left ventricular hypertrophy and WML respectively but this association was not significant when adjusted for age and gender. The
AGES however, were inversely related to renal function. If adjusted for age and gender, an increase of the AGES by 1 unit reflected a decrease of renal function by 20 ml/min/1.73m² ($p=0.02$).

**Figure 1**
IMT in males (left panels) and females (right panels) with classic phenotypes compared to controls, according to age and gender. In males, the slope of the regression line is comparable while the intercept is 0.12 mm higher in classic males compared to controls ($p<0.001$). In females, the intercept and slope did not differ. In classic males, FMD corrected for age is 1.8% lower compared to controls ($p=0.02$) while the slopes are comparable. In classically affected females, FMD is comparable to controls.

**Table 3** IMT and clinical disease manifestations: univariate analysis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Classic phenotypes only (n=57)</th>
<th>Atypical patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>eGFR ml/min/1.73m²</td>
<td>beta -7.6 (SE 2.8)</td>
<td>$p$-value 0.009</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameter</th>
<th>OR (95% CI)</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVH</td>
<td>6.7 (2.4-18.5)</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>WML</td>
<td>4.0 (1.8, 9.1)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Beta represents the change in renal function per 0.1 mm increase in IMT. The odds are reported per 0.1 mm increase in IMT.
Atypical patients. In the atypical patients, no association between IMT, FMD, PWV, AGES, and clinical parameters could be determined.

**IMT, FMD, PWV, AGES and plasma lysoGb3**

Figure 2 shows the IMT and plasma lysoGb3 levels of studied Fabry patients. Classically affected Fabry males all showed markedly elevated lysoGb3 as well as an increase in IMT compared to controls. Classically affected Fabry females showed more modest abnormalities in plasma lysoGb3 and IMT compared to controls. The atypical cases with no increased lysoGb3 showed no clear abnormalities. Neither univariate nor multivariate analysis in the total group of classically affected Fabry males and females, revealed an association between IMT and plasma lysoGb3. A large part of the cohort was treated with ERT and as this reduces lysoGb3 levels, the same analyses were performed using pretreatment lysoGb3, i.e. lipid levels at the time before initiation of ERT. Similarly there were no correlations.

Of interest, a more detailed analysis of female patients with classic Fabry disease alone revealed that their lysoGb3 levels did contribute to IMT thickness. When adjusted for age, lysoGb3 at the time of evaluation (median 6 (range 2-11) nM) was associated with a trend towards increase in IMT ($p=0.08$) and a significant correlation between pretreatment lysoGb3 (median 8 (range 2-24) nM) and IMT was observed; 0.006 mm increase in IMT per 1 nM pretreatment lysoGb3 increase ($p=0.03$). In males, lysoGb3 (median 24 (range 7-87) nM) nor pretreatment lysoGb3 added to the prediction of the IMT thickness.

For FMD a slightly different pattern was observed. Analysis of the classically affected patients only, showed a trend with the lysoGb3 level and FMD ($p=0.07$) but there was a significant

![Figure 2](image_url)

**Figure 2**

Left upper panel shows IMT and lysoGb3 in Fabry disease males and females with classic phenotypes ($n=57$) and the right upper panel in atypical patients ($n=10$). Below FMD and lysoGb3 in Fabry disease males and females with classic phenotypes ($n=46$) on the left and atypical patients ($n=8$) on the right.
correlation with pretreatment lysoGb3; 0.35% decrease in FMD per 10 nM increase of pretreatment lysoGb3 (p<0.01). Correcting for age, gender, and cardiovascular risk factors, there was a trend between pretreatment lysoGb3 and decrease of FMD (p=0.07). Analysis for males and females with classic Fabry disease separately again only showed a significant correlation in females (p=0.01).

There were no correlations between lysoGb3 and pulse wave velocity nor AGES.

**Life-time exposure to lysoGb3 and aging**

There were modest correlations between IMT and life-time exposure to lysoGb3 (R²=0.21, p<0.001) and FMD and life-time exposure to lysoGb3 (R²=0.12, p=0.01) in the classic Fabry cohort. Similar observations were found in males and females separately.

**DISCUSSION**

In this study carotid IMT, FMD, PWV, and AGES were measured in a large and heterogeneous cohort of Fabry patients, including male hemizygotes and female heterozygotes with classical disease manifestations as well as individuals with alfa-Galactosidase A abnormalities that result only in atypical manifestations and minor alterations in lipid abnormalities. Different findings were observed for the various sub-groups that deserve separate discussion. The investigated atypical patients were identified in three families. Besides the index cases, other family members were only very mildly affected. The index cases presented with renal insufficiency (n=2) and multiple TIA's (n=1). The latter patient also presented with the highest IMT, but nevertheless showing very low lysoGb3 levels. Smoking was another known risk factor in this patient. Overall, in the atypical patients, IMT, FMD, and PWV were comparable to matched controls. Only the AGES tended to be increased in males. Although comparable for age, the studied atypical patients showed a trend towards a higher total cholesterol/ high density lipoprotein (TC/HDL) profile compared to the matched controls (p=0.05) as well as a trend towards a higher BMI (p=0.06). This suggests that other factors than Fabry disease contribute to the vascular changes observed in some of these patients. In classically affected males, IMT was significantly increased compared to controls. This has also been reported in previous cross-sectional studies and one longitudinal study in untreated Fabry patients compared to healthy controls.6, 23, 24 In classically affected females IMT was found to be comparable to healthy controls. Barbey and colleagues reported an increased IMT in untreated female patients.6 Possible explanations for these differences are (1) the 6 year age difference between patients and controls in the study by Barbey et al and (2) a possible effect of intervention (ERT) in our cohort. The latter may not be a likely explanation since a longitudinal study during ERT (agalsidase beta 1.0 mg/kg/ 2 weeks) showed no decrease of IMT,24 although this cohort consisted primarily of male patients.
FMD was found to be significantly decreased in classically affected males. In contrast, FMD in female patients was comparable to matched healthy controls. There are few studies on FMD in Fabry disease.\(^\text{23, 37}\) FMD was reported to be impaired, however these studies included males and females and did not differentiate the results for gender.\(^\text{23, 37}\)

PWV was not different between patients and controls, despite an increased IMT in Fabry patients compared to controls. This is in line with previous observations that an increase in IMT does not necessarily lead to increased arterial stiffness.\(^\text{22}\) Another explanation may be that PWV decreases during ERT treatment.\(^\text{24}\) In our study cohort, a large proportion received ERT as well as ACE-ARB medication, lowering blood pressure, which might have contributed to the observed normal PWV. Indeed, treatment with ACE-ARB contributed to a lower PWV beyond blood pressure (data not shown). Furthermore, increase of PWV was associated with an increase in IMT, suggesting that structural macrovascular changes are related to functional changes. The AGES were not different between classically affected patients and controls but an increase in AGES was associated with a decrease of renal function in classic Fabry patients, which is in line with a previous study concerning patients with renal insufficiency.\(^\text{27}\)

We evaluated the clinical relevance of noted abnormalities in vascular parameters. Increased IMT is associated with increased risk of the development of atherosclerosis and cardiovascular disease in general.\(^\text{38}\) In current study, increase of IMT was related to the occurrence of cerebral white matter lesions and stroke. Cerebral white matter lesions seem to be the most prevalent manifestations of disease progression, despite treatment.\(^\text{15, 31}\) In general, an increase in IMT is associated with an increased risk of stroke.\(^\text{39}\) The persistent increase in IMT and occurrence of new WMLs despite ERT are both indicative of ongoing vascular disease manifestations.

The second aim of this study was to evaluate the hypothesis that increased levels lysoGb3 would be associated with an increased IMT. Indeed, in females lysoGb3 appeared to contribute to the increase in IMT. However in males, there was no strict association discovered and there was no additional value of exposure compared to age alone. This result could be due to a ceiling effect. In females, lysoGb3 levels are lower compared to males.\(^\text{30, 31}\) Females also have less increased IMT values compared to males. Classically affected males all have very markedly increased lysoGb3 levels, even after treatment with ERT, as well as an increased IMT. This would suggest that already very low levels of lysoGb3 can add to an increase in IMT and when a certain ceiling is exceeded, higher lysoGb3 values do not add to a higher IMT. Indeed, when the total cohort, including classic and atypical patients was divided into two groups, based on the median lysoGb3 value (lysoGb3 level = 7 nM) patients with lysoGb3 levels above the median showed, on top of age and gender, an 0.044 mm higher IMT compared to patients below the median (\(p=0.034\)). In classically affected patients, there was only an association with the pretreatment lysoGb3: the IMT was 0.056 mm higher in patients with pretreatment lysoGb3 levels above the median (>10 nM) compared to patients with levels below 10 nM independent of age and gender (\(p=0.02\)).
Similarly, in the total cohort of classically affected and atypical patients, a lysoGb3 above 7 nM, was associated with a 2.2% lower FMD as compared to patients with a lysoGb3 below 7 nM after adjustment of age and gender \( (p=0.02) \). In classically affected patients, this was -2.9% in FMD in case of lysoGb3 above 7 nM \( (p=0.02) \).

In atherosclerosis development, carotid IMT, brachial artery FMD, and PWV provide distinct, independent information about this complex process.\(^{40, 41}\) In Fabry disease, lysoGb3 has been shown to induce smooth muscle cell proliferation in vitro and could suggest that an increase in IMT develops first.\(^{30}\) Alternatively, lysoGb3 could induce both an increase in IMT as well as an impaired FMD concomitantly. However, the exact pathophysiology of Fabry disease and the vascular components contributing to disease manifestations are unclear. Previously we hypothesised that excess of lysoGb3 in the circulation gives local depositions in the media layer resulting in smooth muscle cell proliferation and remodeling of both subendothelial layer and extracellular matrix leading to fibrotic structures.\(^{18}\) This may lead to shear that increases expression of both angiotensin 1 receptor (AT1) and angiotensin 2 receptor (AT2) which can activate integrin-mediated signaling, thereby inducing alterations of both extracellular matrix and cytoskeletal composition and organization.\(^{42}\) Increased angiotensin 2 activity before enzyme infusion compared to controls has recently been reported which strengthens this hypothesis.\(^{43}\) An alternative explanation could be elevation of sphingosine-1-phosphate levels in patients with Fabry disease.\(^{44}\) One study showed that high sphingosine-1-phosphate levels in patients with Fabry disease correlate with increased IMT and left ventricular mass, although the mechanism for the increased S1P levels could not be established.\(^{44}\) S1P is also detected in healthy individuals and other diseases and increases during inflammatory responses.\(^{45, 46}\) It is not a disease specific marker, such as lysoGb3, and does therefore not unequivocally explain how the vasculopathy in Fabry disease is induced.

A limitation of our study was that not all PWV and FMD measurements were available for the entire cohort. PWV could not be measured in a subset of patients with cardiac rhythm disturbances. This group was slightly older than the cohort with PWV measurements. However the sensitivity analyses did not alter the overall conclusions.

Another limitation of our investigation is its cross-sectional design and the fact that a large proportion of patients received ERT (21 out of 22 classic Fabry males, and 21 of 35 classic Fabry females), including two different products and in different dosages. In addition, many patients received additional antihypertensive treatment. To come to a better understanding of the pathophysiology and its reversibility/stabilization by ERT and additional therapies, repeated measurements in the same patients should be performed, ideally before start of any intervention (i.e. ERT) and during follow-up. In addition the effect of dosage of ERT should be evaluated.

In conclusion, the present study showed that IMT is increased and FMD decreased in males with classical Fabry disease with elevated lysoGb3 levels. In the female patients such relationships were only detected in more severely affected individuals. We cannot exclude that ERT treatment and/or co-medication ameliorated vascular abnormalities to some extent. However,
the fact that most abnormalities remained increased despite ERT suggests that this treatment modality has only modest effect on vascular abnormalities. Present results suggest that treatment interventions should ideally result in preventing increase of IMT and decrease of FMD by early intervention as well as a more effective lowering of plasma lysoGb3 for example by increasing the dosage of ERT. To obtain a better insight on this matter longitudinal investigations during ERT are warranted in females and in males in case of new treatment strategies, aiming at early as well as more effectively lowering of lysoGb3. These studies may also reveal whether responses in plasma lysoGb3 to enzyme supplementation are predictive for improvement in vascular function.

ACKNOWLEDGEMENTS

We would like to thank the patients and the controls for participation. We would like to thank Johan Gort for performing the carotid and brachial ultrasound scans. Furthermore we would like to thank Dees Klappe and Theo Postma of the Vascular Imaging, the Department of Vascular Medicine, for their image analyses, Els Ormel for her assistance at the clinic, and Sijmen Kuiper and Mina Mirzaian for the lysoGb3 measurements.
REFERENCES


