Fabry disease

The future is in the past

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FABRY DISEASE

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Sanne Jolien van der Veen

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The future is in the past

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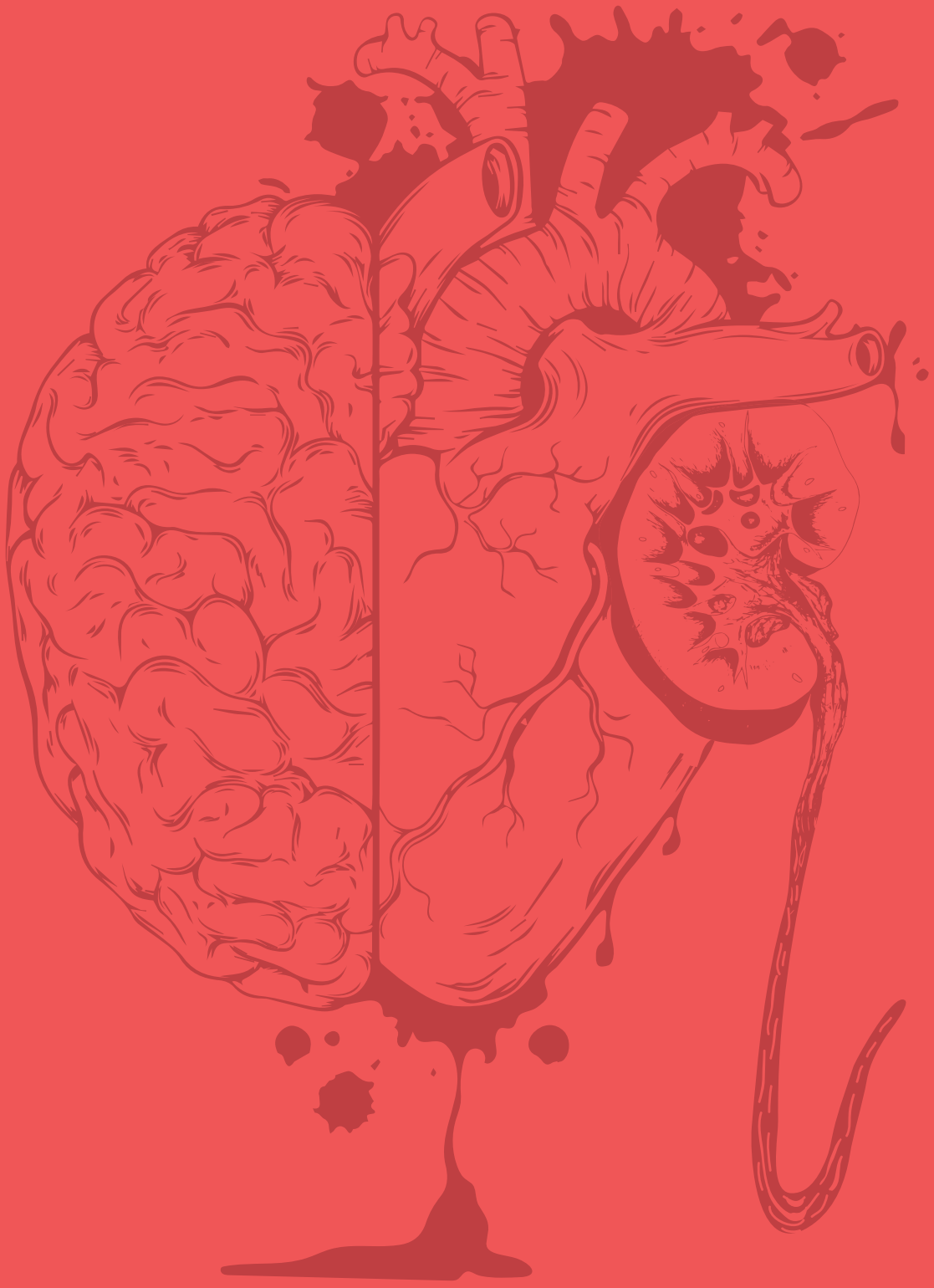


Chapter 1

General Introduction and thesis outline

Background

In 1955, Christian de Duve discovered acidic organelles loaded with hydrolytic enzymes to digest and recycle macromolecules[1]. He named them lysosomes, after the Greek words for cutting/dissolving: lysis and body: sōma. His scientific breakthrough ultimately led to the recognition that the lysosomal accumulation of glycolipids were the underlying cause of several disorders that were already known as clinical syndromes but henceforward grouped and renamed as lysosomal storage diseases (LSDs). One of these diseases is Fabry disease (FD, OMIM 301500), which was first described by William Anderson[2] and Johannes Fabry[3] near the end of the 19th century. In 1965, Hashimoto et al. studied the cells of Fabry patients with electron microscopy and hypothesized that the "extremely overcrowded lysosomes" he saw, could be the result of genetic mutations, leading to dysfunctioning of lysosomal enzymes[4]. The overcrowded lysosomes, later referred to as Zebra bodies, became a hallmark to characterize and diagnose Fabry disease. Soon thereafter alpha galactosidase A (αGalA) deficiency was found to be the underlying cause of FD[5]. Under normal circumstances, αGalA hydrolyzes the terminal α-galactosyl residue from different substrates, predominantly the glycosphingolipid globotriaosylceramide (Gb3)[6]. When the activity of αGalA is reduced or absent, as is the case in Fabry disease, its substrate cannot be broken down and Gb3 accumulates in various cell types and organs[6], ultimately resulting in the clinical presentation of FD. The intracellular glycosphingolipid accumulation initiates several secondary pathological processes, that ultimately lead to the renal, cardiac and cerebral disease manifestations observed in Fabry patients.

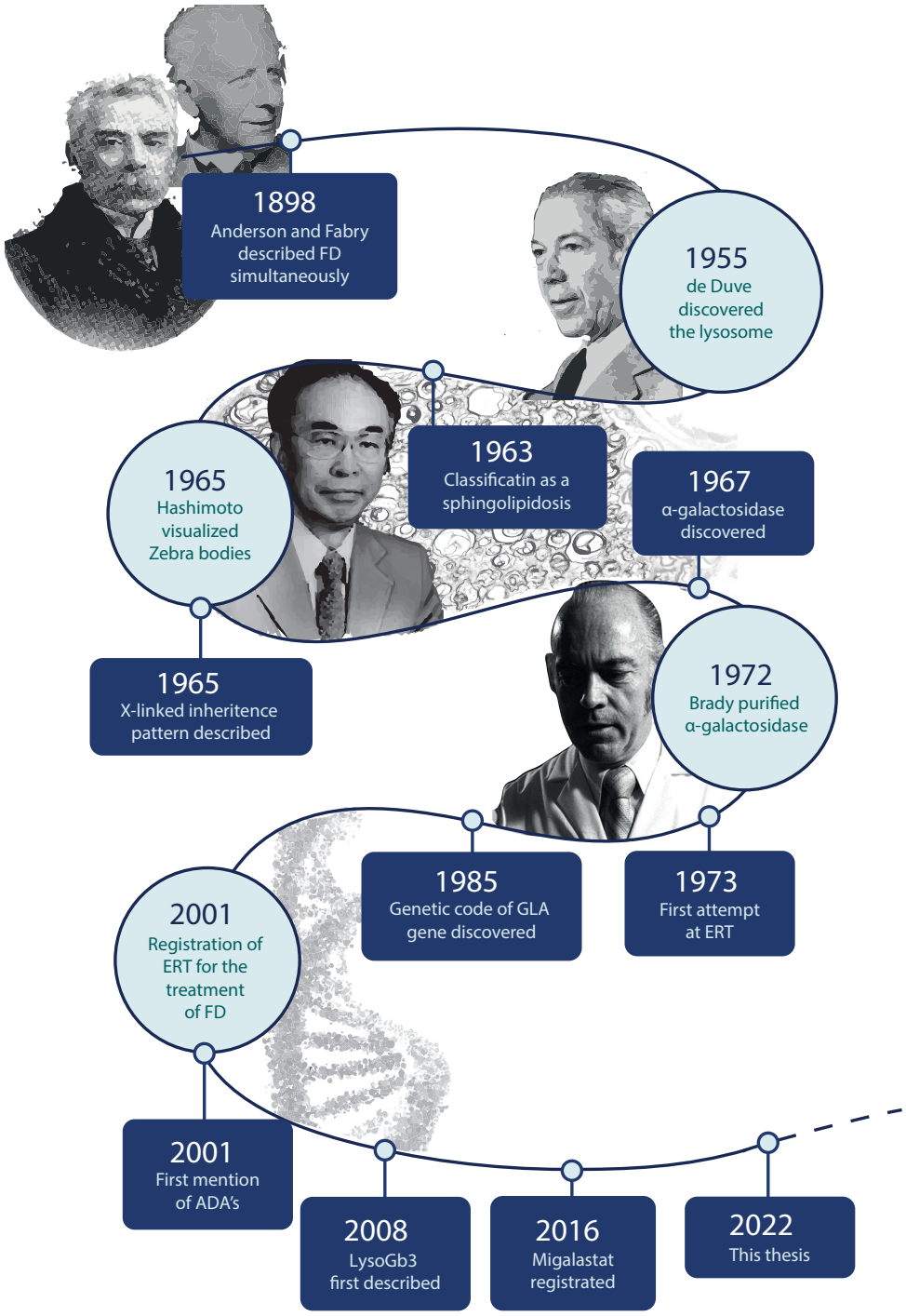


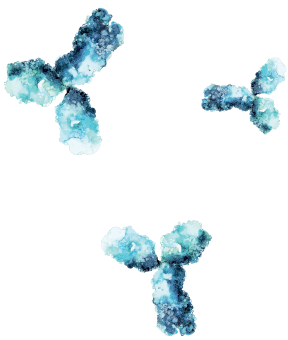
Figure 1: Graphical timeline of key discoveries leading up to this thesis.

Fabry disease phenotypes

In the 1900s, before the clinical availability of enzyme activity assays and genetic testing, Fabry disease was diagnosed by the presence of purple skin lesions (angiokeratomas, hence the alternative name of the disease: angiokeratoma corporis diffusum[2,3]), specific ocular manifestations (called cornea verticillata[7]) and/or the presence of heat induced neuropathic pain (acroparesthesias[8]). This trias of Fabry specific symptoms is now referred to as the 'classical' FD symptoms[9]. In later years, a diagnosis of FD could be confirmed by biopsies[4] (amongst others skin, heart and renal biopsies showing Zebra bodies) or by using enzyme activity assays[10]. In 1985, the genetic code of the gene coding for αGalA (the GLA gene) was deciphered by Calhoun et al.[11], paving the way towards the use of genetic testing in FD diagnostics and the discovery that the phenotypical spectrum of individuals with mutations in the GLA gene is much broader than initially thought. We now know that male patients presenting with one or more classical symptoms tend to have mutations that result in a (near) complete absence of αGalA activity, and a severe clinical phenotype. In fact, classical FD in male patients is nowadays defined by the combination of a classical symptom and a strongly reduced enzymatic activity (<5% of normal[12]). These male classical FD patients develop left ventricular hypertrophy and cardiac fibrosis, leading to heart failure and cardiac arrhythmias. Many of them also suffer from progressive renal failure and strokes. Being an X-linked disease, it was initially thought that only men were affected. Subsequentially, it was shown that females do develop organ manifestations of FD, although clinically relevant FD complications in females do not occur in all patients and manifest later in life compared to their male family members[13]. As a consequence of the increased use of genetic testing in clinical practice, milder (non-classical) phenotypes of FD are increasingly being identified[14]. Most often these patients present with unexplained left ventricular hypertrophy which is why this type of FD is also referred to as the cardiac FD variant. These patients have mutations in the GLA gene that result in misfolded αGalA with higher levels of residual enzyme activity, compared to the classical FD patients. By definition, these patients do not show any of the classical symptoms[12]. Clinically, male patients with non-classical FD were shown to have a disease course similar to female patients with classical FD, while females with non-classical FD rarely seem to develop clinically relevant FD complications[12,13].

The availability of genetic testing also opened the possibility to screen for GLA variants in asymptomatic individuals, either through family- or population screening (such as newborn screening). The upsurge of genetic testing in

asymptomatic individuals vastly increased the number of patients with milder and/or uncertain disease course[15,16], as not every GLA variant results in a decreased αGalA activity and clinical symptoms. Several GLA variants have been described as being benign, based on a lack of specific clinical symptoms[17], high residual enzyme activity and/or normal biochemistry (e.g. normal levels of Gb3 or its deacylated form)[18-20]. These individuals with mutations in the GLA gene are therefore not considered FD patients. We may conclude that the clinical spectrum of Fabry disease is very broad, ranging from patients with a normal life expectancy to patients with severe morbidity and strongly reduced life expectancy. A correct phenotyping of patients into classical and non-classical FD helps to predict disease course, but is not always straightforward or precise. Especially in female patients, disease presentation can vary widely, even between females of the same family, most likely due to random X inactivation[21]. This means that the GLA variant does not predict the disease course in individual female patients. Unfortunately, neither the amount of residual αGalA enzyme activity, nor plasma levels of the αGALA substrate, Gb3, showed a strong relationship with disease severity. In fact, non-classical patients often present with (near) normal plasma Gb3 levels and many female patients become symptomatic despite normal αGalA activity in leukocytes. In 2008, Aerts et al discovered the deacylated form of Gb3, globotriaosylsphingosine (lysoGb3), which was notably increased in all FD patients[22]. This biomarker was later shown to correlate with disease type (classical vs non-classical) as well as sex of the patient[23]. In **chapter 2** of this thesis we studied the course of plasma lysoGb3 levels (in the absence of FD specific treatment) over time in individual FD patients and the relationship of these levels to disease manifestations and progression to see if this biomarker could help improve disease prognostics.



Treatment

Once the underlying cause of Fabry was known, the treatment approach seemed straightforward: supplying patients with the missing enzyme should relieve the symptoms. The idea of administering the deficient enzyme to treat LSDs was first suggested by de Duve in 1964[24]. In the 10 years thereafter, Brady and his group not only discovered alpha galactosidase A (αGalA) deficiency as the cause of Fabry disease[5], but also treated the first Fabry patient with αGalA purified from human placental tissue[25]. This was the first attempt of what we now refer to as enzyme replacement therapy (ERT). After the genetic code of GLA was elucidated recombinant αGalA could be produced en masse in human and animal cell lines. The success of ERT in the treatment for Gaucher disease (another LSD[26]), boosted the expectations regarding ERT for FD and in 2001 recombinant αGalA became commercially available for treatment of FD. Two different preparations were approved, agalsidase-alfa (Replagal, Shire), produced in human fibroblast and dosed at max 0.2mg/kg/eow, and agalsidase-beta (Fabrazyme, Sanofi Genzyme), produced in Chinese hamster ovarian cells and dosed at max 1.0mg/kg/eow. It should be noted that initial marketing authorization was not provided based on improvement on hard clinical endpoints. For agalsidase-alfa[27], a reduction in mean pain score (using the brief pain inventory, BPI) from 3.8 to 2.7 (change of 1.1) in 14 patients in total provided the basis for marketing approval. In comparison, the BPI score in 12 patients treated with placebo went down from 5.4 to 4.7 (mean change of 0.7). For agalsidase-beta[28], marketing authorization was given based on the reduction of Gb3 deposits in endothelial cells from renal biopsies of treated patients, while no effect of treatment on clinical markers was found. More than a decade later, in 2016, a Cochrane review including all the randomized controlled trials (N=9) of these two ERT preparations reported no statistically significant effect of either drug on any clinically relevant endpoint (apart from a small reduction in neuropathic pain score in the agalsidase-alfa study)[29]. A year later the same group published a review including 77 cohort studies with a total of 15,305 patients. For agalsidase-beta, they concluded that there was a significant lower rate of renal, cardiovascular and cerebrovascular complications when compared to placebo cohorts, while no significant effect of treatment on either renal, cardiovascular or cerebral complications was found for agalsidase-alfa[30]. The authors also emphasized that it was not possible to correct for differences in age at start of treatment, sex and FD phenotype in their analyses (besides excluding pediatric patients). The fact that the mean age in the untreated cohorts was 41 years compared to 33 and 34 for agalsidase-beta and agalsidase-alfa cohorts respectively, could in theory have influenced the outcome of the analyses. Also, the authors mentioned that several patients

seemed to be included in more than one study cohort. There are, however, other studies suggesting a benefit of ERT for FD patients. Several studies report that ERT lowers and or stabilizes left ventricular (LV) mass and can stabilize eGFR, but in most patients, disease progression is observed despite treatment[31,32]. One of the causes for the observed limited effect of treatment with ERT could be that patients included in these pivotal trials already suffered from irreversible disease. Several studies following these pivotal trials indeed reported that, when treatment is initiated in the presence of extensive cardiac fibrosis or impaired renal function, disease progression cannot be prevented[33,34]. This led to the international consensus that treatment should be initiated as soon as early signs of organ involvement become present[35]. In this paper authors mention that "The timing of initiation of ERT in young males with classical FD without any symptoms or clinical signs of organ involvement was extensively discussed among panelists". In **chapter 3**, we studied the clinical benefit of starting ERT before the age of 16 on intermediate endpoints in male patients with a classical disease phenotype.

In the years after the introduction of ERT, the search for a more effective and/or less burdensome treatment option for FD continued. These alternative approaches either aimed to further improve ERT (next generation ERT), increase endogenous enzyme activity by improving the folding of mutated enzyme (chaperone therapy), reduce the degree of accumulation of substrate (substrate reduction therapy) or correct the disease at the DNA/mRNA level. In **chapter 4** of this thesis, an in depth review is supplied describing the different treatment approaches and the pros and cons of each approach.

Apart from the limited effectiveness of treatments, another obstacle for patients in many parts of the world are the exceptionally high costs of currently available treatment options, averaging between €200.000 and €400.000 per patient per year[36]. As the patents on the initial ERT preparations have ended, the market is open for biosimilars that could compete in price and perhaps enable the testing of higher doses of ERT in patients to improve treatment efficacy. In **chapter 5** of this thesis, we compared the properties of a generic biosimilar to agalsidase-beta and provide a basis for the future clinical testing of this biosimilar in FD patients.

Anti-drug-antibodies

The downside of infusing patients with any cell-derived protein is the potential immunogenicity of the administered protein, specifically their ability to induce antibody formation (anti-drug-antibodies, ADAs) against the recombinant protein. ADAs can directly inhibit the biological function of a drug, for example by binding to the active site. These ADAs are referred to as inhibiting- or neutralizing antibodies (iADAs) and have been shown to result in treatment failure in several diseases, including Pompe disease[37] and hemophilia[38]. Additionally, ADAs can negatively impact pharmacokinetics of recombinant proteins, as antibody-coated compounds are targeted for cellular clearance. Finally, iADAs can also cause severe allergic reactions and in some cases other serious adverse events, including serum sickness and vasculitis.

In the pivotal trial leading to the marketing authorization of agalsidase-beta for FD, 88% of patients developed ADAs against the drug[28]. More than half of the patients (all antibody positive) experienced infusion-related adverse events, most often fever and cold chills and in some cases hypersensitivity reactions. As reactions could be managed with antihistamines, antipyretics and/or corticosteroids, they were not considered a safety concern by the European Medicine Agency (EMA). Additionally the authors remarked that: "seroconversion did not affect primary or secondary efficacy results, nor did the antibodies have a neutralizing effect"[28]. When taken into account that only 12% of patients (n=7) remained ADA-negative and no effect of treatment on any clinical endpoint was found, evidence for this statement is lacking and in the following years it was shown that ADAs do impact treatment effectiveness in FD. Several studies showed that ADAs did impair the removal of Gb3 depositions from endothelial cells[39], the primary endpoint of the initial clinical study[28]. In 2004 Linthorst et al. found that anti-aGalA ADAs could nearly completely inhibit enzymatic activity in vitro[40]. In the following years it was shown that urinary Gb3 only decreased in ADA negative patients[41,42] and that ADA positive patients had higher levels of the biomarker lysoGb3 during treatment[42]. However, these findings were still not considered convincing enough to prove a negative effect of ADAs on treatment effect by many. In 2016, Lenders et al. showed that ADAs negatively influenced the reduction in LV mass upon treatment[43]. In **chapter 6** of this thesis, we characterized the subclasses of immunoglobulins responsible for the in-vitro inhibition of aGalA in a group of 29 male patients with classical FD. We studied the correlation between the inhibition titer and lysoGb3 reduction in individual patients as well as the effect of iADAs on renal decline.

The recognition of ADA formation as a relevant complication of treatment, prompted the search for a solution. In other LSDs treated with ERT, immunomodulatory protocols using immunosuppressant drugs either failed to induce immune-tolerance in ADA positive patients or required continuous treatment with high doses of immunosuppressant[44-46]. In addition, though ADA titers decreased in Fabry patients that underwent renal transplantation (and the corresponding immunosuppressive treatment protocol), ADA levels rebounded as soon as the immunosuppressive medication was tapered[47]. This suggest that once patients are immunized, tolerization is hard to achieve. An alternative approach would be to try and prevent ADA development in newly treated patients with a high risk for ADA development. Previous studies showed that many patients developed some ADAs against the recombinant enzyme, but the inhibiting ADAs were nearly exclusively present in male patients with a classical disease phenotype. In **chapter 7** of this thesis, we studied additional risk factors for iADA development in male patients with classical FD. Using these risk factors, we build and tested a prediction model to calculate the individual risk of ADA formation in treatment naïve FD patients.

Another approach is the development of recombinant alpha-galactosidase A preparations that are potentially less immunogenic. PEGunigasidase-alfa is a PEGulated version of αGALA and has a substantially increased half-life in plasma compared to the currently available forms of ERT. More continuous exposure to an immunogenic compound could –in theory- lead to reduced immunogenicity. In fact, it's the theory most current immune-tolerance protocols are based on[48,49]. Another variable that influences immunogenicity is epitope availability. In their paper called "Pre-existing anti-drug antibodies in Fabry disease show less affinity for pegunigalsidase alfa", Lenders and co-authors compare the immunogenicity of Pegunigalsidase-alfa to classic ERT and find differences in epitope availability between the two compounds[50]. In **chapter 8** of this thesis we provide an invited commentary on this article in which we highlight the pitfalls in assessing and comparing immunogenicity of different compounds.
In **chapter 9** of this thesis, we provide a summary and discussion of the –in our opinion- most important lessons learned from the research presented in this thesis and elaborate further on the subsequent changes to be implemented in the management of FD in our clinic. We also discuss possible future directions for research, with the ultimate goal to make the management of FD saver, more effective and more efficient.

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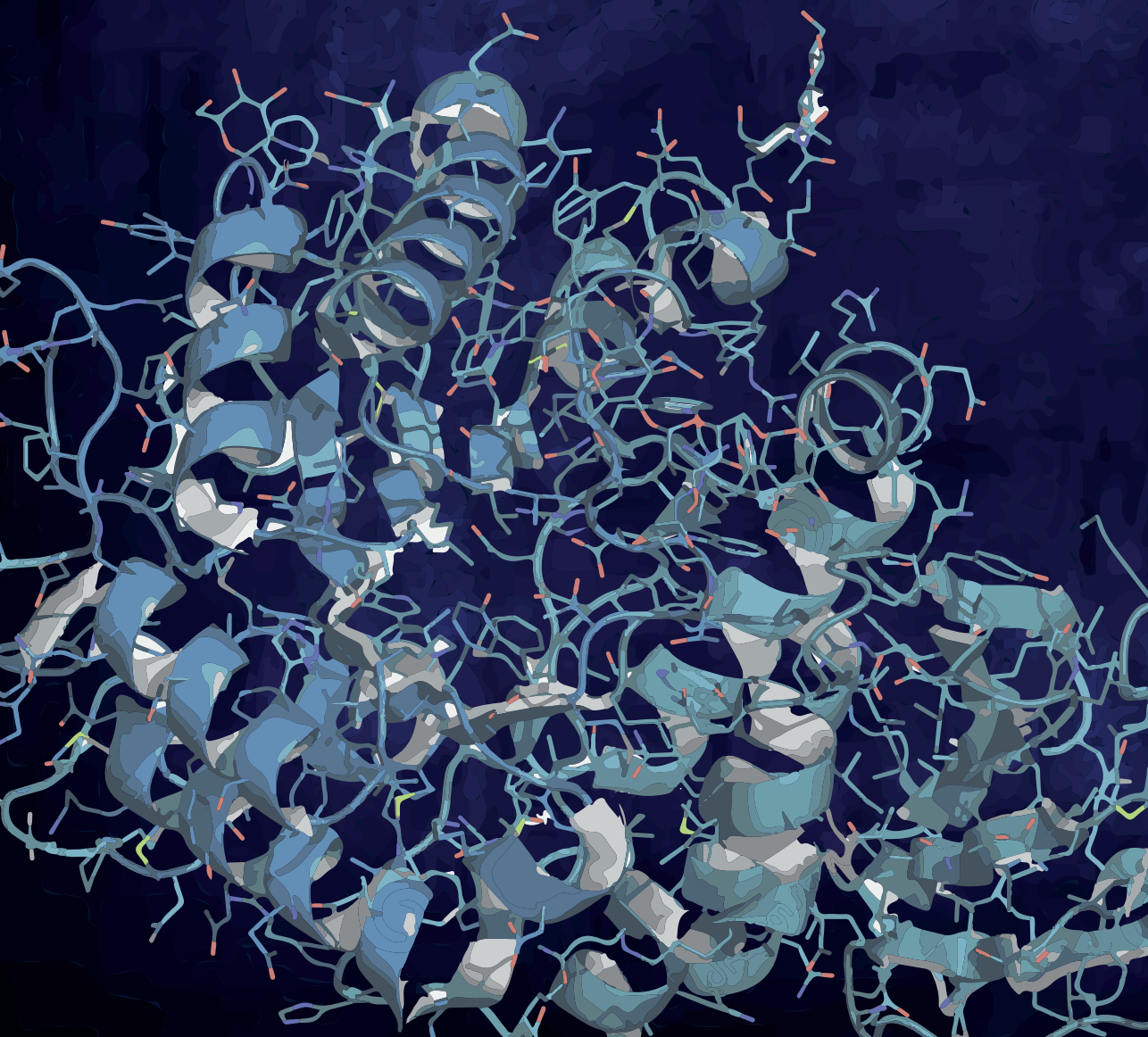
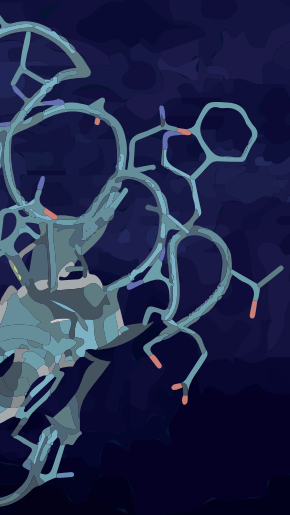
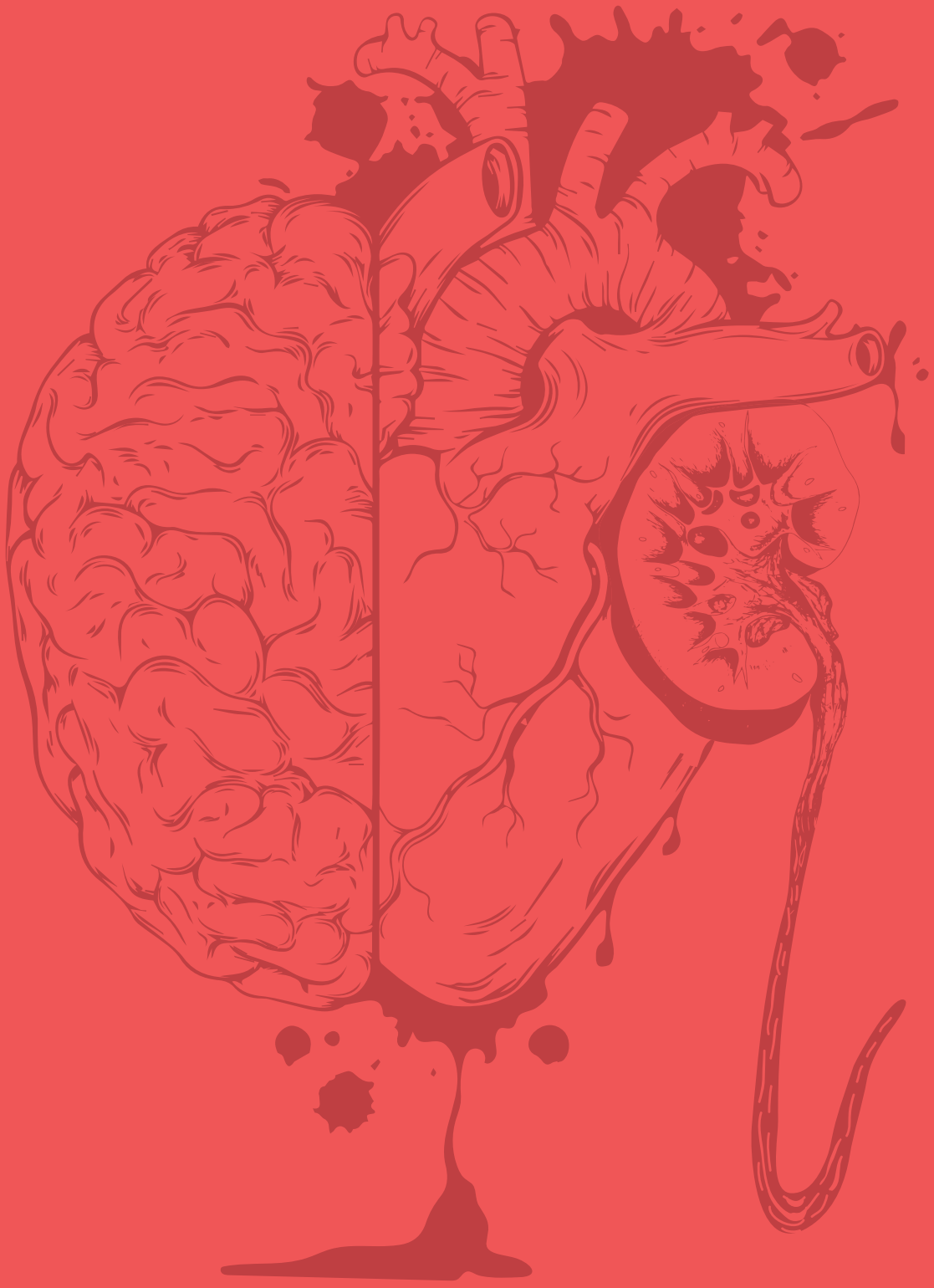
CARE MUST BE TAKEN THAT WE ARE NOT LEFT WITH AN OVERLOAD OF UNDERPOWERED STUDIES OF INSUFFICIENT DURATION, MAKING IT IMPOSSIBLE TO DRAW ANY CONCLUSIONS REGARDING RELATIVE EFFECTIVENESS OF EACH TREATMENT MODALITY.



Part 1

Past & future treatment





Chapter 2

Early risk stratification for natural disease course in Fabry patients using plasma globotriaosylsphingosine levels

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Submitted for publication

Abstract

Background: Fabry disease is a very heterogeneous X-linked lysosomal storage disease. Renal, cardiac and cerebral disease manifestations differ greatly, even between patients of the same sex and with the same disease classification (classical or non-classical). A biomarker with a strong predictive value for the development of disease manifestations is needed to determine the need for Fabry-specific treatment and appropriate frequency of follow-up, since clinical manifestations of the disorder take decennia to develop.

Methods: We investigated the levels of plasma lysoGb3 levels over time and its association with disease -manifestations and -course in 237 untreated FD patients using linear mixed effect models.

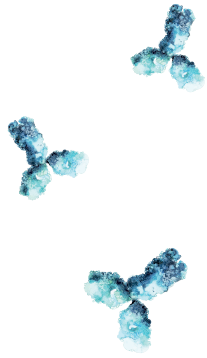
Results: LysoGb3 levels are stable over time in plasma of untreated Fabry patients. Higher levels of lysoGb3 were associated with steeper decline in estimated glomerular filtration rate (eGFR, $p=0.04$) and a faster increase in albuminuria (measured as the urinary albumin to creatinine ratio, UACR, $p<0.001$), left ventricular mass (LVMi, measured on echocardiography, $p<0.001$), left atrial volume index (LAVI, $p=0.003$) and Fazekas score ($p=0.003$). Additionally, regardless of age, higher lysoGb3 levels were associated with higher relative wall thickness (RWT, $p<0.001$) and unfavorable functional markers on echocardiography, including septal mitral annular early diastolic velocity (e', $p<0.001$) and the ratio of early trans mitral velocity (E) to e' (E/e', $p=0.001$).

Conclusion: LysoGb3 is a static, individual FD trait with a close relationship to clinical disease severity. Since it reaches stability well before clinical disease manifestations occur, measuring lysoGb3 at diagnosis provides insight into the expected natural disease course, facilitating clinical decision making.

Introduction

Fabry disease (FD; OMIM 301500) is a rare X-linked lysosomal storage disease, caused by pathogenic mutations in the alpha-galactosidase A (GLA) gene. Depending on the mutation, FD patients can either have total or partial alpha-galactosidase A (αGalA) deficiency, which roughly corresponds with either a classical (more severe) or a non-classical (attenuated) disease phenotype[1]. Due to the X-linked inheritance, female patients develop a milder disease phenotype compared to male patients and some female patients can remain without clinical events up to the 8th decade of life[1,2]. Due to the αGalA deficiency, the lipid Globotriaosylceramide (Gb3) cannot be broken down properly and accumulates in the lysosomes of the cell. The intracellular accumulation of Gb3 and its derivatives is thought to set in motion several pathophysiological processes, ultimately resulting in a variety of clinical symptoms. Left ventricular hypertrophy and myocardial fibrosis often occurs and may result in (diastolic) heart failure and arrhythmias[2], podocytes loss and fibrosis in the kidneys can cause proteinuria and renal failure[3,4] and vascular dysfunction may result in the development of white matter lesions (WMLs), transient ischemic attacks (TIAs) and strokes[5]. Patients with the most severe phenotype (e.g. male patients with a classical disease type), always become symptomatic and seem to benefit from early treatment initiation with enzyme replacement therapy (ERT)[6,8]. Starting ERT in patients with more advanced disease (e.g. with advanced myocardial fibrosis or impaired renal function), no longer seems to alter disease course[9-12]. This supports early treatment initiation. However, while there is no discussion whether or not male patients with classical disease need treatment, the remaining groups contain both patients at risk for complications, as well as those with a (near) normal life expectancy, who will develop no or minimal FD related clinical symptoms[1,2,13]. It's unlikely that this last group would significantly benefit from lifelong biweekly ERT infusions. This poses an important clinical dilemma; not all patients will develop clinically relevant symptoms of FD warranting targeted treatment, but those who will become symptomatic, seem to benefit from early treatment initiation[6]. In addition, for patients presenting with new GLA variants and minor and/or nonspecific symptoms, it's not always clear whether the variant is disease causing- or benign, especially in women, in whom enzyme activity can be normal. Therefore, in current clinical practice, many patients are in routine follow up to identify early signs of organ manifestations so that the window of opportunity for treatment will not be missed. In order to reduce over-medicalization of patients and to improve efficiency of clinical care, there is a need for a tool to stratify FD patients by their risk for developing complications. Levels of Globotriaosylsphingosine (lysoGb3), the water soluble, deacylated form

of Gb3, are strongly associated with both disease type (classical or non-classical) and sex of the patient[14,15]. Additionally, lysoGb3 was found to be associated with several disease severity parameters in small studies, including left ventricular mass[16,17], white matter lesions[17] and overall disease severity measured using the Mainz Severity Score Index (MSSI)[14,16,17]. Interestingly, even in patients that share the same GLA variant, those with higher lysoGb3 levels were more severely affected compared to those with lower plasma lysoGb3 levels[16]. In a more recent study, high lysoGb3 levels were linked to an increased risk for the development of clinically relevant endpoints, including kidney replacement therapy, ICD/pacemaker implantation and cerebrovascular events[18]. However, most studies were small, cross sectional and some included both treated and untreated patients. It is currently unknown whether plasma lysoGb3 levels are stable throughout life and represent an individual patient trait that can be assessed at any time, or that it increases/decreases with age. We hypothesized that, in untreated FD patients, plasma lysoGb3 remains stable and reflects how severely an individual FD patient is or will be affected. In this study, we investigated whether or not plasma lysoGb3 levels remained stable over time in untreated FD patients, we defined optimum cut-off values to classify patients (classical vs non-classical) and we used linear mixed models to test the relation of plasma lysoGb3 levels to disease manifestations and progression.



Materials and Methods

Patients

This study was conducted in accordance with the principles of the Helsinki Declaration, as revised in 2013. Data were collected retrospectively from clinical records at the Amsterdam University Medical Center (Amsterdam UMC), location AMC, the national referral center for FD in the Netherlands. All included patients had a confirmed FD diagnosis and all patients and/or legal guardian signed informed consent. Data included basic diagnostic data, clinical and biochemical parameters, medication use, and the presence of comorbidities and cardiovascular risk factors. The risk factor 'Smoking' was scored as present in case of current or former smoking. Obesity was defined as a body mass index >30kg/m2 at time of lysoGb3 analyses. Hypertension was defined as either a previous diagnosis of hypertension treated with anti-hypertensive medication or an increased systolic or diastolic blood pressure measured on at least two occasions (i.e. 140/90 mmHg).

Patients were classified as having a classical or non-classical phenotype as previously described by Arends et al[1]. A flow chart used to phenotype classification in clinical practice is added in the supplemental material (SM2).
To study the association of plasma lysoGb3 with the natural disease course, only data obtained before the start of ERT or any other Fabry specific treatment were included in this study.

Laboratory measurements

Plasma lysoGb3 (nmol/l) was measured by tandem mass spectrometry, as previously described[19]. For reproduction purposes, a detailed description of our assay is added as supplemental material (SM3). All available untreated lysoGb3 values were used to assess stability in individual patients over time. Only the last available untreated value of lysoGb3 was used to assess its association with markers of disease severity. For the sake of visualization only (figures 1-6) patients were grouped in 4 different groups. Groups are not included in any of the statistical models. The cutoffs of 2.3 nmol/L and 40 nmol/L were chosen as they differentiate between classical and non-classical disease in female and male patients respectively. 7.3 nmol/L was chosen to split the remaining groups (predominately men with non-classical FD and women with classical FD) into equal halves to visualize the effect within patient groups with the same disease phenotype. A separate table of patient characteristics for each visual group is added in the supplemental material (SM1).

Glomerular filtration rate was estimated (eGFR) in mL/min/1.73m2 using CKD-EPI formula. Patients <18 years were excluded from this analysis as the formula can be used in adults only. (Micro)albuminuria was assessed using the urinary albumin to creatinine ratio (uACR, mg/mmol). For analyses, uACR was included as a continuous variable. If urinary albumin was below the level of detection, uACR was set to be 0.

The following patients were excluded from the analysis of all renal outcome parameters: patients with a renal transplantation at time of presentation (n=5), as well as patients (n=3) with a confirmed second renal disease that was considered to be the main reason for the decline in renal function (i,e, renal artery stenosis; acute glomerulonephritis and severe bilateral kidney atrophy of uncertain origin).

Echocardiograms

Left ventricular mass index (LVMI) was calculated using the Devereux formula and was corrected for body surface area (BSA) using the Dubois formula. For the calculation of LVMI and relative wall thickness (RWT), longitudinal data, extracted from clinical echocardiography reports, were used. In a subset of patients (those with at least two echocardiograms with a minimum of 5 years between them) extensive re-evaluation of a large number of echocardiography markers was performed (as part of a different study on the development of cardiac manifestations in FD) by a single observer (MES). If Fabry specific treatment was started between the first and last echocardiogram in this study, only the data of the first echo were used for analysis. From this dataset we selected the following markers of diastolic dysfunction: e' (as a marker of diastolic relaxation), E/e' (As a marker of left atrial filling pressure) and LAVI (as a marker for the duration of the increased LA pressure).

Cerebral MRI

Cerebral MRI data obtained at our site for a previous study were used, see[13] for more detailed information. All MRI data were obtained using 3T scanners. Scans were assessed by an independent neuroradiologist blinded for all identifying data as well as scan order. WMLs were defined as hyper intensities on axial T2-weighted and fluid-attenuated inversion recovery-weighted (FLAIR-weighted) imaging without cavitation. Assessment was done visually using the Fazekas scale[20]. Each scan was given a score between 0 and 6, depending on the severity of WML at 2 different locations: periventricular and deep. Both locations were attributed a score between 0 (no WML) and 3 (severe confluent WMLs).

Statistics

For statistical analysis and model building, R (version 4.0.3) was used. Packages 'ggplot2' and 'ggpubr' were used for visualization, packages 'data.table', 'tidyverse' and 'lubridate' were used to organize data. 'lme4' and 'lmerTest' were used to perform linear mixed effect model analyses. Optimal cutoffs for phenotyping were determined visually and checked with sensitivity and specificity calculations. All other analyses are done using Linear mixed effect models correcting for multiple measurements using patient ID as a random variable (random intercept). LysoGb3 was transformed (Log10) to improve fit of the models. Models were build using manual forward selection of variables. For each variable the interaction between lysoGb3 and age were tested to calculate the effect of lysoGb3 on slope. Next we tested the effect of sex (male/female) and the presence of any cardiovascular risk factor (e.g. one or more of the following risk factors were present: hypertension, obesitas, smoking). Only variables that significantly influenced the model ($p<0.05$) were included in the final model. Assumptions for linear regression were checked. To check if the observations in male patients with classical FD were not the sole factor driving the associations, all analyses were repeated after removing classical male FD patients from the dataset. For some variables (functional markers on echocardiogram and Fazekas score on MRI) insufficient data of untreated male patients with classical FD above the age of 30 were available (since they are usually diagnosed young and start treatment early). For these variables analyses were only performed without this group. A markdown file of the complete statistical approach and outcome in R is included in the supplemental material (SM 4). A complete summary of the analyses and outcomes are presented in Table 2.

Results

Patient characteristics

Patient characteristics of the 237 included FD patients are outlined in table 1.

	MALE FD (N=89)	FEMALE FD (N=148)
Disease classification		
classical	54 (61%)	108 (73%)
non-classical	35 (39%)	40 (27%)
Mutatation type		
Nonsense/frameshift	24 (27%)	44 (30%)
missense	60 (67%)	98 (66%)
others	4 (5%)	6 (4%)
Plasma lysoGb3 (nmol/L)	82 (1 - 178)	5 (0.4 - 41)
Log10(1+lysoGb3)*	1.9 (0.3-2.3)	0.8 (0.1-1.6)
Age of last untreated lysoGb3	40 (3 - 71)	44 (13 - 76)
Plasma lysoGb3 categories**		
< 2.3 nmol/L (n=42)	7 (8%)	35 (24%)
2.3 – 7.3 nmol/L (n=71)	5 (5%)	66 (44%)
7.3 – 40 nmol/L (n=70)	24 (27%)	46 (31%)
> 40 nmol/L (n=54)	53 (60%)	1 (1%)
Cardiovascular risk factors		
Smoker (former or current)	38 (43%)	57 (39%)
Hypertension	22 (25 %)	32 (22 %)
Body mass index >30 kg/m^2	7 (8 %)	30 (20 %)
Any of the above present	50 (56%)	82 (55%)

Table 1. *Baseline characteristics of included FD patients. Data are presented as number (percentage) or median and range, as appropriate.*

Missing data: mutation type (n=1), male patient with clear clinical and biochemical FD characteristics but no mutation found in the coding GLA sequence; smoking (n=26); hypertension (n=6)

** Transformed lysoGb3 values as they are used in the statistical models.*

*** Patients were divided into groups for visualization purposes only. Groups are not included in any of the statistical models.*

Stability of plasma lysoGb3 over time in individual untreated patients

In individual FD patients not treated with FD specific therapy, plasma lysoGb3 levels remain stable over time throughout life (p=0.6 for change over time, fig.1). Median follow up time is 3.7 years (range 0.1-16 years). The exact age at which lysoGb3 stabilizes could not be determined because of the low number of measurements in very young patients.

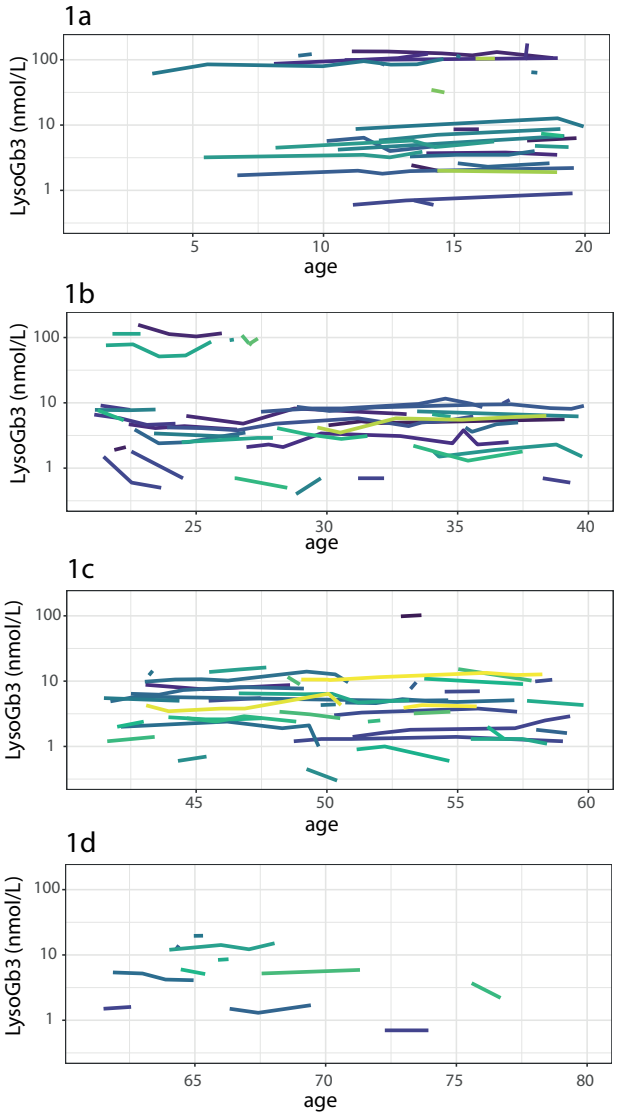


Figure 1. *LysoGb3 in individual untreated FD patients over time. Only patients with at least 2 separate untreated measurements are displayed to visualize change over time.*

The earliest measurements were at the age of 3 years (a) Patients aged 3-20 years (n= 36). (b) Patients aged 21-40 years (n=36). (c) Patients aged 41-60 years (n=40). (d) Patients aged 61-80 years (n=11). Colored lines represent individual patients.

Untreated lysoGb3 in plasma as a marker for disease phenotype

All but one of the 237 FD patients had plasma lysoGb3 levels above the reference range (0.3-0.5 nmol/L). The one female patient with a normal plasma lysoGb3 level (0.3 nmol/L) had a pathogenic GLA variant associated with a non-classical phenotype in her male relatives. At her last evaluation (aged 39) she had no clinical signs or symptoms associated with FD. Plasma lysoGb3 levels >40 nmol/L separated male patients with classical disease from male patients with non-classical disease with 98 % sensitivity and 100 % specificity (P<0.001, PPV= 98%, NPV= 100%, fig 2). Plasma LysoGb3 levels > 2.3 nmol/L separated female patients with classical disease from female patients with non-classical disease with 98% sensitivity and 83% specificity (P<0.001, PPV=94%, NPV= 94%)

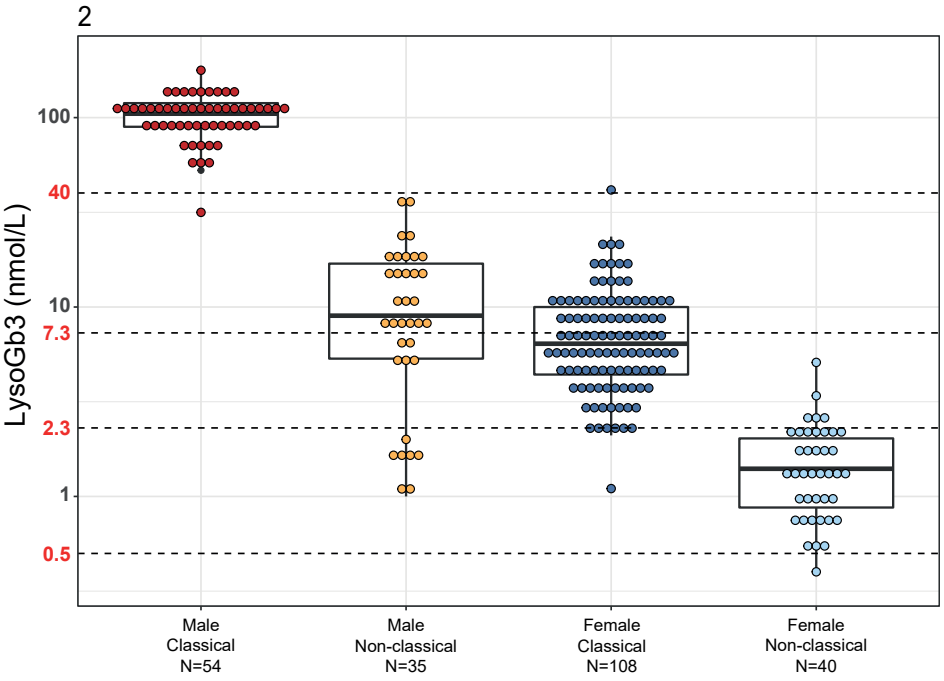
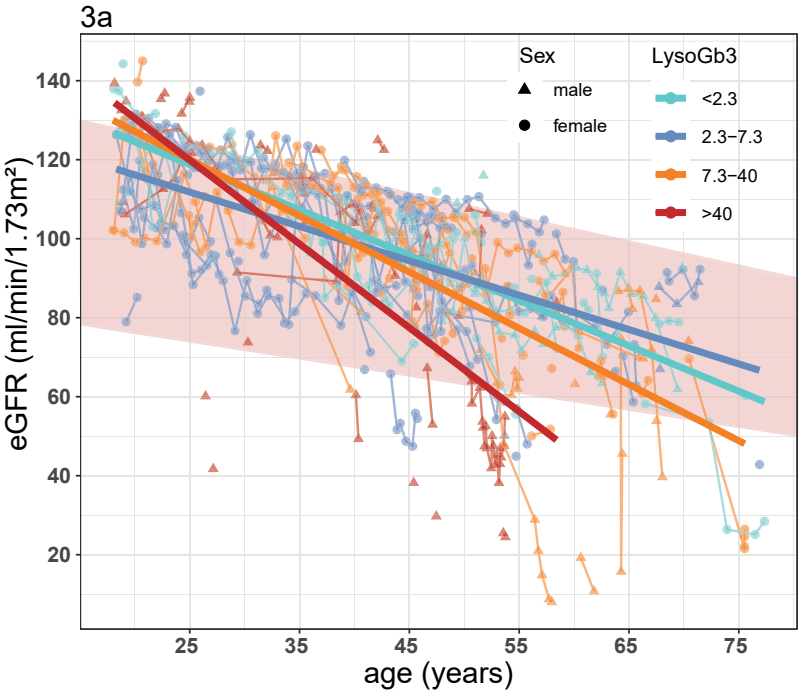


Figure 2. *Plasma lysoGb3 levels in Fabry patients with different phenotypes. Dotted lines represent upper range of normal (0.5 nmol/L), best cut off value to differentiate between classical and non-classical FD in female patients (2.3 nmol/L) and the best cut off value to differentiate between classical and non-classical FD in male patients (40).*

Association of plasma lysoGb3 with renal manifestations in untreated FD patients

Higher lysoGb3 levels were significantly associated with steeper eGFR slopes in all FD patients of 18 years and older (n= 202, median numbers of measurements 2 per patient, range 1-20) (fig 3a, p=0.04). As male patients with classical FD are known to be most at risk for accelerated decline in renal function, analyses were also performed after exclusion of this patient group. The association remained statistically significant (p=0.005).

Higher lysoGb3 levels were also significantly associated with a faster increase in albuminuria (n=198, median 2 measurements per patient, range 1-16) (fig 3b, p<0.001), though this association seemed to be driven largely by male patients with a classical phenotype as the association was no longer statistically significant after exclusion of this group (p=0.09). Adding sex of the patient or the presence of cardiovascular risk factors to either of the models did not significantly impact the outcome and was thus not included in the final analyses. For full results see table 2.


3a
Sex
male
female
LysoGb3
<2.3
2.3–7.3
7.3–40
>40
eGFR (ml/min/1.73m²)
140
120
100
80
60
40
20
25
35
45
55
65
75
age (years)

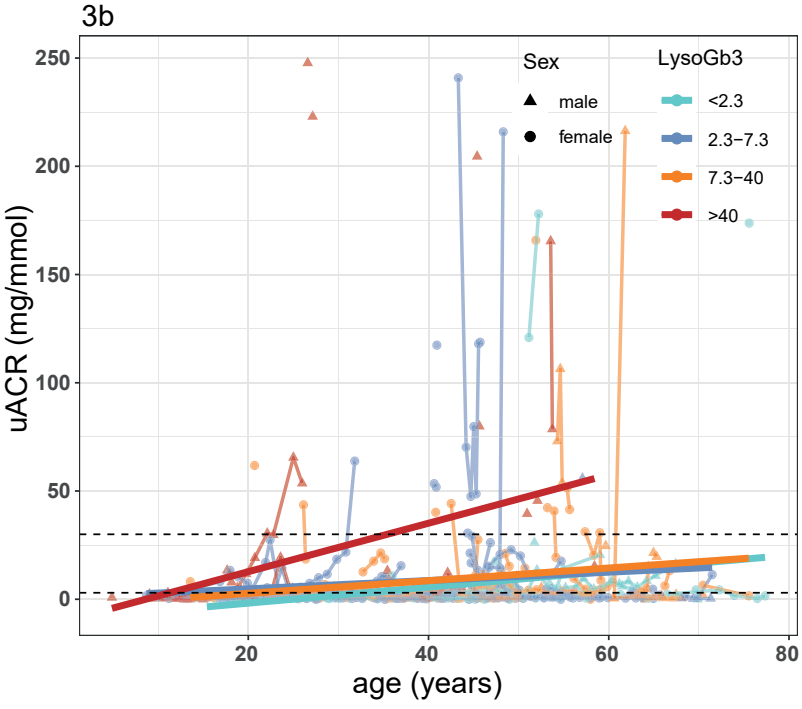
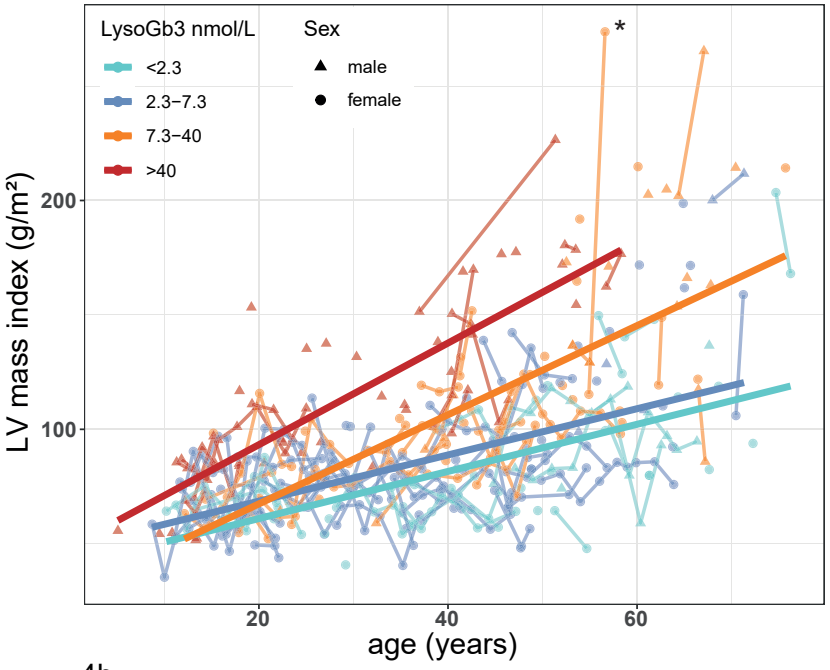

3b
Sex
male
female
LysoGb3
<2.3
2.3–7.3
7.3–40
>40
uACR (mg/mmol)
250
200
150
100
50
0
20
40
60
80
age (years)

Figure 3. *Relation between lysoGb3 and the progression of renal manifestations in untreated Fabry patients. All analyses are performed with actual plasma lysoGb3 levels (after log10 transformation). Grouping is done for visualization purposes only. (a) Association between plasma lysoGb3 and eGFR slope in untreated patients, the pink reference area visualizes the approximate 95th percentile eGFR range in healthy subjects, extrapolated from Baba et all[21]. (b) Association between plasma lysoGb3 levels and progression of albuminuria (uACR) in untreated Fabry patients.*

Association between plasma lysoGb3 and left ventricular mass and relative wall thickness on echocardiography.

In untreated FD patients, plasma lysoGb3 levels were significantly associated with the increase in left ventricular mass over time (indexed to body surface area, LVMi) on echocardiogram ($p<0.001$, n=192, median number of measurements: 1 per patient, range 1-12, fig 4a). Aside from lysoGb3 levels, having one or more cardiovascular risk factors (hypertension, smoking or obesity) was an independent risk factor for higher LVMi ($p=0.003$). Using the estimates in table 2, we can calculate that left ventricular mass of a patient with a plasma lysoGb3 value of 1 increases with 0.78g/m2 every year, compared to a yearly increase of 2.5g/m2 for a patient with a lysoGb3 of 100 nmol/L. Removing male patients with classical FD from the analyses did not change the outcome ($p<0.001$ for lysoGb3, $p=0.005$ for cardiovascular risk factor). Higher lysoGb3 levels were significantly associated with higher relative wall thickness (RWT), as a sign for concentric remodeling, at any age ($p<0.001$, fig. 4b). No association between the plasma lysoGb3 levels and the rate of increase of RWT was found. Female patients had a significantly higher RWT compared to male patients ($p=0.04$). Removing male patients with classical FD from the analyses did not change the outcome ($p=0.001$).

4a


LysoGb3 nmol/L
<2.3
2.3−7.3
7.3−40
>40
Sex
male
female
*
LV mass index (g/m²)
200
100
20
40
60
age (years)

4b

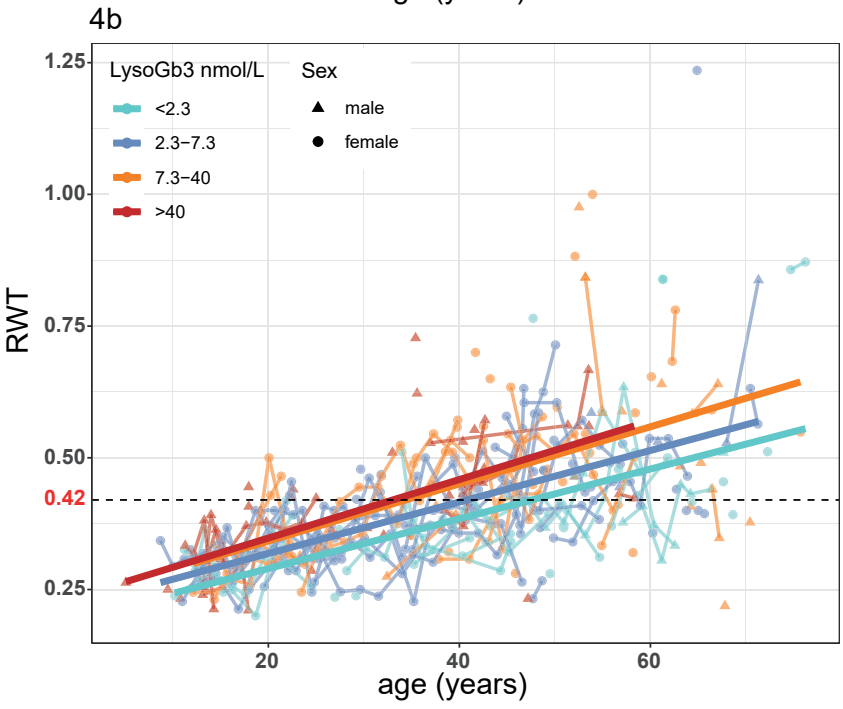

LysoGb3 nmol/L
<2.3
2.3−7.3
7.3−40
>40
Sex
male
female
RWT
1.25
1.00
0.75
0.50
0.42
0.25
20
40
60
age (years)

Figure 4. *(a) Relation between lysoGb3 and the increase (slope) of left ventricular mass (LVMi) over time in untreated FD patients. Reference ranges of normal LVMi are 49-115 g/m2 for males and 43-95 for females, >149g/m2 is considered grossly abnormal in males and >122 g/m2 for females[22]. (b) Association of plasma lysoGb3 with RWT on echocardiography in untreated Fabry patients. RWT was higher in patients with higher lysoGb3 values, but there was no difference in slope over time. Values above >0.42 suggest concentric remodeling[22]. All analyses are performed with actual plasma lysoGb3 levels (after log10 transformation). LysoGb3 levels are grouped for visualization purposes only as described in the legend*

**Patient suffered severe cardiac decompensation between first and last assessment. Patient was repeatedly admitted to ICU after last assessment and passed away 2 years later (due to heart failure). In addition, quality of the echocardiograms in this patient were described as suboptimal. Removing the patient from analyses did not change the outcome of the analysis.*

Plasma lysoGb3 and the functional parameters on echocardiography.

Functional parameters on echocardiography were assessed in a subset of patients. Insufficient data of male patients with classical FD, especially above the age of 30, were available to be able to include them in these analyses on the relationship between plasma lysoGb3 levels and these parameters. In the remaining patient groups (145 patients, median number of measurements per patient was 1, range 1-2), we studied the relationship between plasma lysoGb3 levels and functional echocardiography parameters related to diastolic dysfunction and heart failure with preserved ejection fraction (HFpEF)[23]. Higher plasma lysoGb3 levels were significantly related to lower e' (mitral annular early diastolic velocity, $p<0.001$, fig5a) as well as higher E/e' (the ratio between early mitral inflow velocity and mitral annular early diastolic velocity, $p=0.008$, fig 5b) at any age. No significant association of lysoGb3 on progression over time (slope) for these markers was found. Higher plasma lysoGb3 levels were significantly related to a faster increase in left atrial volume index (LAVI, $p=0.001$) over time.

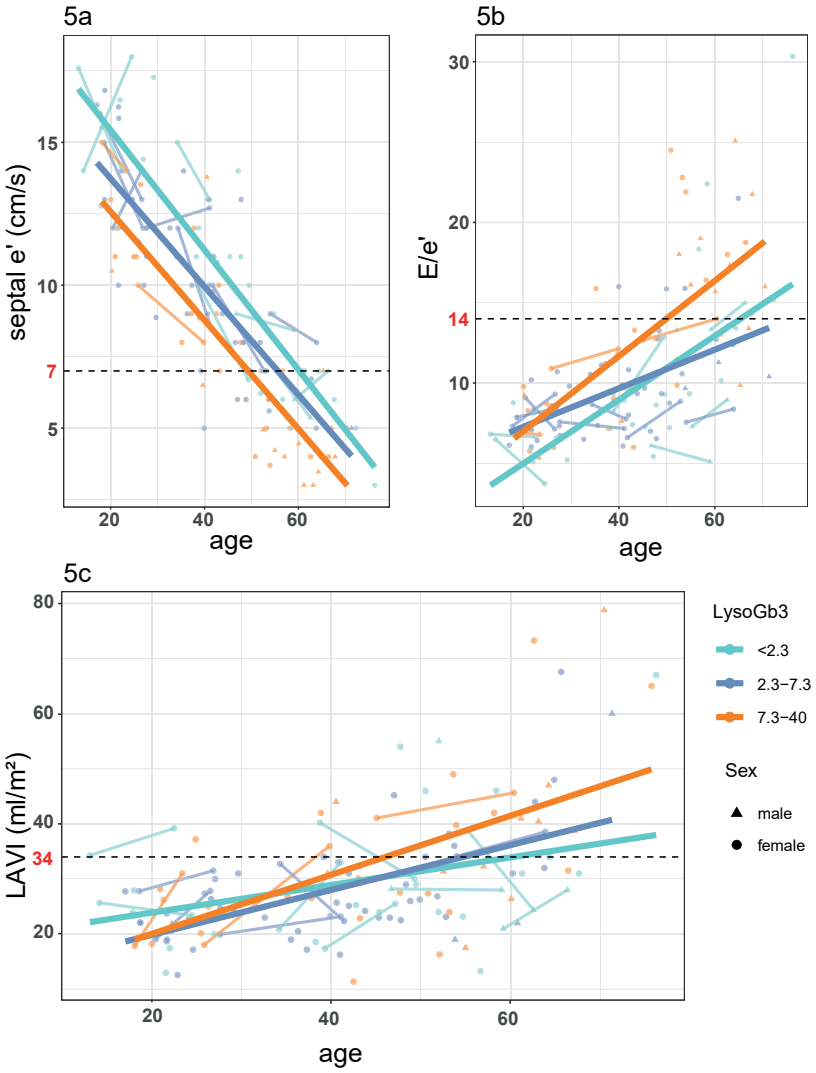


Figure 5. *Association between plasma lysoGb3 levels and functional parameters on echocardiography in untreated FD patients. (a) Association of plasma lysoGb3 with e' (p<0.001). e' represents the velocity of mitral annular motion during early diastole, and is a marker for myocardial relaxation. Patients with higher lysoGb3 values had lower e' (suggesting stiffer LV) at any age, there was no difference in slope (b) Association of plasma lysoGb3 with E/e' (p<0.001). E/e' indicates the ratio between mitral inflow velocity during early diastole (E) and e', and represents a marker for left atrial filling pressure. Patients with higher lysoGb3 values had higher E/e' (suggesting higher filling pressure) at any age, there was no difference in slope (c) Association of plasma lysoGb3 and left atrial volume index (LAVI). Higher lysoGb3 levels were associated with a faster increase over time (p=0.003). The dotted lines in every figure represent the*

cutoff values for diastolic dysfunction as recommended by the European Association of Cardiovascular Imaging/American Society of Echocardiography (EACVI/ASE)[24]. In a healthy heart, relaxation of the LV causes a high velocity of the mitral annulus during early diastole (high e') resulting in blood being 'sucked' from the LA into the LV. Under these circumstances, E/e' is low, usually <8. In the presence of diastolic dysfunction due to LV hypertrophy and stiffening, the LV does not relax properly (e' becomes lower), and as a result E/e' increases. E/e' >14 is highly suggestive of elevated filling pressures. Chronic increase of LA pressure resulting LA dilatation, as indicated by an increased LAVI. All analyses are performed with actual plasma lysoGb3 levels (after log10 transformation). LysoGb3 levels are grouped for visualization purposes only as described in the legend

Correlation between plasma lysoGb3 and Fazekas score on brain MRI

We studied the association between plasma lysoGb3 levels and Fazekas score (1-6) on cerebral MRI, a reflection of the white matter lesion load (WML), in untreated FD patients (n=77, median measurements 2 per patient, range 1-7). Higher plasma lysoGb3 levels were significantly associated with a faster increase in WML over time (fig 6, p=0.003).

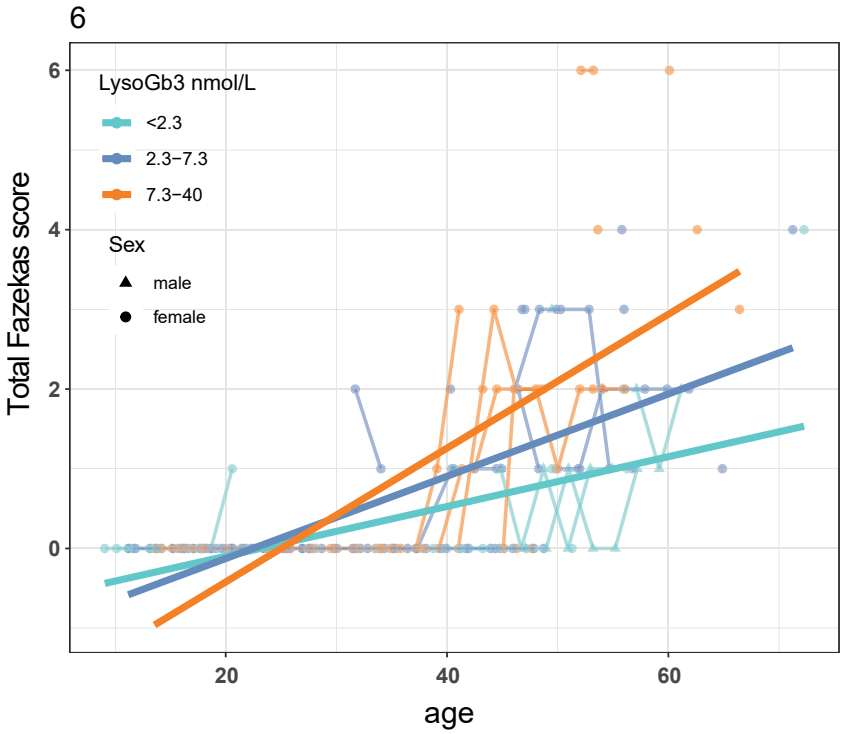
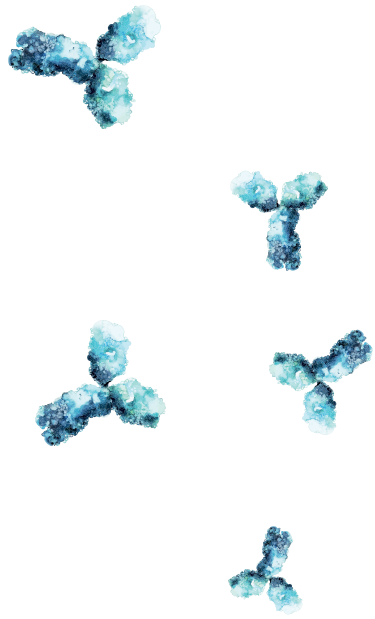


Figure 6. *Association of plasma lysoGb3 with the progression of white matter lesions (measured using Fazekas score) on MRI in untreated patients (n=77).*

FD disease manifestation	*Included variables In analyses*	*Estimate Of effect*	*Confidence Interval (95%)*		*P value*
			Min	*Max*	
eGFR (mL/min/1.73m2)					
- Including CMFD*	$_{Log10}$(LysoGb3)	3.64	-6.95	14.1	0.494
	Age (years)	-1.08	-1.35	-0.83	**<0.001**
	$_{Log10}$(LysoGb3)*Age	-0.26	-0.511	-0.01	**0.037**
- Excluding CMFD*	$_{Log10}$(LysoGb3)	18.6	-1.78	39.1	0.074
	Age (years)	-0.85	-1.17	-0.53	**>0.001**
	$_{Log10}$(LysoGb3)*Age	-0.58	-0.98	-0.18	**0.005**
$_{Log10}$(uACR*)					
- Including CMFD*	$_{Log10}$(LysoGb3)	-0.17	-0.42	0.08	0.190
	Age (years)	-0.001	-0.008	0.006	0.852
	$_{Log10}$(LysoGb3)*Age	0.013	0.006	0.019	**<0.001**
- Excluding CMFD*	$_{Log10}$(LysoGb3)	0.82	0.204	1.433	**0.009**
	Age (years)	0.02	0.006	0.026	**0.002**
	$_{Log10}$(LysoGb3)*Age	-0.01	-0.023	0.002	0.087
LVMi (g/m^2)					
- Including CMFD*	$_{Log10}$(LysoGb3)	-2.839	-16.23	10.39	0.674
	Age (years)	0.471	0.077	0.858	**0.017**
	$_{Log10}$(LysoGb3)*Age	1.022	0.682	1.366	**<0.001**
	Any CV risk factor	11.09	3.760	18.50	**0.003**
- Excluding CMFD*	$_{Log10}$(LysoGb3)	-23.61	-57.53	10.11	0.170
	Age (years)	0.075	-0.500	0.643	0.794
	$_{Log10}$(LysoGb3)*Age	1.582	0.893	2.276	**<0.001**
	Any CV risk factor	12.20	3.834	20.68	**0.005**
RWT					
- Including CMFD*	$_{Log10}$(LysoGb3)	0.079	0.037	0.120	**<0.001**
	Age (years)	0.006	0.005	0.007	**<0.001**
	Sex (male)	-0.053	-0.102	-0004	**0.035**
- Excluding CMFD*	$_{Log10}$(LysoGb3)	0.102	0.040	0.163	**0.001**
	Age (years)	0.006	0.005	0.007	**<0.001**
	Sex (male)	-0.045	-0.102	0.011	0.115
e' (cm/s)					
- Excluding CMFD*	$_{Log10}$(LysoGb3)	-2.780	-0.209	-0.171	**<0.001**
	Age (years)	-0.190	-3.717	-1.844	**0.001**
E/e'					
- Excluding CMFD*	$_{Log10}$(LysoGb3)	0.176	1.269	5.160	**0.001**
	Age (years)	3.212	0.138	0.214	**<0.001**
LAVI (ml/m^2)					
- Excluding CMFD*	$_{Log10}$(LysoGb3)	-18.69	-35.61	-1.778	**0.031**
	Age (years)	0.014	-0.266	0.294	0.921
	$_{Log10}$(LysoGb3)*Age	0.534	0.190	0.879	**0.003**
Fazekas score					
- Excluding CMFD*	$_{Log10}$(LysoGb3)	-1.612	-3.434	0.177	0.079
	Age (years)	0.011	-0.021	0.043	0.485
	$_{Log10}$(LysoGb3)*Age	0.062	0.021	0.103	**0.003**

Table 2. *Summary of statistical analyses for every included disease manifestation of FD. All analyses are done using linear mixed effect models correcting for multiple measurements using patient ID as a random variable (random intercept). LysoGb3 was transformed (Log10) to improve fit of the models. For each analyses the variables 'sex' and 'presence of cardiovascular risk factors' (e.g. one or more of the following*

risk factors were present: hypertension, obesity, smoking) were tested alongside age and individual lysoGb3 values (lysoGb3 was used as a continues variable). Apart from age and lysoGb3 value, only variables that significantly influenced the model ($p<0.05$) were included in the final model. ***P values below 0.05 are underlined****.*
**CMFD classical male Fabry patients, To assess if the effect remains after excluding male patients with classical FD analyses are performed with and without this group. For some variables, we lacked sufficient data of untreated classical male patients above the age of 30.*
**uACR was measured as mg/mmol before transformation to logscale (log10)*



Discussion

In this study, we show for the first time that plasma lysoGb3 stays stable over decades in individual FD patients from childhood onwards (figure 1). Additionally, we showed that plasma lysoGb3 levels are associated with either the severity or progression of nearly all measured FD manifestations. Combined, these findings suggest that plasma lysoGb3 reaches a stable level early on, and indicates which disease burden can be expected later in life. This is in contrast to Gb3 accumulation in -for example- podocytes, which has been shown to slowly increase over time[25]. Interestingly, after start of ERT, plasma lysoGb3 levels reach a new equilibrium within a year after start of treatment (in the absence of anti-drug antibodies)[26]. Once this new level is established, it only changes if the ERT dose is changed[26] or treatment is stopped. Why plasma lysoGb3 remains stable in the untreated state and rapidly reaches a new equilibrium during treatment is currently unknown.

Clinically, FD is a slowly progressive disease and children/young adults do not always show clinical signs of the disorder yet. Our data suggest that measuring plasma lysoGb3 at the time of diagnosis could help predict the disease course in an individual patient, irrespective of the age at diagnosis. The strict association of plasma lysoGb3 with disease type (e.g. classical or non-classical disease) – regardless of the age of analysis– further supports this observation (figure 2[14,17,27,28]). In fact, we showed that plasma lysoGb3 levels can differentiate between the classical and non-classical phenotype with 99% accuracy in male patients and 92% accuracy in female patients and could thus be used as the decisive parameter to determine the disease type in an individual with a GLA variant of unknown significance.

We confirmed the reverse association of plasma lysoGb3 with decline in renal function (figure 3a[14,16]) and verified that this association was not mainly driven by male patients with the classical disease type (who are known to have more renal involvement compared to patients with the other phenotypes). A similar effect was found when looking at albuminuria: higher lysoGb3 levels in plasma were associated with a faster increase in uACR (figure 3b). The strongest association we found, is that between plasma lysoGb3 levels and increase in LVMi (figure 4a[16,17]). In non-FD patients, LVH is strongly associated with overall mortality, myocardial infarction and stroke[29]. In FD patients, higher LVMi on echocardiography was also associated with higher clinical event rate[9]. A new finding is the association of plasma lysoGb3 with markers of diastolic (dys)function, measured by echocardiography (e', E/e' and LAVI). This is particularly relevant since heart

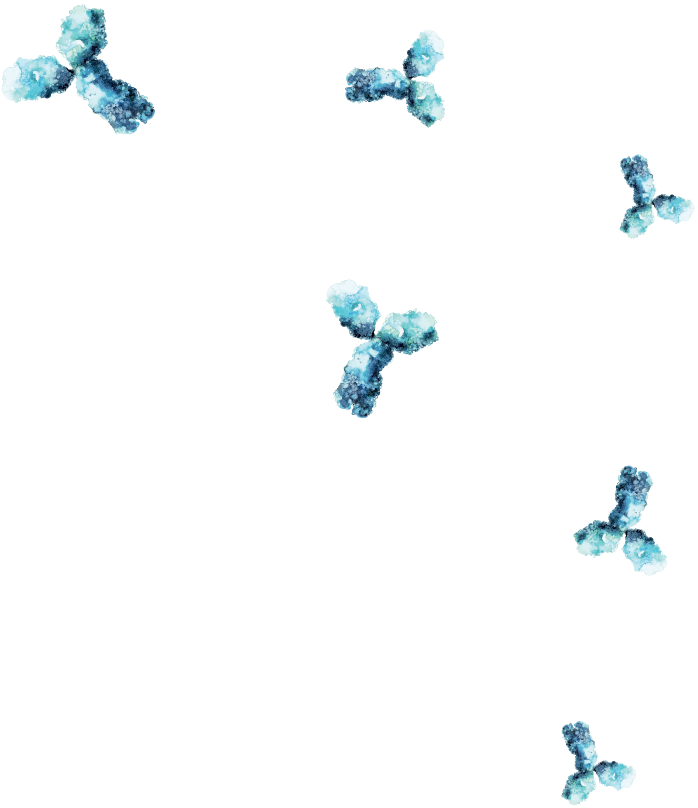
failure with preserved ejection fraction (HFpEF), the clinical syndrome caused by diastolic dysfunction, is a major cause of heart failure in FD[30] and heart failure is currently the leading causes of symptoms and death in FD patients[2]. In many other diseases, higher e', E/e' and LAVI values are strongly associated with increased mortality and increased occurrence of clinical cardiac endpoints[31-34]. The associations indicate that lysoGb3 does not only have a relationship with cardiac morphology, but also with cardiac function and the risk of developing HFpEF. Lastly, we found that higher lysoGb3 levels were associated with a faster progression of white matter lesions in the brain on MRI.

One of the main drawbacks of this study was that this was not a prospective study with longitudinal data collection after an initial lysoGb3 measurement. This represents the real world situation in which many patients were started on treatment and long term untreated data of new patients are simply not available. Strictly speaking, this makes our dataset unfit to be used for prediction modelling. However, we showed the stability of lysoGb3 in individual (untreated) patients, suggesting that the measured lysoGb3 value represents a static individual FD trait which justifies its use in prognostication.

In conclusion, we showed that plasma lysoGb3 values can further fine-tune clinical phenotyping. We confirmed that in patients with lysoGb3 levels below 2.3 nmol/L (with a reference range 0.3-0.5) renal function remains within the normal range (figure 2) and cardiac morphological and functional parameters show slow progression over time and become abnormal only late in life (from the 7th decade of life onwards, figure 4 and 5). These findings are consistent with earlier observations that the risk of cardiovascular complications in female patients with a non-classical disease type (in whom lysoGb3 levels are usually below 2.3 nmol/L) is low and if they do occur it is late in life[2]. The seven male (non-classical) FD patients with lysoGb3 levels below 2.3 in this study showed a similar benign disease course. In our opinion this group is unlikely to benefit from Fabry specific therapy and may not even require routine follow up before the sixth decade of life. Within the group of patients with a plasma lysoGb3 between 2.3 and 40 nmol/L (mostly male patients with non-classical FD and female patients with classical FD), measuring plasma lysoGb3 can help to give additional insight into the expected clinical course. In patients with relatively low lysoGb3 levels and no- or minor clinical FD manifestations, prolonging the interval between clinical evaluations seems justified and –in our opinion- the potential effect of treatment should be weighed against the burden of lifelong infusions in consultation with the patient. While in patients with higher lysoGb3 levels, more rigorous follow up is warranted and treatment

may be started with less restraint. Further multicenter studies are needed to confirm these findings and identify other biomarkers to improve clinical risk stratification of FD patients.

Based on the results from this study, we propose that measuring plasma lysoGb3 at time of diagnosis can be a useful tool to help diagnose and classify FD. Additionally, the early stabilization of lysoGb3 in plasma over time makes it a suitable marker to aid clinical decision making, more specifically to help determine the needed frequency of follow and the timing of treatment initiation in asymptomatic patients.



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Supplemental Material 1

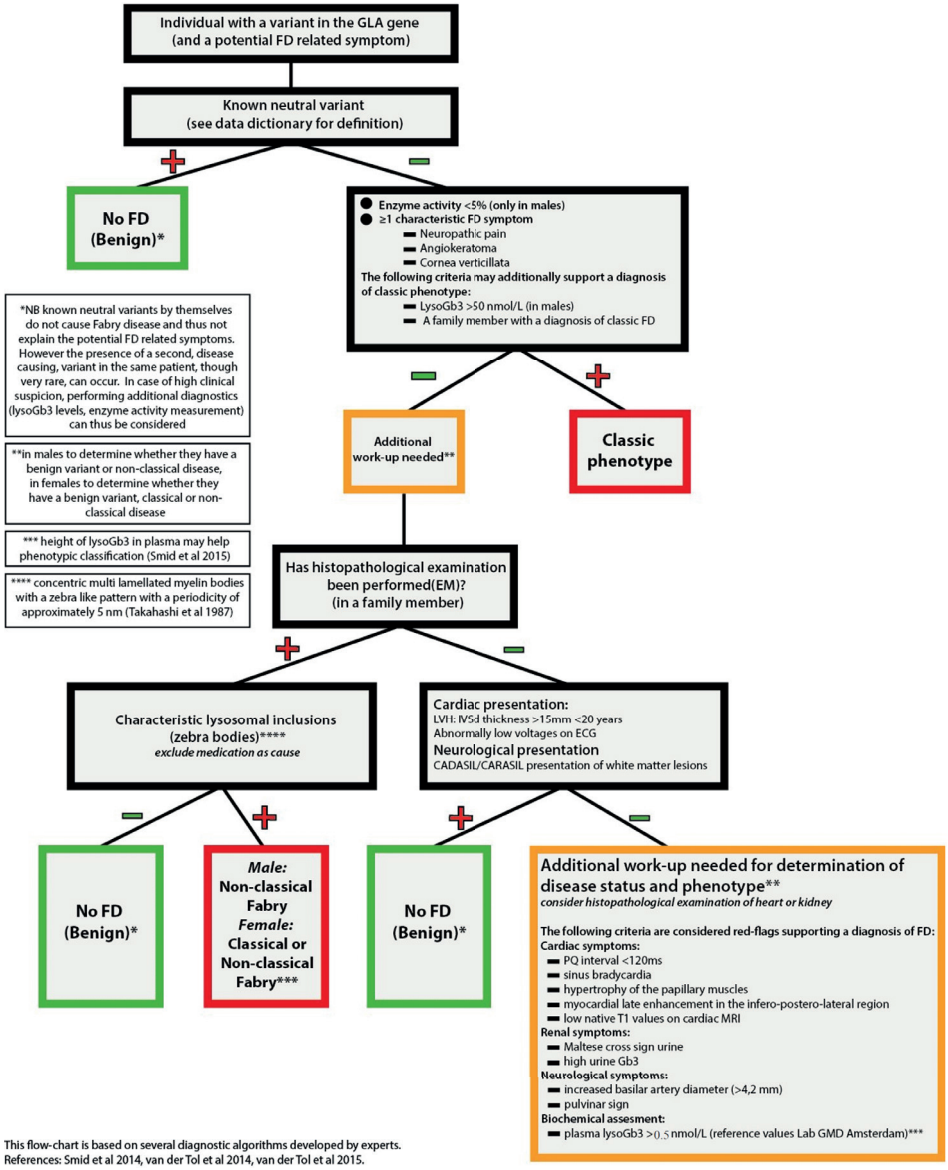
Patient characteristics per visualized lysoGb3 group

LysoGb3 group:	Low (n=42)	Low mid (n=71)	High mid (n=70)	High (n=54)
Sex				
- Male	7 (17%)	5 (7%)	24 (34%)	53 (98%)
- female	35 (83%)	66 (93%)	46 (66%)	1 (2%)
Phenotype				
- classical	2 (5%)	59 (83%)	47 (67%)	54 (100%)
- non-classical	40 (95%)	12 (17%)	23 (33%)	0 (0%)
Age	49 (19 - 76)	44 (13 - 76)	48 (14 - 70)	27 (3 - 58)
Mutatation type				
- nonsense	1 (2%)	19 (27%)	23 (33%)	25 (46%)
- missense	41 (98%)	47 (66%)	45 (64%)	25 (46%)
- other	0 (0%)	4 (5%)	2 (3%)	4 (8%)
LysoGb3 (nmol/L)	1.3 (0.4 – 2.2)	4.8 (2.4 - 7.2)	11 (7.3 - 36.2)	105 (38 - 166)
Additional risk factors				
- ever smoked	20 (53%)	26 (43%)	29 (45%)	20 (42%)
- Hypertension*	13 (32%)	16 (24%)	17 (24%)	8 (15%)
- BMI >30 kg/m2	10 (24%)	17 (24%)	8 (11%)	2 (4%)
Any known risk factor	28 (67%)	38 (54%)	41 (59%)	25 (47%)

Table 1. *Baseline characteristics of included FD patients per visualized group. Grouping was done for visualization purposes only. Low group (<2.3 nmol/L) and high group (>40 nmol/L) were chosen based on the best cut-off value for classification (classical vs non-classical disease in females and males respectively). The remaining group was cut in half at the median of 7.3 nmol/L to create 2 equally divided groups. Data are presented as number (percentage) or median and range, as appropriate.*

Supplemental Material 2

Current flowchart used to diagnose and phenotype FD at the AUMC



This flow-chart is based on several diagnostic algorithms developed by experts.
References: Smid et al 2014, van der Tol et al 2014, van der Tol et al 2015.

Supplemental Material 3

Detailed procedure of Lab GMD (Amsterdam UMC) lysoGb3 assay

LysoGb3 is measured using LC-MS/MS ((Xevo TQ MS, Waters Inc.) in multiple reaction monitoring (MRM) mode.

Sample preparation

- Pipette 50 µL plasma into a 2 mL vial.
- Add 25 µL Internal Standard (IS) (concentration: 0.1 µM).
- Add 25 µL of MilliQ water.
- Add 300 µL MeOH and 150 µL CHCl3 and vortex for 30 sec.
- Centrifuge at 16060 g (14000 rpm) at 4°C for 10 min.
- Transfer the supernatant to a 2 mL vial.
- Add 150 µL CHCl3 and 225 µL MilliQ water and vortex for 30 sec.
- Centrifuge at 16060 g (14000 rpm) at 4°C for 2 min.
- Take 400 µL of the supernatant and transfer to a 1.5 mL screw cap vial.
- Evaporate to dryness at 35°C under N2.
- Take up the residue in 60 µL MeOH by vortexing (30 sec) and sonicating (1 min) in a sonication bath.
- Centrifuge for 10 min at 16060 g at 4°C.
- Transfer 50 µL to a gilson vial and cap and add 50 µL MilliQ water.
- Mix by vortexing briefly.

(Internal) standards and LC-column

- Internal standard: Gly-lysoGb3 (Matreya, 1530)
- Standard: LysoGb3 (Sigma, G9534)
- LC-column: Acquity BEH C18 200x2.1 mm, 1.7µm particles (Waters, 186002350)

Liquid chromatography settings

Run time (min):	5.50
Solvent selection A:	A1
Solvent selection B:	B1
Low pressure limit (bar):	0
High pressure limit (bar):	134
Solvent name A:	Water
Solvent name B:	Methanol
Seal wash (min):	5.0
Gradient start:	At injection
Gradient table:	

Time (min)	Flow rate	%A	%B	Curve
Initial	0.250	100.0	0.0	Initial
2.50	0.250	0.0	100.0	6
4.00	0.250	0.0	100.0	6
5.00	0.250	100.0	0.0	6
5.50	0.250	100.0	0.0	6

MS/MS settings

Type:	MRM
Ion mode:	ES+
Inter channel delay (sec):	- 1.000
Inter scan time (sec):	- 1.000
Span (Da):	0.0
Start time (min):	0.0
End time (min):	5.5

Ch	Parent (Da)	Daughter (Da)	Dwell (sec)	Cone (V)	Coll (eV)	Delay (s)	Compound	
1	786.40	282.30	0.100	45.00	35.00	- 1.000	Lyso-CTH	

Type:			MRM					
Ion mode:			ES+					
Inter channel delay (sec):			- 1.000					
Inter scan time (sec):			- 1.000					
Span (Da):			0.0					
Start time (min):			0.0					
End time (min):			5.5					
Ch	Parent (Da)	Daughter (Da)	Dwell (sec)	Cone (V)	Coll (eV)	Delay (s)	Compound	
1	843.5	264.40	0.100	45.00	55.00	- 1.000	Gly-lyso-CTH	

NB.

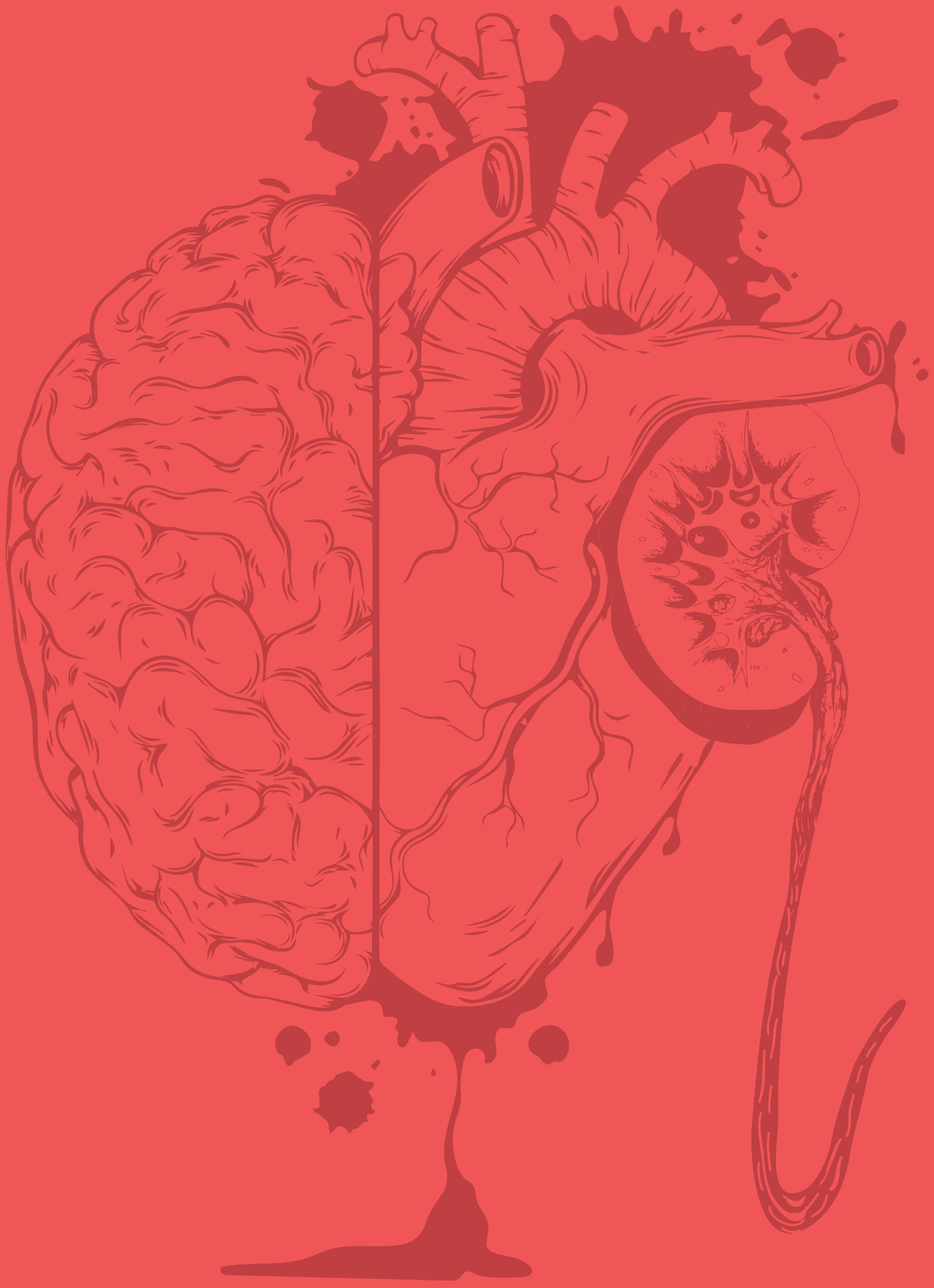
- Lyso-CTH=LysoGb3
- Gly-Lyso-CTH=gly-lysoGb3 (IS)

Calculation

Concentrations are calculated in Targetlynx (Waters Inc.) using a lysoGb3 standard line prepared in plasma: (concentrations: 0, 2, 5, 10, 20, 50, 100, 200 nmol/L).

Supplemental Material 4

Full Markdown file of statistical analyses performed in R will be published online.



Chapter 3

Early start of enzyme replacement therapy in pediatric male patients with classical Fabry disease is associated with attenuated disease progression

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Abstract

Background: Enzyme replacement therapy (ERT) slows disease progression of Fabry disease (FD), especially when initiated before the onset of irreversible organ damage. However, with the clinically asymptomatic progression of renal, cardiac and cerebral disease manifestations spanning decades, optimal timing of ERT initiation remains unclear.

Methods: In this cross-sectional retrospective study, seven male FD patients with a classical disease phenotype (cFD) who started treatment with agalsidase-beta in childhood were evaluated after 10 years of treatment (median age at evaluation 24 years, range 14–26). Cardiac imaging (echocardiography and MRI), electrophysiological and biochemical data of these patients were compared to those of untreated male cFD patients (n = 23, median age 22 years, range 13–27).

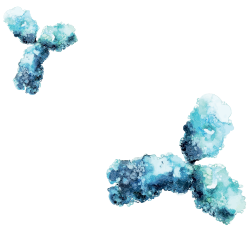
Results: Albuminuria was less common and less severe in treated patients (albumin to creatinine ratio, ACR 0–8.8 mg/mmol, median 0.4) compared to untreated patients (ACR 0–248 mg/mmol, median 3.7, p = 0.02). The treated group had a lower left ventricular mass, measured using echocardiography (median 80 g/m2 versus 94 g/m2, p = 0.02) and MRI (median 53 g/m2 versus 68 g/m2, p = 0.02). Myocardial fibrosis was absent in all included patients. eGFR was normal in all treated patients whereas 7/23 (30%) of untreated patients had abnormal eGFR. Cerebral manifestations did not differ.

Conclusions: Start of treatment with ERT before age 16, in male cFD patients is associated with reduced occurrence of renal and cardiac manifestations of FD, as assessed by intermediate endpoints. Confirmation that this approach delays or even prevents renal failure and cardiac events requires another decade of follow-up.

Introduction

Fabry disease (FD, OMIM 301500) is a rare hereditary lysosomal storage disease (LSD) caused by a mutation in the X chromosome-located GLA gene, leading to a deficiency of the enzyme alpha-galactosidase A (αGAL A, EC 3.2.1.22). The accumulation of its substrate, globotriaosylceramide (Gb3), and its deacylated form, globotriaosylsphingosine (lysoGb3), is associated with progressive damage to small nerve fibers, vascular endothelium, renal and cardiac cells[1]. Male FD patients with the classical disease phenotype (cFD), in whom there is absent or very little residual αGAL A activity, are the most severely affected[2]. These patients predominantly present with neuropathic pain in hands and feet in childhood. In this phase of the disease there is increasing tissue accumulation of Gb3, but clinical renal, cardiac and cerebral complications do not yet occur[3,4]. During adolescence and early adulthood, cardiac left ventricular (LV) mass gradually increases and many male cFD patients develop albuminuria[5,6]. Later on, ECG changes become apparent and cerebral white matter lesions (WML) appear on MRI, this is followed by the development of myocardial- and glomerular sclerosis, during the 3rd and 4th decade of life, leading to cardiac complications and renal failure[7]. Ultimately the cardiac, renal and cerebrovascular complications cause severe morbidity and premature death, in the vast majority of patients before the age of 60[5]. Almost two decades ago, two recombinant preparations of αGAL A became available for treatment of FD. Agalsidase-alfa (Replagal, Shire/Takeda) registered at a dose of 0.2 mg/kg/every other week (eow) and agalsidase-beta (Fabrazyme, Sanofi-Genzyme) registered at a dose of 1 mg/kg/eow. In the pivotal trials that led to the registration of agalsidase-alfa and -beta it was shown that enzyme-replacement therapy (ERT) resulted in a clear biochemical response with reduction of Gb3 in plasma and urine[8-10]. ERT was shown to clear storage material from endothelial cells and several renal cell types including vascular, interstitial and mesangial cells[11,12]. Podocyte clearance was also observed in several patients, especially in patients treated with agalsidase-beta that started treatment at a young age[13] and in patients that were treated for a longer period of time[14]. In a subset of patients, treatment with ERT resulted in a stabilization of renal function, a reduction of cardiac mass[15] and a delay of clinical events[16], but response to treatment was highly variable and in most patients the disease progressed despite treatment[15]. Especially in patients with declined renal function, proteinuria and/or myocardial fibrosis at the time of treatment initiation, disease markedly progressed despite treatment with ERT[15,17]. Patients that started treatment relatively early in their disease course, that is to say with an estimated glomerular filtration rate (eGFR) > 60 ml/min/1.73 m2 and no significant myocardial fibrosis at the time of treatment initiation, tended to have

more favorable outcomes[18-24]. This led to the international clinical consensus[5,25,26] that in male patients with the classical disease phenotype, treatment with ERT should be started early, even before clear clinical disease manifestations in kidney or heart are present. To date, a more precise advice regarding optimal age of treatment initiation in this patient group is not possible, as there are no trials comparing effects of starting treatment in early childhood versus adolescence/ young adulthood. Some studies suggested a beneficial effect of starting ERT in childhood, since a more pronounced substrate reduction in podocytes was observed in FD patients that started ERT in early childhood[23]. However, ERT is costly and the biweekly infusions may pose a significant treatment burden, especially for young children. In addition, male cFD patients are at risk to develop infusion related reactions such as hyperthermia, cold chills, skin rashes, dyspnea or in the worst case scenario anaphylactic shock, requiring treatment with immunosuppressive drugs to tolerate the infusions[8,27,28]. Therefore, clinical evidence to guide timing of ERT initiation is clearly needed. In 2019 the results of the FIELD study, evaluating the effect of childhood initiation of low dose ERT (5 years of treatment) were published. The FIELD study showed treatment resulted in reductions in plasma and urine Gb3 levels and a mixed response on histopathological endpoints (e.g. reduction in intracellular Gb3 depositions in most, but not all patients). The downside of this study was that it lacked an untreated control group[12], hampering a solid conclusion on treatment effect. The patients that were originally treated in this study at our centers have now been treated for a decade (continuation after study ended). They form a unique cohort of FD patients that started treatment in childhood and in the current study we compared clinical, imaging, biochemical and electrophysiological parameters of these patients after 10 years of treatment with ERT to a group of untreated Fabry patients with the same phenotype (classical) and of comparable age.



Materials and Methods

Patients

All included patients were male and classified as having the classical Fabry disease phenotype based on the residual enzymatic activity (leucocyte α-Gal A activity ≤5% of the median of the reference range) and the presence of one or more characteristic symptoms of FD (Fabry specific neuropathic pain, angiokeratoma, and/or cornea verticillata)[2,29].

Treated group

This group consists of male cFD patients who participated in the FIELD study[12]. The FIELD study was a randomized controlled trial assessing the effect of different dosing regimens of agalsidase-beta in pediatric FD patients. There was no untreated group in the original study. All patients were treated with a lower then registered dose, only the interval differed. One group was treated with 0.5 mg/kg biweekly (3 patients in our study) the other with 1.0 mg/kg once a month (4 patients in our study), no differences in outcome were found between these 2 groups). The study ran from September 2008 to June 2015 (NCT00701415)[12]. All patients that were enrolled in the FIELD study at the Amsterdam University Medical Centers (UMC) Amsterdam, the Netherlands (n = 5) and Haukeland University Hospital (UH), Bergen, Norway (n = 2) were included in the current analysis. All patients continued treatment on study dose directly after the study ended and had been treated for approximately 10 years at time of assessment. 6/7 patients switched to full dose (1 mg/kg biweekly) after a median treatment duration of 9 years (range 8.3–10).

Untreated group

We conducted a search in the clinical database at the Amsterdam UMC for patients with the same sex and phenotype as the treated patients (male FD patients with the classical disease phenotype), of whom treatment naive data were available between the age of 12 and 27 (±2 years of the age range in the treated group). Twenty-three patients fitted these criteria. If data of more than one time point was available for an individual patient (n = 3), the time point was chosen to best match the median age at evaluation of the treated patients. In three untreated patients, reduced renal function or severe proteinuria was present at the time of referral to the Fabry expertise center and the main reason for performing diagnostics. No other reason than FD for kidney disease was found. As we could not rule out potential inclusion bias, we ran two analyses on renal parameters, one including and one excluding these patients.

This retrospective, cross-sectional study compared clinical manifestations of FD in adolescent and young adult classical male Fabry patients after 10 years of treatment with ERT (the treated group) to untreated classical male FD patients in the same age range (untreated group). The study was conducted in accordance with the principles of the Helsinki Declaration, as revised in 2013. All included patients signed informed consent for the use of their data at start of clinical follow up.

Imaging: Echocardiography

Echocardiography was performed at the Amsterdam UMC (NL) and Haukeland UH (NO). If original images were available (for 20 patients), they were reassessed by a specialized cardiologist (AH) blinded for treatment status. If images were not available, data from the original report were used (6 patients). Echocardiography data were missing from 4 patients. LV mass was estimated with the Devereux and Reichek "cube" formula as recommended by the American Society of Echocardiography's Guidelines[30] and corrected for BSA using the DuBois formula (recommended and best validated according to the American Society of Echocardiography's Guidelines).

Imaging: Cardiac MRI

Cardiac MRIs were performed at the Amsterdam UMC on a 1.5 T clinical MR system (Magnetom Avanto, Siemens, Erlangen, Germany) and reassessed by a specialized cardiologist (AH) blinded for treatment status. The protocol included a complete cine short-axis stack covering the whole LV from base to apex, acquired using a balanced steady-state free precession sequence. Furthermore, 2-dimensional late gadolinium enhancement images were performed 10–20 min after contrast and visually scored for the presence or absence of late gadolinium enhancement. For the assessment of LV mass, endocardial and epicardial contours were manually traced in end-diastole and end-systole on the cine short-axis stack according to Society for Cardiovascular Magnetic Resonance guidelines on image post-processing[31]. Papillary muscles were separately traced and included in the LV mass. LV mass was corrected for BSA using the DuBois formula. Analyses were performed using QMass software version 8.1 (Medis Medical Imaging Systems bv). Due to the potential influence of post-processing software, inter-observer variations as well as differences in the in- or exclusion of papillary muscles on LV mass, only patients of whom original images were available for re-evaluation (n = 17) were included. Cardiac MRI data were missing for 3 treated patients (no cardiac MRI performed because the patient was either <18 years old or original imaging not available for re-evaluation) and 10 untreated patients (no MRIs were performed before 2008 or in patients <18 years old).

Imaging: Brain MRI

Brain MRIs were performed locally at the Amsterdam UMC (NL) and Haukeland UH (NO). MRI data from the Amsterdam UMC were obtained using 3 T scanners. Scans before October 2012 on the Philips Intera system (Philips Medical Systems, Best, The Netherlands) and scans after October 2012 on the Philips Ingenia system (Philips Medical Systems, Best, The Netherlands). Data from the original clinical reports were used. White matter lesions (WML), defined as hyper intensities on axial T2-weighed and FLAIR-weighed imaging, were visually scored using the Fazekas scale (ranging from 0, no WMLs to 6, severe confluent WMLs)[32,33]. MRI brain was missing in 1 patient.

ECGs

If original ECG was available (21 patients), these were analyzed by a specialized cardiologist (AH). If original ECG was not available (N = 7), the clinical report of the ECG was checked to see if any anomalies were described. ECG data were missing for one patient.

Biochemical analyses

To account for the inclusion of both adolescence and adults, the Full Age Spectrum equation for eGFR was used with a correction for age (FASage) to estimate the glomerular filtration rate[34,35]. Serum creatinine values were obtained from the electronic patient records. Full formula and table with age adjusted correction are added to the supplemental material. Normal eGFR range was defined as 90–140 ml/min/1.73 m2. Albuminuria was depicted as Urinary albumin/creatinine ratio (uACR, mg/mmol) and categorized into A1, A2 and A3 according to the Kidney Disease Improving Global Outcomes (KDIGO) guidelines[36]. For analyses uACR was included as a continues variable. If the urinary albumin levels were below the level of detection (<3 mg/l), uACR was entered as 0.

Plasma lysoGb3 (nmol/l) was measured with tandem mass spectrometry, as previous described[3,4]. The presence of inhibiting anti-drug-antibodies (iADAs) to r-αGAL A activity was measured as previously described[5]. iADA titers represent the dilution factor of plasma resulting in 50% inhibition of the r-αGAL A activity. Patients are considered iADA-positive if the inhibition titer was >6.

Statistical analyses

We used R (version 3.4.3) for all statistical analysis. Non-normal distribution was assumed in all analyses due to the small number of observations. Continuous variables were assessed using Mann-Whitney-Wilcoxon test and categorical variables with Fisher exact test. Spearman's rank was used to assess correlations. Missing values could be explained by known variables in the majority of cases

(missing at random) and had no relation to disease severity. Missing values were dealt with through case wise deletion. Reasons for missing data are mentioned in the results. P values < 0.05 were considered statistically significant.

Results

Patient characteristics

Patient characteristics are outlined in Table 1. There were no significant differences in age, mutation type, classical FD features, reason for diagnosis (based on clinical features or through family screening) or height of untreated lysoGb3 concentrations (although there was a trend for higher baseline lysoGb3 in the treated group).

	Treated	Untreated	P-value
No of patients	7	23	
Age at evaluation (years)	24 (14-26)	22 (13-27)	0.7
Treatment duration (years)	10.4 (9.5-10.7)	-	
Mutation type			
- Nonsense/Frameshift	4/7 (57%)	10/23 (43%)	1.0
- Missense	3/7 (43%)	11/23 (48%)	
- Other	0/7 (0%)	2/23 (9%)	
Reason for diagnosis			
- Family screening	5/7 (71%)	10/23 (43%)	0.5
- Acroparesthesia	2/7 (29%)	10/23 (43%)	
- Renal insufficiency or albuminuria*	0/7 (0%)	3/23 (13%)	
Untreated plasma lysoGb3 (nmol/L)	118 (89-215)**	102 (65-137)	0.07
Clinical features of classical FD (at diagnosis)			
- Acroparesthesia	7/7 (100%)	22/23 (96%)	1.0
- Angiokeratomas	4/7 (57%)	17/23 (74%)	0.6
- Cornea verticillata	6/7 (86%)	17/21 (81%)	1.0
Hypertension	0/7 (0%)	1/23 (4%)***	1.0
Use of ACEi or ARB	0/7 (0%)	4/23 (17%)	0.5

Table 1. *Patient characteristics. Categorical variables are depicted as number (percentage) and continuous variables as median (range). Missing values (if any): presence of cornea verticillata (n=2), reason unknown.*

** Renal insufficiency or albuminuria was the reason for performing Fabry diagnostics in these patients. The presence of renal involvement as the primary reason for Fabry diagnostics was deemed a potential risk factor for bias of patient selection between the treated and untreated patient groups. Therefore all analyses were done with and without inclusion of these patients.*
*** Untreated plasma LysoGb3 levels were used. In the treated group lysoGb3 levels at start of treatment initiation are represented. LysoGb3 levels from Haukeland UH were measured in ng/ml and converted using the formula: (lysoGb3(ng/ml)*1000) / 785,9.*
**** One patient was diagnosed with hypertension, eGFR of this patient was normal (118 mL/min/1.73 m^2). Echocardiography and cardiac MRI data were missing for this patient.*

Renal disease manifestations

Albuminuria (Fig. 1a) occurred at a younger age and was more pronounced in the untreated patients (median: 3.7 mg/mmol, range: 0–248 mg/mmol) compared to treated patients (median: 0.4 mg/mmol, range: 0–8.8 mg/mmol, p = 0.02). For three patients in the untreated group renal insufficiency and/or severe proteinuria was the main cause for performing FD diagnostics. To remove the effect of a potential inclusion bias, we ran the test again excluding these patients resulting in a median ACR of 3.3 mg/mmol (range: 0–200 mg/mmol) in untreated patients, with the same distribution in treated patients. Results remained statistically significant (p = 0.04). Median eGFR, as a representation of renal function did not differ between groups. The treated group had a median eGFR of 116 ml/min/1.73 m2 (range: 92–132) while the untreated patients also had a median eGFR of 116 ml/min/1.73 m2 (range: 46–165 p = 0.96). It should be noted however that both reduced renal function (defined as an eGFR <90, n = 4) and hyperfiltration (defined as an eGFR >140, n = 3) only occurred in the untreated group (Fig. 1b). Exclusion of patients that were diagnosed because of renal involvement had no significant effect on the results. ACR was missing in 1 patient (untreated group, no urine sample at time of evaluation). Additional disease influencing renal function were excluded in all 3 patients that were diagnosed due to renal involvement.

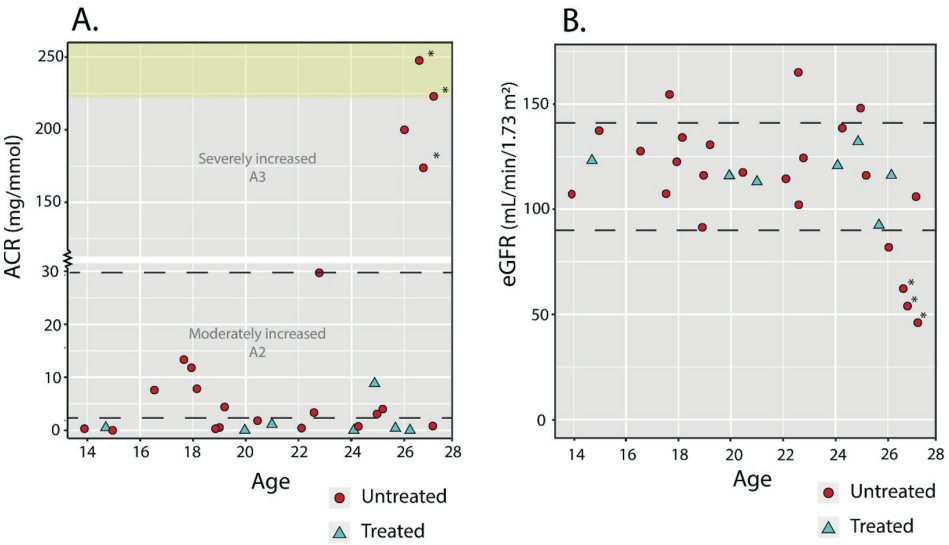


Figure 1. *Renal disease manifestations. (a) albumin to creatinine ratio (ACR) in urine in mg albumin/mmol creatinine (p = 0.02). Dashed lines represent the shifts in classification of albuminuria; A1 (<3 mg/mmol, normal to mildly increased), A2 (3-30mg/mmol, moderately increased) and A3 (>30 mg/mmol, severely increased). ACR >220 mg/mmol (arced in yellow) falls within the nephrotic range. (b) eGFR calculated by FASage (p = 0.96). Dashed lines represent upper (140 ml/min/1.73 m2) and lower (90 ml/min/1.73 m2) limit of normal eGFR. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)*
** For three patients, renal disease was the presenting symptom leading to the FD diagnosis. Analyses were performed with and without inclusion of these patients. Excluding these patients did not significantly change the outcome of the analyses.*

Cardiac disease manifestations

Estimated median LV mass based on echocardiography measurements was 94 g/m2 (59–149 g/m2) in the untreated group versus 80 g/m2 (67–84 g/m2) in the treated group (p = 0.02) (Fig. 2a). Median LV mass measured by MRI (including papillary muscles) was 68 g/m2 (53–99 g/m2) in the untreated group versus 53 g/m2 (46–59 g/m2) in the treated group (Fig. 2b, p = 0.02). Analyses were repeated after excluding patients diagnosed due to renal involvement, this did not change the outcome. Mass measured by echocardiography and MRI correlated well (ϱ = 0.72, p = 0.002). None of the patients had late gadolinium enhancement on MRI. Cardiac MRIs were missing in 13 patients. Reason for missing were a) original data not available for re-evaluation (n = 2) or b) analyses performed before 2008 (n = 11) since cardiac MRI as part of the routine follow-up of FD patients was introduced in 2008 at the Amsterdam UMC. All ECGs that

were reevaluated (n = 21) showed sinus rhythm and normal PR-, QRS- and QTc-intervals were observed. An incomplete right bundle branch block was present in five of 16 patients (31%) in the untreated group and in 0 of 5 patients (0%) in the treated group (p = 0.3). Sinus bradycardia (defined as a resting heart rate below 60 bpm) was present in 1 of 7 patients (14%) in the treated group, compared to 11 of 21 patients (52%) in the untreated group. Median heart rate did not differ between groups (p = 1). No anomalies were described in the clinical reports from patients of whom original ECG was not available for reevaluation.

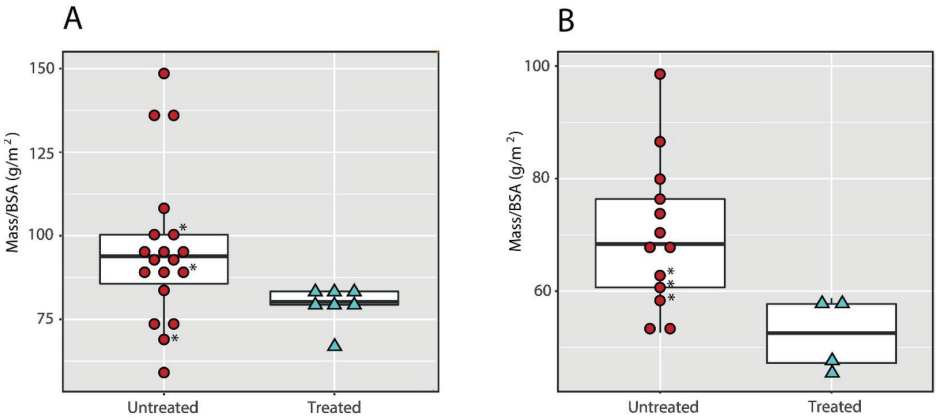


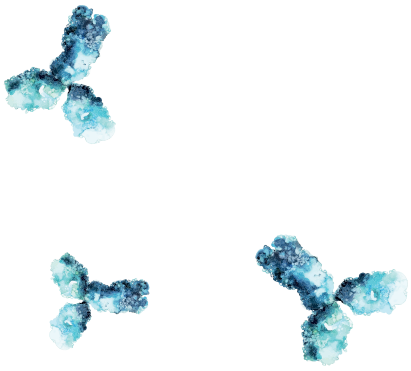
Figure 2. *Cardiac mass measured by echocardiography (A) and MRI (B), in both panels corrected for BSA. Differences between untreated and treated patient groups were significant in both comparisons (A: p = 0.02, B: p = 0.02).*
** For three patients, marked in the figure, renal disease was the presenting symptom leading to the FD diagnosis. Analyses were performed with and without inclusion of these patients. Excluding these patients did not significantly change the outcome of the analyses.*

Cerebral disease manifestations

Cerebral involvement was minimal in both groups. Six out of 22 untreated patients had white matter lesions (all Fazekas 1). In the treated group one out of seven patients had white matter lesions (Fazekas 1) (p = 0.6). In one patient (untreated group) a lacunar infarction was described, in another patient (untreated group) microbleeds were found. Cerebral MRI was missing in one patient (reason unknown).

Adverse events of treatment with ERT

Four of the seven treated patients developed inhibiting anti-drug antibodies (iADA) during treatment. These patients showed a trend for higher plasma lysoGb3 levels during treatment compared to the three treated patients without iADAs ($p = 0.06$). In one of these patients treatment with ERT caused serious infusion related reactions with pyrexia and cold chills, requiring treatment with antihistamines and corticosteroids prior to infusions to continue treatment.



Discussion

This study shows that young adult male cFD patients that have been treated with ERT for 10 years have significantly less albuminuria and a lower cardiac mass compared to untreated patients from the same age group. No difference was found in median eGFR between the treated and untreated group. However, it deserves to be mentioned that seven out of 23 patients (30%) in the untreated group had eGFR values outside the normal reference range (e.g. 90–140 ml/min/1.73 m2) versus none of the patients in the treated group. Three of these patients had hyperfiltration at the time of evaluation and four had reduced renal function. It should be mentioned, however, that for 3 of the 4 patients with reduced renal function, renal manifestations were the reason for referral and Fabry diagnostics. To reduce potential inclusion bias, all analyses were repeated excluding these patients. This did not affect the outcome.

Previous studies showed that higher uACR is the strongest risk factor for the development of renal failure in FD[6]. For nephropathy caused by more common disorders, such as diabetes and hypertension, positive effects of early interventions (e.g. normalization of blood glucose and blood pressure) are well established. Most nephropathies have a 'point of no return', after which renal decline progresses despite adequate treatment of the underlying condition[37]. This is in concordance with the observation in FD that ERT has a limited benefit on renal decline in patients with reduced eGFR at start of ERT (Fig. 3)[15]. Renal biopsy studies suggest that higher doses and a younger age at start of ERT are related to more sustained reduction of Gb3 in podocytes[13,23]. This may be clinically relevant since podocyte Gb3 inclusions in male cFD patients correlate with albuminuria, progressive podocytes loss and worse renal outcome[4,38] and a greater reduction of podocyte inclusions is related to a reduction in microalbuminuria[13]. In other nephropathies albuminuria is also strongly related to an accelerated renal decline and a reduction of albuminuria after treatment with anti-proteinuric drugs generally correlates with the preservation of renal function[39]. Finally, the occurrence of hyperfiltration may represent another early FD disease manifestation[40] and has been found to predispose for renal decline in other nephropathies[41], but in FD this has not been evaluated yet. In summary, the previous mentioned findings in literature combined with the observations from our study, demonstrate a beneficial effect of starting ERT in childhood on albuminuria, with a funded expectation for preservation of, or less decline in, renal function.

Evaluation of the benefit of early treatment initiation on cardiac manifestations of FD is more complicated for several reasons. Most importantly, although

cardiac LV mass was on average higher in the untreated group, for most included patients, cardiac mass was still within the reference ranges of normal[30,42-44]. Up to date there are no long-term longitudinal studies showing the prognostic value of (mildly) increased left ventricular mass on the occurrence of cardiac complications in FD. However, both in the general population[45] and in FD, LVH is an independent short term predictor for cardiac events such as arrhythmias, heart failure and cardiac death[46]. But the fact that female patients can still develop cardiac fibrosis and complications despite having a normal LV mass indicates that maintenance of a normal cardiac mass does not automatically means protection from the development of fibrosis and/or the occurrence of clinical complications[47]. Furthermore, the effect of ERT on cardiac fibrosis formation is not yet known. In patients with fibrosis at start of treatment, its formation progresses despite treatment[17]. Whether or not ERT slows down the progression of fibrosis or prevents it in patients without detectable fibrosis at treatment initiation, is yet unknown. Finally, cardiac biopsies showed no clear reduction of Gb3 inclusions in cardiomyocytes in response to ERT[48], questioning the accessibility of these cells for recombinant enzyme. However, Gb3 accumulation alone does not explain all aspects of the pathophysiology in FD cardiomyopathy[49]. Circulating lysoGb3, for example, has been shown to promote inflammation[50] and hypertrophy[51-53] and is effectively lowered upon treatment with ERT. As secondary disease processes are set in motion well before they lead to clinical manifestations[49], we hypothesize that earlier intervention will modify progression, leading to a more attenuated disease course (Fig. 3). Nonetheless, to confirm the effect of early treatment on the development clinical endpoints of FD such as conduction disorders, arrhythmias and heart failure, validation of intermediate endpoints and longer follow-up are required[54].

The development of anti-drug antibodies (ADAs) against recombinant αGAL A can limit the effect of ERT. ADAs limit cellular uptake of the recombinant enzyme[55], reduce cellular Gb3 clearance[12] and result in an accelerated renal decline[56]. In addition, ADAs are responsible for the majority of adverse reaction to ERT[14] and may even result in anaphylactic reactions. The risk of ADA development and potential infusion reactions should also be taken into account when weighing the pros and cons of ERT, especially in pediatric patients. Future studies assessing different approaches to minimize ADA risks may also help in this regard.

This study was not without its limitations: a relatively small number of early treated patients are included and patients were treated with a lower than registered dose at the time of treatment initiation (e.g. 0.5 mg/kg/biweekly or 1 mg/kg/month).

However, the fact that these results were found whilst the treated group received a suboptimal dose, does not weaken the conclusion. In fact, based on earlier studies showing a better effect of higher dosed ERT[57], using the full registered dose can be expected to lead to a more pronounced treatment effect. Potential reasons for (selection) bias were reduced as much as possible by comparing all known variables that could be related to disease severity (a.o. untreated plasma lysoGb3, mutation type, the presence of classical symptoms etc.) and by having original imaging reassessed by a blinded physician. Unfortunately we could not assess the effect of early treatment initiation on native T1 values[58], as most cardiac MRIs in the untreated group were conducted before the routine native T1 assessment was implemented at our site.

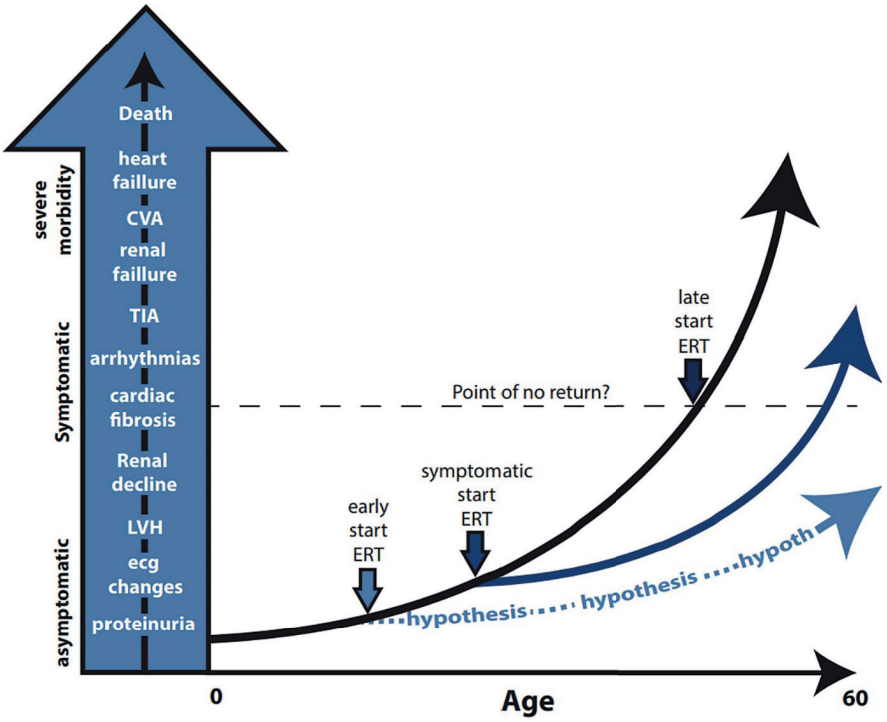


Figure 3. *Influence of treatment with ERT on disease course. From previous studies we know that treatment initiation after the onset of irreversible organ damage does not alter disease course. Starting treatment in an early symptomatic phase (e.g. first signs of organ involvement) slows disease progression. Based on the findings in this study combined with published results of early treatment on histopathological endpoints, we expect that earlier start of treatment (e.g. before the age of 16) in male FD patients with the classical disease phenotype will lead to a more pronounced inhibition of disease progression.*

Strengths of the study are the precise phenotyping of the patients, making them as comparable as possible, the long treatment duration (10 years) and the standardized analysis of disease manifestations in both patient groups.

Overall, this study is the first to provide clinical evidence that treatment of male cFD patients before the age of 16 has a beneficial impact on progression of clinical renal and cardiac manifestations of FD, as assessed by intermediate endpoints.

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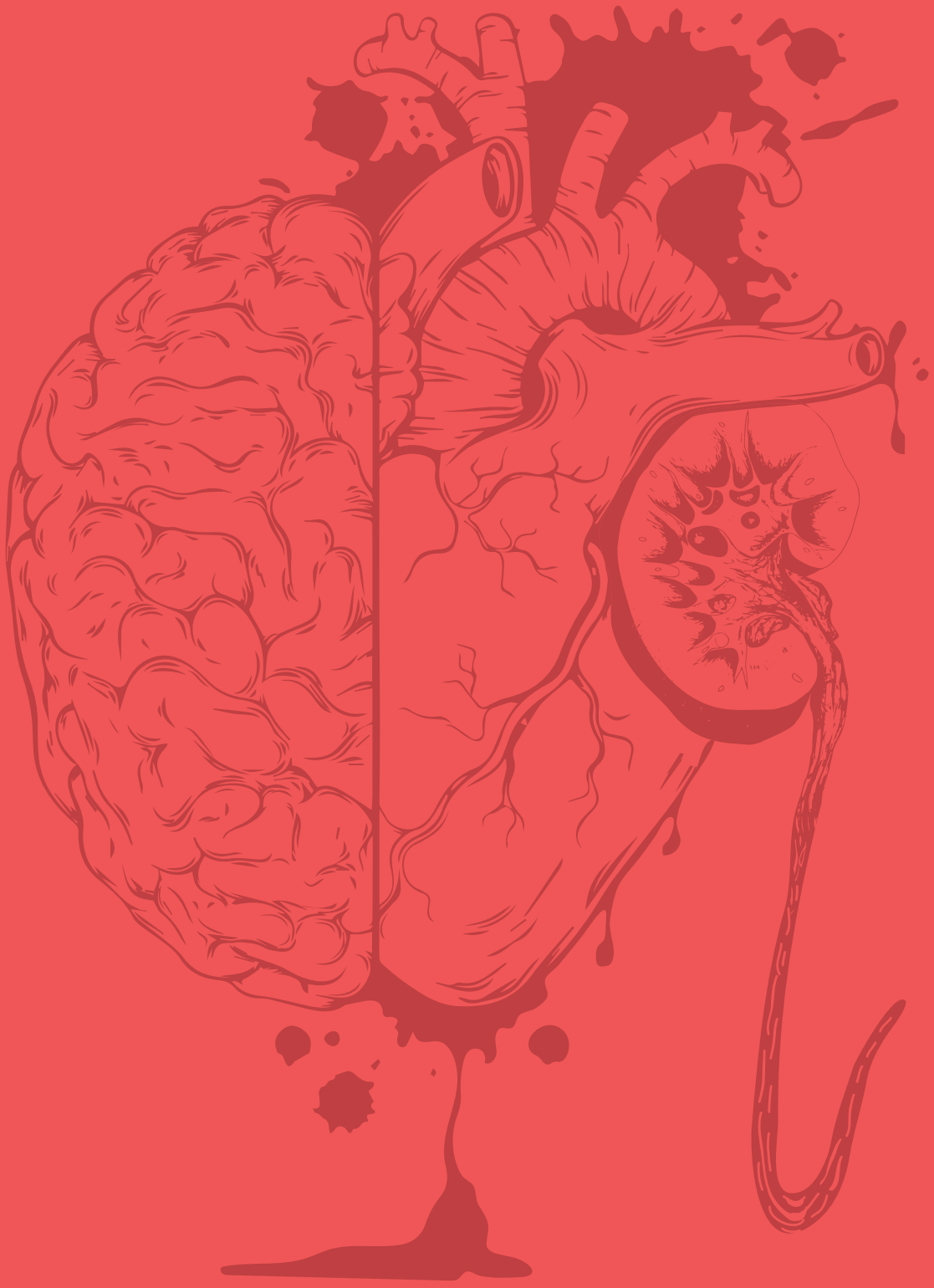
Supplemental material

FAS formula and Q-values (for age 2-40)

Formula:
FASeGFR=107.3/(*S*Cr/*Q*)

Age, years	Q, µmol/L
1	23
2	26
3	27
4	30
5	34
6	36
7	39
8	41
9	43
10	45
11	47
12	50
13	52
14	54
15	64
16	69
17	72
18	75
19	78
≥20	80

Table 1. *Q values (=median serum creatinine in µmol/L) for the FAS equation, according to age. Values from age 15 and older are gender specific (only for male adolecents/adults)*



Chapter 4

Developments in the treatment of Fabry disease

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Abstract

Enzyme replacement therapy (ERT) with recombinant α-galactosidase A (r-αGAL A) for the treatment of Fabry disease has been available for over 15 years. Long-term treatment may slow down disease progression, but cardiac, renal, and cerebral complications still develop in most patients. In addition, lifelong intravenous treatment is burdensome. Therefore, several new treatment approaches have been explored over the past decade. Chaperone therapy (Migalastat; 1-deoxygalactonojirimycin) is the only other currently approved therapy for Fabry disease. This oral small molecule aims to improve enzyme activity of mutated α-galactosidase A and can only be used in patients with specific mutations. Treatments currently under evaluation in (pre)clinical trials are second generation enzyme replacement therapies (Pegunigalsidasealfa, Moss-aGal), substrate reduction therapies (Venglustat and Lucerastat), mRNA- and gene-based therapy. This review summarises the knowledge on currently available and potential future options for the treatment of Fabry disease.

Introduction

To date, more than 50 genetic lysosomal storage disorders (LSDs) have been identified, of which Fabry disease (FD) (OMIM number: 301500) is probably the most prevalent. FD is caused by the presence of a deleterious mutation in the GLA gene coding for the enzyme alpha-galactosidase A (αGAL A) on the X chromosome, resulting in progressive accumulation of the enzyme's substrate. Accumulation of, predominantly, globotriaosylceramide (Gb3) and its derivatives such as globotriaosylsphingosine (lysoGb3) in many different cell types is thought to be responsible for the pathology observed in FD. The lysosomal Gb3 inclusions are most prominent in the endothelium, cardiomyocytes, peripheral neurons, and various renal cell types[1,2]. The disease is characterised by a broad phenotypic spectrum. Variety in disease expression is largely determined by the type of mutation in the GLA gene and sex of the patient. Due to the X-linked inheritance pattern, males are generally more severely affected than females and develop disease symptoms and complications earlier in life[3]. Male patients can be classified as having the classical- or non-classical form of FD based on residual enzyme activity (if appropriately measured in preferably leucocytes) and the presence of characteristic classical symptoms. In females, enzyme activity does not distinguish between the classical and non-classical phenotype and classification is based on mutation, family history, and clinical as well as biochemical characteristics[4,5]. Symptoms of classical FD are angiokeratomas, cornea verticillata, and heat and exercise induced neuropathic pain (acroparesthesia)[6]. These symptoms often become apparent in childhood. At a later age, proteinuria, renal function loss, white matter lesions in the brain, electrocardiographic changes and left ventricular hypertrophy may occur. In the absence of treatment, life expectancy of Fabry patients with classic disease is approximately 60 years in males and 75 years in females, with the most common causes of death being sudden cardiac death, renal failure, and stroke[7]. For over 15 years, two recombinant enzyme preparations have been available to treat patients with FD. The effectiveness of these two recombinant enzyme preparations is variable, probably depending on timing of treatment initiation and phenotype[8]. This emphasizes the need to improve our understanding of disease course, as well as develop alternative therapies. In the past years, several new treatment modalities for FD have been developed, of which only Migalastat (chaperone therapy) is currently approved. Several other new approaches are being explored in clinical and preclinical studies. Amongst these are second generation enzyme replacement therapies, substrate reduction therapy (SRT), gene- and mRNA based therapy (Figure 1). In the end we briefly elaborate on the potential future approach to stimulate the egress of storage material from the

cell (Figure 4). The goal of this review is to provide an overview of the current status of the various therapeutic approaches for the treatment of FD.

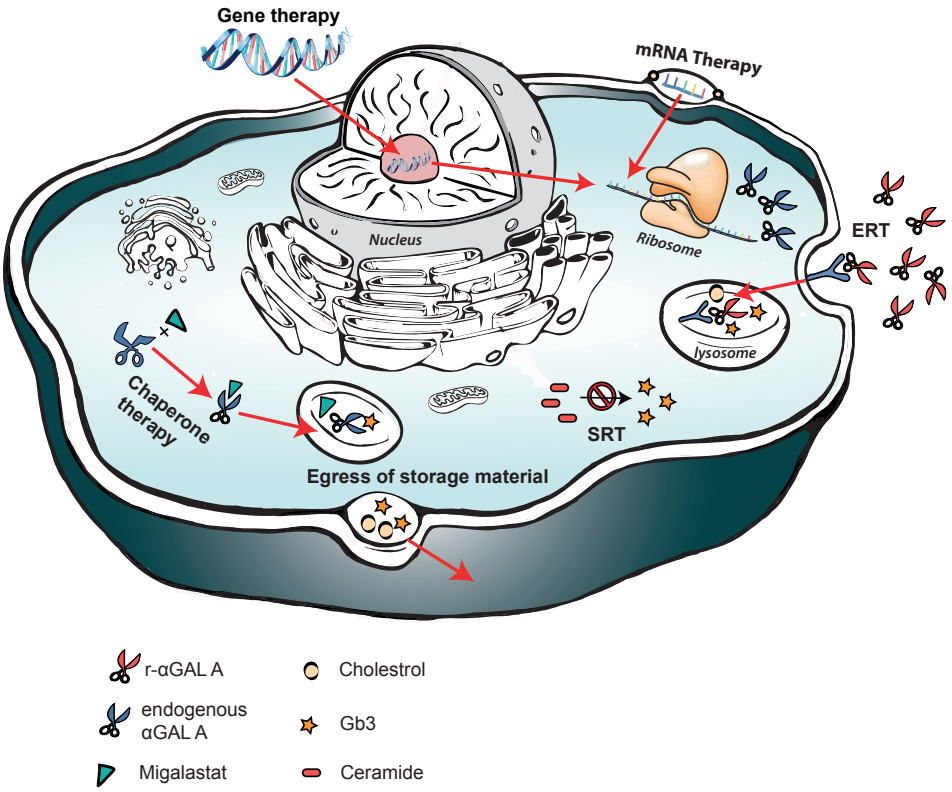


Figure 1. *Overview of different approaches in treating Fabry disease; Enzyme replacement therapy (ERT) aims to restitute defective αGAL A. Chaperones bind to the active site of the unstable αGAL A to aid proper folding. Substrate reduction therapy targets the glycosphingolipid synthesis to reduce formation of Gb3 and its derivatives. Gene therapy aims to correct the underlying genetic defect of FD. MRNA therapy induces transient endogenous αGAL A production. The egress of Gb3 can potentially be stimulated by enhancing cholesterol efflux (Figure 4). FD, Fabry disease*

Currently available treatment options

Enzyme replacement therapy, developments, and challenges

Currently, two different forms of enzyme replacement therapy (ERT) are available; agalsidase-alfa (Replagal, Takeda), produced in human fibroblasts and registered at a dose of 0.2 mg/kg biweekly, and agalsidase-beta (Fabrazyme, Sanofi Genzyme), produced in Chinese hamster ovary cells and registered at a dose of 1.0 mg/kg biweekly. Short term pathological studies on the effect of agalsidase-beta treatment mainly focused on renal biopsies and showed that treatment resulted in clearance of Gb3 from endothelial cells, mesangial cells and podocytes[9,10]. In a study with agalsidase-alfa, studying different cell types, decreased Gb3 accumulation in liver and in tubular epithelial cells was observed, as well as a reduction of Gb3 excretion in urine[11]. Tøndel et al showed that 5 years of treatment with either preparation resulted in complete clearance of glomerular endothelial as well as mesangial inclusions. Patients treated with the highest dose also had a notable reduction in podocytes inclusions[12]. Long-term clinical studies showed a small but significant effect of ERT on cardiovascular and renal complication rate, with some superiority of the higher dosed agalsidase-beta compared to agalsidase-alfa[13,14]. Especially loss of renal function, occurring in the vast majority of male patients with classic FD, is attenuated by ERT[8]. These clinical benefits were mainly observed in patients who started ERT before the presence of irreversible organ damage[12,15-17]. The presence of decreased renal function, proteinuria and/or cardiac fibrosis at the time of treatment initiation was associated with disease progression despite treatment with ERT[8]. In 2008 the deacylated form of Gb3, lysoGb3, was discovered as FD biomarker. Plasma lysoGb3 levels are strongly related to disease phenotype, with high levels in classical patients and lower levels in non-classical patients[4,18,19]. In non-classical FD patients and in female patients with classical FD, plasma lysoGb3 levels correlate with disease severity[20-25]. This relation is not present in the most severely affected group with the highest lysoGb3 levels (male patients with classical FD)[13,18], probably due to a non-linear relationship or a plateaued response. Plasma levels of lysoGb3 decrease substantially during treatment with ERT in male patients with classic FD[18,19], often into the ranges of non-classical or female patients[13]. During treatment, lysoGb3 levels were lower in patients who started treatment before the age of 25 years, compared to those who started later in life[15]. In light of these findings, starting treatment early, before signs of organ damage become apparent, especially in male patients with classical FD seems a logical approach. However, the exact timing of treatment initiation is unclear, since no randomized controlled studies have so far been performed and the follow-up of early treated classically affected boys is still too short to draw

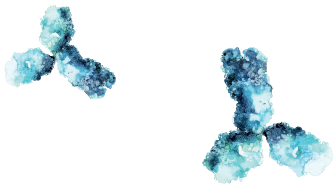
firm conclusions[26,27]. For the other patient groups (females with classical FD and patients with non-classical FD), timing of treatment initiation is even more complex given the great variability in both disease severity and age of onset of disease manifestations. This also makes evaluation of treatment effectiveness in these groups much more difficult. In patients with non-classical FD, as well as in female patients with classical FD, the heart is often the most prominently affected organ. The fact that in cardiac biopsies of male and female FD patients Gb3 was not cleared from cardiomyocytes during treatment with agalsidase-beta[28], is worrying. Several studies reported an initial reduction in cardiac mass in response to ERT in women[29]. However, most included studies were of short duration (2-36 months) and used echocardiography to measure cardiac mass. Unfortunately, echocardiography is a suboptimal technique to assess changes in cardiac mass because of the large inter- and intra-observer variability in the measurements[30,31]. Cardiac fibrosis is a well-known consequence of FD and predisposes for arrhythmias and cardiac dysfunction. It is unknown whether ERT slows progression of fibrosis development and assessment of development of fibrosis is especially difficult in women, where this complication can occur even in the absence of left ventricular hypertrophy[32,33]. In addition to gender, phenotype and timing of ERT, another factor influencing the response to ERT is the formation of anti-drug antibodies (ADAs) against recombinant alpha-galactosidase A (r-αGAL A). Depending on the used assay, estimations on the prevalence of ADAs against r-αGAL A may vary[34,35]. ADAs can negatively influence treatment efficacy by changing distribution, cellular uptake, cellular localization and/or catalytic activity of the administered enzyme[36]. ADAs that inhibit the catalytic activity of r-αGAL A in vitro (iADAs), occur exclusively in approximately 50% of male patients with the classical phenotype and are associated with a less robust decline in plasma lysoGb3 levels[13,37-39] as well as with increased urinary Gb3 levels[40,41]. We recently showed that there was an antibody dose effect: higher iADA titers were associated with less reduction in plasma lysoGb3[42]. A reduction in lysoGb3 does not guarantee a clinical response, however, a loss of the lysoGb3 response suggests a concomitant loss of therapeutic effectiveness. In fact, the presence of iADAs also resulted in an accelerated decline in renal function[42]. Increasing the ERT dose in patients with established iADAs may attenuate the negative effect of those antibodies by saturation of the present ADAs, leaving excess of enzyme to perform its catalytic function[23,41]. In patients that received a kidney transplant, the immunosuppressive agents administered (most often tacrolimus, prednisolone and/or myophenolate-mofetil/mycophenolic acid) prevented ADA formation in formerly treatment naive patients and temporarily reduced ADA titers in ERT treated patients[43]. Whether ADAs inhibit enzyme uptake in the target cells in FD remains to be investigated.

Another factor that could contribute to the limited effectiveness of ERT is the inefficient bio-distribution. The majority of administered recombinant enzyme ends up in the liver, whereas cardiomyocytes and podocytes, two of the most severely affected cell types in FD, take up very limited amounts of recombinant enzyme[10,28,44]. The variation in r-αGAL A uptake between different cell types is not yet fully understood. Although it is traditionally thought that mannose 6-phosphate (M6P) mediated endocytosis is the main mechanism of r-αGAL A uptake, more recent studies show that other pathways are involved as well. Blocking the M6P receptor inhibited r-αGAL A uptake in fibroblasts, but it did not affect uptake in endothelial cells, indicating a different uptake mechanism of r-αGAL A in the latter cell type. This observation is further supported by the lack of M6P receptors on the plasma membrane of endothelial cells[45]. Currently available forms of ERT do clear storage material from endothelial cells[9,46], indicating that these non-M6P dependent endocytic pathways can be adequately utilized. In podocytes, enzyme uptake is in part mediated by M6P receptors, along with two other receptors: megalin and sortilin. However, blocking all three receptors only inhibited recombinant enzyme uptake by 39%[47], again indicating the existence of additional uptake mechanisms. Finally, none of the recombinant enzyme preparations can pass the blood-brain barrier. Whether or not this is relevant for FD, remains a subject of debate. Although there is some accumulation of Gb3 in the brain of FD patients[48,49], the clinical significance remains unclear, as the main complications like TIAs and CVAs most likely result from vascular pathology[50].

Chaperone therapy

Several missense mutations in FD patients have been shown to result in a mutant protein with normal αGAL A catalytic activity. The reduction in overall αGAL A enzymatic activity in patients carrying these GLA mutations has been attributed to the strongly reduced stability of the mutated protein. This is caused by protein misfolding and subsequent premature degradation[51,52]. The goal of chaperone therapy is to enhance correct folding of the mutated protein to improve its stability. The first in vitro studies on the effect of a chaperone in FD used galactose. Adding galactose to the culture medium of COS-1 cells with the p.Q279E mutation increased the enzyme activity in vitro[51]. The follow-up study showed that galactose increased enzyme activity in COS-1 cells and lymphoblasts for several, but not all GLA mutations (eg, no response for the p.G328R mutation)[53]. To date, the only study on the clinical use of galactose describes a male FD patient with the p.G328R mutation who received 1 g/kg galactose intravenously every other day for 2 years. In apparent contrast with the in vitro results, a 180% increase in enzymatic activity in endo-myocardial biopsy

specimens and reduction in cardiac mass was reported[54]. Subsequent chaperone studies mostly used the galactose analogue 1-deoxygalactonojirimycin (now known as Migalastat, Amicus Therapeutics), in which the oxygen is replaced by a nitrogen atom in the ring, resulting in an iminosugar (Figure 2). Migalastat is a potent inhibitor of αGAL A, but at lower doses increases enzymatic activity for some GLA mutations (Figure 3)[55]. It is believed that due to binding of the iminosugar to the catalytic domain of αGAL A, the enzyme is properly folded and after transportation to the lysosome the competitive inhibitor is replaced by the natural substrate of αGAL A. Patients eligibility for treatment with Migalastat is determined using an in vitro enzyme activity assay. In short, wild type HEK-293 cells are transiently transfected with plasmids containing mutant GLA DNA and incubated with Migalastat. Empty vector-transfected cells are used to determine endogenous enzyme activity and this is subtracted from the total enzyme activity of cells transfected with mutant DNA. If the corrected αGAL A activity increases at least 1.2-fold, with an absolute increase in activity of >3%, patients with such mutations in the GLA gene are deemed eligible for treatment with Migalastat. Within the eligible group, the increase in αGAL A activity ranges from 1.2 up to 30.4-fold[56] and is positively related to baseline enzyme activity. The broad range of αGAL A activity increase in response to treatment with Migalastat may, at least partially, explain the highly variable (biochemical) response to Migalastat treatment in the clinical studies described below. Furthermore, the fact that the analyses were done in wild-type cells instead of GLA-knock out cells may warrant some caution in the interpretation of the results as endogenous αGAL A activity may vary per cell and cell count may vary per plate.



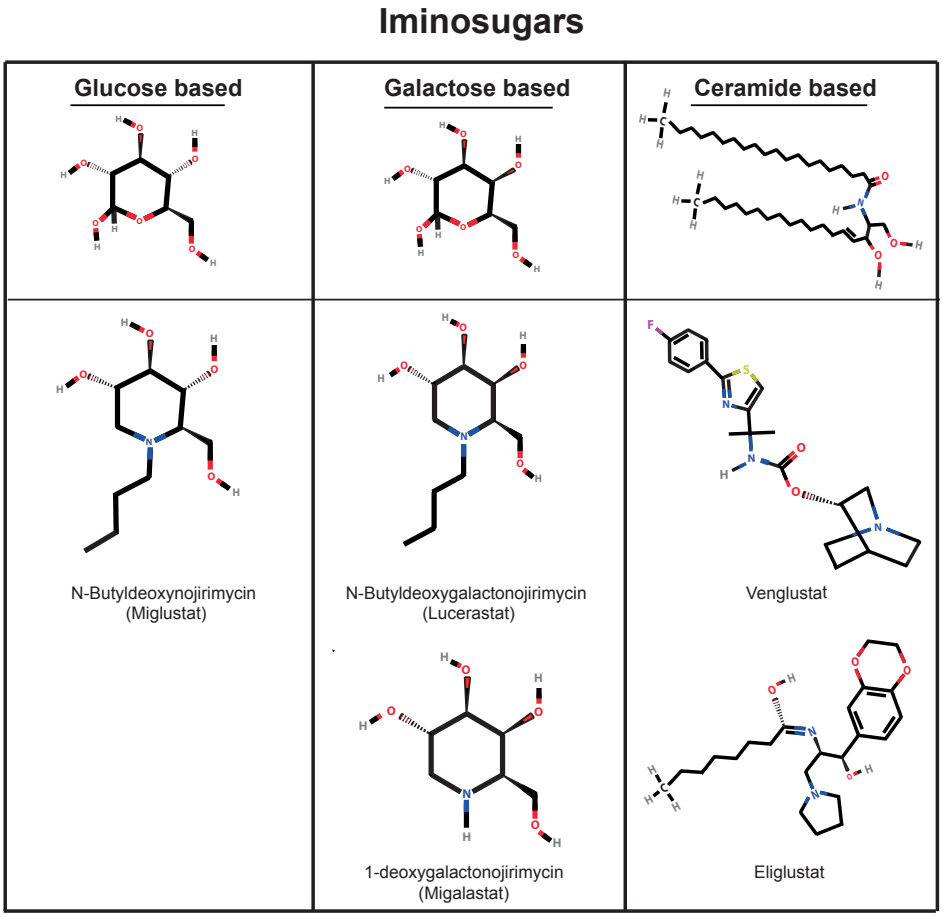


Figure 2. *Similarities and differences in chemical structures of different iminosugars (both chaperone and substrate reduction therapy)*

Migalastat is currently the only oral treatment for FD approved by the US Food Drug Administration (FDA) and European Medicines Agency (EMA)[56,57,58]. Countries outside the USA and EU that have also approved Migalastat are Israel, Australia and Canada. A recent study showed that Migalastat may reduce the accumulation of Gb3 in podocytes after 6 months of treatment[59]. In the same study, gastrointestinal symptoms improved in Migalastat treated patients compared to placebo treated patients[60]. In an 18-month open label study in 57 FD patients, comparing a switch from Fabrazyme or Replagal to Migalastat, no significant differences in kidney function decline (primary endpoint of the study) were observed[58]. A recent publication about Migalastat described a reduction of cardiac mass of 5% on echocardiography in FD patients, a mean increase in αGAL A activity in patient leucocytes and a 45% reduction of plasma lysoGb3

in the previously untreated group[61]. However, as previously pointed out[62], echocardiographic measurements of cardiac mass are highly variable (inter-observer variability 15%-19%, de Simone et al 1999), making it very difficult to draw any conclusion in only 14 patients. For a more precise evaluation of cardiac mass[63], cMRI (inter-observer variability 4%-10%) should be considered for future studies. In addition, native T1 values, late gadolinium enhancement and extracellular volume fractions (ECV) could be measured to assess the development and progress of cardiac fibrosis, although the latter should only be performed in patients with an eGFR >30 mL/min.[64]. Furthermore the increase in enzymatic activity as well as the reduction in plasma lysoGb3 did not occur in all patients, in fact one patient showed an increase in lysoGb3 after switch from ERT. Although the rate of reduction of lysoGb3 may differ between treatments, as they differ in bio-distribution and working mechanism, a rise in lysoGb3 after switch from ERT to chaperone therapy does suggests re-accumulation of the substrate. Lenders et al. recently reported that patients with the p.N215S mutation had a significant reduction of plasma lysoGb3 levels, as well as an increase in leucocyte αGAL A activity upon treatment with Migalastat. In contrast, enzyme activity did not increase in FD patients with the p.L294S mutation and lysoGb3 levels rose significantly in these patients after switch from ERT. Further investigation showed that when amenability was tested in GLA-knockout HEK-293 cells and patient derived cell lines, there was indeed no response to Migalastat in cells carrying the p.L294S mutation, strengthening the clinical observations[65]. Careful monitoring of the in-vivo response, including leukocyte αGAL A activity, plasma lysoGb3 changes and detailed clinical evaluation, will help to select patients that are most likely to benefit from treatment with Migalastat.

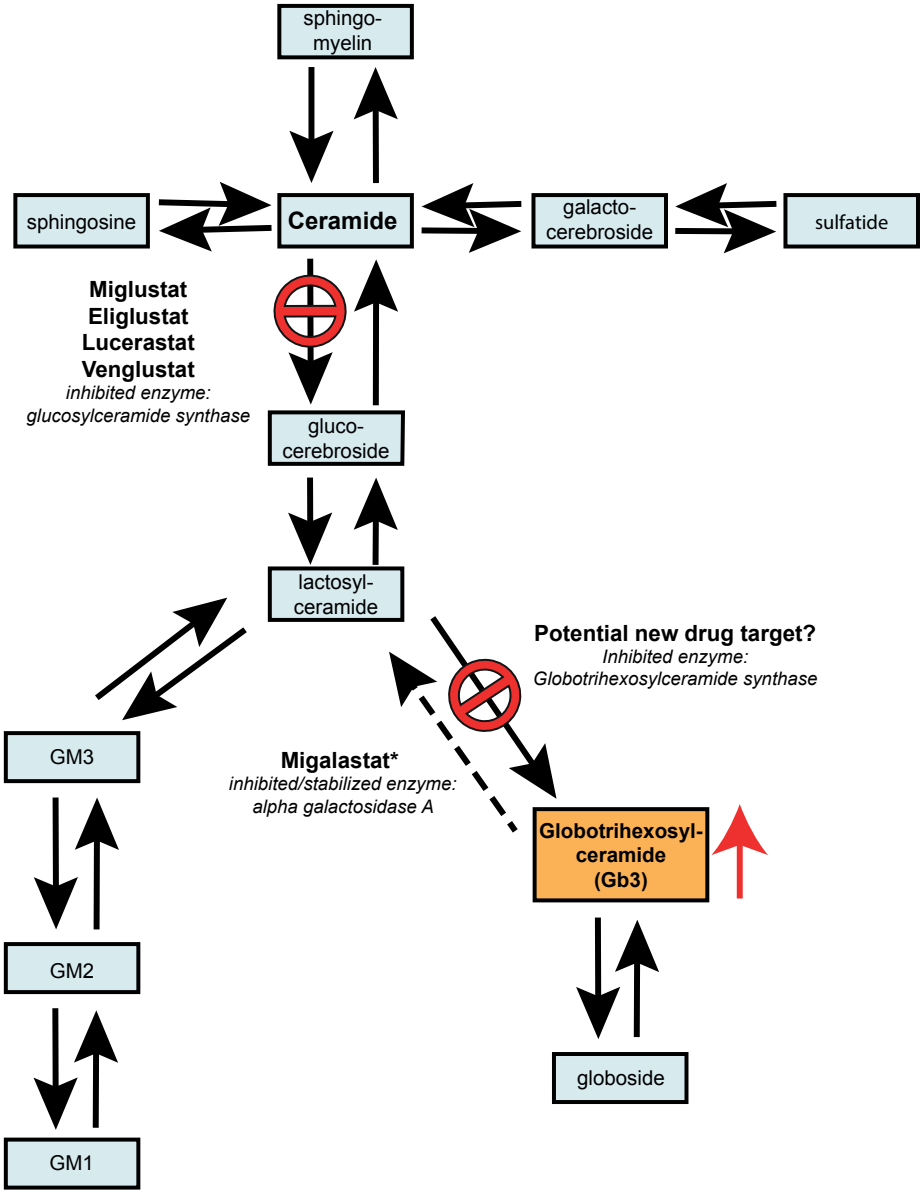


Figure 3. *Glycosphingolipids pathways and location of intervention of substrate reduction therapy and chaperone therapy preparations. Dashed arrow indicates the mutated enzyme in FD. GM 1, 2, and 3 are gangliosides. Arrows indicate specific enzymatic reactions. Stop signs indicate point of intervention for each iminosugar. Red arrow indicates accumulation. *Migalastat inhibits αGAL A in high doses but aids proper folding of unstable αGAL A, resulting from certain amenable mutations, thus increasing enzymatic activity. FD, Fabry disease*

Potential future treatment options

Second generation enzyme therapies

Recently, two new forms of ERT for the treatment of FD have been developed; Pegunigalsidase-alfa (Protalix Biotherapeutics, Israel) and moss-aGal (Greenovation biopharmaceuticals, Germany). Being plant-derived, these enzymes do not carry M6P on their surface[66,67], which may result in a different bio distribution compared to the first generation enzymes.

Pegunigalsidase-alfa (PRX-102) is produced in tobacco cells and has been chemically modified with polyethylene glycol (PEG) to reduce clearance and increase the stability of the enzyme. The increased stability of the enzyme was confirmed in vitro in human plasma and under lysosomal conditions (eg, pH 4.6), as well as in vivo in a murine model. Uptake assays in patients fibroblasts showed co-localization of PRX-102 with lysosomes, but the concentrations used in vitro (160 mg/L) exceeded the expected concentration of PRX-102 in plasma several fold (assuming the dose is 1 mg/kg, which is currently used in clinical trials). The fact that uptake of protein into cells is often concentration dependent makes it difficult to draw any conclusions regarding the expected in vivo uptake[66]. The murine FD model showed different pharmacokinetics and improved bio distribution of PRX-102 compared to agalsidase-alfa and substantial reduction in Gb3 accumulation in heart and kidneys[66]. Data from the phase I/II safety and dose-ranging trial show a mean half-life of 80 hours for PRX-102 (compared to <1 hour for agalsidase-beta) in plasma of patients as well as a reduction of Gb3 in human kidney biopsies in response to treatment[68]. PRX-102 is currently being tested in phase 3 clinical trials (NCT02795676, NCT03018730, NCT03180840). Concerns regarding immunogenicity are that either the altered glycosylation pattern of a plant derived protein[69] and/or the PEG group may serve as an epitope, eliciting an immune response. The latter has been described for other pegylated biologicals[70]. On the other hand, because of the extended half-life in plasma, exposure of the immune system to the enzyme will be much greater, which may lead to immunological tolerance[71]. In the phase I/II trial, treatment was terminated in one patient because of a hypersensitive reaction during the first infusion. Three out of eleven male patients developed ADAs against PRX-102, all of which tolerized after 1 year of treatment[68]. It should be noted that the increased plasma half-life of PRX-102 might interfere with the results of both the Elisa and the inhibitory assays currently used to detect ADAs. Circulating r-αGAL A in plasma at the moment of sampling could bind the ADAs and thereby prevent their detection, as has been shown for other intravenously administered proteins[72]. However, the fact that the profound effect of the ADAs

on pharmacokinetics and pharmacodynamics of PRX-102 in the three ADA positive patients was also transient is promising[68].

Another plant derived form of r-αGAL A is moss-aGal. In vitro, moss-aGal was shown to be adequately taken up in endothelial cells but not in fibroblasts. R-αGAL A enzymatic activity was higher in kidney biopsies from moss-aGal treated FD mice compared to agalsidase-alfa treated FD mice, though no differences were found in Gb3 clearance from these cell types. The half-life of the administered recombinant enzyme in heart and kidney were generally lower for moss-aGal compared to agalsidase-alfa[67]. A phase I clinical trial, in which a single dose of moss-aGal (0.2 mg/kg) was administered to 7 females with GLA mutations (4 classical, 2 late-onset, 1 benign phenotype) (NCT02995993) has recently been completed. No serious adverse events were reported and pharmacokinetic evaluation shows a half-life of 14 minutes in plasma after a single infusion[73].

Substrate reduction therapy

The rationale behind substrate reduction therapy (SRT), another oral therapy for FD, is to limit the formation of metabolites that cannot be degraded due to the underlying enzymatic defect. Precaution in dosing should be taken, for the complete abrogation of a single enzymatic reaction could potentially disrupt the homeostasis of the cell, affecting processes such as apoptosis, cell growth and differentiation[74]. In patients with residual enzyme activity, SRT might be sufficient to reduce the production of the substrate to a level compatible with the remaining enzyme activity. Additional mechanisms to clear accumulated Gb3 in FD patients, such as excretion in bile, may also contribute to the balance between accumulation and degradation of Gb3 in FD patients as has been shown for glucosylceramide in Gaucher disease[74,75]. In patients with minimal to no residual enzyme activity, SRT may not suffice as a single therapy but could still be of added value in addition to ERT[76]. One of the additional potential benefits of iminosugars (SRT's and chaperone therapy) is that they are small molecules which, unlike ERT, do not induce ADA development and may be capable of passing the blood-brain barrier[77].

The first SRT used to treat an LSD (eg, Gaucher disease) was the glucose based iminosugar N-butyldeoxynojirimycin (Figure 2). N-butyldeoxynojirimycin inhibits glucosylceramide synthase (GCS), the first step in glycosphingolipid syntheses (Figure 3) and the drug was introduced as Miglustat (Actelion Pharmaceuticals) for the treatment of Gaucher disease[78,79]. Later on, the more selective GCS inhibitor Eliglustat (Sanofi Genzyme) was introduced, which is successfully used for the treatment of Gaucher disease[80], but is unsuitable for the treatment of

Fabry disease because of its effect on cardiac conduction. Subsequently, novel SRT molecules were developed and tested for FD, such as the ceramide based Venglustat (Sanofi Genzyme) and the galactose derivative Lucerastat (Idorsia Pharmaceuticals, Switzerland), both inhibiting GCS (Figures (Figures22 and and33)[81,82]. Differences in inhibitory capacity and specificity are mentioned in the supplemental file. Preliminary data from clinical trials evaluating the effect of Venglustat in treatment-naïve Fabry patients suggest a slow but gradual clearance of Gb3 from superficial skin capillary endothelium and a gradual decrease of plasma lysoGb3 in most included patients over the course of 3 years of treatment[83,84]. Lucerastat is the galactose form of Miglustat (Figure 2). In a 12 week open label clinical safety trial in a small group of Fabry patients combining ERT with Lucerastat, Gb3 serum levels and urine Gb3 concentrations were lower in the group treated with both ERT and Lucerastat (N = 10) compared to the group treated with ERT alone (N = 4)[84]. Because the above mentioned SRTs all inhibit GCS, the formation of a large number of glycosphingolipids is suppressed, whilst for the treatment of F D only the formation of Gb3 needs to be inhibited (Figure 3). The only study examining the inhibition of Gb3 synthase crossed aGAL A deficient (Fabry) mice with mice lacking Gb3 synthase activity. This resulted in a reduction in globosides like Gb3 in, amongst others, cells of the heart and kidney as well as restoration of lysosomal morphology in these organs[85]. Finally, some general characteristics of iminosugars are good to keep in mind. I) It has been shown for several iminosugars that they can both function as inhibitor as well as chaperone on the same enzyme, depending on their concentration (lower concentrations tend to stabilise mutant enzyme in cell lines with specific mutations, higher concentrations tend to inhibit enzyme activity)[86,87]. Thus, for clinical applications, an estimate of the dose that results in the right intracellular concentration of the iminosugar needs to be made. II) Iminosugars are not fully specific and may also affect other reactions (supplemental file).

Stem cell, gene, and mRNA based therapies

The first bone-marrow transplantations in LSD patients were performed in the 1980s in a mucopolysaccharidosis type I (MPS I) and a Gaucher patient[88,89]. Subsequent clinical experience showed that this treatment can partially (MPSI) or fully (type I Gaucher disease) prevent or reverse disease symptoms[89]. Though new techniques greatly reduced transplantation related mortality, the slowly progressive nature of FD may not justify the risk of severe complications such as graft vs host disease and infections[90]. Especially since the transplantation would preferably be performed in young FD patients, before the onset of organ damage. Furthermore, bone-marrow transplantation is most likely not the optimal route

to target cells of the kidney and the heart. In the last decades, many pre-clinical gene therapy studies for FD have been reported. Both in vivo approaches as well as ex vivo approaches have been explored with several different vectors (retroviral, lentiviral, adenoviral, adeno-associated viral, and non-viral vectors), which have been summarized previously[91]. The main challenge of gene therapy in FD is the targeting of all affected cell types and tissues. As this is unlikely to be accomplished, the efficacy of this approach will mostly depend on the ability of transfected cells to release αGal A into the circulation. The fact that heterozygous female FD patients are still symptomatic despite the fact that they express a normal copy of the GLA gene in, on average, half their cells, demonstrates that the enzyme is either not excreted sufficiently or not taken up efficiently enough by neighboring cells under normal conditions. Whether or not inducing overexpression of αGal A could result in sufficient release of the enzyme into the circulation and adequate uptake by affected tissues in humans remains to be determined[92,94]. Very recently, the first Fabry patients have been treated in phase I and II clinical trials using an ex vivo approach, in which hematopoietic stem cells of the patient are recruited, transfected using lentiviruses (AVR-RD-01, Avrobio) and re-administered to the patient (NCT02800070 and NCT03454893). Next in line are pending clinical trials using adeno-associated viral (AAV) mediated gene therapy, aiming at enzyme production and secretion by the liver using liver specific promoters. Transfection of mice using FLT190 (Freeline therapeutics, UK) and ST-920 (Sangamo Therapeutics) indeed resulted in overexpression and measurable increases in plasma αGAL A[95,96]. Amongst others, there are immunological challenges that accompany gene therapy. In AAV mediated gene therapy, AAV-neutralizing antibodies can directly limit transduction and CD8+ T cells may target AAV-transduced cells, causing loss of transfected cells[97]. Finally, for all forms of gene therapy in FD, the question that remains to be answered is whether or not male patients with classical FD will go on to develop antibodies and/or immunological reactions against the expressed enzyme. It is likely that the continuous exposure and endogenous glycosylation will result in tolerance in most, if not all, transfected patients. However, no conclusions can be drawn from gene therapy trials in Fabry or other protein deficiencies to date, since both high titers of pre-existing ADAs and the use of immunomodulatory drugs under ERT have been an exclusion criterion[98]. Ongoing and pending clinical trials in treatment-naïve male patients with classical FD will give more insight in the risk of antibody development in gene therapy treated patients.

In addition to gene therapy, systemic messenger RNA (mRNA) therapy is currently being developed for FD (Moderna Inc and Translate Bio). mRNA-based therapy has the advantage over DNA-based therapy that it is not at risk for

insertional mutagenesis. A downside of mRNA-based therapy is that the effect is transient, and thus requires repeated administration. Potential advantages over ERT could be that the endogenous protein translation system ensures proper folding, glycosylation and intracellular trafficking of αGAL A[99]. mRNA therapy, encapsulated in lipid nanoparticles, primarily targets hepatocytes in which the enzyme is produced, secreted into the circulation and taken up by tissues. The steady production of enzyme after a single infusion of mRNA in mice and non-human primates resulted in a plasma half-life of αGAL A of 7.5 hours[100,101]. Further murine and non-human primate studies showed a dose-dependent elevation of enzyme levels in plasma, kidney and heart with a half-life of over 100 hours as well as a reduction of Gb3 and lysoGb3 up to 90% and 70% in heart and kidney, respectively, after repeated administration with mRNA.101 mRNA based therapy is relatively new and clinical experience in humans is limited. Within the field of inborn errors of metabolism a phase I/II clinical trial has recently started for methyl malonic acidemia (NCT03810690).

Removal of storage material

An alternative approach to reduce intra-lysosomal storage would be to stimulate the egress of storage material from the lysosomal compartment and subsequently the cell. Studies investigating this approach are based on the association between Gb3 and cholesterol homeostasis, which interact in several manners. Gb3, amongst the other glycosphingolipids, is a component of plasma lipoproteins, specifically low-density lipoprotein (LDL), and can thus be transported into endothelial cells through the LDL receptor[102,103,104]. Storage of glycosphingolipids (GSL) have been shown to result in intracellular accumulation of cholesterol due to upregulation of the LDL receptor. Thus, Gb3 storage may induce further influx of Gb3, in the context of LDL, into the endothelial cell. The influx of cholesterol results in mistargeting of GSLs to the lysosome instead of the Golgi, further increasing lysosomal storage[105,106,107]. Furthermore, Gb3 accumulation has been shown to inhibit cholesterol efflux mediated by apolipoprotein A1 (apoA1)[108]. Therefore, targeting cholesterol metabolism might be a good way to alter glycosphingolipid homeostasis in LSDs such as FD. Incubation of Fabry fibroblasts with HDL or a synthetic replica of apoA1 reduced Gb3 accumulation, by promoting the efflux of both cholesterol and Gb3 from the cell (Figure 4)[107].

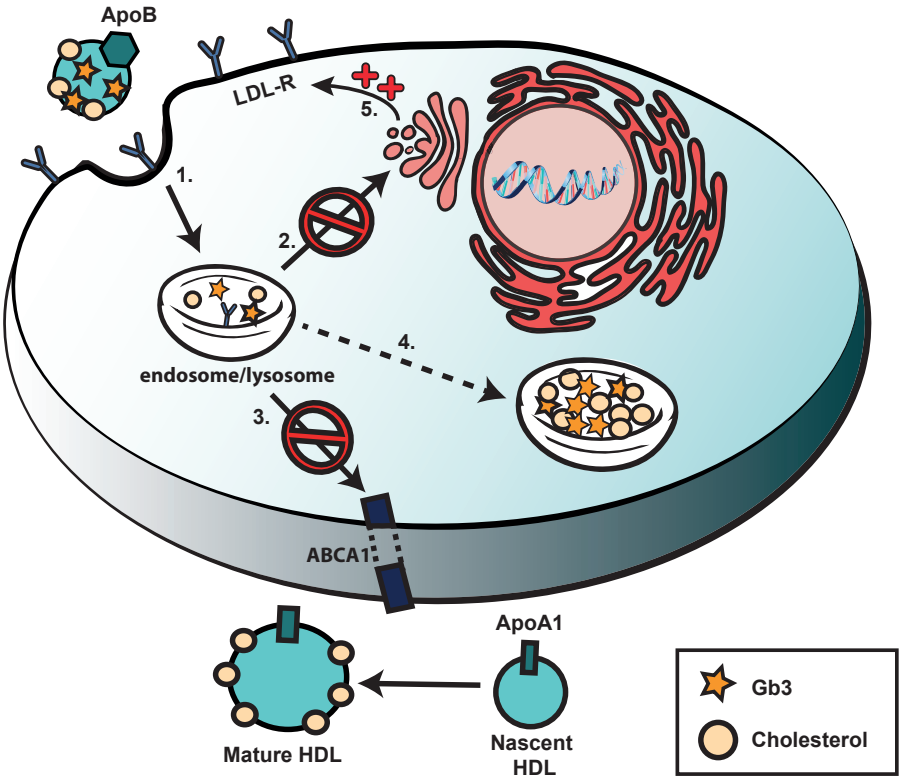


Figure 4. *Hypothesized method of altered lipid homeostasis in Fabry disease.*

1. *Gb3 and cholesterol are taken up through the LDL receptor*[102-104].
2. *Gb3 is mistargeted to lysosomes instead of Golgi and other membranes*[105-107].
3. *Gb3 causes inhibition of ApoA1 mediated efflux of cholesterol*[108].
4. *Storage of GSLs causes upregulation of the LDL receptor thus increasing intracellular uptake of cholesterol and additional GSLs*[105-107].
5. *Increased uptake, mistargeting, and decreased efflux results in lysosomal accumulation of lipids. GSLs, glycosphingolipids; LDL, low-density lipoprotein.*

Non-Fabry-specific therapies

Besides the FD specific treatment, many patients require treatment of their FD symptoms with adjuvant medication or interventions. Patients with pain caused by small fibre neuropathy benefit most from treatment with carbamazepine[109]. In patients with proteinuria treatment with anti-proteinuric agents like ACEi or ARBs reduces proteinuria and slows down renal decline[110]. Treatment with anti-platelet drugs like carbasalate calcium or clopidogrel may be considered, specifically in patients with evident white matter lesions, although evidence on their effectiveness in primary prevention of stroke in the general population is

still lacking[111]. Renal protective and cardiovascular risk management measures should be in place, with particular emphasis on smoking cessation, dietary salt restriction and the treatment of hypertension and dyslipidemia. Some patients require pacemaker implantation because of conductions disorders or sinus node dysfunction. ICD implantation is considered mostly in patients with extensive fibrosis and/or non-sustained ventricular tachycardia on holter examination. No validated sustained ventricular arrhythmia risk calculation tool exists for Fabry disease. Finally, it should be noted that differences in the use of these additional treatments amongst treatment centres may significantly influence trial outcomes. Furthermore, the start of clinical trials in FD has led to a more intensive follow up in patients and more rigorous symptomatic treatment, as well as an attention to life style interventions such as smoking cessation plans. Therefore, any comparison of currently treated to historical untreated FD patients should be interpreted bearing this in mind.

The whole might be greater than the sum of its parts

In conclusion, although important steps have been taken to improve the treatment of Fabry disease, a cure is not yet in sight. For a small subset of patients with specific mutations, treatment with chaperones might be a suitable approach. For the remaining patients, combining different approaches such as ERT with substrate reduction, might be beneficial, but the high costs of the individual therapies currently form an important barrier for this approach. Gene therapy options have been awaited for some time now and the results of the human trials are highly anticipated. Finally, the first results of mRNA based therapies seem promising. However, the small number of FD patients combined with the heterogeneity and slow progression of the disease, makes it difficult to perform well-powered trials of sufficient duration to draw valid conclusions regarding the therapeutic efficacy. This emphasizes the need for clinically validated biomarkers that predict clinical outcome. With the arrival of new treatment options, care must be taken that we are not left with an overload of underpowered studies of insufficient duration, making it impossible to draw any conclusions regarding relative effectiveness of each treatment modality. To tackle this, independent international registries in which data of patients, both untreated and on different treatments, are systematically collected, are essential.

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COMPOUND	SCIENTIFIC NAME	EXTERNAL ID'S/ SYNONYMS	COMPANY (TRADENAME)	SOURCE	INHIBITION OF GCS	INHIBITION OF OTHER ENZYMES (IC_{50}, RANGE)	INHIBITION OF OTHER ENZYMES (K_I, RANGE)
MIGLUSTAT	N-butyl-1-deoxynojirimycin	OGT 918 OGT-918 C 48334 SC-48334 SC48334 NB-DNJ	Actelion (Zavesca)	Platt et al., 1994[2] Wennekes et al., 2010[1,2] Gu et al., 2016[3] Ridley et al., 2013[1] Andersson et al., 2000[1]	IC_{50}: 20.4 µM IC_{50}: 50.0 µM - - -	GBA1 (74-520 µM), GBA2 (0.06 – 0.3 µM) α-Glucosidase (0.1 – 0.4 µM) sucrase (0.5 µM) maltase (10 µM) Lactase (100 µM) Debranching enzyme -	GBA1 (34 µM) GBA2 (48 µM) α-Glucosidase (515 µM) sucrase (0.26 µM) maltase (0.37 µM) Lactase (4000 µM) Debranching enzyme -
ELIGLUSTAT	N-[(1R,2R)-1-(2,3-Dihydro-1,4-benzodioxin-6-yl)-1-hydroxy-3-(1-pyrrolidinyl)-2-propanyl]octanamide	GENZ 99067 GENZ-99067 GENZ-112638	Genzyme (Cerdelga)	McEachern et al., 2007[2]	IC_{50}: 0.024 - 0.04 µM**	GBA1 (>2500 µM) GBA2 (1600 µM) α-Glucosidase (>2500 µM) sucrase (undetectable) maltase (undetectable) Lactase - Debranching enzyme (>2500 µM)	No published data found
LUCERATAT	N-Butyl-1-deoxygalactono-jirimycin	OGT-923 ACT-434964 AA009DW5 NB-DGJ	Idorsia	Platt et al., 1994[2] Wennekes et al., 2010[1,2] Welford et al., 2018[2] Ridley et al., 2013[1] Gu et al., 2016[3] Andersson et al., 2000[1]	IC_{50}: 41.4 µM IC_{50}: 10.0 µM IC_{50}: 11 - 42.4 µM** - - -	GBA1 (320 µM - undetectable) GBA2 (0.3 - 3.5µM) α-Glucosidase (1.5-2100 µM) sucrase (0.5 µM) maltase (10 µM) Lactase (1000 µM) Debranching enzyme (10 µM)	GBA1 (>1000 µM) GBA2 (8.2 µM) α-Glucosidase (5 µM) sucrase (2000 µM) maltase (undetectable) Lactase (85 µM) Debranching enzyme -
VENGLUSTAT (IBIGLUSTAT)	[(3S)-1-azabicyclo[2.2.2]octan-3-yl] N-[2-[2-(4-fluorophenyl)-1,3-thiazol-4-yl]propan-2-yl]carbamate	Genz-682452 GZ-SAR402671 GZ402671	Genzyme	Marshall et al, 2016 Ashe et al., 2015 Marshall et al., 2019	No published data found	No published data found	No published data found

Table 1. *Differences in inhibitory capacity and specificity of different immunosugars currently tested for the treatment of FD.*
NB. Differences in inhibitory effect found may be in part explained by the use of different assays, cell types, techniques and outcome parameters. All represented values were measured in situ.
** IC_{50} represents the half maximal inhibitory concentration: The amount of a given compound needed (ex vivo) to inhibit an enzymatic activity by 50%.*
*** Several methods/cell lines used within the same study*
[1] Studies were done in vitro by directly combining inhibitor and substrate
[2] studies were done in living cells
[3] Studies were done using microsome preparations from animals

Abbreviations: GCS: glucosylceramide synthase/ Ceramide glucosyltransferase, GBA1: glucocerebrosidase/lysosomal acid glucosylceramidase/acid β-glucosidase, GBA2: non-lysosomal glucosylceramidase/ β-glucosidase 2

Enzymes measured:
Andersson et al.: sucrase (K_i), maltase (K_i), lactase (K_i)
Gu et al.: GBA1, GBA2, α-Glucosidase
McEachern et al.: GCS, α-Glucosidase, GBA1, GBA2, glycogen debranching enzyme, sucrose, maltase
Platt et al.: α-Glucosidase, GBA1, GCS
Ridley et al.: GBA1, GBA2
Welford et al.: GCS
Wennekes et al.: GCS, GBA1, GBA2, sucrase, maltase, lactase, α-Glucosidase, debranching enzyme

Supplemental sources

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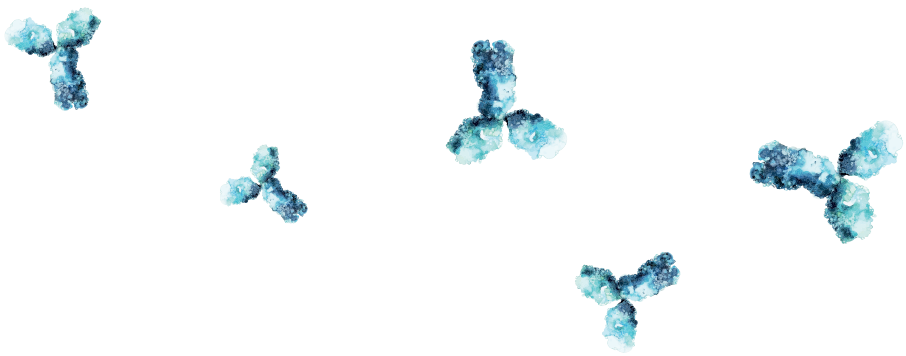

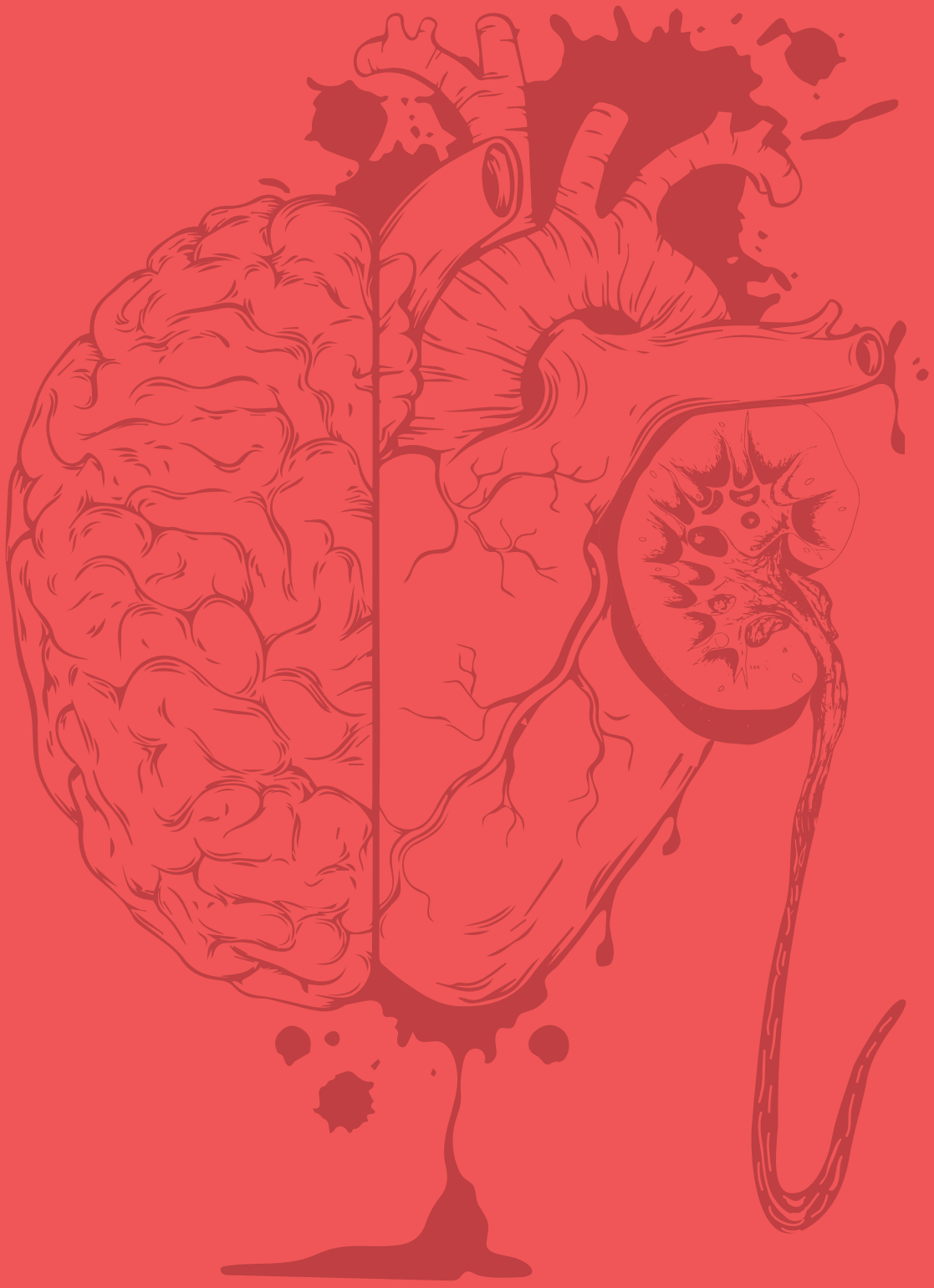

Chapter 5

Development of a biosimilar of agalsidase beta for the treatment of Fabry disease: preclinical evaluation

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Abstract

Background: Fabry Disease (FD) is a rare lysosomal storage disorder caused by a deficiency of the enzyme α-galactosidase A (aGal A). Since 2001, two different enzyme replacement therapies have been authorized, with agalsidase beta being used in most parts of the Western world. Currently, biosimilars of several expensive enzyme therapies are under development to improve their accessibility for patients. We present the pre-clinical results of the development of a biosimilar to agalsidase beta.

Methods: Produced in a CHO-cell system, the biosimilar aGal A Biosidus (AGABIO), was compared to agalsidase beta with respect to aminoacid sequence, glycosylation, specific a-galactosidase activity, stability in plasma, and effects on cultured human Fabry fibroblasts and Fabry mice.

Results: AGABIO had a similar amino acid composition, glycosylation, enzymatic activity and stability as compared to agalsidase beta. After uptake in fibroblasts, α-galactosidase A activity increased in a dose dependent manner with maximum uptake observed after 24 h which remained stable until at least 48 h. Both enzymes were localized to lysosomes. Reduction of accumulated globotriaosylceramide (Gb3) and lysoGb3 in cultured Fabry fibroblasts by AGABIO and agalsidase beta showed comparable dose-response curves. In Fabry knockout mice, after a single injection, both enzymes were rapidly cleared from the plasma and showed equal reductions in tissue and plasma sphingolipids. Repeated dose studies in rats did not raise any safety concerns. Anti-drug-antibodies from FD patients treated with agalsidase beta showed equal neutralization activity towards AGABIO.

Conclusion: These findings support the biosimilarity of AGABIO in comparison to agalsidase beta. The clinical study-phase is currently under development.

Introduction

Fabry disease (FD; MIM 301500) is one of the most prevalent lysosomal storage disorders affecting around 1 in 40.000 people[1]. Deficiency of the enzyme α-galactosidase A (aGalA; (EC 3.2.1.22)) results in storage of glycosphingolipids, mainly globotriaosylceramide (Gb3) in multiple cell types[2]. The disorder is X-linked, resulting in differences in phenotypic expression: the most severe manifestations are present in male patients without any residual aGalA activity[1,3]. The disease is much more variable in female carriers and in males with some residual aGalA activity. These latter patients show predominant involvement of the heart and an increased risk for central nervous system injury[3]. The pathophysiology of FD relates to storage of Gb3 and other lipids that are not or incompletely degraded due to the aGalA deficiency. Storage in vascular cells, podocytes and cardiomyocytes may result in progressive kidney, heart and brain injury[1]. However, the pathophysiology is only partially understood and for example abnormal cell signaling and impaired autophagy may play a role in irreversible manifestations of the disease[4]. Treatment consist of supportive care as well as timely intervention with a fixed dose of enzyme replacement therapy, either with agalsidase alfa 0.2 mg/kg eow (Replagal, Takeda Shire) or agalsidase beta 1.0 mg/kg eow (Fabrazyme, Sanofi Genzyme). In the pivotal trial that led to the registration of agalsidase beta it was shown that enzyme-replacement therapy (ERT) resulted in reduction of Gb3 in plasma, urine and different cell types[5,6]. In general, treatment with ERT can result in a stabilization of renal function, a reduction in cardiac mass and may delay the onset of new clinical events[7]. However, response differs depending on the timing of initiation of therapy, phenotype and the presence of irreversible damage[3,8]. In addition, dose matters: the biochemical response is clearly better with agalsidase beta compared to agalsidase alfa, the first being given at a 5 times higher dose[9]. Effects of anti-drug antibodies (ADA) are relevant in this aspect. In most classical males, development of ADA may partially neutralize the effectiveness of ERT[10]. Indeed, a higher dose of 115 enzyme can partially overcome the inhibitory effect of circulating ADA[11]. Restrictions in reimbursement of this expensive therapy hamper further dose finding in patients with high antibody titers. A cheaper alternative would allow finetuning of dosing in classical males with ADA's who do not benefit from the standard dose. Hence, development of biosimilars at lower costs is of interest. In addition, cheaper alternatives could improve access to treatment for patients in low and middle income countries that are unable to afford the high prices. Currently, new enzymes are being investigated[12], but so far biosimilars of agalsidase beta have only become available in Korea and Japan[13]. Biosidus is a pharmaceutical company based in Argentina aiming to provide

high quality biosimilar products at a fair price. A biosimilar of agalsidase beta was developed according to the European Medicines Agency (EMA) guideline on similar biological medicinal products (https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-similar-biologicalmedicinal-products-rev1_en.pdf) and the biophysical and biochemical properties of this AGABIO are described here.

Materials and Methods

Alpha Galactosidase A and Fabry fibroblasts

Different lots of the recombinant agalsidase originator, agalsidase beta (Fabrazyme®, Sanofi Genzyme) were used for the analyses. The putative biosimilar product, agalsidase beta BIOSIDUS (AGABIO), was manufactured by Biosidus SA. Like agalsidase beta, AGABIO was produced from an established CHO cell line. Full amino acid sequence and disulphide linkage analysis of both agalsidase beta and AGABIO was performed by RP-HPLC/ESI-MS/MS (Protagen, Germany). The protein concentration for each product batch was determined using a high-performance liquid chromatography (HPLC)-based concentration assay calibrated with a control of a known concentration, determined by time resolved amino acid analysis (Protagen, Germany). From this analysis, the extinction coefficient for agalsidase was determined using the Lambert Beer Law, given a value of 2.55 L/(g x cm). The Fabry fibroblasts cell lines used in this publication were GM00107 (Coriell Institute, NIGMS Human Genetic Cell Repository NJ, USA) and a Fabry patient-derived fibroblasts cell line.

Enzyme Activity Measurement

The enzymatic activity of agalsidase was measured using the fluorogenic substrate 4-methylumbelliferyl a-Dgalactopyranoside (4-MU-αGal; Sigma) in a final concentration of 3.0 mmol/L in 0.05 mol/L citrate phosphate buffer (pH 4.6), containing 0.1% (w/v) BSA, as described previously[14]. Reactions were terminated after 15 min by the addition of Glycine/NaOH buffer, pH 10.6, and fluorescent 4-methyl umbelliferone was measured with a fluorimeter (Synergy H1 Multi-Mode Reader BioTek at 455 nm). Specific activity of each sample was measured using the calculated concentration.

Glycosylation analysis

The glycan micro-heterogeneity was characterized by HPAEC-PAD (anion exchange chromatography with pulsed amperometric detection). The study was performed at Alvotech (Germany). Briefly, 200 µg of each sample was reduced in 0.3% SDS/50 mM 2-mercaptoethanol for 30 min at 65°C prior to enzymatic release of the N-linked carbohydrate chains from the protein in 50 mM Tris-HCl, pH 7.4 by adding polypeptide N-glycosidase F (Agilent). Quantitative high resolution HPAEC-PAD mapping of native N-glycans released from samples was performed on an ICS 5000+ ion chromatography system of the Thermo Fisher Scientific Inc. (Waltham, MA, USA). Native N-glycans were applied to high resolution CarboPac PA200 columns at a constant flow rate of 0.4 ml/min and at 30°C column temperature and were eluted by using a concentration gradient consisting of 0.04

M sodium hydroxide, 0.4 M sodium hydroxide and 0.04 M sodium hydroxide/1.2 M sodium acetate. N-glycans were detected via electrochemical detection and the data were collected and processed by using Chromeleon Chromatography Management System Version 7.2 SR5. Characterization and quantification of native N168 glycans via HPAEC-PAD was performed by analyzing appropriate oligosaccharide reference standards.

Stability of agalsidase in different matrices and temperatures

AGABIO and agalsidase beta were separately spiked into human plasma to a final concentration of 1 μg/mL, and incubated at either on ice or at 37°C for 0, 15, 30, 45, 60, 120 and 180 min. Following exposure to plasma, the samples were analyzed for residual enzymatic activity (compared to t=0). The same procedure was done in different matrices and temperatures: normal human serum, phosphate citrate buffer (pH 4.6) and culture medium supplemented with 10% Fetal Bovine Serum (FBS).

Agalsidase cellular uptake and localization

Fabry fibroblasts were cultured at 37°C in 35 mm CELLview dishes from Greiner Bio-One (50% confluency) with 2 ml of DMEM culture medium containing 10% FBS, 21 mM HEPES and fungizone/ penicillin/streptomycin. After overnight adherence medium was replaced with 1 ml DMEM medium containing Alexa 555 labeled AGABIO or Alexa 555 labeled agalsidase beta (2.5 μg enzyme/ml DMEM) and were cultured at 37°C for 24 hr. Forty-five minutes before analyzing the cells LysoTracker™ Green DND-26 was added to the medium in a final concentration of 60 nM. Cells were analyzed using confocal microscopy with the use of a Leica TCS SP8 SMD mounted on a Leica DMI6000 inverted microscope.

Fabry fibroblasts agalsidase activity uptake

Fabry fibroblasts were cultured in 35 mm dishes (200.000 cells/well) with 1 ml of MEM culture medium containing FBS at 37 °C. After overnight adherence and subsequent 24 h of culturing, AGABIO or agalsidase beta (5 μl) was added to 1 ml of fresh culture medium and the cells were cultured for up to 48 h. Agalsidase final concentration evaluated were 0.1, 1 and 10 μg/ml. Untreated cells were used as control. Each sample was analyzed in 6 culture dishes. Cells were trypsinized after extensive washing with PBS. The cell pellet was disrupted by freeze thawing in lysis buffer (27 mM citric acid, 46 mM sodium phosphate dibasic, 1% Triton X-100, pH 4.7). Debris was pelleted by centrifugation, and the soluble fractions were assayed for α-galactosidase activity using 4-MU-αGal as a substrate. Cell lysate total protein content was measured by BCA Protein Assay Kit (Thermo Scientific).

Agalsidase treatment and Gb3 levels

To examine intracellular Gb3 degradation activity in agalsidase treated cells, Fabry fibroblasts were seeded on 24 mm dishes. N-dodecanoyl-NBD-ceramide trihexoside (NBD-Gb3, Abcam, USA), a green fluorescent Gb3 analog, was added (3 μM in serum-free culture medium) and incubated 203 for 24h[15,16]. After cells were washed with PBS, fresh culture media without (control) or with aGalA (agalsidase beta or AGABIO) was added for a further 24h incubation. aGalA final concentration in medium was 0.1 μg/ml. After incubation, the culture medium was replaced with fresh medium and maintained for 48h. Cells were washed with PBS and fixed with PFA 4%. Nuclei were visualized with DAPI (4′,6-diamidino-2-phenylindole). Intracellular fluorescence was visualized using microscopy (Nikon Eclipse TS100) and image was taken by the manufacturer's software (NIS Elements F version 4.0 Nikon).

Dose-response curve of NBD-Gb3 degradation through flow cytometry

NBD-Gb3 fluorescence was measured using a flow cytometry analyzer (Accuri C6 Plus BD Biosciences). Fibroblasts were cultured in 24 well plates (75000 cells/well). After overnight adherence and subsequent 72 h of culturing in medium without FBS, cells were loaded with 0.75 μM NBD-Gb3 and incubated 24h. Medium was replaced by 1 ml of fresh culture medium containing AGABIO or agalsidase beta (0.001 to 1 μg/mL) and incubated for 24h. Cells were trypsinized and suspended in cytometry buffer. Cell fluorescent signal was quantified by FACS. Fibroblasts population (P1) was selected by Dot plot of forward scatter versus side scatter. P1 fluorescence histogram was acquired in FL1 channel (green). Results were expressed as % of intracellular remaining Gb3: this is calculated as the sample MFI (Mean Fluorescence Intensity) relative to the positive control MFI. Positive control is prepared using fibroblasts loaded with NBD-Gb3 without agalsidase treatment.

Dose-response curve of Gb3 degradation through MS/MS analysis

Fibroblasts were cultured in 6 well plates. After overnight adherence, the culture medium was replaced with culture medium containing AGABIO (0.5 to 5.0 μg/mL) or agalsidase beta (0.54 to 5.4 μg/mL). Subsequently, the cells were cultured for 24h, washed with PBS and sonicated. The cell suspension was used for analysis of glycosphingolipids and their sphingoid bases. The 227 time-dependent degradation of Gb3 was studied using fibroblasts cultured in the presence of 1 μg/ml AGABIO or 1.08 μg/ml agalsidase beta for up to 6 days. Gb3 was dissolved in culture medium using either sonication or supplementation with BSA. Loading of the fibroblasts with Gb3 was performed by incubating the cells with culture medium containing Gb3 for 5 days. Subsequently, the culture medium was

replaced by fresh medium containing 5 μg/ml AGABIO or 5.4 μg/ml agalsidase beta and the cells were cultured for another 24 h. Cell lysate total protein content was measured by BCA Protein Assay Kit (Thermo Scientific).

Sphingolipid analysis: Chemicals

Glucosylsphingosine (lysoGlcCer), lactosylsphingosine (lysoLacCer) and d5-glucosylsphingosine (d5-lysoGlcCer) (internal) standards were obtained from Avanti Polar Lipids Inc. and all organic solvents were obtained from Biosolve (LC-MS/MS quality). N-Glycinated globotriaosylsphingosine (Gly-lysoGb3) internal standard was obtained from Matreya LCC. Formic acid, butanol, and hydrochloric acid were obtained from Merck and globotriaosylsphingosine (lysoGb3) standard, sodium hydroxide and ammonium formate from Sigma.

Extraction of sphingolipids from fibroblasts

The metabolites were extracted from fibroblast homogenates by a modification of the method of Bligh and Dyer, see[17] and Online resource 1. Briefly, 600 μL methanol, 600 μL chloroform and 450 μL water was added to 150 μL homogenate. 500 μL of the lower phase, containing Gb3 and LacCer, was dried under a stream of nitrogen at 40°C and the residue was dissolved in 500 μL 0.1 M sodiumhydroxide in methanol. Samples were deacylated (microwave) for analysis of derived lysoGb3 and lysoLacCer asdescribed earlier[17]. After hydrolysis, samples were neutralized by addition of 50 μL 0.1 M hydrochloric acid and 25 μL 1 μM d5-lysoGlcCer in methanol (internal standard) was added. Next, samples were dried (N2, 40°C) and dissolved in butanol and water (1:1, v/v). After drying the butanol phase (N2, 40°C), samples were dissolved in 120 μL methanol and analyzed by LC-MS/MS. For the upper phase (1 mL), containing lysoGb3, 25 μL 0.1 μM Gly-lysoGb3 in methanol (internal standard) was added, samples were dried (N2, 40°C), dissolved in 120 μL methanol and analyzed by LC-MS/MS.

Extraction of sphingolipids from mouse tissues

Pieces of tissue were kept at -80˚C until use. After transferring the tissue pieces to 2 mL safe lock tubes (Eppendorf), 9 parts of water and a metal ball were added and tissues were homogenized using a MagNA Lyser Instrument (Roche Diagnostics GmbH) at a speed of 5000 rpm for 30 seconds. This was repeated until consistent homogenates were obtained. LysoGb3 was extracted from plasma and tissue homogenates by a modification of the method of Bligh and Dyer (methanol:chloroform: water, 1:1:1, v/v/v , see[17] and supplementary fig. 1. Prior to protein precipitation, 25 μL 1 μM Gly-lysoGb3 in methanol was added to each sample. The upper phase, containing LysoGb3, was taken to dryness (N2, 40°C), dissolved in methanol and analyzed by LC-MS/MS. For extraction of Gb3

and LacCer, protein was precipitated and separation of phases was induced by addition of water (methanol:chloroform:water, 1:1:0.9, v/v/v)[17]. Next, the lower phase was transferred to a microwave tube and the upper phase was washed with chloroform. The lower phase was added to the first and combined phases were dried under a stream of nitrogen at 40°C. The residue was dissolved in 500 µL 0.1 M sodiumhydroxide in methanol and samples were deacylated[18]. After this, samples were neutralized by addition of 50 µL 0.1 M hydrochloric acid, 25 µL 1 µM d5-lysoGlcCer (internal standard) was added to each sample and samples were dried 275 and dissolved in butanol and water (1:1, v/v). After drying the butanol phase, samples were dissolved in methanol and analyzed by LC-MS/MS.

LC-MS/MS measurement of sphingolipids

Metabolites were separated by RP-UPLC using an Acquity I-Class UPLC with BEH C18 column, 2.1 × 50 mm with 1.7-µm particle size (Waters Inc.) and detected by electron spray ionization in positive mode (ESI+) and MS/MS-instrument (Xevo TQ MS, Waters Inc.) in multiple reaction monitoring (MRM) mode (see supplementary table 1). In both upper and lower phases, metabolites were calculated using calibration lines within the appropriate concentration range, according to the internal standard ratio. For details on the used LC-MS/MS methods and settings, see supplementary table 1 and[19].

Substrate reduction in GLA-knockout mice organs

Animal experiments were carried out at the Jackson Laboratory In Vivo Services (Bar Harbor ME, USA). This study complied with all applicable sections of the Guide for the Care and Use of Laboratory Animals from the National Research Council. The protocol and any amendments or procedures involving the care or use of animals in this study were approved by the Testing Facility Institutional Animal Care and Use Committee. Fabry model mice (also known as aGal A KO) B6;129-Glatm1Kul/J mice, hemizygous) were used (JAX stock# 003535). Hemizygous mice were grouped into three dosing groups. Each group consisted of four mice. All animals used in this study were 12-week-old males. In one group AGABIO was administered at 1 mg/kg body weight, in a second group agalsidase beta was administered at 1 mg/kg body weight and the third group received vehicle (50 mM Phosphate buffer pH 6.8, 0.6 mg/ml mannitol) All substances were administered intravenously in the tail vein. A wild type group 299 mice (B6;129-Glatm1Kul/J) were administered vehicle and used as control. Necropsies were performed 7 days after dosing. Mice were euthanized by CO2 asphyxiation. Terminal blood was collected by cardiocentesis immediately after euthanasia. Plasma was separated by centrifugation at 10,000 rpm for 10min at 4°C and stored at -80°C until analysis. The following tissues were collected from each mouse:

shaved skin sample, liver, spleen, kidneys and heart. Each tissue was cut into 5 pieces. Each piece weight was recorded and snap frozen in dry ice in separate tubes. Frozen tissues were stored at -80°C until sphingolipid analysis.

Pharmacokinetics

AGABIO or agalsidase beta was intravenously administered in mice, 8 weeks of age, 5 animals in each group, at a dose of 1.0 mg/kg body weight. Whole blood was collected by tail bleed at 1, 10, 30, 60 min after injection. The enzyme activity of agalsidase beta in plasma was measured using the substrate fluorescent analogue, 4MU-Gal.

Safety evaluations

Safety evaluations was performed in a repeated-dose toxicity study in rats compliant with Good Laboratory Practice regulations. AGABIO or agalsidase beta was administered to Wistar rats (8-9 weeks of age, 6 males and 6 females per group) intravenously at 1.0 mg/kg, 2.2 mg/kg and 5.0 mg/kg once every other week for 12 weeks. Recorded clinical observations included standard behavior, body weight and food consumption. Necropsy was conducted on all animals. Weights and histopathology of the following organs and tissues were analyzed: site of injection (to evaluate local tolerance), brain, cerebellum, lung, heart, liver, spleen, thymus, bone marrow, stomach, duodenum, colon, kidney and lymph nodes. Complementary analyses of hematology and serum biochemistry were done for all animals.

Patient anti-αGAL A immunoglobulin cross-reactivity assay

Plasma samples from 10 anti r-αGAL A antibody positive patients were pooled and used to assess cross reactivity of agalsidase beta versus AGABIO. 96-wells microtiter plates (Nunc/Maxisorp) were coated overnight at 4 °C with 100 µl 2 µg/ml recombinant aGal A (agalsidase beta and AGABIO) in PBS (pH 7.4). Concentration of the AGABIO and agalsidase beta batches before dilutions were determined using a bichinchonic acid assay. After washing, the plates were incubated at 37 °C for 1.5 h with 200 µl blocking buffer composed of PBS containing 2% (w/v) BSA fraction V (Merck). Subsequently, plasma samples were diluted in PBS containing 0.1% (v/v) Tween-20 and 2% (w/v) BSA fraction V (dilution buffer). Plasma dilutions ranged from 1:20.000 to 1:2.560.000. Of each dilution, 100 µl was incubated on the plates at 37 °C for 1.5 h, after which plates were washed. Next, plates were incubated with the secondary antibodies; either mouse-anti-human IgG (no differentiation between subtypes) or mouse-anti-human IgG4, labeled with horseradish peroxidase (HRP) (Sanquin Reagents). Secondary antibodies were diluted 1:2500 in dilution buffer. Plates were washed

before 100 µl TMB-substrate was added and plates were incubated approximately 3 min at room temperature and the reaction was stopped using 1 M H2SO4. Absorbance was measured using a microtiter plate reader (Spectramax plus 384) at 450 nm using 540 nm as a reference for background absorption. A pool containing plasma of 35 healthy donors (Sanquin, Amsterdam, the Netherlands) was used as a negative control. All measurements were performed in duplicates. All wash steps consisted of 5 rinsing cycles with PBS 0.1% (v/v) Tween-20.

Statistical analysis

Data were presented as mean ± SD. Statistical significance was determined by the Student's t-test.

Results and discussion

In vitro characterization

Agalsidase beta and AGABIO are stably expressed in CHO cells. Both products have the same amino acid sequence and disulphide linkages (data not shown). Enzymatic analysis, carried out with an artificial substrate, did not reveal any significant differences in the specific activity between the two preparations (supplementary fig. 2). The structure of the carbohydrate chains of aGal A is highly complex[20,21]. Similar to agalsidase beta, AGABIO contains three N-linked oligosaccharide binding sites (Asn108, Asn161, and Asn184). Glycosylation profiling using normal-phase HPLC after glycosidase digestions showed similar peak patterns for AGABIO and agalsidase beta. Similar content of total complex glycans were found in AGABIO and agalsidase beta samples. Complex glycans constituted approximately 60% of the total glycans. In all samples, N-glycan structures with 0 to 4 terminal sialic acid residues (α2,3-linked Neu5Ac) were found. Sialylated isoforms are important for the half-life of circulating agalsidase. Subtle sialylated glycan distribution differences were observed among agalsidase beta and AGABIO samples (fig. 1). Neutral glycan content was less than 10% in all the samples. However, agalsidase beta showed higher amounts of neutral N-glycans (8-9%) compared to the AGABIO preparations (2–4%). The Man5GlcNAc2 structure was identified as the main glycan component in the neutral N-glycan group. Phosphorylated hybrid-type N-glycans and phosphorylated oligomannosidic N-glycan chains bearing one and two phosphate residues, respectively, were present in each AGABIO and agalsidase beta sample. N-glycan phosphorylated structures detected in the three agalsidase beta samples represent about 40% of the total N-glycans. Compared to agalsidase beta, the AGABIO samples showed similar proportions of phosphorylated N-glycans. Importantly, the M6P receptor has higher affinity for biphosphorylated glycans than it does for monophosphorylated glycans. All samples contain 6-8% of biphosphorylated glycans (fig. 1).

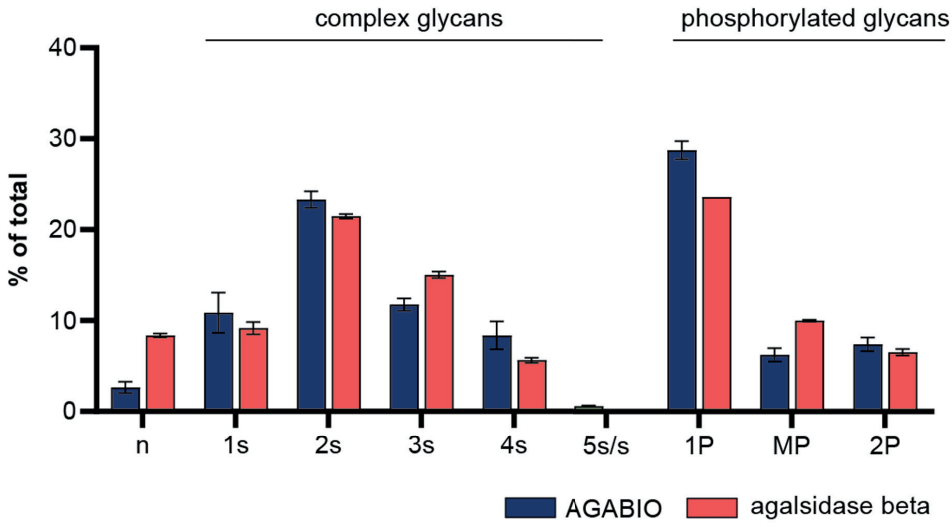

Figure 1. *Quantitative analysis by of native N-glycans released from AGABIO (4 separate batches) and agalsidase beta (3 different batches) samples. Data are shown as mean ± standard deviation. Glycan references are n: neutral, 1s: monosialylated, 2s: disialylated, 3s: trisialylated, 4s: tetrasialylated, 5s/s: pentasialylated and sulphated, 1P, phosphorylated oligomanosidic, MP: phosphorylated hybrid type 2P: diphosphorylated oligomannosidic AGABIO: α-galactosidase A Biosidus*

Stability of AGA in different matrices and temperatures

A time course of changes in enzyme activity of AGABIO and agalsidase beta in plasma at different temperatures and other matrices revealed that there was no difference in stability between AGABIO and agalsidase beta under these conditions (supplementary fig. 3).

Subcellular localization

The subcellular localization of AGABIO and agalsidase beta was determined by confocal microscopy. Intracellular accumulation of Alexa 555 labeled AGABIO or Alexa 555 labeled agalsidase beta was observed as a punctuated form in cytoplasmic area. To verify lysosomal localization, cells were exposed to LysoTracker™ Green. When merged, red fluorescence from Alexa 555 labeled agalsidase and green fluorescence from "LysoTracker™ Green" showed apparent co-localization and presented as yellow dots, showing the same localization of AGABIO and agalsidase beta in lysosomes (fig. 2).

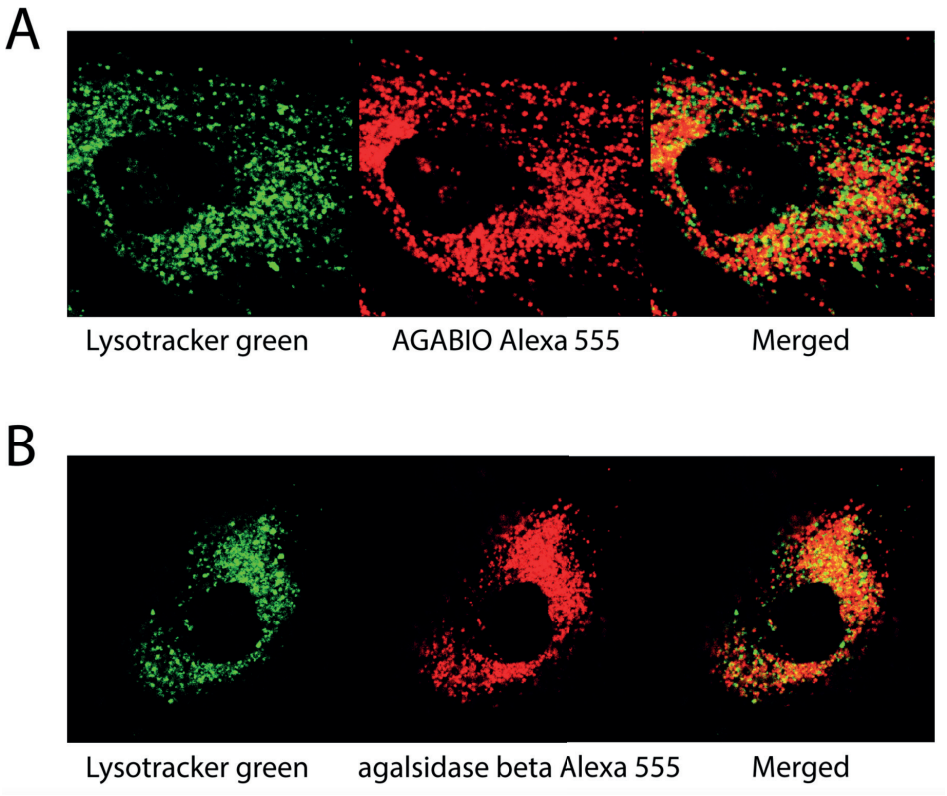

Figure 2. *Internalization and lysosomal localization of Alexa 555 labelled agalsidase into Fabry fibroblasts. Cells were grown over night on glass coverslips and incubated for 24 h in the presence of Alexa 555 labeled AGABIO (red color in panel A) or Alexa 555 labeled agalsidase beta (red color in panel B). Lysosome labeling was achieved using LysoTracker™ Green DND-26. The apparent co-localization of the lysosomal staining (green) and the agalsidase containing granules (red) is represented in yellow when the images are superimposed (Merged).*

Agalsidase activity in cultured human Fabry fibroblasts

The intracellular enzymatic activity in cultured Fabry fibroblasts increased in a dose dependent manner after 24 h. Dose response curves were similar for AGABIO and agalsidase beta. (Fig. 3A). The maximum α-galactosidase A activity uptake was observed after 24 h and remained stable until at least 48 h (fig. 3B). Incubation of culture medium, containing agalsidase beta or AGABIO, at 37°C for 3 hours resulted in an almost complete loss of agalsidase A activity (fig. 4). However, the addition of pre-incubated culture medium (37°C, 3 hours) containing agalsidase beta or AGABIO, to Fabry fibroblasts, restored the agalsidase A activity (fig. 4).

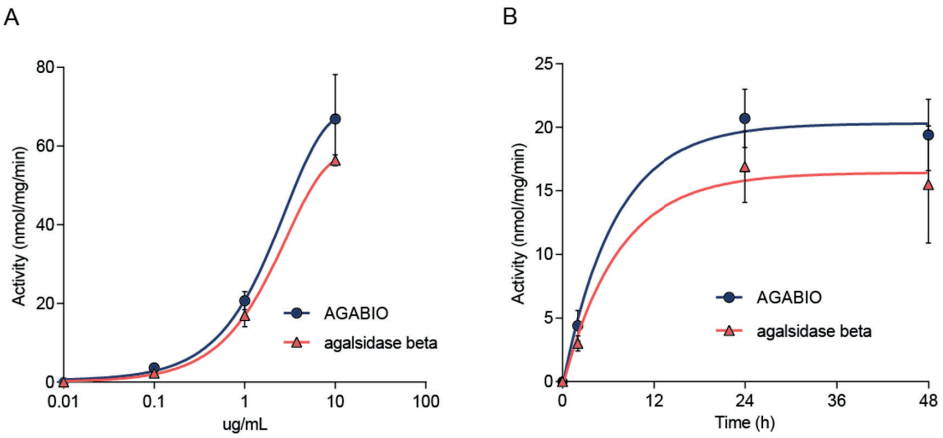

Figure 3. *Quantitative cellular uptake – A) dose response curve and B) Time dependent cellular uptake. Data are means with standard deviation bars from four replicates of one representative lot of AGABIO and agalsidase beta, respectively. AGABIO: α-galactosidase A Biosidus*

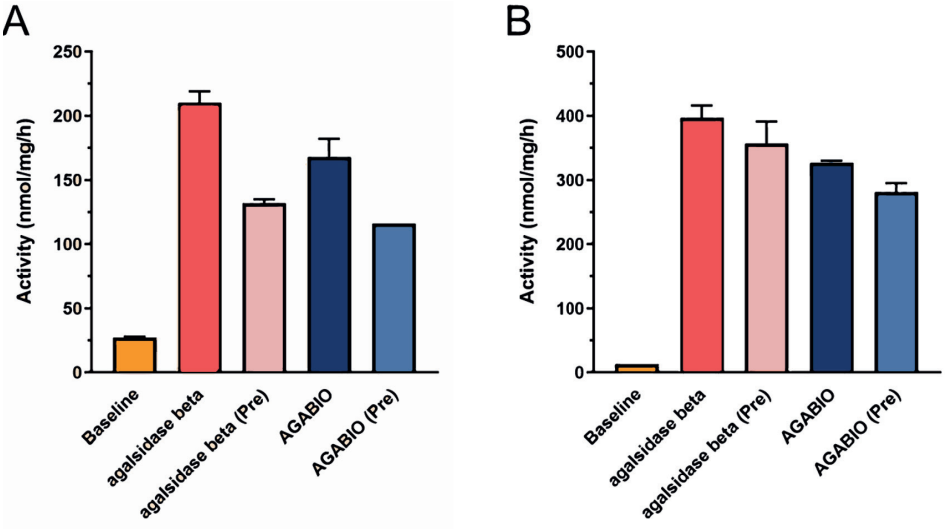

Figure 4. *Quantitative cellular uptake of agalsidase beta and AGABIO 550 enzymatic activity after preincubation (Pre) of the culture medium containing agalsidase beta or AGABIO at 37 oC for 3 hours, followed by culturing the fibroblasts with the preincubated medium for 3 hours (panel A) or 24 hours (panel B). AGABIO: α-galactosidase A Biosidus*

Degradation of accumulated Gb3 in cultured Fabry fibroblasts

The uptake of agalsidase beta and AGABIO resulted in a marked reduction in Gb3 content. Gb3 uptake was identified by treating Fabry fibroblasts with a medium

containing a fluorescently labeled Gb3 (NBD397 Gb3). Intracellular NBD-Gb3 was detected within 4 h after treatment, and persisted up to 96 h. In the presence of agalsidase the fluorescence of the substrate completely disappeared after 48 hours (fig. 5). In addition, NBD-Gb3 degradation at different agalsidase beta and AGABIO doses was quantified using FACS. The incorporated enzymes degraded Gb3 and lowered accumulation of GB3 in cultured Fabry fibroblasts in a dose dependent manner. Dose response curves were similar for AGABIO and agalsidase beta. After 24h, almost all initial NBD-Gb3 was degraded with either product at 0.1 μg/mL concentration (fig. 6). Fibroblasts loaded with Gb3 showed increased intracellular levels of Gb3 and lysoGb3 when compared to that observed in the non-loaded cells. Treatment of the non-loaded and GB3-loaded fibroblasts with AGABIO or agalsidase beta resulted in decreased intracellular Gb3 and lysoGb3 levels and increased lactosylceramide levels (supplementary figures 4-6).

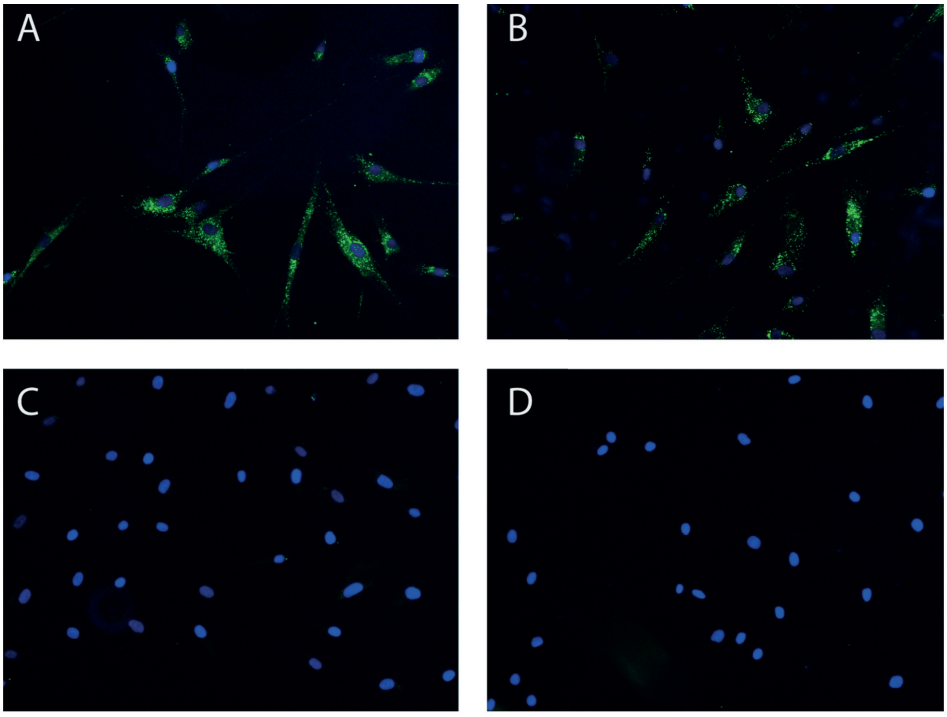

Figure 5. *Intracellular NBD-Gb3 fluorescence. Fluorescent uptake analysis of NBD-Gb3 (green) after incubation in Fabry cells (A). After 48 h treatment with media (control) green fluorescence remained unchanged (B). After 48 h treatment either with AGABIO (C) or agalsidase beta (D), all accumulated NBD558 Gb3 was degraded. Nuclei are stained with DAPI (blue). AGABIO: α-galactosidase A Biosidus DAPI: 4′,6-diamidino-2-phenylindole NBD-Gb3: N-dodecanoyl-NBD-ceramide trihexoside.*

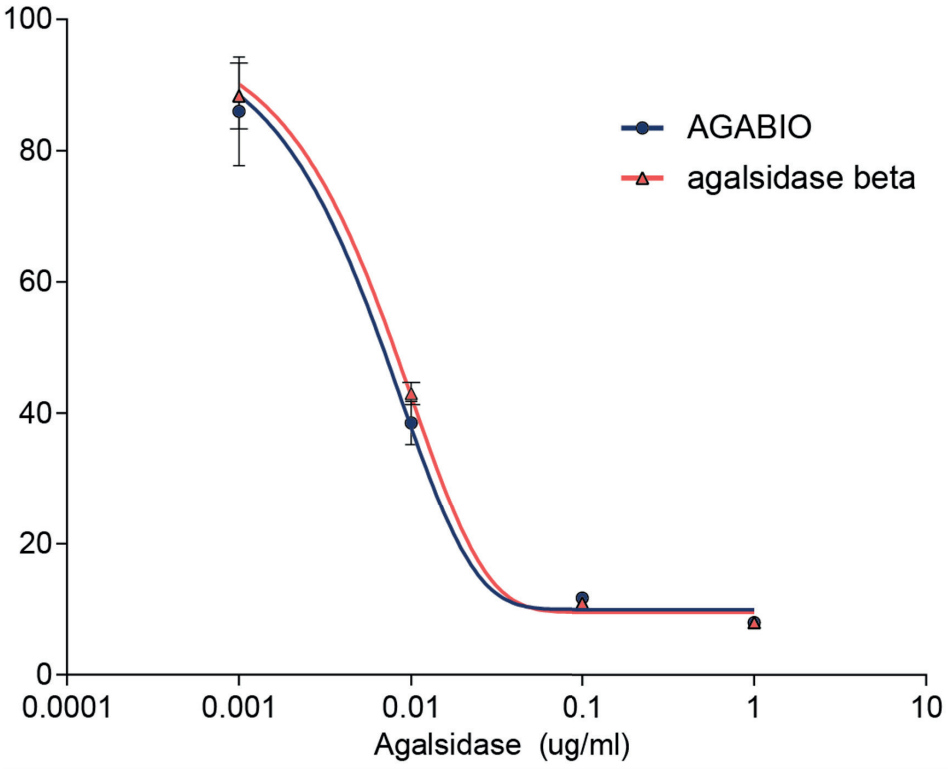

Figure 6. *Intracellular NBD-Gb3 degradation by agalsidase treatment- dose response curve. Data are means with standard deviation bars from four and three lots of AGABIO and agalsidase beta, respectively. AGABIO: α-galactosidase A Biosidus NBD-Gb3: N-dodecanoyl-NBD-ceramide trihexoside*

Patient anti-αGAL A immunoglobulin cross-reactivity

Anti-drug antibodies that developed in 10 male patients with classical FD upon treatment with agalsidase beta showed complete cross reactivity in vitro with AGABIO compared to agalsidase beta. Both the assay measuring total IgGs (fig. 7a) as the assay measuring IgG4 (fig. 7b) showed no differences between agalsidase beta and AGABIO.

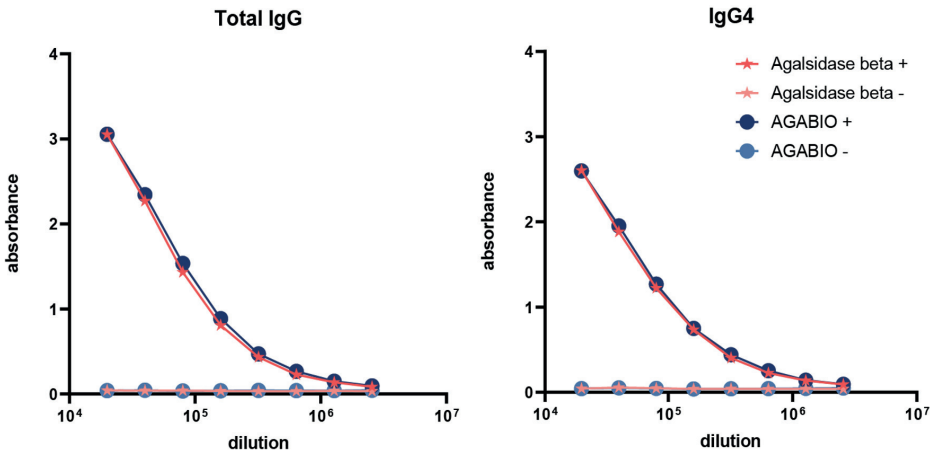

Figure 7. *In vitro cross reactivity of anti-aGal A antibodies (anti-drug antibodies, ADA) from a pool of 10 ADA positive male FD patients. Elisas were performed using HRP labeled mouse-anti human antibodies directed at all IgGs (a) as well as IgG4 specifically (b). Darker colored lines (+) represent the cross reactivity of various dilutions of the Fabry serum pool, consisting of 10 ADA positive FD patients, with either Agalsidase beta (Red) or AGABIO (Blue). Lighter colors represent negative controls using a serum pool derived from 35 healthy donors. Dilutions of serum started at 1:20000 up to 1:2560000. All measurements were performed in duplicate on the same plate. ADA: anti-drug antibodies AGABIO: α-galactosidase A Biosidus*

Pharmacodynamics in Fabry mice

Preclinical studies of enzyme-replacement therapy for FD were performed in agalsidase–deficient mice. Despite having a normal lifespan without organ failure, biochemically, Fabry knockout mice have been shown to reproduce abnormalities found in Fabry patients, including significant Gb3[22] and lyso-Gb3 accumulation[23] in plasma and in the lysosomes of most tissues (in particular, the liver, spleen, heart, skin, and kidneys). Indeed, the Gb3 and lysoGb3 content in the organs of untreated Fabry mice was markedly higher than that in those of wild type mice. Following one single injection (1 mg/kg) of either AGABIO or agalsidase beta into the blood vessels of Fabry mice, the degradation of substrates in the kidneys, heart, skin, spleen and liver was comparable between wild-type and knockout mice. Tissue and plasma sphingolipids decreased with both products in a similar manner (fig. 8).

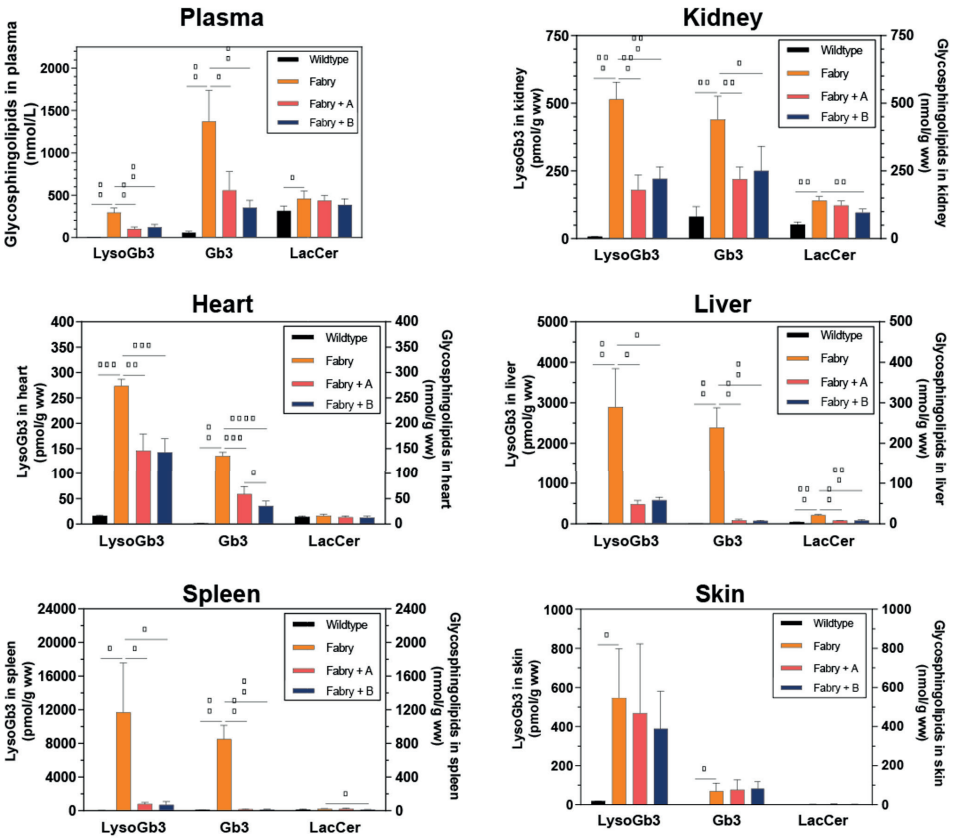

Figure 8. *Glycosphingolipids in plasma and tissues of wild-type mice. Fabry mice and Fabry mice treated with A (agalsidase beta) or B (AGABIO). The bars represent the mean + SD values. Statistical analysis was performed using the Welch's t-test, P<0.05 *, P<0.01 **, P<0.001 ***, P<0.0001 ****. AGABIO: α-galactosidase A Biosidus GB3: Globotriaosylceramide LAcCer: lactosylsphingosine LysoGB3: globotriaosylsphingosine*

Pharmacokinetics

Plasma activity of AGABIO and agalsidase beta (three batches each) showed no marked difference throughout the study period (supplementary fig. 7). After a single intravenous dose of 1 mg/kg, product was rapidly cleared from the circulation with a T½ of 12-13 min. These results indicate that both AGABIO and agalsidase beta have similar pharmacokinetic profiles.

Repeated-dose toxicity study in rats

AGABIO or agalsidase beta was administered to Wistar rats (8-9 weeks of age, 6 males and 6 females per group) intravenously at 1.0 mg/kg, 2.2 mg/kg and 5.0

mg/kg once every other week for 12 weeks. During the experimental period, mortality and body mass were monitored, and necropsy was undertaken at the end of the experimental period. No deaths or clinical signs were observed. No abnormalities were found during necropsy. There were no significant effects of the treatment on body weight, food consumption, biochemistry, necropsy, organ weight, or histopathology in either males or females in all test groups. No pathological signs were observed. There were no notable differences in safety profile between AGABIO and agalsidase beta.

Summary and conclusions

We show in this biochemical and pre-clinical comparative study that the characteristics of AGABIO are similar to that of agalsidase beta. In addition, an identical uptake and distribution in cells and ability to degrade accumulated substrates was observed in both fibroblasts and in mice. No toxicity was observed in a rat study. We feel that this product may improve the access of enzyme therapy to patients in need at a lower price. At the same time, we expect that further clinical analysis of individualized dosing in patients with neutralizing antibodies may lead to improved benefit in these patients.

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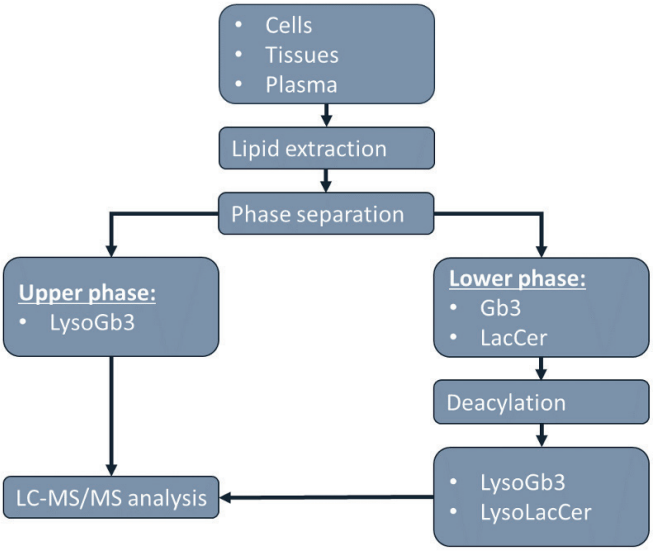
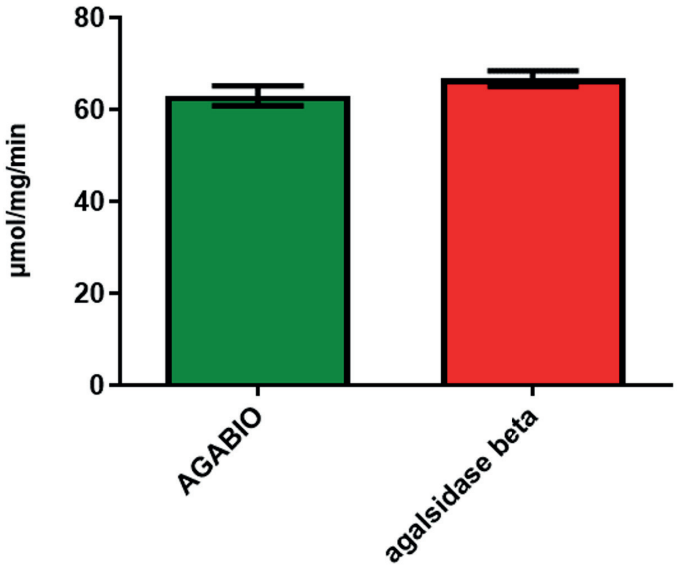
Supplemental Material



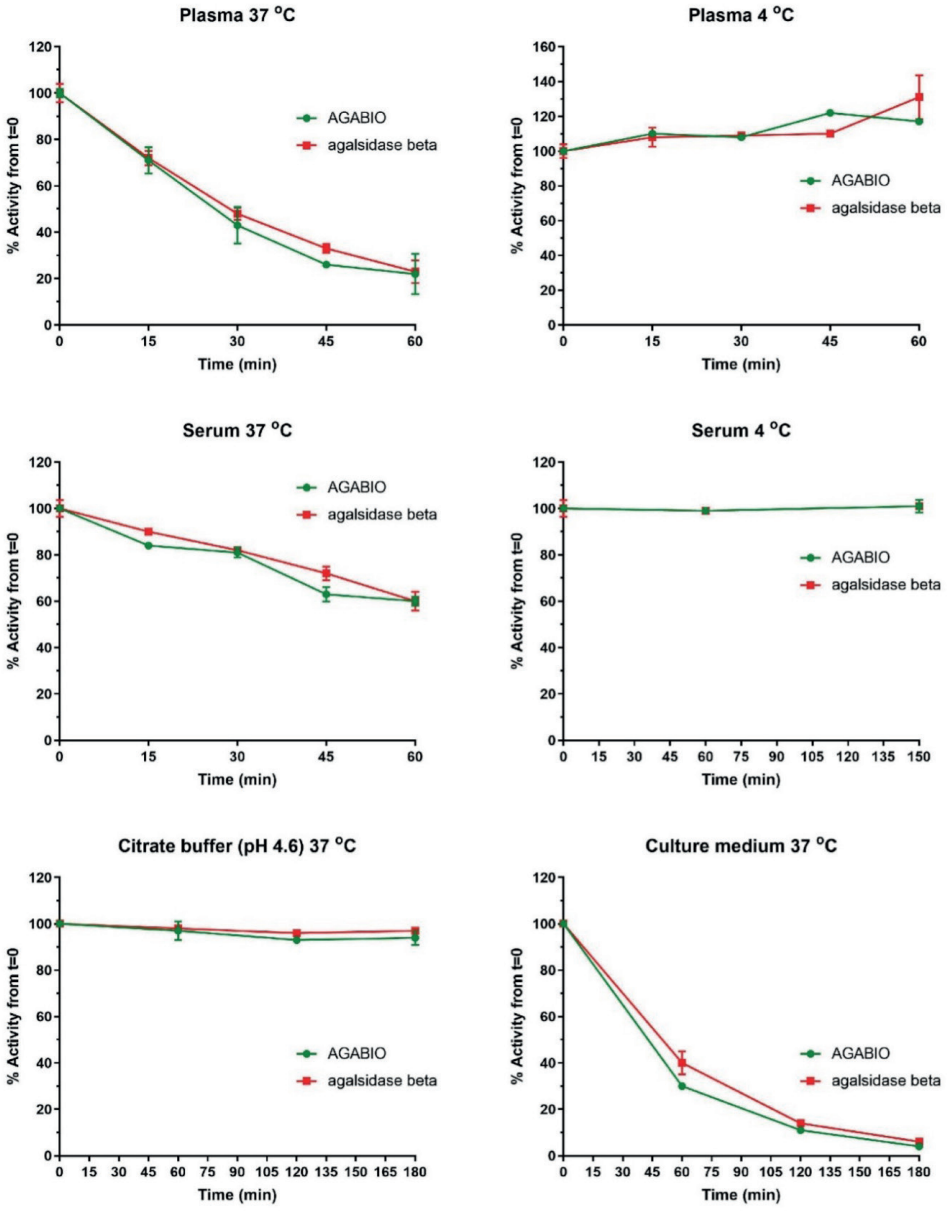
Supplementary figure 1. *Scheme for determination of LysoGb3, Gb3 and LacCer*

Metabolite	Calibration curve	Internal standard	MRM (m/z)	Cone voltage (V)	Collision voltage (V)	Retention time (min)
Gb3	LysoGb3	d5-LysoGlcCer	786.5 > 282.3	45	35	3.08
LacCer	LysoLacCer	d5-LysoGlcCer	624.4 > 282.3	35	30	3.10
LysoGb3	LysoGb3	Gly-lysoGb3	786.5 > 282.3	45	35	3.08
d5-LysoGlcCer	Not applicable	Not applicable	467.4 > 291.5	25	20	3.12
Gly-lysoGb3	Not applicable	Not applicable	843.5 > 264.4	40	55	3.09

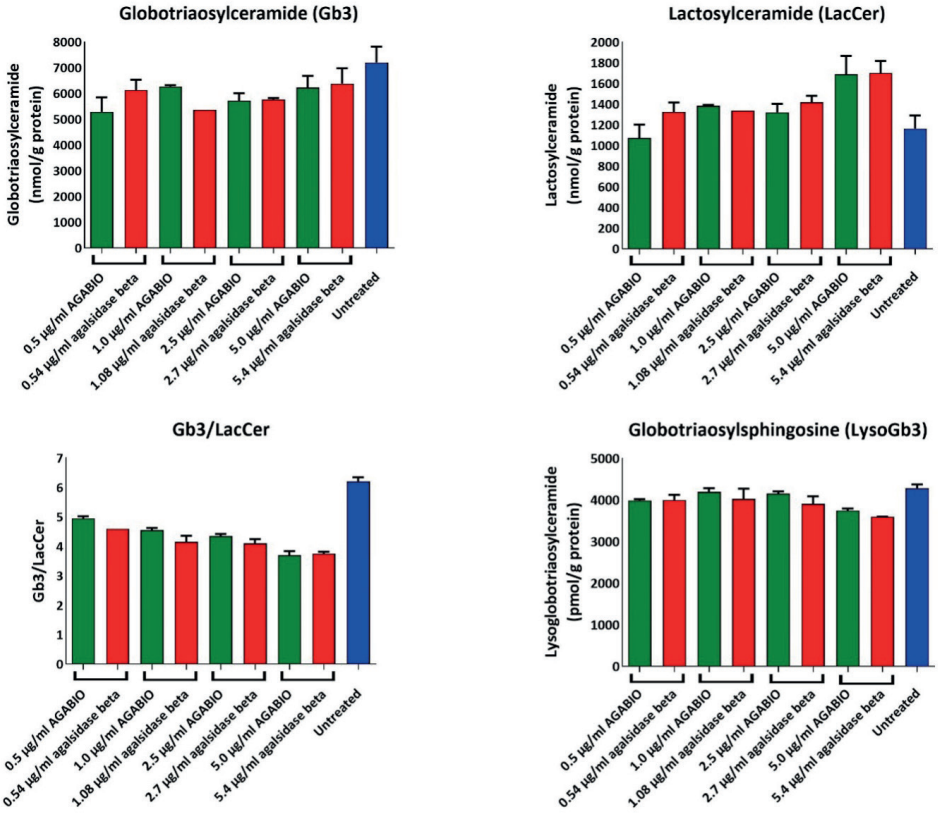
Supplementary table 1. *LC-MS/MS settings*



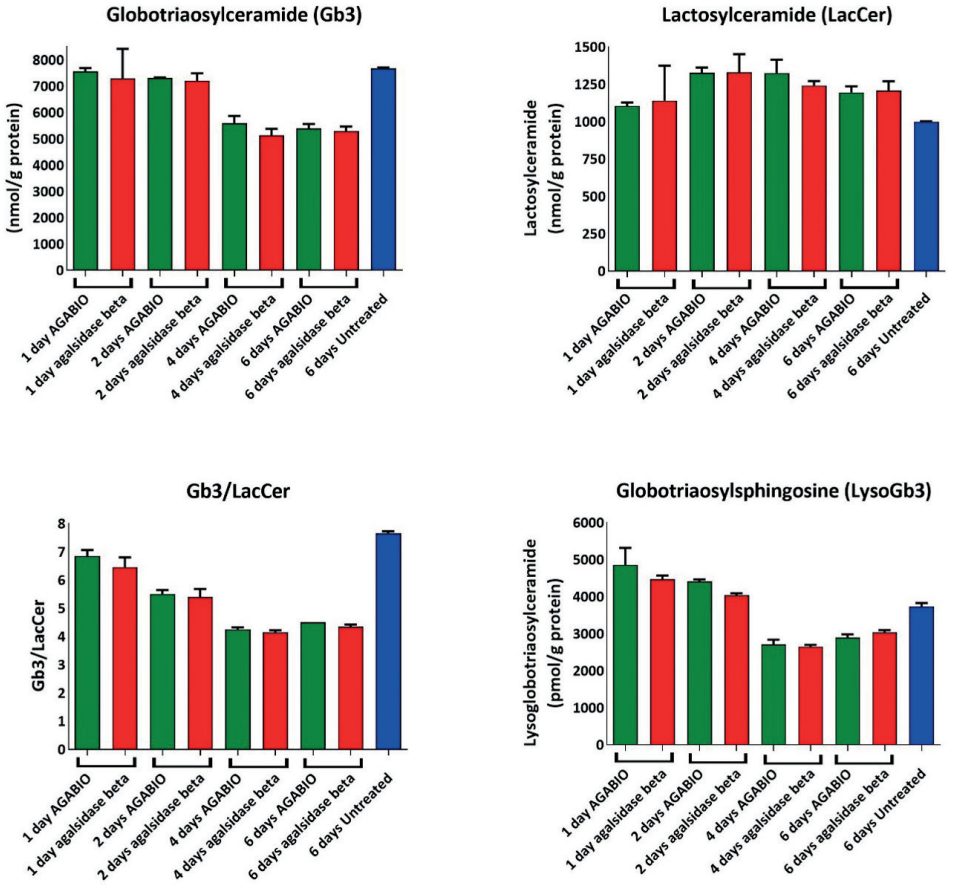
Supplementary figure 2. *Specific activity agalsidase samples. Data are means with SD bars from four lots of AGABIO and agalsidase beta, respectively. The statistical difference was examined by Student's t-test.*



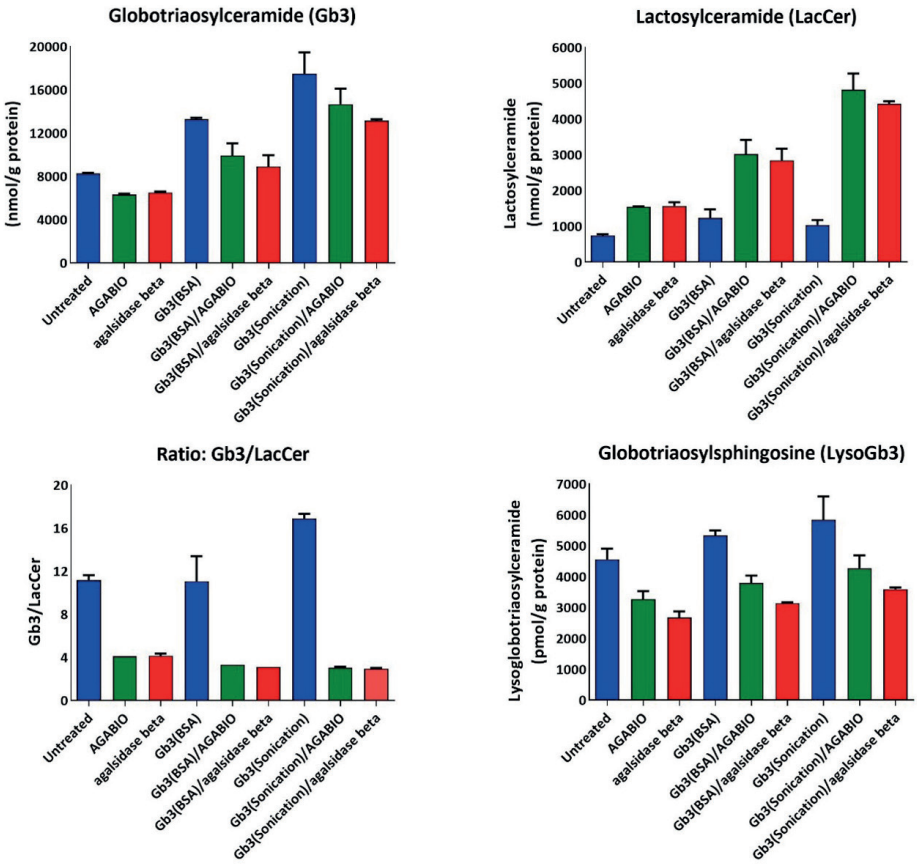
Supplementary figure 3. *In vitro stability of AGABIO and agalsidase beta. Stability was measured as the remaining activity of the enzymes analyzed by the hydrolysis of the synthetic substrate 4-MU-αGal in human plasma (A) 37 °C (B) 4 °C (on ice). The results are expressed as the percentage of the initial enzymatic activity at the beginning of the experiment. Results are means of 2 measurements ± S.D.*



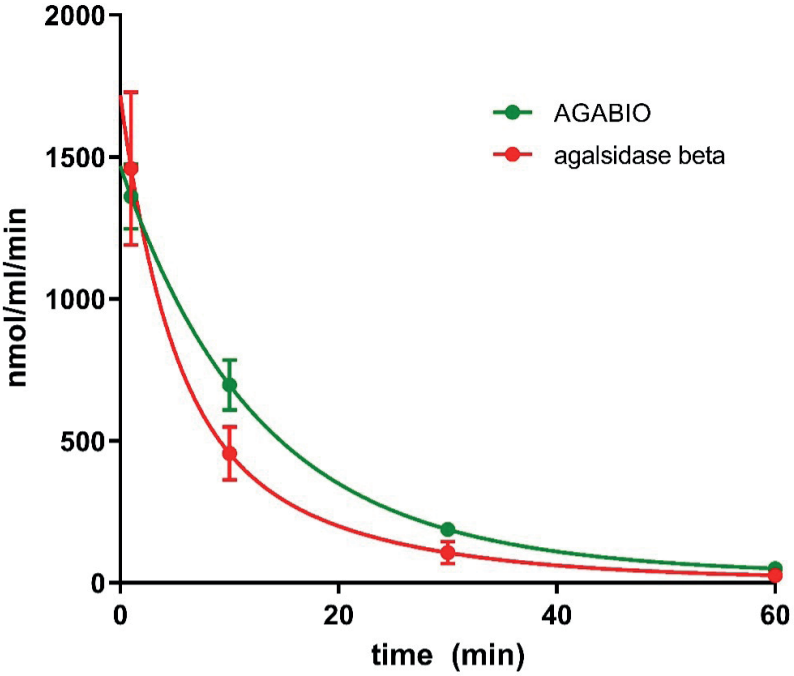
Supplementary Figure 4. *Concentration dependent intracellular degradation of intracellular endogenous Gb3, LacCer, Gb3/LacCer ratio and lysoGb3 after treatment with different concentrations of recombinant AGABIO (0.5 to 5.0 µg/mL) or agalsidase beta (0.54 to 5.4 µg/mL). Cells were cultured in 6-well culture plates with 2 ml of DMEM culture medium. After overnight adherence medium was replaced with medium containing agalsidase beta or AGABIO and the cells were cultured for 24h, harvested and the sphingolipids GB3, lysoGB3 and lacCer were quantified.*



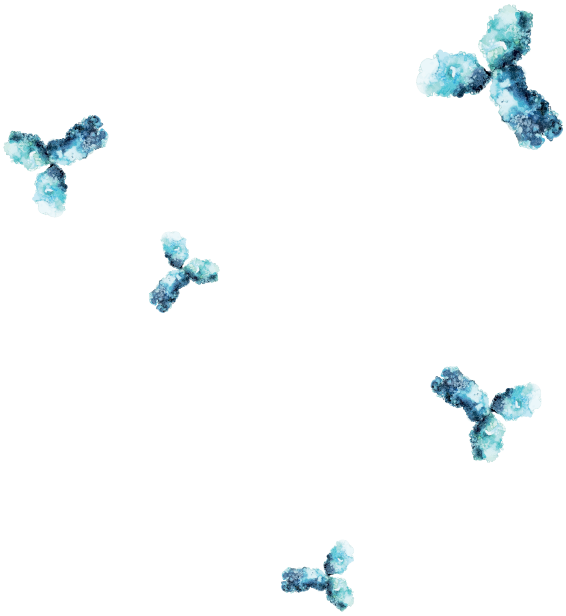
Supplementary figure 5. *Time dependent degradation of intracellular endogenous Gb3, LacCer, Gb3/LacCer ratio and lysoGb3. Cells were cultured in 6-well culture plates with 2 ml of DMEM culture medium. After overnight adherence, the medium was replaced with medium containing 1 µg/ml AGABIO or 1.08 µg/ml agalsidase beta and the cells were cultured for 1-6 days. Subsequently, the cells were harvested and analyzed for the sphingolipids GB3, lysoGB3 and lacCer.*



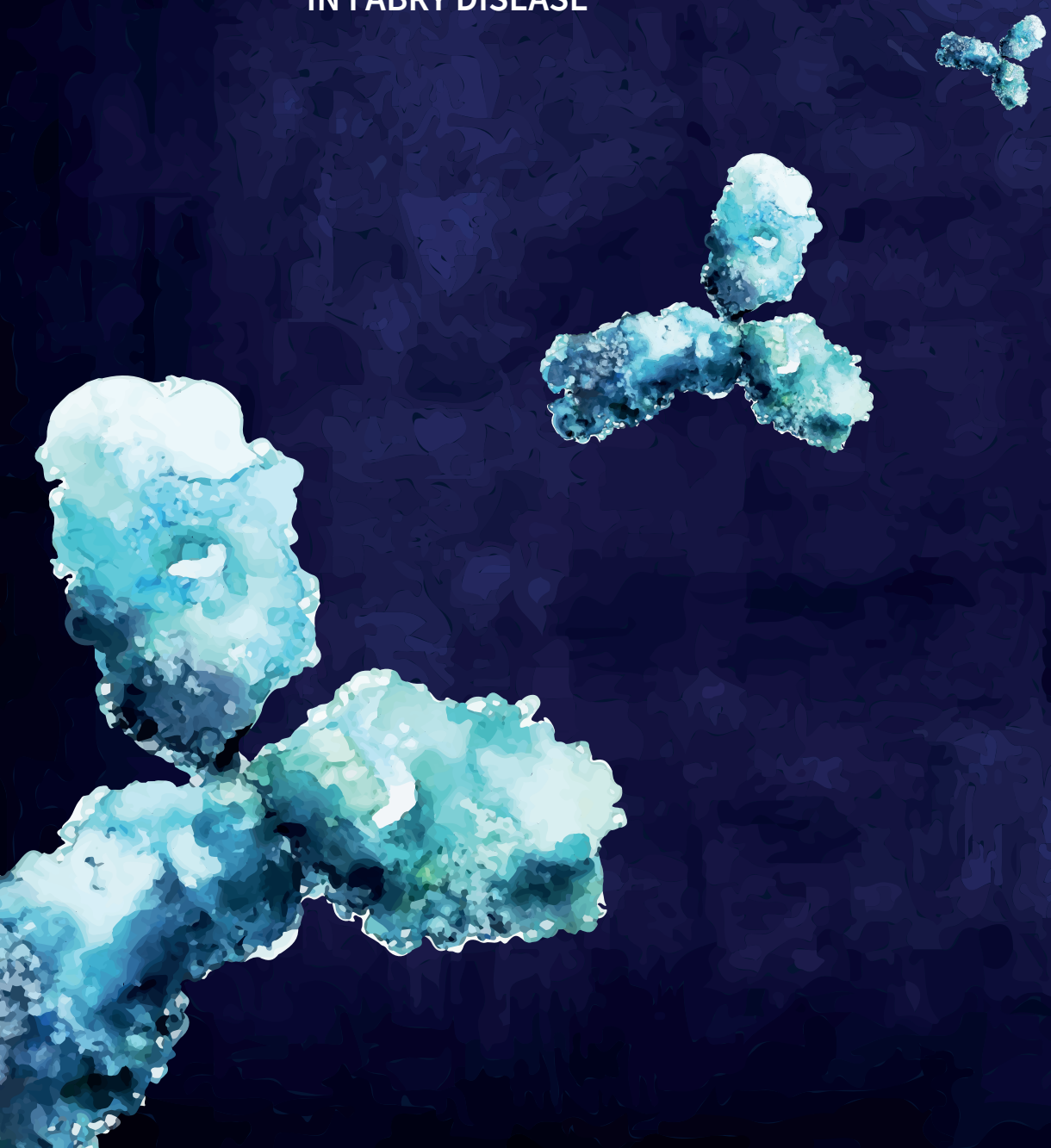
Supplementary figure 6. *intracellular degradation of intracellular Gb3, LacCer, Gb3/LacCer ratio and lysoGb3 after treatment of GB3-loaded fibroblasts with recombinant agalsidase beta and AGALBIO: comparison with controls. Cells were cultured in 6-well culture plates with 2 ml of DMEM culture medium. After overnight adherence medium was replaced with DMEM, DMEM containing GB3 and sonication [GB3(sonication)] or DMEM containing GB3 mixed with BSA [GB3(BSA)]. After 5 days of incubation, medium was replaced with 2 ml DMEM medium containing 5 µg/ml AGABIO or 5.4 µg/ml agalsidase beta and the cells were cultured for 24h. Subsequently, the cells were harvested and analyzed for the sphingolipids GB3, lysoGB3 and lacCer.*



Supplementary figure 7. *Pharmacokinetic profile of AGABIO and agalsidase beta in mice plasma. Mice (5 per group per batch of product tested) were injected with a single intravenous dose of 1 mg/mL of AGABIO or agalsidase beta. Plasma was tested at different time points post-injection for enzymatic activity, using 4-MU-Gal. Results are the mean of three different AGABIO batches and three different agalsidase beta batches measurements ± S.D.*

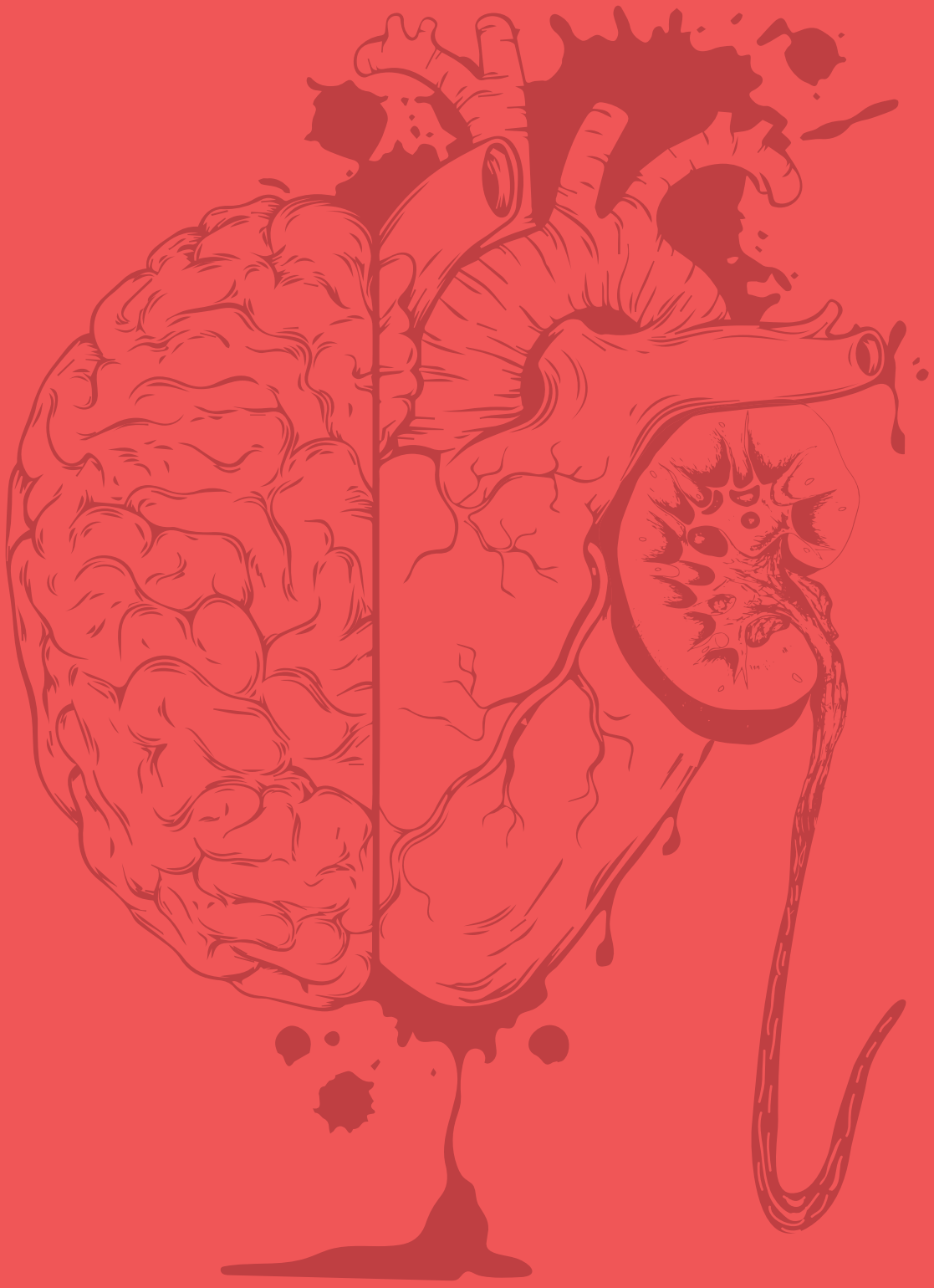


THERE IS A NEED FOR INTERNATIONAL CONSENSUS ON HOW BEST TO MONITOR ADAs AND THEIR EFFECT ON TREATMENT IN FABRY DISEASE



Part 2

Past & future treatment complications



Chapter 6

Antibodies against recombinant alpha-galactosidase A in Fabry disease: Subclass analysis and impact on response to treatment

Sanne J. van der Veen, André B.P. van Kuilenburg,
Carla E.M. Hollak, Paul H.P. Kaijen,
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Abstract

Background: Treatment of Fabry disease (FD) with recombinant alpha-galactosidase A (r-αGAL A) is complicated by the formation of anti-drug antibodies in the majority of male patients with the classical disease phenotype. Detailed information regarding antibody subtypes, onset and persistence of antibody development and their effect on treatment efficacy is sparse.

Methods: A retrospective study was carried out in 39 male patients with classical FD, treated with either agalsidase-alfa or agalsidase-beta (mean follow up of 10 years). With six to twelve months intervals plasma-induced in vitro inhibition of enzyme activity, globotriaosylsphingosine (lysoGb3) levels and renal function were assessed. In a subset of 12 patients, additionally anti- r-αGAL A IgM, IgA and IgG1, 2, 3 and 4 levels were analyzed.

Results: In 23 out of 39 patients, plasma-induced in vitro inhibition of r-αGAL A activity was observed (inhibition-positive). The inhibition titer was strongly negatively correlated to the decrease in lysoGb3: agalsidase-alfa (FElog10(inhibition) = −10.3, P ≤.001), agalsidase-beta (FElog10(inhibition) = −4.7, P ≤.001). Inhibition-positive patients had an accelerated decline in renal function (FE = 1.21, p = .042). During treatment IgG1 anti-r-αGAL A levels increased only in inhibition-positive patients (p = .0045). IgG4 anti-r-αGAL A antibodies developed in 7 out of 9 inhibition-positive patients. Other antibody subclasses were either not present or too low to quantify.

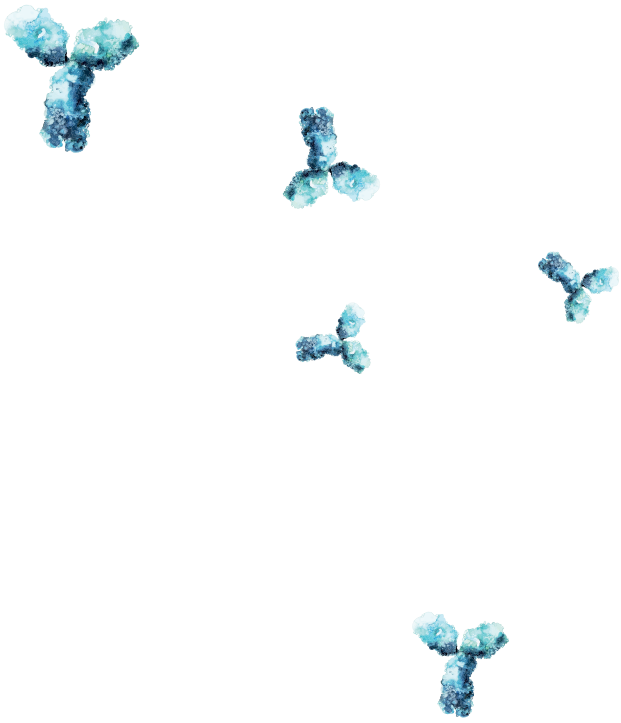
Conclusion: Development of inhibiting antibodies against r-αGAL A negatively affects the biochemical response to ERT and resulted in an accelerated decline in renal function. The presence of IgG1 and IgG4 anti-r-αGAL A antibodies is associated with in vitro αGAL A activity inhibition.

Introduction

Fabry disease (OMIM 301500) is an X-linked lysosomal storage disorder resulting from a deficiency of the enzyme alpha-galactosidase A (αGAL A, EC 3.2.1.22). The resulting failure to hydrolyze the terminal alpha-galactosyl moiety from globotriaosylceramide (Gb3) causes accumulation of Gb3 in lysosomes and elsewhere in the cell. Early characteristic clinical manifestations include severe neuropathic pain (acroparesthesia), skin lesions (angiokeratomas) and ocular signs (cornea verticillata). Later in life, cardiac, renal and cerebrovascular complications are responsible for severe morbidity and a shortened lifespan[1]. The phenotypic spectrum of Fabry disease is broad. Mutations in the GLA gene that result in complete absence of αGAL A activity generally results in classical, more severe disease, especially in male patients. Less severe mutations, predominantly missense mutations, result in non-classical disease phenotypes, with later onset and variable disease progression. Because of the X-linked inheritance, women have residual enzyme activity and disease manifestations are usually less extensive and develop later in life compared to male patients[2].

At present, two recombinant preparations of alpha-galactosidase A are available for treatment, agalsidase-alfa (Replagal), manufactured by Shire in human fibroblasts and registered at a dose of 0.2 mg/kg/eow and agalsidase-beta (Fabrazyme), manufactured by Sanofi-Genzyme in Chinese Hamster ovarian (CHO) cells and registered at a dose of 1 mg/kg/eow. Treatment with enzyme-replacement therapy (ERT) results in a notable reduction of Gb3 and its deacylated form lysoglobotriaosylsphingosine (lysoGb3) in plasma and urine[3,4] as well as morphological clearance of storage material in endothelial cells and, to a lesser extent, podocytes5[,6]. In male patients with classical Fabry disease, treatment with ERT delays the occurrence of complications, especially when treatment is initiated before the onset of irreversible organ damage[7,8]. However, more than half of classically affected male patients treated with ERT develop anti-drug-antibodies (ADAs)[4,9,10]. In female patients and patients with a non-classical disease phenotype, antibody formation against the administered recombinant enzyme is rarely observed[4,9,10]. In addition to hypersensitivity reactions, ADAs can cause inhibition of αGAL A activity. The impact of ADAs on ERT effectiveness has been addressed previously and a clear effect on (lyso)Gb3 clearance has been described by several groups[9,11-13]. Recently, the influence of ADAs on clinical outcomes was investigated in 41 male Fabry patients[9]. The ADA positive group had a lower eGFR and higher MSSI- and disease severity score, compared to the ADA negative group. Although analyses were corrected for mutation type (nonsense or missense), drawing conclusions regarding the effect of ADAs based

on this data is difficult, since there was no stratification for classical versus non-classical phenotype. Correcting for mutation type does not solve this problem, since missense-mutations can result in both classical and non-classical disease. Given the negative effect of the presence of ADAs on clearance of storage materials from endothelial cells[14], a deleterious influence of inhibiting ADAs on therapy efficacy is likely. The aim of this longitudinal retrospective cohort study is to characterize the antibody response in Fabry disease and determine its effect on both biochemical and clinical response to treatment in male Fabry patients with a classical disease phenotype.



Methods

Patients

This study was conducted in accordance with the principles of the Helsinki Declaration, as revised in 2000. Informed consent was obtained from all included patients. From the total Fabry patient population followed at the Amsterdam Lysosome Center (SPHINX), only male patients with a classical phenotype who were treated with enzyme replacement therapy (n = 39) were included in this study. Classification of patients as having classical or non-classical Fabry disease was based on the residual enzymatic activity and the presence or absence of characteristic symptoms, as described by Arends et al.[2]. During follow up, clinical data, as well as plasma samples, were collected at baseline and at every six months during treatment. In vitro plasma-induced enzyme activity inhibition (inhibition titer), lysoGb3 levels and renal function were determined as part of routine care in our hospital.

Samples were centrifuged and plasma aliquoted and stored at −80 °C. Data and samples from an average treatment duration of 9.7 years (range 1.5 to 16.6 years) were available. 6 patients were treated with agalsidase-alfa only, 15 with agalsidase-beta only and 18 alternated between agalsidase-beta and agalsidase-alfa. To avoid potential influence of the different enzyme preparations on antibody formation due to switching, only patients who started treatment with agalsidase-beta and stayed on this treatment for at least 4 years (mean treatment duration of 7 years) were studied for the presence and titers of the different Ig subclasses. Twelve out of 39 patients fulfilled these criteria.

Biochemistry and in vitro inhibition

LysoGb3 (nmol/l) was analyzed as previously described[15,16]. Samples collected before august 2015 were analyzed with isotope-labeled lysoGb3 as a standard. Subsequent analyses were performed with glycine-labeled lysoGb3 as a standard. Results of both methods correlate closely[2]. Biochemical response to treatment at any given time was determined as follows: decrease LysoGb3X = (LysoGb3X/LysoGb3baseline)*100, in which X stands for an individual time point. In vitro plasma-induced inhibition of r-αGAL A activity was measured as previously described by Linthorst et al.[13]. In short, inhibition titers represent the dilution factor of plasma resulting in 50% inhibition of the r-αGAL A activity. Patients are considered inhibition-positive if they had an inhibition titer >6 at any point during treatment.

Renal function and albuminuria

Creatinine values were obtained from electronic medical records of patients and used to estimate glomerular filtration rate using the CKD-EPI formula. Albuminuria was categorized into A1, A2 and A3 according to the Kidney Disease Improving Global Outcomes (KDIGO) guidelines[17].

Anti-αGAL A immunoglobulin ELISA's

Plasma samples from different time points of 10 anti r-αGAL A antibody positive patients were pooled and used as a reference sample to quantify levels of immunoglobulin subclasses in arbitrary units (AU). 96-wells microtiter plates (Nunc/Maxisorp) were coated overnight at 4 °C with 100 µl 1 µg/ml alpha-galactosidase beta (Fabrazyme, Genzyme) in sodium bicarbonate buffer (pH 9.8). Plates were washed five times and incubated for 1.5 h at 37 °C with 200 µl blocking buffer composed of PBS containing 2% (w/v) BSA fraction V (Merck). Subsequently, plates were washed five times. Plasma samples were diluted in PBS containing 0.1% (v/v) Tween-20 and 2% (w/v) BSA fraction V (Dilution buffer). 100 µl of each dilution was incubated for 1.5 h at 37 °C after which plates were washed five times. Next, plates were incubated with horseradish peroxidase (HRP) labeled anti-human IgG, IgG1, IgG2, IgG3, IgG4, IgA and IgM antibodies, respectively (Sanquin Reagents) diluted 2500× in dilution buffer. Plates were washed five times again before 100 µl TMB-substrate was added and plates were incubated 5 min at room temperature before the reaction was stopped using 1 M H2SO4. Absorbance was measured using a microtiter plate reader (Spectramax plus 384) at 450 nm using 540 nm as a reference for background absorption. A plasma pool of healthy donors (Sanquin, Amsterdam, the Netherlands) was used as a negative control. Patient samples were measured in duplicates per plate and on two different plates. All wash steps were done with PBS 0.1% (v/v) Tween-20.

Statistical analyses

For statistical analysis R (version 3.4.3) was used. Distributions were tested visually as well as by using Shapiro-Wilk test of normality and homogeneity of variances was tested using Bartlett test. Depending on the distribution, differences in baseline characteristics were tested using Mann-Whitney-Wilcoxon test or unpaired t-test for continuous variables. Fisher exact test was used for categorical variables. Baseline IgG1 of patients was compared to IgG1 level of a plasma pool of healthy blood donors using a one sample t-test. Fisher exact test was used to compare the number of patients with a rise in IgG1 during treatment in inhibition-positive and -negative patients. Correlation between immunoglobulin (Ig) subclasses and inhibition titer were assessed using non-parametric correlation analyses (Spearman's Rho). A linear mixed effect model

(package lme4) was used to determine the effect of inhibition titer on decrease in lysoGb3 from baseline (start of treatment) in each patient. Inhibition titer was transformed to log10 to optimize fit. Analyses were performed separately on data from patients treated with agalsidase-alfa 0.2 mg/kg/eow and those treated with agalsidase-beta 1 mg/kg/eow. Samples collected on doses other than the recommended doses and samples measured within 1 year after start of treatment or within one year after any dose switch were excluded from analysis. This was done since the nadir of the plasma lysoGb3 concentration is reached within the first year of treatment, and on stable dose of ERT, lysoGb3 concentrations remain stable thereafter (see supplemental material B, fig. 2). In supplemental material B, Fig. 1, the repeated measurements within the patients are depicted and measurements before and after the first dose or treatment switch are depicted differently. To correct for repeated measurements, patient number was used as a random effect. The model was corrected for age of the patients at the start of ERT. The effect of inhibition status on renal function was assessed using a linear mixed model (package lme4)[18]. Random intercept and random slope were added to correct for repeated measurements. The model was corrected for the cumulative dose of ERT received at each time point as well as age, eGFR and the grade of proteinuria at start treatment. P values <.05 were considered statistically significant. Full model specifications and R syntax for models and visualization are added as supplemental material A.

Results

Patient characteristics

Patient characteristics of the 39 included patients are outlined in Table 1. Inhibition-positive patients (n = 23) more often had a nonsense or frameshift mutation, whereas missense mutations were more prevalent in the inhibition-negative group (n = 16). There was also a significant difference in treatment type and dose: inhibition-positive patients were more often treated with agalsidase-beta only and inhibition-negative patients more often with agalsidase-alfa only, resulting in a higher mean and cumulative dose in the inhibition-positive group. Baseline lysoGb3, enzyme activity, age at start of ERT, albuminuria, smoking and hypertension status were not significantly different between these two groups. However, there was a trend for older age and lower eGFR at baseline in the inhibition-positive group.

	Inhibition positive	Inhibition negative	P value
Nr of patients	23 (59%)	16 (41%)	-
Mutation type:			0.02
- Nonsense/ frameshift	14 (61%)	3 (19%)	
- Missense/other	9 (39%)	13 (81%)	
LysoGb3 at baseline	115.5 (53-178)	95.3 (61-149)	0.14
Enzyme activity (% of mean reference)	1.1% (0-3)	0.3% (0-5)	0.35*
Age at start ERT (years)	35.4 (9-58)	20.1 (13-52)	0.07
Treatment			
- Agalsidase alfa only	0 (0%)	6 (15%)	>0.001
- Agasidase beta only	13 (33%)	2 (5%)	
- Switched	10 (26%)	8 (21%)	
Cumulative dose received (at end of follow up in mg/kg)	121 (29-360)	79 (37-308)	0.07*
Mean dose per infusion (mg/kg/eow)	0.7 (0.2-1)	0.3 (0.2-1)	0.004*
eGFR (at baseline in ml/min/1.73^2)	108.5 (24-172)	132.1 (38-158)	0.09
Baseline albuminuria			0.47
No albuminuria (<30mg/24h)	8 (35%)	9 (56%)	
Mild albuminuria (30-300mg/24h)	10 (43%)	5 (31%)	
Severe albuminuria (>300mg/24h)	5 (22%)	2 (13%)	
Treated with ACEi/ARB**	11/23 (48%)	7/16 (44%)	1
Smoker	4/13 (31%)	4/8 (50%)	0.46
Hypertension	3/22 (14%)	2/12 (17%)	1

Table 1. *Characteristics of 39 male patients with classic Fabry disease. Continuous variables are depicted as median (range), categorical variables are depicted as number (percentage). Missing values: lysoGb3 (n = 1), smoker (n = 18), hypertension (n = 5), enzyme activity (n = 6). Fisher exact was performed on categorical variables, continuous variables were analyzed using Mann-Whitney-Wilcoxon test or unpaired two-tailed t-test, depending on distribution.*
** Exact p-value could not be computed due to ties.*
*** For at least one year during treatment with ERT.*

Relationship between in vitro inhibition and biochemical response to treatment

Twenty three out of 39 patients were inhibition-positive at any point during treatment (59%). Ten out of those 23 patients became inhibition-negative or alternated between inhibition-positive and inhibition-negative status during treatment. In general, these were patients with low inhibition titers. In patients with a persistent antibody response the highest titer in each individual patient ranged from 130 to 15,000 (mean 2188), while in patient with a fluctuating antibody response the highest measured titer ranged from 8 to 375 (mean 107).

There was a clear negative correlation between inhibition titer and the decrease in lysoGb3 in response to treatment. This relation was most pronounced in patients treated with agalsidase-alfa (0.2 mg/kg/eow). With each tenfold increase in inhibition titer there is an estimated 10% less decrease in lysoGb3 in patients treated with agalsidase-alfa ($FE_{log10(inhibition)}$) = −10.3, SE = 1.9, P ≤ 0.001) (fig. 1B). In patients treated with agalsidase-beta this was 5% ($FE_{log10(inhibition)}$) = −4.7, SE = 0.9, P ≤ 0.001) (fig. 1A).

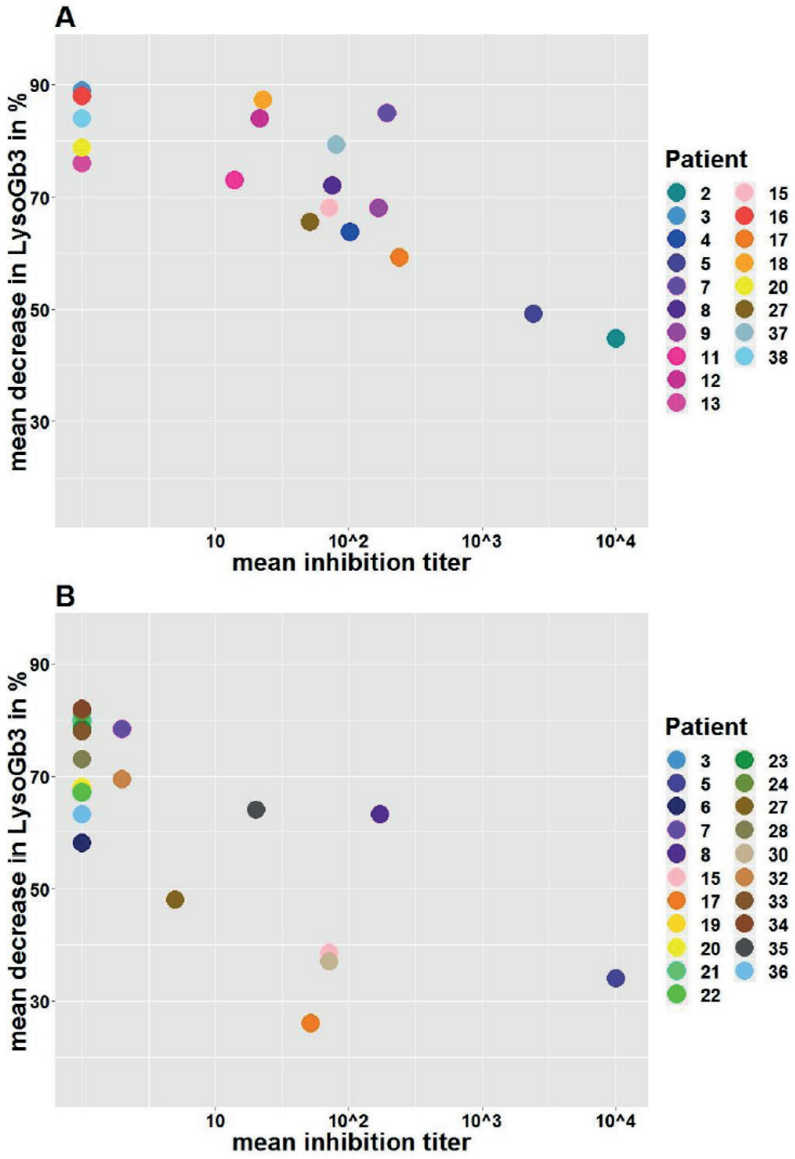


Figure 1. *Effect of in vitro inhibition on biochemical response to treatment with ERT. Main in vitro inhibition and mean decrease in lysoGb3 from baseline are depicted per patient under treatment with agalsidase-beta 1.0 mg/kg/eow (A) or agalsidase-alfa 0.2 mg/kg/eow (B). Time points within 1 year after start treatment as well as time points within 1 year after any dose switch were excluded from analyses. Samples from 31 patients remained. Seven out of these 31 patients appear in both graphs. Each color represents an individual patient. All measured time points are depicted in supplemental material B, figure 1.*

Relationship between in vitro inhibition and decline in renal function during ERT

Using a linear mixed effect model correcting for the cumulative ERT dose received at each time point as well as age, eGFR and the category of proteinuria at baseline, we found an accelerated decline in renal function in inhibition-positive patients of approximately 1.2 ml/min/1,73m2 per year while on treatment compared to patients that were inhibition-negative (FE = 1.21, SE = 0.59, p = .042) (fig. 2). Adding treatment with ACEi or ARB or mutation type as covariates to the model did not result in a better fit or different results.

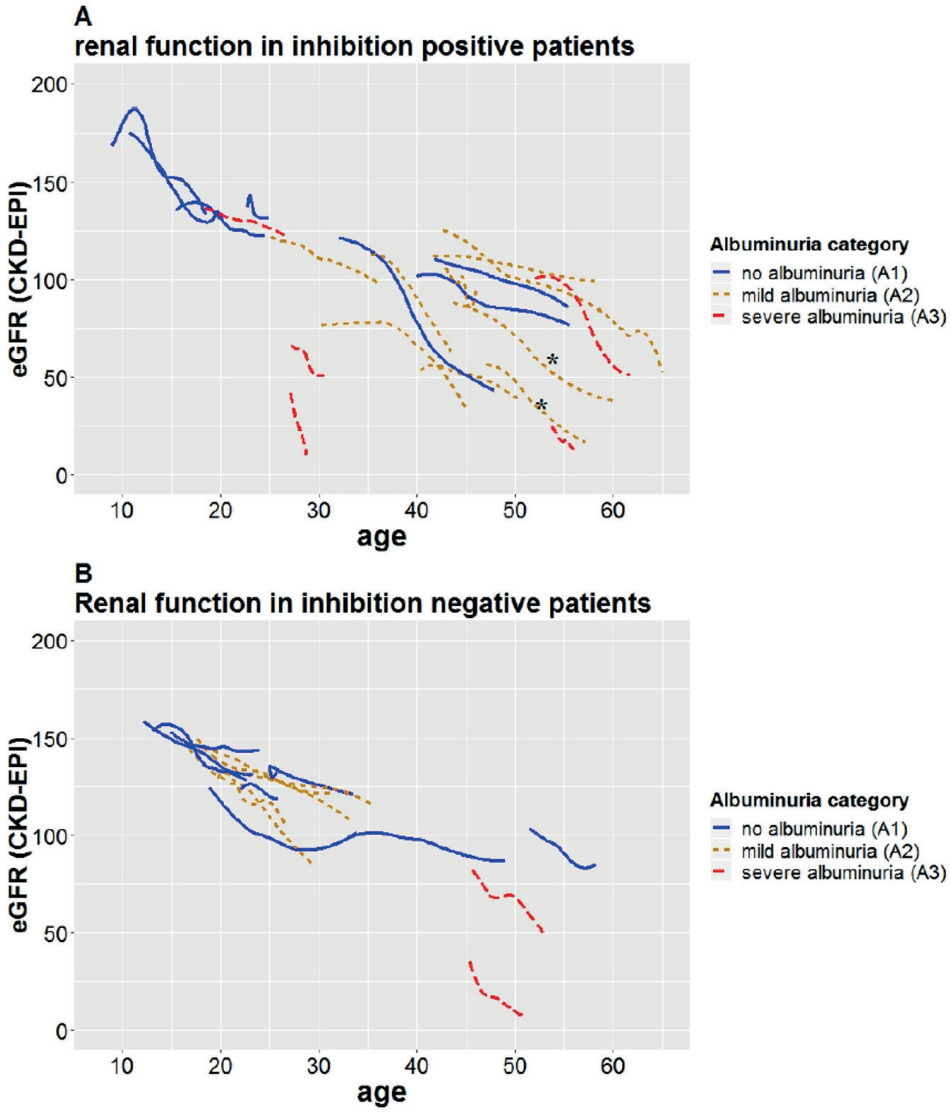


Figure 2. *Effect of in vitro inhibition on renal function (GFR estimated by CKD-EPI in ml/min/1,73m2) during treatment with ERT in classical male Fabry patients. Each line represents an individual patient. Panel A depicts the slope of renal function in inhibition-positive patients, panel B depicts inhibition-negative patients. Differences in treatment are depicted in table 1. Color and line type represent the stage of proteinuria before start with ERT.*
** The 2 patients in fig. 2A who were found to have a sustained IgG4 response were marked with an asterisk.*

Total anti r-αGALA immunoglobulin and Ig subclass titers and their relation to in vitro enzyme activity inhibition

Of the 39 Fabry patients, 12 patients were solely treated with agalsidase-beta and stayed on this treatment for at least 4 years. Before start of ERT, all 12 patients had low titers of anti r-αGAL A IgG1 antibodies, mean titers were higher in treatment naïve patients compared to the background measured in healthy donor plasma pool (39 vs 10 au, p = .0032). During treatment, IgG1 anti r-αGAL A levels increased during treatment in all 9 inhibition-positive patients (range: 3–24-fold increase from baseline), but not in the 3 inhibition-negative patients (p = 0.0045, Fig. 3). IgG4 anti r-αGAL A antibodies developed in 7 out of 9 inhibition-positive patients (range 3–2082 au) and none of the 3 inhibition negative patients. Only 2 patients had a sustained IgG4 response, these patients also showed the highest levels of in vitro inhibition of r-αGAL A activity (fig. 3). In one of these patients ERT was discontinued because of pronounced disease progression during treatment with ERT (fig. 3D), the other patient died at the age of 57 due to complications of a myocardial infarction (fig. 3K). A different patient underwent renal transplantation during follow up at a time point at which a significant IgG1 r-αGAL A antibody and inhibition titer was present. After transplantation, the IgG1 r-αGAL A antibody and inhibition titers went down to pretreatment level (fig. 3E).

Levels of anti r-αGAL A IgG2 and IgA were present in some of the inhibition-positive patients but were too low to reliably quantify. Anti r-αGAL A IgM and IgG3 were not detectable in any of the plasma samples. Total anti r-αGAL A IgG, as well as anti r-αGALA IgG1 and IgG4 correlated well with in vitro measured plasma inhibition of r-αGAL A enzyme activity (ρ = 0.71, 0.60 and 0.67 respectively).

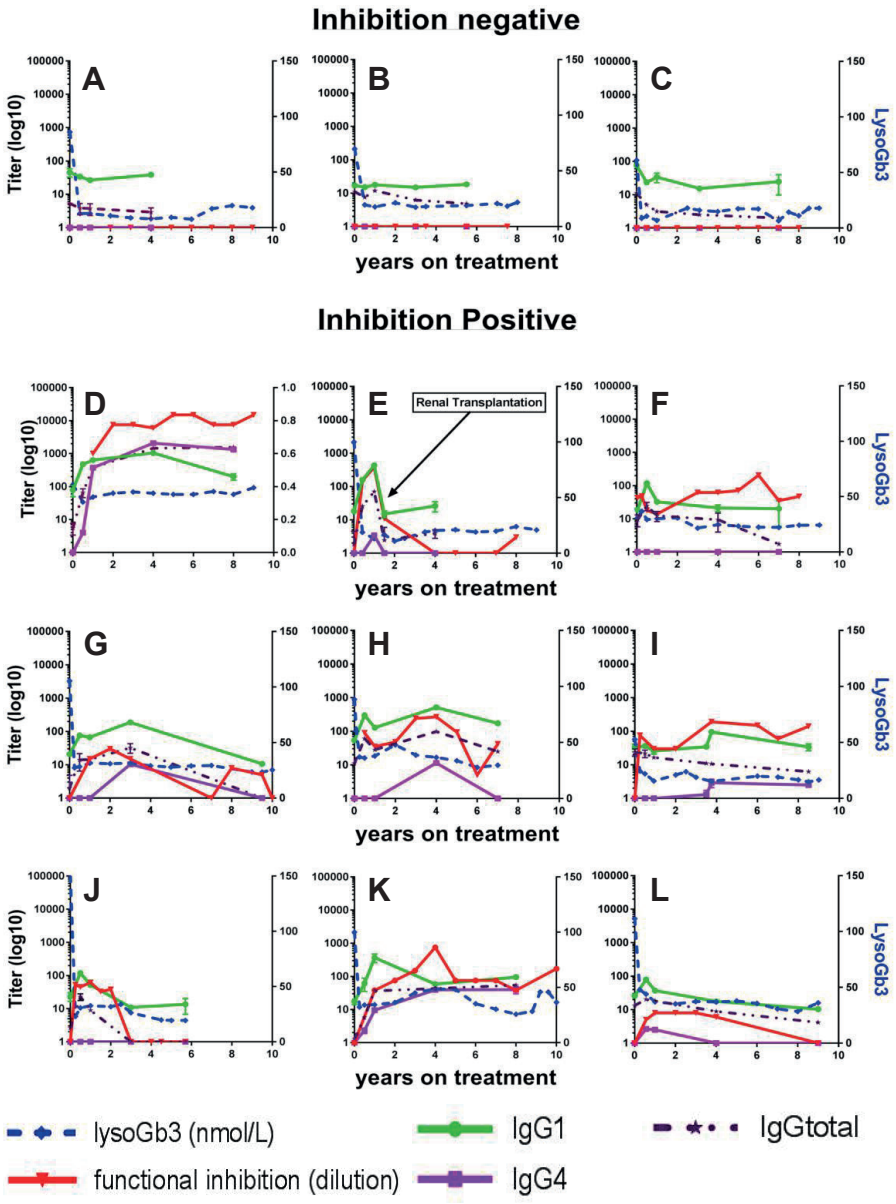


Figure 3. *Titers of in vitro inhibition, IgG1, IgG4, total IgG (arbitrary units) and lysoGb3 (nmol/L). Patients A-C are inhibition-negative, patients D-L are inhibition-positive. In patients G, J and L inhibition disappeared spontaneously during treatment. In Patient E inhibition disappeared after renal transplantation and treatment with immunosuppressive therapy. Values of antibodies and inhibition are depicted on the left y-axis in Log (10). LysoGb3 (nmol/L) is depicted on the right y-axis. X axis depicts the years since treatment initiation.*

Discussion

In this study, in vitro inhibition of r-αGAL A activity by ADAs in plasma of Fabry patients was clearly associated with a less robust reduction in lysoGb3 in response to treatment with ERT. In this study we showed for the first time that this effect was titer dependent. Higher inhibition titers led to an inferior biochemical response. This effect was most pronounced during treatment with agalsidase-alfa and most likely caused by the difference in dose between agalsidase-alfa and -beta, as previously described[11]. The proposed explanation is that a higher proportion of the lower concentration of agalsidase-alfa (dose 0.2 mg/kg/eow in contrast to 1.0 mg/kg/eow for agalsidase-beta) of enzyme is inhibited when antibodies are present.

LysoGb3 is thought to be directly involved in the development of glomerular injury[19], induction of fibrosis[20] as well as neuropathic pain[21]. Despite the known effect of ADAs on the biochemical response to treatment, investigations showing a clinical meaningful effect of ADAs in Fabry disease are scarce. In the current study we assessed the effect of inhibiting antibodies on renal function with a linear mixed effect analysis correcting for cumulative ERT dose at each time point as well as age, renal function and proteinuria at treatment initiation. Inhibition-positive patients had an accelerated decline in renal function of 1.2 ml/min/1,73m2 per year compared to inhibition-negative patients. Although the observed effect was on average modest, it is clinically highly relevant as a more rapid loss of renal function implies an earlier need for dialyses or renal transplantation. Confirmation of the negative effect of antibodies on renal function, correcting for the abovementioned factors, in a second patient cohort would strengthen our observation. Previously Lenders et al. reported higher lysoGb3 levels, greater left ventricular mass and worse renal function in inhibition-positive compared to inhibition-negative patients[9]. However, the fact that classical and non-classical patients were studied as one cohort hampers the interpretation of the results, since there are significant differences in disease course between these patient groups[2]. More recently a prospective French study reported no clinical difference between inhibition-positive and inhibition-negative patients[12]. Although, due to the relative slow disease progression and small patient group (29 treated classical males), no meaningful changes could have been expected during the 2 year follow up. To overcome these limitations, the current study was carried out in a relatively large cohort of male patients with the classical disease phenotype during a mean follow up duration of 10 years. However, our study still has some limitations. In the inhibition-positive group more patients had a nonsense

or frameshift mutation compared to the inhibition-negative group, in which missense mutations were more prevalent. This can have two effects: 1. the nature of nonsense and frameshift mutation (leading to truncated protein) may make the patients more prone to ADA development 2. nonsense and frameshift mutation may cause more severe disease, leading to an overestimation of the effect of the ADAs on treatment outcome. The latter was not confirmed, since adding mutation type to the model of the effect of ADAs on renal function did not improve the model or change the outcome. Moreover, all 39 patients were classical male Fabry patients and no statistical differences were found in lysoGb3 or enzyme activity at baseline, thus the unfavorable effect of ADAs on disease outcome is likely to be caused by the development of inhibiting antibodies. Another limitation was that the cohort was too small to be able to study the effects of ADAs on clinical events (e.g. myocardial infarction or cerebrovascular event) due to the clinically relevant trend for younger age and the accompanying very low rate of events in the inhibition-negative patients.

What stands out is the highly relevant effect on lysoGb3 and the relation with (persisting) high titers of ADA. We argue that a biochemical response is of importance and relates to clinical responses. The fact that there is not always a relationship between reductions in lysoGb3 and clinical effects has to do with the slow progressive nature and the different stages of the disease: in patients with advanced disease at start of ERT, treatment may not sufficiently influence the disease course, even if a robust decline in lysoGb3 is achieved. Vice versa, in those in whom progression can still be halted, a decline in lysoGb3 supports a beneficial effect of therapy. As such, reduction in lysoGb3 is a prerequisite for any clinical effect[8] and interfering ADAs, blunting the lysoGb3 decline, are thus of clinical importance.

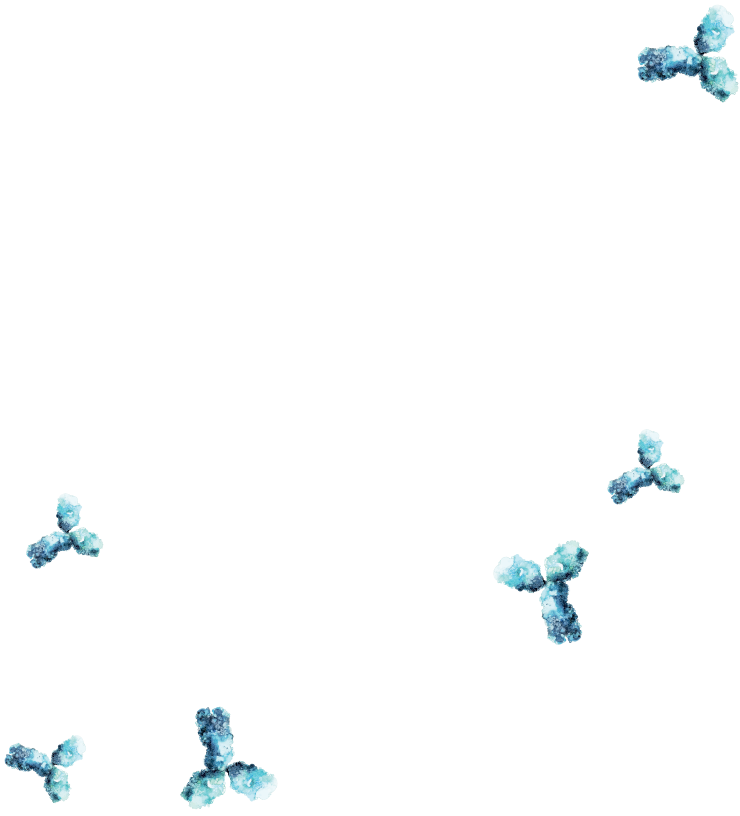
Longitudinal analysis of immunoglobulin subclasses against recombinant αGAL A showed that anti r-αGAL A antibodies in our studied sub-cohort were mainly of the IgG1 and IgG4 subclasses. This is in accordance with the findings of Mauhin et al.[12]. Low levels of IgG1 were also found in the plasma pool of healthy controls. Interestingly, baseline levels of anti-αGAL A IgG1, i.e. before any exposure to exogenous administered enzyme, were higher in all 12 measured patients compared to healthy control subjects. However, no relation between baseline IgG1 titer and the development of in vitro inhibition during treatment was found. Presence of anti-drug antibodies in protein replacement therapies prior to start of treatment have been described in for example hemophilia A[22,23] and mucopolysaccharidosis IVA[24]. Suggested mechanisms of development of these antibodies include early antigen exposure (e.g. from the maternal circulation

during birth), exposure to mutated protein and antibody producing B cell clone maturing independent from antigen exposure[22,25].

Previously, Lenders et al. demonstrated that IgG4 isolated from patient plasma was capable of inhibiting enzymatic activity[26]. Furthermore, they found that the inhibitory capacity per microgram total IgG differed per patient indicating that total IgG levels per se may not be indicative of the effect of ADAs on disease course. In our study, the occurrence of in vitro inhibition of enzyme activity during treatment was associated with an increase in IgG1 or both IgG1 and IgG4 anti-αGAL A, other antibody subclasses were not detected in significant amounts. The two patients that developed a sustained IgG4 response were also the two patients with the highest inhibition titers. This is consistent with finding in hemophilia A, where low-titer inhibition patients had primarily IgG1 anti-FVIII antibodies, whereas IgG4 antibodies were more prominent in patients with high inhibition titers[27,28]. From an immunological point of view this distribution makes sense. While antibody responses to soluble proteins primarily induce a IgG1 response, repeated exposure to antigens in non-infectious settings are known to induce IgG4 formation[29]. IgG4 is often referred to as a 'blocking' antibody because they bind the epitope, but do not initiate a pathogenic immunological response due to its lack of binding to C1q and poor binding to Fcɣ receptors[29]. In this manner they may prevent the negative immunological effect of other immunoglobulins by competing for epitope binding. In addition, autoimmune disease associated with IgG4 subclasses are attributed to the inappropriate activation or blockage of endogenous enzymes or receptors by IgG4 antibodies, for example in muscle-specific kinase myasthenia gravis[30,31]. In the same manner, IgG ADAs in Fabry could inhibit r- αGAL A function. However, inhibition of enzymatic activity is not the only way ADAs can influence treatment efficacy, pharmacokinetics and uptake in target cells may also be altered. A protein coated with antibodies is cleared more rapidly from the circulation by phagocytotic cells resulting in decreased availability for other cell types[32,33]. In Pompe disease, patients with high ADA titers had a 50% increase in clearance rate of Myozyme[34]. Complex formation and increased clearance of r-αGALA when ADAs are present have also been demonstrated in Fabry disease[13]. We hypothesize that enzyme uptake by target cells in Fabry disease (e.g. cardiomyocytes, podocytes and endothelial cells) is also negatively affected by anti r-αGAL A antibodies.

Ways forward could include development of strategies to reduce or prevent the occurrence of ADAs. Immunomodulation before treatment initiation may be considered to prevent antibody formation. However, given the potential side effects, precise prediction of which patients are at risk for ADA development

is necessary. The finding that patients that started ERT treatment after renal transplantation (and were thus treated with immunosuppressive drugs) did not develop antibodies against ERT suggests this approach holds promise. In the same study, patients transplanted while already on ERT had an initial reduction in inhibition titer, but in some patients the titer rose again after tapering of the immunosuppressive medication[35]. Therefore, immunomodulation before start of ERT in patients most at risk for clinically significant antibody development might be the best way forward.



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Supplemental material A

R syntax

In vitro inhibition and decrease in lysoGb3

Type of analysis
- Linear mixed model

Covariates included
- Age of start ERT = ERTstart

R syntax
- Lmer('decrease lysoGb3' ~ log10('*in vitro* inhibition')+ (1|Patient) + ERTstart)

Figure 1 (mean data)
- ggplot(data, aes (x=log10('mean in vitro inhibition'), y='mean decrease lysoGb3')+ geom_point(aes(color=patient))

Supplemental Figure 1 (full data)
- ggplot(data, aes (x= log10('in vitro inhibition'), y='decrease lysoGb3')+ geom_point(aes(color=patient, shape='dose switch'))

Inhibition status and renal function (eGFR)

Type of analysis
- Linear mixed model

Covariates included
- Age of start ERT = ERTstart
- eGFR (CKD-EPI) at start therapy = eGFRstart
- category of proteinuria (A1, A2, A3) at start ERT = proteinuria
- cumulative dose received at each time point included = dose

R syntax
- Lmer(eGFR ~ 'years on ERT' * 'inhibition status'+ (1+'years on ERT'|Patient)+ ERTstart
  +eGFRstart + proteinuria+dose)

Figure

- ggplot(data, aes(x=age, y=eGFR(CKDEPI, group=patient, color=p, linetype=proteinuria) geom_smooth (se=false) method=loess

Correlation immunoglobulin subclasses

Type of analysis

- Spearman correlation

R syntax

- Cor (IgGsubclass, 'inhibition titer', use = "complete.obs", method="spearman")

Baseline IgG1 vs healthy controls

Type of analysis

- One sample T test

R syntax

- T.test (baseline IgG1, mu=9.88, conf=0.95)

Supplemental material B

Supplemental figure 1.

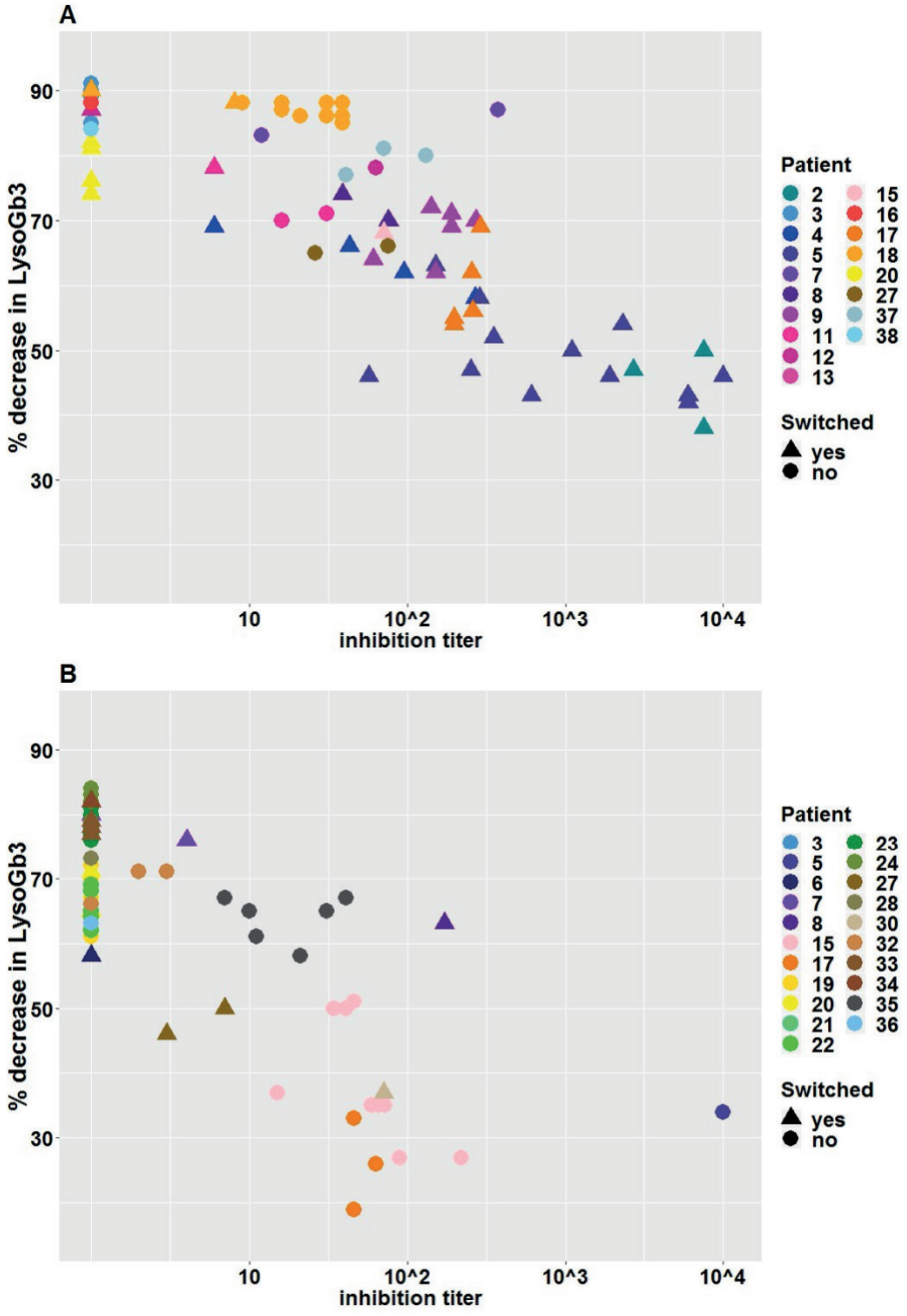


Figure 1. *In vitro inhibition and decrease in lysoGb3 from baseline during treatment. Samples measured while on treatment with agalsidase-beta 1.0 mg/kg/eow (A) and while on treatment with agalsidase- alfa 0.2mg/kg/eow (B) are depicted separately. Time points within 1 year after start treatment as well as time points within 1 year after any dose switch were excluded from analyses. Samples from 31 patients remained. Seven out of these 31 appear in both graphs. Each color represents an individual patient. Samples measured after any dose switch are depicted as a triangle.*

Supplemental figure 2.

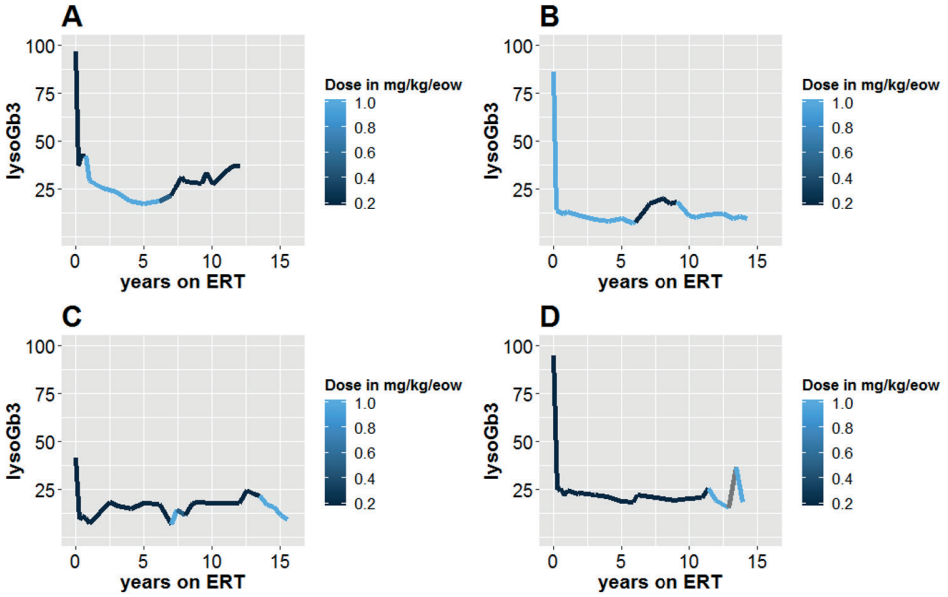
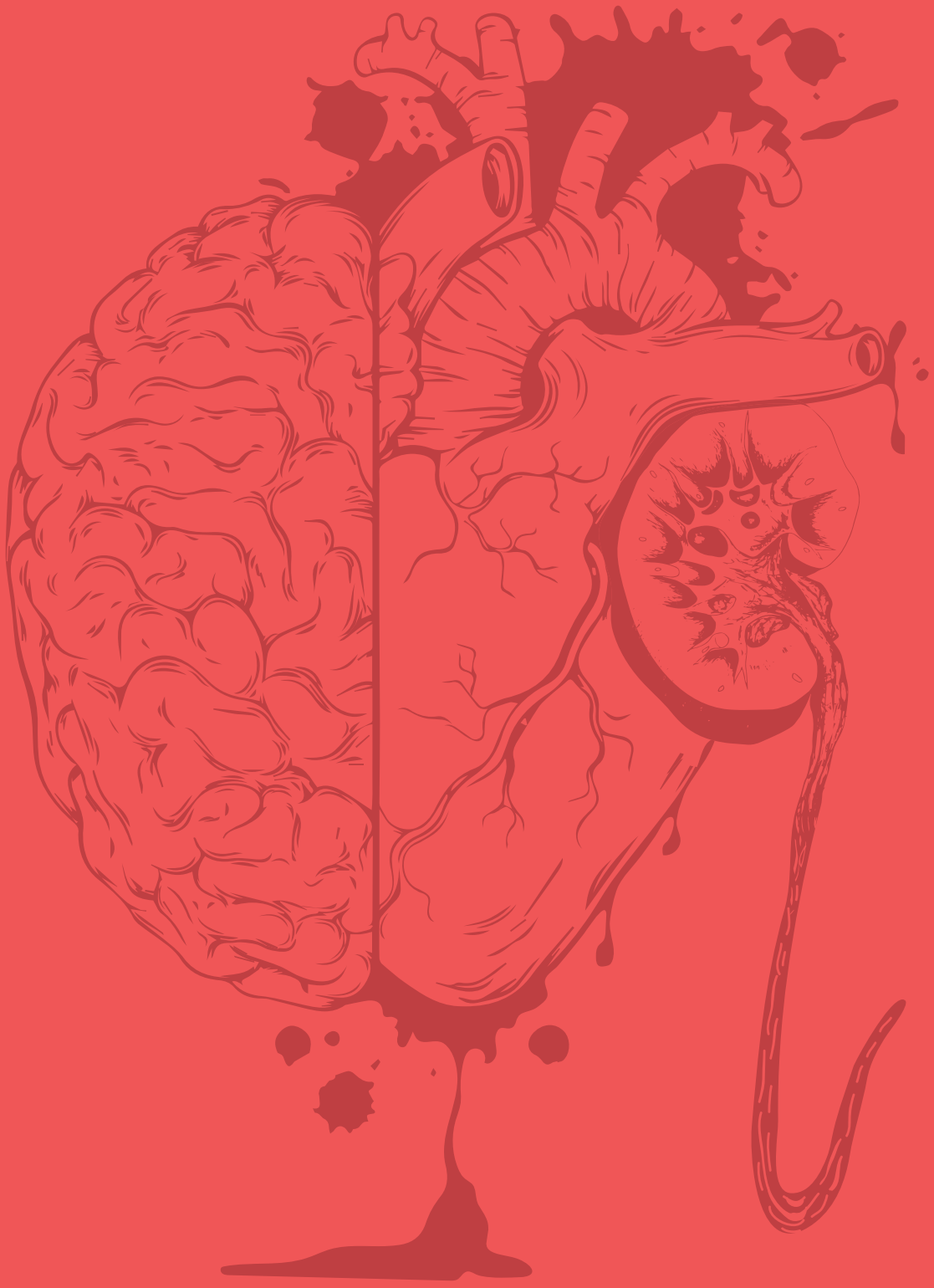


Figure 2. *Effect of treatment with consecutive different doses and/or different enzyme preparations on plasma lysoGb3 concentrations in individual inhibition-negative patients. Lighter color represents higher dose. X axis: time in years, Y axis: lysoGb3 in nmol/L.*
Patient A: switched from agalsidase-alfa 0.2 mg/kg/eow to agalsidase-beta 1.0 mg/kg/eow to agalsidase-beta 0.5 mg/kg/eow and back to agalsidase-alfa 0.2 mg/kg/eow.
Patient B: switched from agalsidase-beta 1.0 mg/kg/eow to agalsidase-alfa 0.2 mg/kg/eow and back to agalsidase-beta 1.0 mg/kg/eow.
Patient C: switched from agalsidase-beta 0.2 mg/kg/eow to 1.0 mg/kg/eow to 0.5 mg/kg/eow, after which he switched to agalsidase-alfa 0.2 mg/kg/eow and back to agalsidase-beta 1.0 mg/kg/eow.
Patient D: Switched from agalsidase-alfa to agalsidase-beta. The grey color represents a period of a year with severe lack of treatment compliance.



Chapter 7

Predicting the development of anti-drug antibodies against recombinant alpha-galactosidase A in male patients with classical Fabry disease

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Abstract

Fabry Disease (FD) is a rare, X-linked, lysosomal storage disease that mainly causes renal, cardiac and cerebral complications. Enzyme replacement therapy (ERT) with recombinant alpha-galactosidase A is available, but approximately 50% of male patients with classical FD develop inhibiting anti-drug antibodies (iADAs) that lead to reduced biochemical responses and an accelerated loss of renal function. Once immunization has occurred, iADAs tend to persist and tolerization is hard to achieve. Here we developed a pre-treatment prediction model for iADA development in FD using existing data from 120 classical male FD patients from three European centers, treated with ERT. We found that nonsense and frameshift mutations in the α-galactosidase A gene ($p = 0.05$), higher plasma lysoGb3 at baseline ($p < 0.001$) and agalsidase beta as first treatment ($p = 0.006$) were significantly associated with iADA development. Prediction performance of a Random Forest model, using multiple variables (AUC-ROC: 0.77) was compared to a logistic regression (LR) model using the three significantly associated variables (AUC-ROC: 0.77). The LR model can be used to determine iADA risk in individual FD patients prior to treatment initiation. This helps to determine in which patients adjusted treatment and/or immunomodulatory regimes may be considered to minimize iADA development risk.

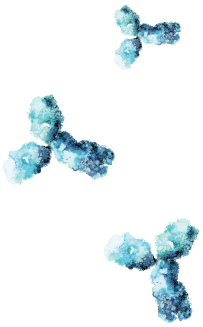
Introduction

Fabry disease (FD; OMIM 301500) is a rare, X-linked, lysosomal storage disease caused by mutations in the alpha-galactosidase A (GLA) gene. This leads to absent or reduced alpha-galactosidase A enzyme activity and the subsequent accumulation of its substrate globotriaosylceramide (Gb3). Accumulation of Gb3 and its deacylated form globotriaosylsphingosine (lysoGb3) results in progressive damage to heart, kidneys and brain[1]. The disease is most often treated with biweekly infusions of recombinant alpha-galactosidase A (r-αGAL A), also referred to as enzyme replacement therapy (ERT). Two ERT preparations are currently available. One is agalsidase beta (Fabrazyme, Sanofi Genzyme), produced in Chinese hamster ovary cells (CHO) and most often dosed at 1 mg/kg biweekly. The other is agalsidase-alfa (Replagal, Takeda), produced in human fibroblasts and dosed at 0.2 mg/kg biweekly. In patients with the most severe disease phenotype, male patients with classical FD, treatment with ERT often results in the development of anti-drug antibodies (ADAs). Because these patients have little to no native enzyme, the immune system recognizes the exogenously administered enzyme as foreign. ADAs are thought to be responsible for both the infusion-related reactions including fever, chills and chest pain as well as the more classical allergic reactions with edema, dyspnea, rash, itching and (rarely) anaphylactic shock[2,3,4]. In approximately half of the classical male FD patients, the formed ADAs are capable of inhibiting αGAL A activity (iADAs) in vitro[5,6] as well as inhibiting enzyme uptake into cells[7]. In vivo, iADAs negatively influence pharmacokinetics of the recombinant enzyme[5,8,9] and -titer dependently- limit the biochemical response to treatment with ERT[6]. Additionally, the development of ADAs is linked to incomplete clearance of substrate in endothelial cells or re-accumulation after initial clearance[10,11]. Clinically, male FD patients with established iADAs had higher disease severity scores[12], although the inclusion of male patients with classical as well as non-classical disease may have influenced the outcome. The negative effect of iADAs on disease outcome was confirmed in classical male patients, as iADA positive patients had an accelerated renal decline compared to iADA negative patients[6].

Experience in other disorders in which treatment with recombinant proteins is hampered by iADA formation (e.g., hemophilia, Pompe disease and MPS1) [13,14,15,16], shows that once iADAs occur, they tend to persist despite treatment with immunosuppressants or tolerization protocols (e.g., immune tolerance is hard to achieve)[17,18,19,20]. Therefore, it is important to develop protocols that can prevent or treat iADAs. Methods to prevent iADA development should primarily be tested in patients with a high risk of developing these antibodies. Comparing different

pre-treatment immunomodulatory interventions on high-risk patients, rather than all Fabry patients, is expected to improve the efficiency and interpretation of these future studies, and reduce the required sample sizes.

Several variables were found to be related to the immunogenicity of other biotherapeutics. These included: the dose and frequency of administration[21], the origin and glycosylation of the product (e.g., in which cell line is the product produced)[22,23], the age of the recipient[21,24], mutation type (nonsense vs. missense)[25] and the presence of residual native protein in the patient, also referred to as cross- reactive immunologic material (CRIM) status[15,25]. These factors may play a role in iADA formation in Fabry disease as well. Within our population of male Fabry patients with classical disease we noticed disparity in the risk of iADA formation, with families with high and low risk, suggesting genetic predilection. In this study we set out to answer the following two questions: (1) Which factors predispose male patients with classical FD for iADA development? (2). How accurately can the development of iADAs be predicted prior to treatment initiation in an individual patient? To address these questions we used previously collected[23] demographic, medical and biochemical data from 120 classical male FD patients and used these data to build and validate predictive models.



Results

Patient Characteristics

Patient characteristics of the 120 included patients are outlined in table 1.

	iADA+	iADA-
Site (N, % of total)		
- Amsterdam UMC	23 (40%)	16 (26%)
- The Royal Free Hospital	24 (41%)	26 (42%)
- Universitätsklinikum Würzburg	11 (19%)	20 (32%)
Mutation type (N, % of total)		
- Nonsense/frameshift	33 (57%)	17 (27%)
- Missense	21 (36%)	37 (60%)
- Other	4 (7%)	8 (13%)
Age at ERT start (years, median, range)	37 (9-58)	35 (13-63)
LysoGb3 (nmol/L, median, range)	123 (38-178)	96 (48-149)
First treatment (N, % of total)		
- Agalsidase alfa 0.2 mg/kg	14 (24%)	31 (50%)
- Agalsidase beta 0.2 mg/kg	4 (7%)	2 (3%)
- Agalsidase beta 0.5 mg/kg	2 (3%)	2 (3%)
- Agalsidase beta 1.0mg/kg	38 (66%)	27 (44%)
Inhibition titer (median, range)	113 (7-32645)	0 (0-5)

Table 1. *Characteristics of 120 male patients with classic Fabry disease*

Logistic Regression Model

To identify factors associated with iADAs and assess their ability to predict iADA formation, we built a logistic regression (LR) model, using backwards selection of variables. The final model was validated internally using repeated cross validation. In this model, three variables were included that were found to be associated with an increased risk for iADA development: higher levels of the biomarker lysoGb3 before start of treatment ($p < 0.001$), the presence of a nonsense or frameshift mutation ($p = 0.053$) and starting treatment with agalsidase beta ($p = 0.006$). Distribution of individual variables is visualized in Figure 1a-c. Predictive performance was assessed with the area under the receiver operating characteristic curve (AUC-ROC) which was 0.77 (Figure 2a). Optimal accuracy (0.73, Figure 2b) was determined at a cutoff of 0.53 (sensitivity: 0.69, specificity:

0.76, Figure 2c and d). Figure 2e shows the predicted vs. observed outcome for each patient.

Age at start treatment did not influence the risk of iADA development. Both initial treatment type (agalsidase alfa vs. agalsidase beta) and treatment dose (0.2, 0.5 or 1.0 mg/kg every other week) were significantly associated with a higher risk for iADA development. Since these variables are strongly related, only treatment type was included in the model. Location of the mutation as a numeric factor was not associated with an increased risk. However one location of missense mutations seemed especially prone for iADA development and is explored separately in a post-hoc analysis.

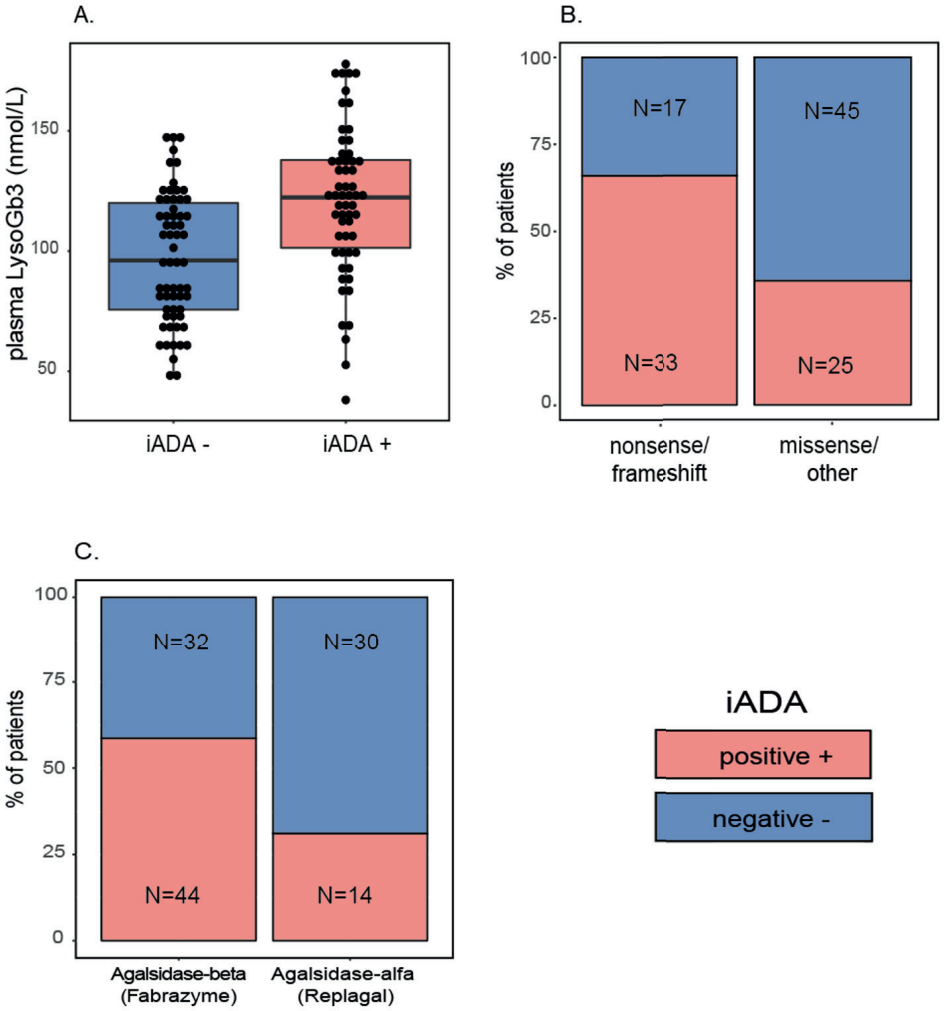


Figure 1. *Distribution of variables significantly associated with an increased risk of iADA development in male Fabry patients with classical disease. Color represents iADA status. (a) Baseline plasma lysoGb3 levels. (b) Mutation type (n.b. eight out of 25 iADA positive patients in the missense group had a mutation at location c.1025). (c) Treatment type at start of treatment: agalsidase beta (0.2–1 mg/kg) versus agalsidase alfa (0.2 mg/kg)*

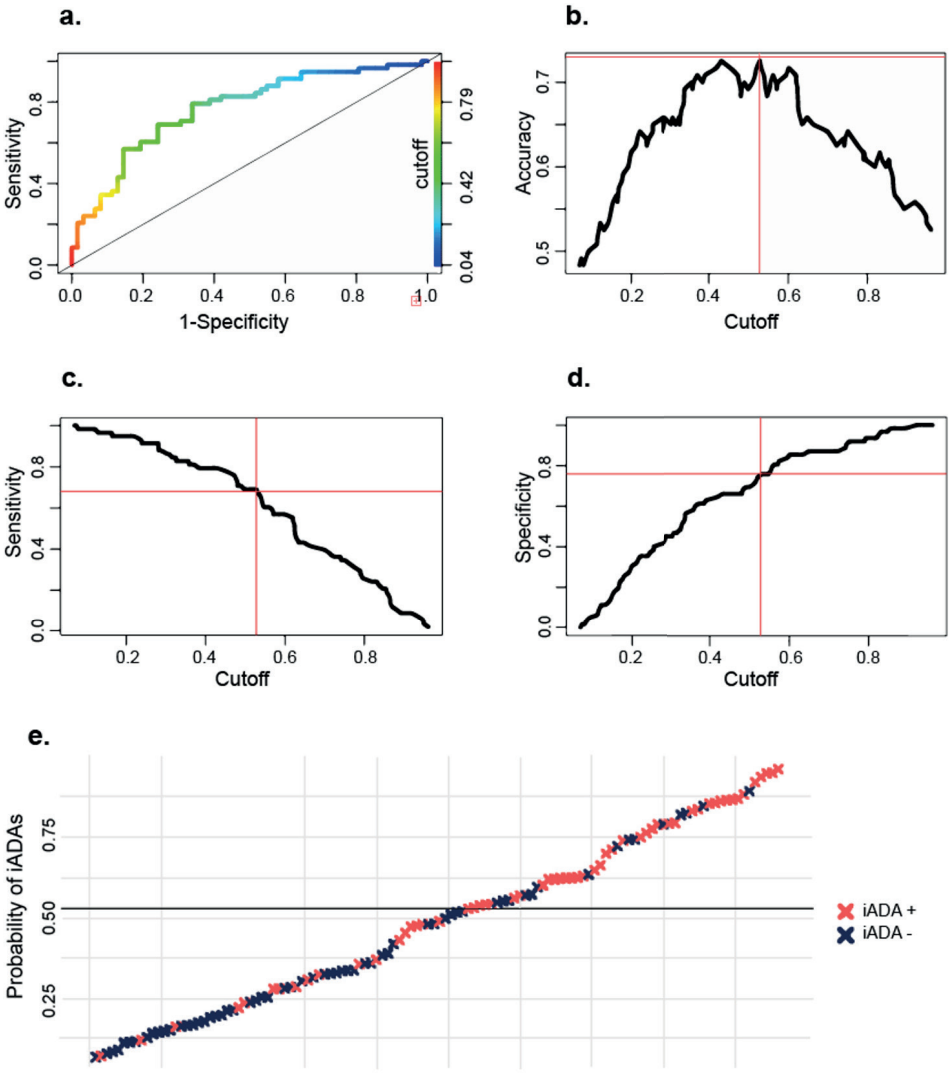


Figure 2. *Results from the LR model. (a) ROC curve, AUC = 0.77. Colors represent the different possible cutoff* values; (b) Accuracy of the model at different cutoffs* (maximum of 0.73 at cutoff 0.53); (c) Sensitivity of the model. At the chosen cutoff*

*sensitivity is 0.69; (d) Specificity of the model at the chosen cutoff is 0.76; (e) Visualization of the predicted (Y axis) versus observed outcome (color coded) per patient. The line drawn shows the chosen cutoff. * The cutoff is a chosen decision threshold above which patients are predicted as positive (will develop ADAs). Lower cutoffs favor sensitivity, higher cutoffs favor specificity.*

Random Forest Model

A second model was built using the ensemble learning method 'Random Forest' (RF). Ensemble learning models can deal with co-linearity and are able to handle many variables. Furthermore, these models can handle variables with relative low contribution to the calculated risk. We build a RF model using the following variables: baseline plasma lysoGb3, mutation type and location, age of start, first treatment type and first treatment dose. Compared to the LR model, the fit of the RF model was similar (AUC-ROC 0.77) and it did not improve prediction accuracy. For details see Supplemental Materials.

Post Hoc Analyses

Patients with missense mutations were less likely to develop iADAs compared to patients with nonsense and frameshift mutations. To check whether, within the missense group, the location of the mutation influenced iADA risk, we visualized the location of missense mutation and the presence of iADAs (Supplemental Materials). Mutation location as a numeric variable was not associated with iADA risk. However, eight out of 11 patients with a mutation at the c.1025 position developed iADAs (73%) compared to 17 out of 59 (29%) of patients with missense mutations at other positions ($p = 0.01$, OR 6.3). Most other mutations in this cohort were unique mutations, thus an effect of location for these mutations could not be established. In silico analysis did not suggest that variants at position c.1025 affected splicing of the pre-mRNA GLA.

Second Cohort

Due to differences in the iADA detection method (sample dilution vs. enzyme saturation) as well as lysoGb3 analyses (dried blood spot vs. plasma), we were not able to test our model on the second, independent, cohort of patients. We therefore performed the same steps for imputation and build a LR prediction model using the same three variables on this second cohort to check reproducibility of the results. In this group of 30 classical male patients, only mutation-type was significantly correlated ($p = 0.01$) to the risk of iADA development. Initial treatment type ($p = 0.3$) and baseline lysoGb3 ($p = 0.4$) were not significantly related to iADA risk in this smaller cohort. For details see Supplemental Materials.

Discussion

Our large international Fabry cohort study identified the following variables to be associated with an increased risk of iADA development in male patients with classical FD: (1) having a nonsense or frameshift mutation. (2) starting treatment with agalsidase beta and (3) higher levels of the disease biomarker (lysoGb3) in plasma before start of treatment. In the second control cohort, the importance of mutation type was confirmed, but type of recombinant enzyme and plasma lysoGb3 levels showed only a trend, which may have been due to the smaller sample size and/or the differences in lysoGb3 measurements.

Mutation type has previously been found to be related to iADA risk in FD[6,26], which is in accordance with observations in Pompe disease and hemophilia[27,28]. Although all classical patients have minimal or no residual enzyme activity, it is possible that the mutated protein is still produced in small amounts. Thus, the immune system of patients with missense mutations may still be exposed to the protein, leading to central tolerance induction. In patients with a large deletion or early frameshift mutations, the protein will either not be produced at all, or it will be truncated. These patients are less likely to develop central tolerance and are more likely to develop an immunological reaction to exogenously administered enzyme. The increased occurrence of iADAs in patients who started treatment with agalsidase beta vs. agalsidase alfa could either be attributed to the higher dose (1 mg/kg vs. 0.2 mg/kg), the difference in production cell-line and thus in glycosylation pattern (Chinese hamster ovary cells for agalsidase beta vs. human fibroblasts for agalsidase alfa) or a combination of both. The group of patients that started with a lower than recommended dose of agalsidase beta (see Table 1) was too small to draw conclusions on the dose effect. In this study, the plasma lysoGb3 level was identified as an independent predictive variable for iADA formation. We hypothesize that either plasma lysoGb3 levels reflect subtle differences in enzyme activity (not detectable in the enzyme activity assay) or that the pro-inflammatory effects of (lyso)Gb3[29] serve as an adjuvant to prime the immune system.

Detrimental effects of iADAs on treatment effectiveness in FD is becoming increasingly clear over the last decade[5,7,10,11,12]. Overcoming iADA development is therefore essential to improve treatment outcome and can hypothetically be achieved in two ways: (1) by achieving tolerization through immune tolerance induction (ITI) in iADA positive patients or (2) by preventing iADA formation prior to treatment. Both strategies have previously been tried in other diseases.

The first approach, ITI, has been studied extensively in hemophilia. Most ITI protocols require long term, frequent (>3 times a week) administration of the recombinant protein and are still only successful in 60–70% of cases for hemophilia A and only 30% of cases for hemophilia B[19,30]. In Pompe disease, ITI has been tried with intensive immune-modulatory protocols (including rituximab, methotrexate, bortezomib and intraveneus immunoglobulines) resulting in a steady decrease in iADAs and improvement of therapeutic effectiveness, but full tolerization was not achieved[17,18]. This is in accordance with the findings from Lenders et al. in FD patients who underwent kidney or heart transplantation (and were thus treated with immunosuppressive therapy). Patients with established iADAs demonstrated an initial reduction in their iADA titer[31]. However, after tapering of the immunosuppressive medication (specifically corticosteroids), the iADA titers increased again[31]. Therefore, patients would require continuous exposure to immunosuppressive drugs to maintain immune tolerance, with unacceptable side effects for such a slowly progressive disease as FD.

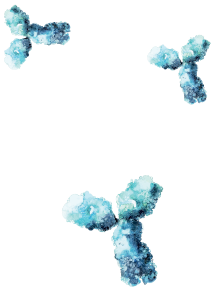
The second approach (preventing iADA formation) has been tried using immunosuppressive medication in infantile Pompe disease and MPS1 patients simultaneously with—or shortly before— ERT initiation. This approach has proven to be difficult, as iADAs often still arise after tapering of the immunosuppressive medication[20,32,33]. This may be explained by the fact that immune suppression was not optimal at the time ERT was started (in infantile Pompe disease ERT initiation cannot be delayed). In six classical male FD patients that started ERT after a transplantation, iADAs did not develop[31].

Another approach to iADA prevention is treatment initiation with lower (and more regular) doses of recombinant protein. In hemophilia, starting treatment with lower-dosed prophylactic treatment at regular intervals was associated with a 60% lower risk of iADA development compared to patients that started treatment with high doses and continued to get high doses 'on demand' (e.g., at irregular intervals)[34]. This is in accordance with the findings in our study that starting treatment with the lower dosed agalsidase alfa is associated with a lower risk for iADA development. Thus starting treatment with lower than registered doses of recombinant enzyme, in combination with shortening administration intervals, might be a way to induce central tolerance. Once central tolerance is induced, doses could gradually be increased and dosing intervals reduced. Future studies will focus on finding an optimal build up schedule for this patient group.

Our prediction model was built on a selective patient group (male patients with classical FD). We chose this approach as female patients and patients with non-

classical disease do not tend to develop iADAs. Using this model we are able to correctly predict iADA formation in 73% of male FD patients with the classical disease phenotype. To further optimize predictive accuracy in future models other variables could be included, that were not present in our current dataset. Studies in Pompe disease and hemophilia describe a potential influence of certain gene polymorphisms, such as the HLA haplotype[35,36,37]. In hemophilia, the presence of so-called danger signals before or during the first infusions (e.g., recent surgery, bleeds, vaccinations and active infections) were associated with an increased risk for iADA development[38]. The explanation is that the danger or stress signals that are released work as an adjuvants to induce immunogenicity. Although active infection is already a contra-indication for ERT administration, it may be wise to avoid initiating ERT soon after other stressors as well (e.g., surgery or vaccinations).

The outcome of this model can be translated to clinical care as the individual risk of iADAs in new patients helps physicians to decide which patients are eligible for pre-ERT immunomodulatory interventions. In addition, knowing the a-priori risk aides the evaluation of the effectiveness of these interventions, as the pre-intervention risk can be included in the outcome analysis.



Methods

Patients

This study was conducted in accordance with the principles of the Helsinki Declaration, as revised in 2013. To build the models, retrospective collected data from three European FD centers of excellence (Amsterdam University Medical Center, The Netherlands; Royal Free London NHS Foundation Trust, United Kingdom; and the University Hospital Würzburg, Germany) were used[39]. Data included basic diagnostic data, clinical and biochemical parameters, comorbidities and medication use. In this study, only male patients with a classical disease phenotype were included. Male patients were classified as having a classical phenotype based on both a residual enzymatic activity of less than 5% and the presence of one or more of the characteristic classic FD symptoms (acroparesthesia, clustered angiokeratoma, cornea verticillata), as described by Arends et al.[40]. All included patients were treated with ERT (agalsidase alfa or -beta). Patients who switched dose or treatment type were not excluded. Agalsidase alfa was always dosed at 0.2 mg/kg/eow, Agalsidase beta was predominantly dosed at 1 mg/kg/eow, but patients on 0.2 mg/kg (n = 4) and 0.5 mg/kg (n = 6) were also included.

Variables and Development of the Prediction Models

The Tripod checklist for prediction model development was followed where possible[41]. The following variables were included and tested: age at start ERT, mutation location and type, plasma lysoGb3 at baseline and the initial dose and type of ERT.

Laboratory Measurements

Plasma lysoGb3 and iADAs were measured at the AMC. Plasma lysoGb3 values were obtained within one year before start of treatment with ERT. LysoGb3 levels were analyzed using tandem mass spectrometry, as described previously[42,43]. AMC samples from before August 2015 used a different internal standard than samples from later time points at the AMC as well as samples from the Royal Free Hospital and the University Clinic Würzburg. After application of a correction factor, outcomes using both internal standards correlated well[40].

IADAs were measured as previously described[5]. In short, patient plasma in various dilutions is added to the recombinant protein. The titer represents the amount of dilutions needed to recover at least 50% of enzymatic activity in vitro. Outcome was determined as iADA positive (iADA+) or iADA negative (iADA−). Patients were considered iADA+ if they tested positive at one or more time points

for αGAL A inhibition with a titer of 6 or higher. iADA titers were measured after at least 1 year of treatment, with the exception of two patients for whom only iADA titers at 9 months after ERT initiation were available. 23/120 patients had only one iADA measurement, 24/120 had two measurements, all other patients had three or more iADA measurements (range 3–16).

Statistics

For statistical analysis and model building, R (version 3.4.3) was used. A markdown file containing the full code and results was added as Supplemental Materials. Package 'MICE' was used for data imputation. Package 'caret' was used to build and validate predictive models. Package 'ggplot2' was used for data visualization.

Algorithms

The performance of two different machine learning algorithms for the prediction of iADAs were compared: Logistic Regression (LR) and the most common ensemble method, i.e., Random Forest (RF). Overall LR and RF are known to give comparable results, but accuracy may vary depending on the number of subjects, number of explanatory variables and amount of noise variables[44]. LR is a form of binomial regression and models the probabilities for classification problems with two possible outcomes, in this case iADA+ and iADA−. It uses the logarithm of the odds (i.e., the logarithm of the probability of iADA+ divided by the probability of iADA− status), resulting in a linear combination of the independent variables (predictors) (Figure 3a). All assumptions for LR were met, details can be found in the Supplemental Materials. RF utilizes "ensemble learning". In brief, it creates many decision trees from random samples and averages out the results to get a clear model (Figure 3b). Unlike LR, RF does not assume a linear relationship between variables and therefore outcome and model are not influenced by co-linearity. Furthermore RF models can handle many different variables and do not require a significant contribution to the predicted risk for individual variables[44].

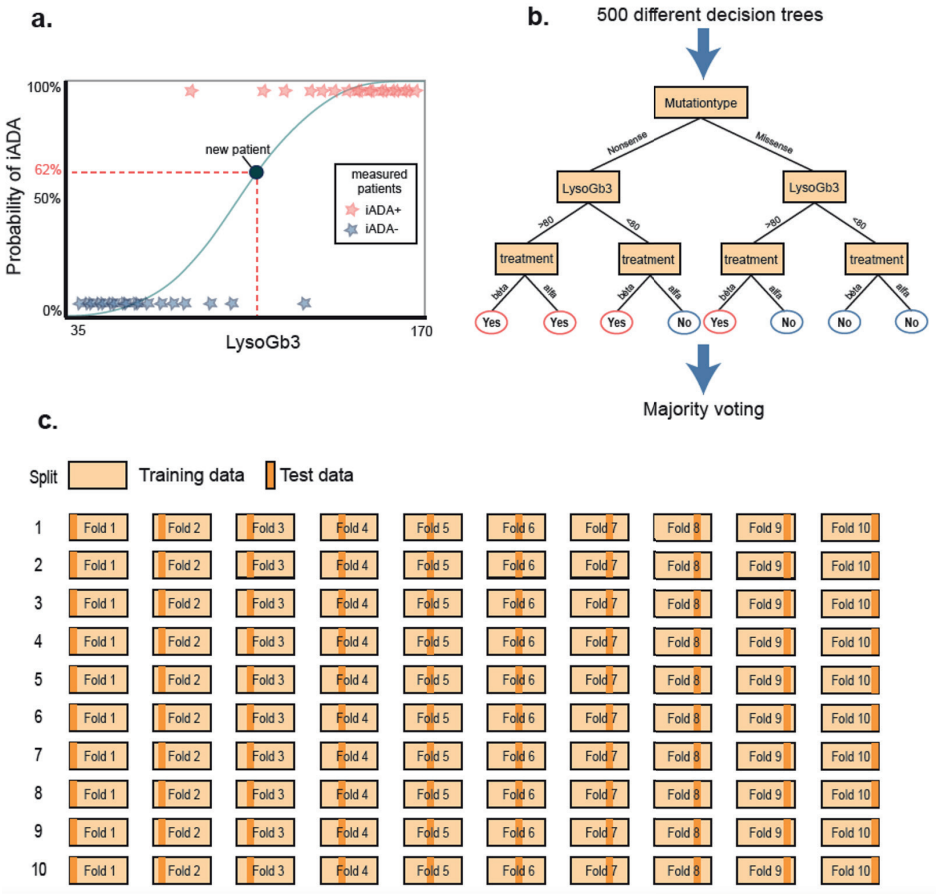


Figure 3. *Visualization of used methods. (a) Logistic regression uses a combination of independent variables to draw a sigmoid curve that fits best with the training data. New subjects are plotted on the curve to calculate the risk of iADA development (in schematic presentation this is based on a single variable (lysoGb3), in reality all contributing variables weigh in in the predicted outcome). Blue stars resemble iADA negative subjects and the red stars represent iADA positive patients in the data set used to build the model. The blue dot represents a subject in the test set. (b) Random forest is a classification algorithm. It randomly creates multiple decision trees (default is 500). Each tree results in a conclusion (e.g., iADA yes or no), majority voting of all trees is used to determine risk of iADA development; (c) Cross-validation is a resampling procedure used to evaluate predictive models with limited data. The goal is to optimize usage of data and minimize overestimation of predictive accuracy. The data was randomly split in 10 subsets. For each iteration 9 sets are used to build the model and one to test the model, until every subject has been in both groups. This procedure is repeated 10 times, until 100 models are built. Outcome of each individual patient were averaged and used to build the final model.*

Handling of Missing Data

Missing data (3%) consisted mostly of missing lysoGb3 values (28% of lysoGb3 values were missing) and were imputed using multiple imputation (package MICE)[45]. It is important to note that the MICE algorithm assumes missing data to be either Missing At Random (MAR), meaning that the probability that a value is missing can be explained by the observed values, or Completely Missing At Random (CMAR), meaning that there is no other reason for missing data than chance. In our study the treatment center and date of start ERT were the main explanatory variables for missing lysoGb3 measurements. We assumed that the year of ERT initiations and the location where patients were treated did not influence the risk for iADA development and thus considered the data to be MAR. To assess reproducibility, imputation was repeated five times and distribution of imputed variables as well as differences in mean and SD for the imputed variables were visualized and added in the Supplemental Materials. Five individual imputed datasets were created, each consisting of 5 cycles. Imputation was deemed consistent based on the comparable means and low standard deviation for the imputed values. Imputed datasets were merged and the mean of all 5 imputations was used as the final imputed value.

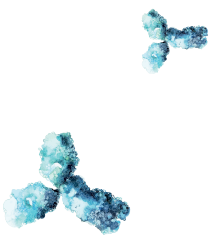
Experiment and Intrinsic Validation

For a detailed description of steps and full code see the markdown file in Supplemental Materials. In short R package CARET was used to build two prediction models. To prevent overfitting, without losing data, validation was done using 10-fold cross validation, meaning that the dataset was divided into 10 subsets. For every round nine folds were used to train the model and this model was then validated on the remaining folds. This was repeated until every set was used in both the training as the testing group. These steps were then repeated 10 times and results of each individual patient were aggregated to form the final model (Figure 3c). All variables used in the LR model met LR assumptions (visualization in SM.1). The area under the curve (AUC) of the receiver operating characteristic curve (ROC) was used to compare classification performance. The cutoff was decided based on optimal accuracy.

External Cohort

A second dataset of 30 classical male patients was provided by the University Hospital Muenster, Germany to validate the results of our model. Phenotyping was done locally and based on classical FD symptoms and enzyme activity. In six patients, no baseline enzyme activity was available, all presented with classical symptoms and had a mutation associated with classical disease. Patients were treated with agalsidase alfa (dosed at 0.2 mg/kg/eow) or agalsidase beta (dosed

at 0.2–1.37 mg/kg/eow). LysoGb3 measurements were performed at Centogene (Rockstock, Germany) in dry blood-spot. Lyso-Ceramide was used as reference (Matreya, LLC, Pleasant Gap, PA, USA) and D5-Fluticasone Propionate (EJY Tech, Inc., Rockville, MD, USA) served as internal standard. LysoGb3 values at baseline were missing in 10/30 patients. IADAs were measured as described previously[29]. All patients with >50% inhibition are considered iADA positive. Titers represent the amount of enzyme necessary to overcome the neutralizing capacity of iADAs in a patient. Due to differences in measuring techniques for both iADA status, lysoGb3 and differences in ERT dosing the data could not be used to validate the initial model, to show reproducibility model building was repeated with the same variables and outcome is reported separately.



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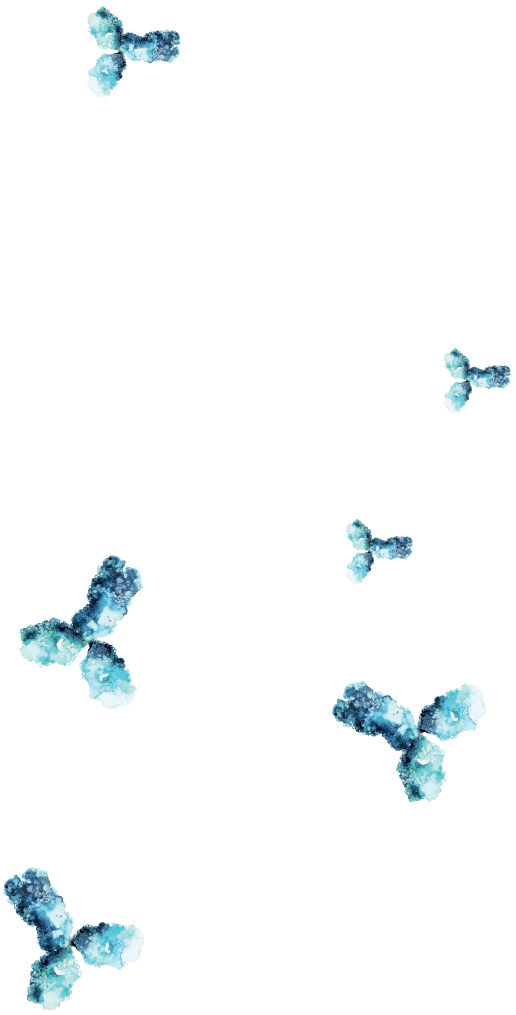
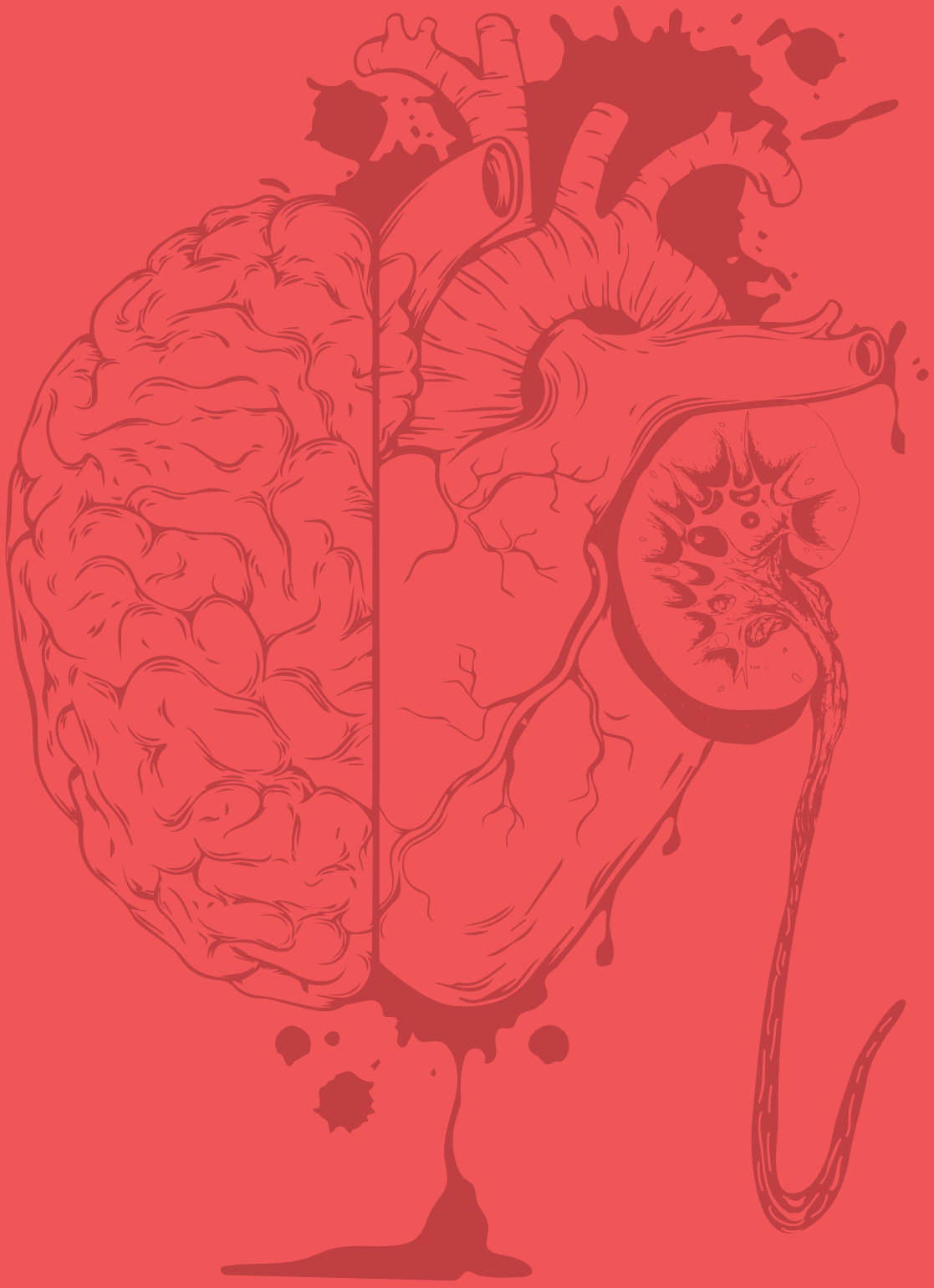
Supplemental material

Full markdown file of the R code can be downloaded along with the publication





Chapter 8

Antibodies against recombinant enzyme in the treatment of Fabry disease: Now you see them, now you don't

Sanne J. van der Veen and Mirjam Langeveld

Molecular Therapy Methods & Clinical Development
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In their article published in this issue of Molecular Therapy – Methods & Clinical Development[1], Lenders and co-workers study the affinity of pre-existing anti-drug antibodies (ADAs), which developed in patients with Fabry disease (FD) after treatment with recombinant alpha-galactosidase-A (αGALA), to a new PEGylated form of the enzyme (Pegunigalsidase-α, PRX-102). For the majority of patients, pre-existing ADAs showed less affinity to PRX-102 compared with the unPEGylated compounds. Since the amino acid sequence of the unPEGylated and PEGylated enzyme is identical, these results suggest an altered epitope availability.

FD is a rare lysosomal storage disease caused by pathogenic variants in the gene coding for the enzyme αGALA. Treatment most often consists of enzyme replacement therapy (ERT) with recombinant αGALA (agalsidase-α or agalsidase-β), so-called biologicals. Biologicals are drugs produced in living organisms (e.g., enzymes, hormones, monoclonal antibodies), and their development has greatly changed the outlook for many diseases. The downside of biologicals is their immunogenicity and, specifically, their ability to induce anti-drug-antibody (ADA) formation. ADAs can impact pharmacokinetics, inhibit the biological function of the proteins, and/or cause severe anaphylactic and anaphylactoid reactions. In particular, the inhibiting/neutralizing ADAs (iADAs) have been proven to cause therapy failure in many diseases, including Pompe disease and hemophilia. In these disorders, the impaired efficacy results in direct observable treatment failure. In FD, it has taken decades before the deleterious effect of iADAs on treatment efficacy was also recognized[2]. This is partially explained by the disease heterogeneity and slow progression (requiring long follow up of a large patient cohort to detect the effect) but probably also by insufficient access to ADA assays and independent disease registries.

Now that the negative effect of ADAs against αGALA on treatment effectiveness is finally recognized, several approaches are being tried by different specialized centers to lower existing ADA titers or prevent ADA formation in newly treated patients. In other metabolic diseases treated with ERT, immunomodulatory protocols using immunosuppressant drugs were either unsuccessful and/or required very high doses of immunosuppressants[3-5]. Immunomodulation protocols focusing on a more constant drug exposure, for example those used in hemophilia, look more promising[6]. With this in mind, our group is currently testing an alternative treatment initiation protocol in patients at high risk for developing ADAs[7], aiming to induce tolerance by giving more frequent infusions with gradually increasing doses of ERT. However, the necessity of more frequent infusions can be a burden for patients and can theoretically be solved by increasing the half-life of the drug.

PEGylated αGALA (PRX-102) has a plasma half-life of several days as opposed to minutes for the non-PEGylated enzymes. Additionally, epitope exposure may be different for PRX-102 compared with the currently used recombinant enzymes since polyclonal rabbit-anti-αGALA reacted stronger to normal recombinant-GLA compared to PRX-102[8].

In the paper from Lenders et al., a set of sophisticated experiments were performed, showing that ADAs that developed in patients with FD after exposure to agalsidase-α and/or -β indeed tend to bind with less affinity to the PEGylated enzyme PRX-102. The results from the pull-down experiment and cross-ELISAs suggest that PRX-102 has a reduced exposition of some of the immunogenic epitopes. However, the conclusion that this results in reduced immunogenicity needs to be confirmed in future clinical studies. The fact that a small subset of patients have ADAs that bind to PRX-102 with more affinity suggests that the PEGylated compound displays different epitopes but not necessarily less immunogenic ones. And, as all patients were immunized with either agalsidase-α or -β, it makes sense that most of their ADAs react more strongly to those compounds when there is a difference in epitope availability between the enzymes. The only way to test this would be to do the reversed experiment with patients immunized with PRX-102. Nonetheless, the idea of switching treatment in patients with ADAs with a favorable in vitro response to PRX-102 seems appealing, provided that the ADA response after switch is closely monitored.

The latter sounds a lot easier than it is in practice. As the authors accurately described in the discussion, measuring ADAs when the immunogenic compound is still in the circulation is very difficult since the ADAs will remain bound and currently used assays only measure free ADAs (figure 1). ADA levels in patients treated with PEGylated enzymes can therefore not be compared with levels in patients treated with agalsidase-α or β. The authors' suggestion to measure ADA-drug complexes or use assays that dissociate the complexes first also come with pitfalls: the pH alterations needed to break ADA-drug interactions can potentially alter ADA binding capacity, and the unbound drug needs to be removed to prevent rebinding. Additionally, as the epitopes differ between compounds, we need assays using PRX-102 as antigen instead of the currently available assays that use the unPEGylated enzyme. Finally, the clinical effect of ADAs in patients may be different for different compounds, especially if the compounds greatly differ in half-life (figure 2).

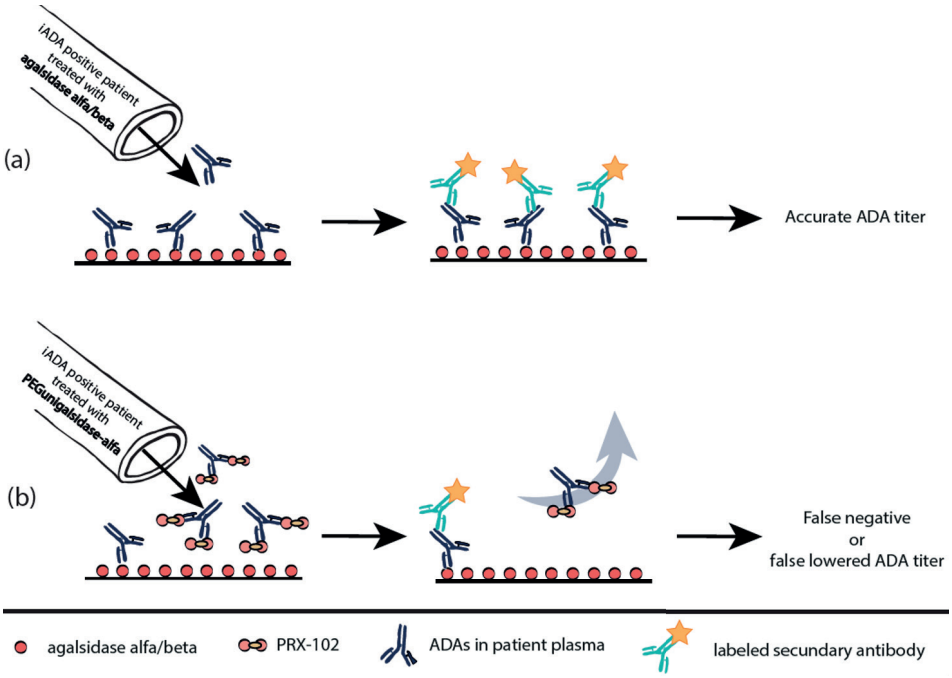

Figure 1. *Interference of residual drug antigens in patient plasma with ADA measurements using ELISAs (A) For patients treated with agalsidase-α or -β, drug concentrations in plasma are unmeasurable within hours after infusion ended. Circulating ADAs are unbound, and titers can be adequately measured using conventional ELISAs. (B) For patients treated with PEGunigalsidase-α (PRX-102), the drug is continuously present in patient plasma, and an unknown amount of ADAs will remain bound to PRX-102 and will thus not be detected, resulting in falsely low titers or false negative results.*

In the phase 1/2 study of PRX-102, 3 out of 11 male patients had measurable ADA levels, and increasing ADA titers resulted in a vastly reduced half-life of the drug[9]. Interestingly, when ADA titers went down, the half-life of the drug recovered. Pharmacokinetic measurements may therefore be a good (though time-consuming) way to monitor the effect of ADAs in patients after switch (figure 3). In addition to measuring the effect of PRX-102 on ADA titers, it should be confirmed that the potential lower immunogenicity of the drug leads to a measurable improvement of biochemical (plasma lysoGb3 concentrations), and ultimately clinical, therapeutic effectiveness.

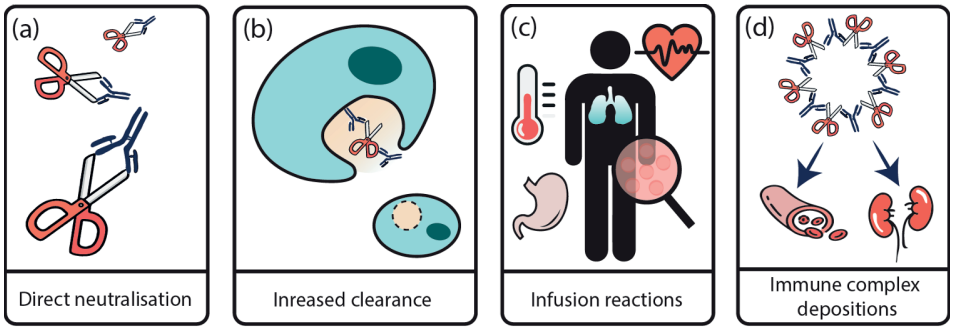

Figure 2. *Potential detrimental effects of ADAs against biologicals. (A) ADAs against biologicals can impair effectiveness of treatment by directly neutralizing the drug, rendering it incapable to perform its function. Neutralizing antibodies work immediately and are most strongly linked to reduced treatment efficacy. (B) Non-neutralizing antibodies can impact the pharmacokinetic profile of a drug by increasing clearance from the circulation, though its effect on treatment efficacy is more variable. It is presumed that with increased circulatory half-life, the relative importance of these non-neutralizing ADAs increases. (C) In addition to the effect on treatment efficacy, ADAs can induce infusion reactions. Classical infusion reactions are common in male patients with classical FD and are characterized by a sudden onset of fever, cold chills, nausea, and general malaise, which recover quickly after stopping the infusion. (D) Finally, it has been described for biologicals other than ERT for Fabry disease that ADA-drug aggregates can accumulate in the vascular wall or in the kidney, causing vasculitis, thrombosis, and renal failure. Although rare, the occurrence of this complication should be monitored for every new biological.*

In conclusion, the potential for a less immunogenic therapy for the treatment of male patients with classical FD is promising, and the idea to switch treatment in patients whose pre-existing ADAs show a significant lower affinity to PRX-102 is justifiable. However, there is a need for international consensus on how best to monitor ADAs and their effect on treatment after switch to PRX-102 to be able to confirm the benefit in a timely manner.

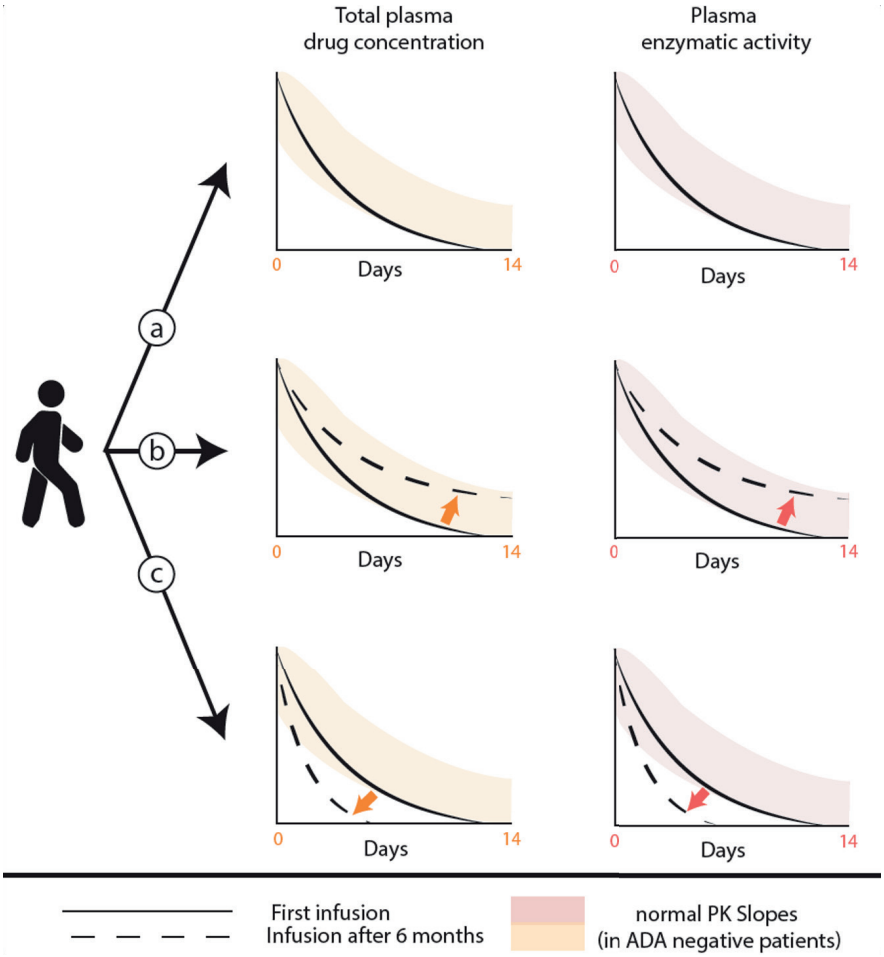

Figure 3. *Proposition for analyses of the effect of ADAs on treatment after switching ADA positive patients from agalsidase-β to PRX-102. As ADA titers against PRX-102 cannot simply be compared with those against agalsidase-α and -β, it may be considered to also monitor the pharmacokinetic profile in ADA-positive patients after switch. One could measure the total concentration of PRX-102 in plasma (yellow), to assess total drug clearance, as well as enzyme activity in plasma (pink), to assess in vivo enzyme inhibition by ADAs. After switching patients whose ADAs have low in vitro cross reactivity to PRX-102 from agalsidase-β to PRX-102, there are three possible scenarios, described as follows. (A) No response: PK curve is normal or slightly lower than normal. PK slope remains unchanged, suggesting no changes in the ADA response (titer and affinity). (B) Most favorable response: PK curves improve to fully normal, suggesting that constant exposure induced further tolerization (ADAs disappear). (C) Least favorable response: PK curves worsen over time, suggesting new ADAs develop (higher titers) with specific affinity to PRX102.*

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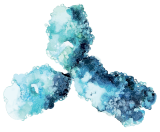
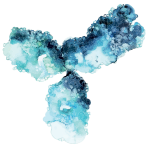
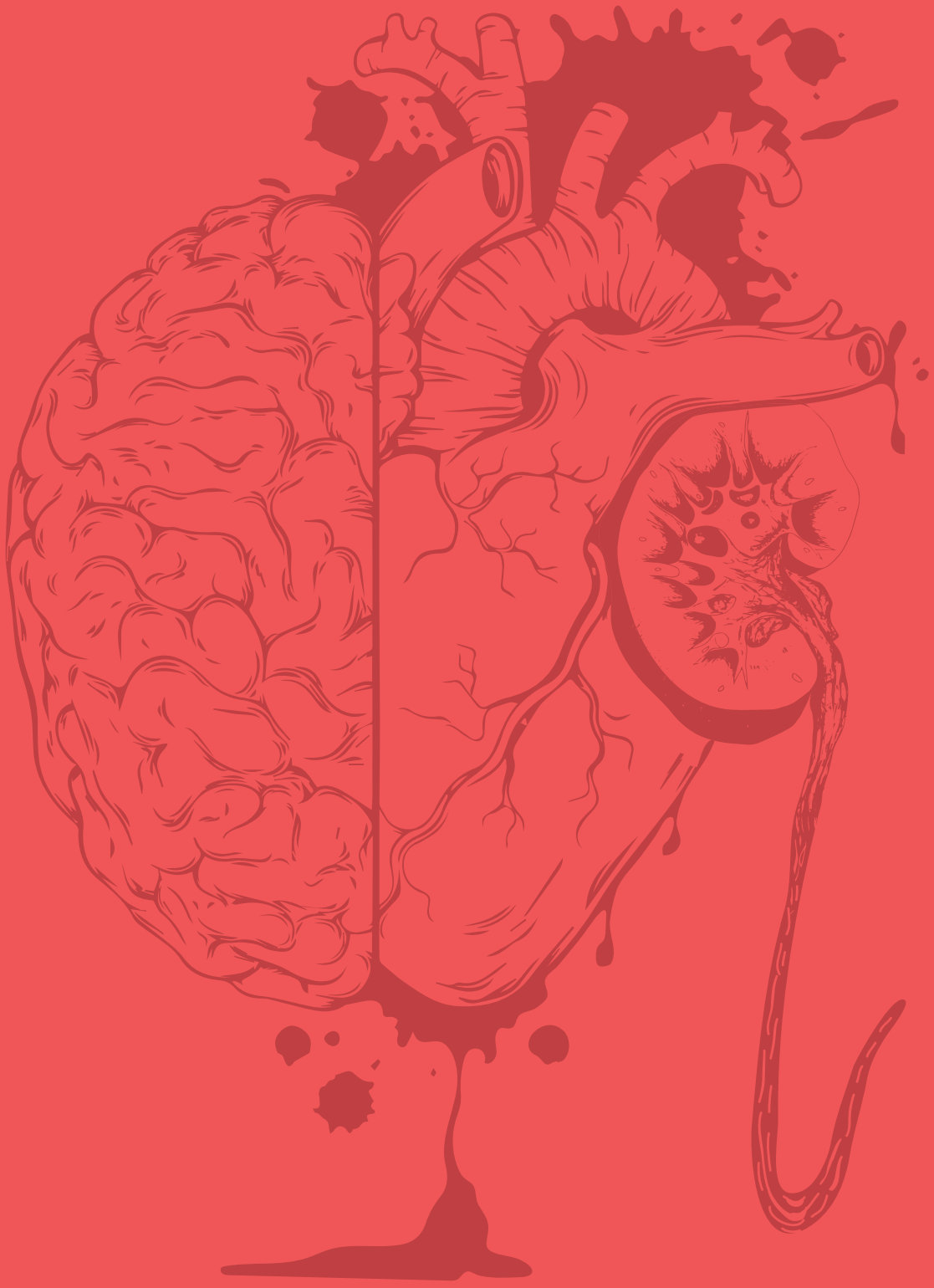






Chapter 9

Summary and general discussion

Summary

Despite its rare occurrence, much attention, time and money has been devoted into research of Fabry disease (FD). This effort led to the simultaneous development of two forms of enzyme replacement therapy (ERT) two decades ago, agalsidase-alfa and agalsidase-beta. In the years since, hundreds of studies have been conducted to study the effectiveness of these drugs, but although there is increasing evidence that the treatment could slightly slow the disease process, it also became clear that in most patients, disease progressed, despite treatment. In recent years, research into new treatment methods has spiked and there are dozens of new drugs that are currently undergoing clinical investigation. At the same time, the increased attention for FD has led to an increase in (genetic) diagnostics resulting in the discovery of many mutations in GLA that are associated with a milder disease course. The clinical distinction between 'classical' disease (more severe form of FD) and 'non-classical' disease (milder form of FD) has helped to improve prognostic information and clinical decision making. In reality, however, Fabry disease appears to be more of a spectrum rather than a dichotomous disease, ranging from mildly elevated cardiovascular risk to severe multi-organ disease. Thus raising the need for more precise prognostic tools. Finally, the most common complication of the treatment of FD with ERT, the development of antibodies against the recombinant enzyme, has long been underexposed.
The aim of this thesis is therefore threefold;

I. to improve prognostic information in newly diagnosed patients regarding their expected disease course.
II. Examining the clinical effects of antibodies against ERT and analyzing which patients are most at risk.
III. Finding leads to make the treatment of FD more effective by optimizing the timing of treatment initiation and by preventing antibody formation.

In **chapter 2**, we investigated the levels of globotriaosylsphingosine (lysoGb3) In plasma of 237 untreated FD patients over time and determined its association with disease -manifestations and -course using linear mixed effect models. We showed that in the absence of FD specific treatment, the plasma FD biomarker lysoGb3 remains stable over decades in FD patients and that it thus represents a static, individual FD trait. Additionally, the untreated plasma lysoGb3 levels were associated with the progression and/or severity of nearly all clinical FD manifestations (including eGFR, albuminuria, cardiac morphology and function and cerebral manifestations). The fact that this biomarker reaches stability well before clinical disease manifestations occur suggests that measuring lysoGb3 at diagnosis provides insight into the expected natural disease course.

This knowledge facilitates clinical decision making as it may aid to determine the needed frequency of follow-up and the timing of treatment initiation in asymptomatic patients.

In **chapter 3**, we showed that the start of treatment with ERT before the age of 16, in male patients with the most severe phenotype -classical disease- is associated with reduced occurrence and severity of renal and cardiac manifestations of FD. We collected data from seven male patients with classical disease that started treatment in childhood and received continuous treatment with agalsidase-beta for 10 years and compared them to 23 age matched male patients with classical FD that remained untreated. Compared to untreated patients, albuminuria was less common and less severe in treated patients and left ventricular cardiac mass was lower in the treated group compared to the untreated group.

In **chapter 4** we evaluated the currently available treatment modalities: ERT and chaperone therapy, as well as upcoming treatment modalities such as second generation ERT, substrate reduction therapy, mRNA- and gene- based therapies. The aim of this review was to provide insight in the currently available evidence on efficacy, the mechanism of action and potential pitfalls of each treatment modality.

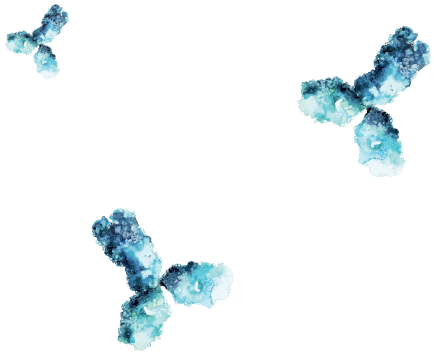
As the patent on the available ERT preparation has expired, the market is open for more affordable biosimilars. In **chapter 5** we describe the successful development of a biosimilar of agalsidase-beta, aGal A biosidus (AGABIO) and show the complete resemblance of AGABIO to agalsidase-beta.

One of the main complications of treatment with ERT is the development of inhibiting anti-drug-antibodies (iADAs) against recombinant αGalA. In **chapter 6**, we analyzed data of 39 classical male Fabry patients over a mean period of ten years to determine the effect of iADAs on treatment efficacy. We showed that the ADA inhibition titer was strongly negatively correlated to the decrease in plasma lysoGb3 upon treatment. Clinically, patients with iADAs had an accelerated decline in renal function compared to those without iADAs. In addition we characterized the iADAs and found that they were of the IgG1 and IgG4 subclasses. The outcome of this study highlights the importance of evaluating the iADA response towards recombinant αGalA and emphasizes the need for better protocols to deal with this complication.

To establish the risk of iADA development before treatment initiation we set out to build an iADA prediction model in **chapter 7**. As inhibiting ADAs nearly

exclusively develop in male patients with the classical disease phenotype, we only included this subgroup of patients. We determined that having a nonsense mutation and high baseline levels of the biomarker lysoGb3 were associated with higher risk for iADA development. Starting treatment with the higher dosed agalsidase-beta (compared to agalsidase-alfa) was also related to an increased iADA risk. Using these variables we build a logistic regression model that predicts the iADA risk in individual patients.

One of the new treatment modalities described in **chapter 4** is a new PEGylated form of αGalA, Pegunigalsidase-α (PRX-102). The increased half-life of PRX-102 and the differences in epitope availability compared to agalsidase-alfa and agalsidase-beta, may result in reduced immunogenicity. In **chapter 8** we provide an invited commentary on a paper published by Lenders et al discussing the pitfalls in comparing the immunogenicity of different compounds.



General discussion

Disease or risk factor? The risk of labels

Fabry disease is a highly heterogeneous disease and this heterogeneity is currently one of the biggest challenges in the management, evaluation and treatment of Fabry patients. Firstly, mutations in GLA can either cause complete absence of αGalA enzymatic activity (resulting in the most severe 'classical' disease phenotype) or cause reduced enzymatic activity (resulting in the milder 'non-classical' disease phenotype). Especially in the latter group, the clinical presentation differs greatly depending on the residual enzymatic activity. Secondly, due to the X-linked inheritance, there is a clear difference in the age of onset and severity of disease manifestations between men and woman. Due to a process called random X-inactivation (meaning that every cell is thought to only use one copy of the X- chromosome), the clinical presentation in female FD patients is even greater, though on average they are less severely affected then their male relatives carrying the same mutation. Finally there are benign GLA mutations that do not seem to cause any symptoms, but the distinction between benign mutation and 'mild disease is not always easy to make and the pathogenicity of some mutations is still subject to debate[1-3]. Although the clinical distinction between classical and non classical FD is favorable in cinical practice, in reality FD is more a spectrum ranging from individuals with a mildly increased cardiovascular risk profile compared to the healthy population to patients with a severe monogenetic disease. The majority of patients on the milder end of the clinical spectrum (female patients with a non-classical phenotype) seem to remain free of clinical events[4], although they may develop some left ventricular hypertrophy or cardiac conduction defects in the 7th or 8th decade of life[5]. While all female FD patients (classical and non-classical) combined lose on average 5 years in life expectancy[6], it is our clinical experience that female FD patients with the non-classical phenotype have a (near) normal life expectancy. To put this in perspective, analyzing up to date data from statistics Netherlands (CBS) and the Netherlands Institute of Mental health and Addiction (TRIMBOS institute) shows that smoking reduces life expectancy with 9 to 13 years. Another study shows that obesity in non-smokers reduces life expectancy with 6-7 years while obese smokers lost an additional 13-14 years compared to normal weight smokers[7]. Hypertension, another cardiovascular risk factor that, like FD, can cause left ventricular hypertrophy, proteinuria, renal failure and stroke, reduces life expectancy with approximately 5 years[8]. It therefor seems logical to put more emphasis on optimizing the treatment of 'regular' cardiovascular risk factors and life style interventions regardless of the decision to start with FD specific treatment.

On the other end of the spectrum are the male patients with classical FD who are described in all the initial publications on the disease, whom, even in the absence of other risk factors, develop serious clinical complications early in adult life[4] and who have a strongly reduced life expectancy. This is usually the group of patients that people have in mind when discussing FD. In between these groups at either end of the spectrum of disease severity are the female patients with classical FD and the male patients with non-classical FD, for whom the natural disease course and with it the necessity to start FD specific treatment may be the most difficult to predict. In current clinical practice, most patients are under routine clinical follow up to be able to notice early signs of FD (e.g. proteinuria, LV mass increase, ECG abnormalities) to not miss the window of opportunity to start treatment. This is important as on one hand we must avoid treating patients that will not develop any clinical relevant complications, while on the other hand we learned that treatment seems to become less effective when it is started after irreversible organ damage is already present. However, this approach of routine follow up comes with several drawbacks. The first is the uncertainty whether or not the observed symptoms can be (fully) attributed to FD in that individual. Apart from smoking, obesity and hypertension, which are mentioned before, many other factors, including high cholesterol levels and high alcohol consumption contribute to an increased risk of cardiovascular death and can cause symptoms that resemble FD (e.g. proteinuria, renal decline, left ventricular hypertrophy, stroke, myocardial infarctions). In many cases it is impossible to decide which factor contributes most to the observed clinical manifestations, making it difficult to establish the relative contribution of the GLA mutation to the observed clinical phenotype, which in turn complicates the decision whether or not to start FD specific treatment in that individual. Another, important downside of routine follow-up of asymptomatic individuals is the issue of over-medicalization and the effect it can have on 'patients'. As healthcare workers we sometimes forget that diagnostic labels can have a significant impact on psychological well-being[9,10]. In a large cohort of patients with hypertension, the ones that were aware of the diagnosis reported higher levels of psychological stress then patients with similar blood pressures who were not (yet) aware of the diagnosis[11]. Additionally, asymptomatic patients with a diagnosis of glucose intolerance, hyperlipidemia, or hypertension reported a lower health related quality of life compared to patients that did not yet receive the diagnosis[12]. Not only a diagnosis itself but even the words used to describe a diagnosis impacts psychological well-being. The more medical and precise the terminology, the more severe patients perceive their disease and the higher the anxiety levels in patients. Additionally, the more difficult the terminology used in describing a disease to patients, the more invasive interventions were deemed acceptable

by patients[13]. Although proper studies on the effects of increased medical testing (routine MRIs, echocardiography etc) on mental health are lacking, one can imagine that this may further strengthen the patients belief that their disease is severe. Additionally, while hypertension, glucose intolerance and hyperlipidemia are well known conditions with well described risks, this is not always the case for genetic diagnosis, including FD. Thus far not much is known regarding the potential impact of a diagnosis with uncertain prognostic implications on patients mental health[14]. Previous studies show that nearly half of FD patients suffer from objectified depressive symptoms[15,16]. Yet, unlike all the other FD disease manifestations, the severity of depressive symptoms was not related to age, sex, phenotype or the severity of organ involvement and thus not related to objective disease severity[16]. This suggests that it may not be the disease itself, but rather the diagnosis that has the biggest impact on the mental health of our patients.

Further optimization of clinical prediction of the severity of FD at the time of diagnosis, especially in the group of female patients with classical FD and male patients with non-classical FD would be a first step to improve prognostic information and help individual patients to establish a realistic conception of their disease. In **chapter 2** we show that measuring plasma levels of the biomarker lysoGb3 gives us additional insight in the expected natural disease course. If we could make use of large international datasets to verify these results and find additional markers to further optimize clinical prediction; we could start treatment timely in those patients most in need, we could properly counsel milder patients about the relative risk of their FD variant as well as the impact of potential other risk factors on their life expectancy to come to the best treatment/follow-up plan for that individual patient.

Improving current treatment - can we learn from other diseases?

Apart from the decision in whom to start FD specific treatment, another challenge in the treatment of FD is to improve treatment outcome. Currently the golden standard of treatment is enzyme replacement therapy (ERT), which has been available for over 2 decades in two forms (agalsidase-alfa and agalsidase-beta). Nonetheless, evidence to the effect of ERT on clinical relevant endpoints is still limited. The relatively short follow-up duration and low number of included patients of the initial randomized controlled trials on ERT are plausible explanations for the lack of evidence they provided for clinical effect[17], yet the treatment was approved based on mostly biochemical and functional endpoints. Though driven by the desire to provide a treatment for a group of patients that thus far had no treatment options available and possibly

motivated by the success of ERT in Gaucher disease patients, in hindsight the rushed approval of ERT for FD has led to some clinical and methodological obstacles. First, the expected clinical effect of treatment remained unclear, but as it would be unethical to withhold an available treatment from symptomatic patients, the approval of ERT resulted in a strong treatment bias. Meaning that the only unbiased untreated cohorts we could compare treatment to are historical cohorts. However, in parallel to the use of Fabry specific treatment, supporting treatment has improved dramatically in the last two decades: ACE inhibition to preserve renal and cardiac function, improved transplantation outcomes and improved cardiac devices to prevent sudden cardiac death. This renders the comparison to historical cohorts invalid since it is unclear what part of the improvement is due to Fabry specific treatment and what part to the improved supportive therapy. Secondly, as most patient registries are pharma-controlled, they only contained data on patients treated with one of the two available ERT preparations, making it difficult to compare the 2 compounds directly. The 2017 Cochrane review including 77 cohort studies on a total of 15.000 FD patients concluded that there is some benefit on clinical endpoints of starting agalsidase-beta, but not agalsidase-alfa, compared to natural history cohorts[18]. One possible explanation for the lack of clinical effect found in these studies could be the late start of treatment initiation. It seems that once certain secondary pathophysiological processes are set in motion, disease progresses regardless of treatment. The -so called- point of no return. Due to the slow disease progression, it took a long time to learn the importance of early treatment initiation in FD[19]. This was less the case with infantile Pompe disease, another lysosomal storage disease with a much faster clinical course. The lack of the lysosomal enzyme alpha-glucosidase (αGlu) in these patients results in the intracellular accumulation of glycogen in mainly skeletal and cardiac muscle. When left untreated, patients usually die within the first year of life due to cardiac failure. Due to the rapid clinical course, it was much easier to study effect of treatment in these patients and the importance of timing of treatment became evident much faster. Earlier intervention with ERT improved clinical outcome dramatically. Another interesting observation in these patients was the effect of different dosing regimens. Infantile pompe patients were initially treated with 20 or 40mg/kg of αGlu every other week (eow)[20], which resulted in a fast reduction of lysosomal substrate accumulation in cardiac tissue of patients as well as a profound effect on clinical cardiac outcome in patients. Skeletal muscle tissue was found more resistant to treatment, but increasing the dose up to 40mg/kg weekly further improved the clinical outcome of patients[21,22]. In contrast, the recommended doses for ERT in the treatment of FD are 0.2mg/kg/eow (agalsidase-alfa) and 1mg/kg/eow (agalsidase-beta) and the effect of higher

doses on treatment efficacy have thus far not been tested. The explanation behind the differences in chosen dosage between these two lysosomal storage disorders, both affecting the heart, remains elusive and cannot be satisfactory explained by the differences in normal enzymatic activity in healthy individuals or the different PK characteristics of the different compounds. Treatment of FD patients with the recommended doses of ERT results in a rapid reduction of substrate in mainly the endothelial cells. Podocytes however[23,24], have proven to be more difficult to clear and Gb3 accumulation was hardly –if at all- reduced in cardiomyocytes, despite years of treatment[25]. Both recombinant αGalA, and recombinant αGlu are primarily taken up into cells by the mannose 6-phosphate (M6P)-receptor[26]. As the high doses of recombinant αGlu do seem to enter the cardiomyocytes and reduce substrate accumulation, unlike the lower doses of recombinant αGalA, it seems logical to also try the Pompe treatments approach in the treatment of FD: early intervention with high doses of ERT (But only in patients that are known to become symptomatic). It is good to keep in mind that while earlier treatment initiation and higher doses of ERT improved the outcome in children with Pompe disease significantly, disease progression can still not be fully halted. Meaning that ERT changes the clinical phenotype in a dose dependent manner, but does not cure the underlying disease.

There are several possible hypotheses as to why ERT is less effective in treating FD when started in later phases of the disease. One of the hypotheses is that Gb3 accumulation in those most relevant celltypes (e.g. podocytes and cardiomyocytes) takes many years to clear completely, and might come too late for more advanced patients. In theory, this could be solved by initially starting treatment with high doses ERT and reducing to a lower maintenance dose after accumulation has been sufficiently reduced. The other hypotheses is that with progressing disease, cells become increasingly hard to clear with ERT. Either due to reduced availability of the M6P receptor and reduced uptake of the recombinant enzyme in more advanced disease[27] or because of an overflow to extralysosomal accumulation in more advanced disease, where the recombinant enzyme cannot reach it[25]. The first of which may also be partly overcome with higher doses of treatment. The main drawback for trials with high doses of ERT is the excessively high cost of the product. However, given that the patent for ERT has long expired, this issue might be overcome by using (lower priced) biosimilars. In **chapter 5** we show the biological and functional comparability of one such biosimilar to agalsidase-beta. The other drawback is the concern for potentially increased immunogenicity that can come from using higher doses of ERT in a subgroup of patients.

Unfortunately, the group of FD patients who are most in need of treatment (e.g. male patients with classical FD) are also the ones most at risk for treatment complications. As they (nearly) completely lack in endogenous αGalA production, their body does not recognize the enzyme as a self-protein and treatment may thus result in immunological reactions. Antibodies towards the recombinant enzyme (anti-drug-antibodies, ADAs) are capable of evoking infusion related reactions. The most observed reaction is a fast rise in body temperature causing rigorous shaking of the body (cold chills) often accompanied with nausea and/or vomiting. Fortunately, stopping the infusion and starting corticosteroid treatment usually results in a quick recovery. Other potential reactions include the more classical allergic reaction ranging from mild rash to anaphylactic shock. In most cases reactions can ultimately be managed by slowing down infusion speed and pre-treatment with immunomodulatory drugs (corticosteroids and/or antihistamines). The other detrimental effect of ADAs is less acute but more difficult to manage. ADAs are capable of nearly completely inhibiting αGalA activity both *in-vivo* as *in-vitro*. In **Chapter 6** we showed that these inhibiting ADAs (iADAs) not only result in a weaker biochemical response to treatment –e.g. less pronounced reduction of lysoGb3 in plasma upon treatment- but also in an accelerated renal decline. Therefore, another approach to optimize treatment effect would be to overcome the issue of iADA formation either by preventing iADA development in patients that are starting with ERT or by trying to induce immune tolerance in patients with an already established immune response to ERT. As iADAs are difficult to eradicate once an immune response has occurred, the former approach would be preferred in patients with a high risk for iADA development. In **Chapter 7** we showed that within the group of male patients with classical FD, several factors contributed to a further increased risk for iADA development, including higher baseline levels of lysoGb3, having a nonsense mutation and starting treatment with –the higher dosed- agalsidase-beta. Using these variants, we build a prediction model to calculate an individual's iADA risk before start of treatment initiation. We determined the optimal cutoff point to divide patients into relatively high and low risk for iADA development.

In Hemophilia patients, starting treatment with more frequently administered lower doses of factor VIII was associated with lower risk for iADA development compared to high doses with longer intervals[28]. As mentioned, the latter may be an issue when we consider that there may also be an expected benefit for treatment with higher doses of ERT in FD patients. Additionally, apart from iADA development, infusion related reactions to ERT seem to be concentration – and thus dose – dependent, which is illustrated by the fact that they can often be clinically managed by slowing down infusion rate. Simply increasing the ERT

dose in new patients may thus come with an additional immunological risk. A potential solution for this problem is to initiate treatment with more frequent administrations of ERT. With this in mind, we are currently testing an alternative treatment protocol in our high risk patients to try and prevent infusion reactions to increase safety and prevent ADA development to improve efficacy of treatment. The protocol consists of initiating treatment with more frequently administered low doses of ERT. Doses are gradually increased over the course of one year after which we will evaluate the effect on iADA formation, infusion reaction and biochemical response to treatment.

FD patients that are already on treatment and developed iADA are in need of safe and effective protocols to induce immune tolerization. As mentioned in the introduction, the expected contribution of immunosuppressive drugs in this regard is limited as even in patients that received organ transplants and are under intensive treatment with immunosuppressive drugs, ADAs towards αGalA either persisted or reappeared as soon as treatment with immunosuppressants was tapered[29]. However, again looking at clinical experience from treating hemophilia, protocols using intensive treatment schedules with frequent administrations of either very low or very high doses of the recombinant protein to induce tolerization have proven to be safe and relatively effective[30-32], which may be an interesting approach to try.

New treatment options – Learning from past mistakes?

In **Chapter 4** we discuss current and upcoming treatment options for FD, including new (improved?) forms of ERT, chaperone therapy, many options for substrate reduction therapy, gene- and mRNA- therapy. At first sight, the avalanche of potential treatment options seems like a positive development; more options to choose from and thus more individualized treatment. However, we concluded our chapter with the warning that "care must be taken that we are not left with an overload of underpowered studies of insufficient duration, making it impossible to draw any conclusions regarding the relative effectiveness of each treatment modality". As it is no longer ethically defensible to compare treatment to placebo, all new treatments are compared to the 'golden standard' of treatment with ERT. New treatment options, including the chaperone Migalastat, are being considered or accepted as alternative treatment options based on non-inferiority compared to ERT on clinical effect[33]. Taking into regard the limited effect of ERT on disease progression and the amount –and years- of research needed to prove it, non-inferiority based on a single trial of two years does not prove a clinical effect. Moreover, the more treatment options become available and the more often patients switch treatment, the harder it will become to make

any claim regarding the relative effect of each treatment modality on disease progression. The solution is simple in concept: New compounds need to prove definite superiority compared to ERT on clinical disease course before being accepted for treatment. In real life, this proves to be more difficult due to the slow disease progression and the fact that treatment is most effective when initiated early (when clinical complications are decades away), requiring long term follow up of large patient groups, which is not always feasible in the form of an RCT. We therefore emphasize the need for large international registries, independent from pharmaceutical companies, in which data of both untreated patients as well as patients on different treatments are systematically collected. The current disease course in patients under treatment with ERT could be used as a benchmark against which the effect of new treatment modalities are compared. Future research should also focus on finding and validating clinical biomarkers and intermediate endpoints to be able to assess and evaluate clinical effect of treatment in a more timely manner.

Supportive treatment – targeted approached to intervene in later disease stages

The lack of alpha-galactosidase A activity in FD patients results in intracellular Gb3 depositions which sets in motion a variety of pathophysiological processes that ultimately lead to the observed clinical manifestations of FD (fig. 1). Disease specific treatment options (ERT, substrate reduction, chaperones) aim to reduce GB3 accumulation but fail to alter clinical disease course when initiated in later stages of the disease, despite a reduction of substrate accumulation. Most likely because the initial accumulation has set in motion secondary pathophysiological processes that –at some point- enter a self-propagating loop. An alternative approach for patients with more advanced disease could be to intervene in the key secondary disease processes of FD including inflammation, oxidative stress, autophagic dysfunction and upregulation of the renin–angiotensin–aldosterone system (RAAS). Though the initial cause for FD is unique, there is increasing evidence that it shares later-stage pathogenic pathways with other diseases, most notably diabetes[34]. Similar to diabetic nephropathy, Fabry nephropathy starts early with signs of podocytes injury, thickening of the glomerular basement membrane due to increased production of extra cellular matrix and ultimately activation of fibroblasts resulting in focal and segmental glomerular fibrosis and sclerosis[34]. The clinical presentation of increasing proteinuria and ultimately (slowly) progressive renal decline is similar as well. In addition, diabetic cardiomyopathy is characterized by left ventricular hypertrophy, fibrosis and cell signaling abnormalities. Initially subclinical diastolic dysfunction occurs, often evolving to heart failure with preserved ejection fraction (HFPEF) and finally to systolic heart failure[35]. Again, a disease course very similar to FD cardiomyopathy[5].

In the search for compounds that may help alter more advanced stages of FD, the similarities between the two diseases make it appealing to primarily look at compounds that are already successfully used in the treatment of late-stage DM nephropathy and cardiomyopathy. One example that is already used in both are drugs that intervene with the RAAS, like ACEi and ARBs, mainly due to their anti-proteinuric and anti-fibrotic properties[36]. Although the main focus in treating diabetes is lowering blood glucose levels, some compounds have proven to improve cardiovascular outcome through a direct effect on the myocardium and have since also successfully been tested in non-diabetic patients. A compound worth mentioning in this regard is the sodium-glucose cotransporter 2 inhibitor (SGLT2i). The use of SGLT2i in non-diabetic heart failure patients was shown to reduce levels of N-terminal pro-B-type natriuretic peptide (NT-proBNP), improved left ventricular ejection fraction and led to an improvement in maximal oxygen consumption[37]. The effect of SGLT2i in non- diabetic patients is thought to emerge from the increase of sodium excretion as well as its anti-inflammatory and oxidative stress reducing properties[38,39].

Another drug that may be interesting to test is melatonin, an endocrine hormone released by the pineal gland. Melatonin has been used for many decades to treat circadian rhythm disturbances and to improve quality of sleep[40]. It has since become a popular compound to test in various disease-models due to its anti-inflammatory and anti-oxidant properties[41-43]. Nevertheless, proper clinical studies are scarce, as is often the case with food supplements and over the counter drugs. Though there are indications that this may be an interesting compound to test in FD: Animal models showed a potential beneficial effect of melatonin on HfPEF[44] and more recently, melatonin supplementation was also shown to improve Nt-proBNP levels as well as clinical outcome in a small group of patients with HfPEF[41,45]. Regarding its relation with renal disease, impaired endogenous melatonin release was strongly related to the degree of chronic kidney disease in humans[46] while many animal studies suggest a direct protective effect of melatonin on the kidney[47-49]. Another compound of interest is Carbamazepine, an anticonvulsant and mood-stabilising drug that is already successfully used in a large group of classical FD patients to treat their acroparesthesias[50]. As Carbamazepine is capable of increasing autophagic flux in an mTOR independent matter[51,52], it would be interesting to test whether or not it might alleviate some of the autophagic stress observed in FD. These are just a few of many interesting compounds worth mentioning. More detailed mechanism of action profiles of readily available and affordable compounds are emerging rapidly and combined with our progressive understanding of FD pathophysiology I am confident that we will find new, safe and affordable ways to improve patient care and outcome.

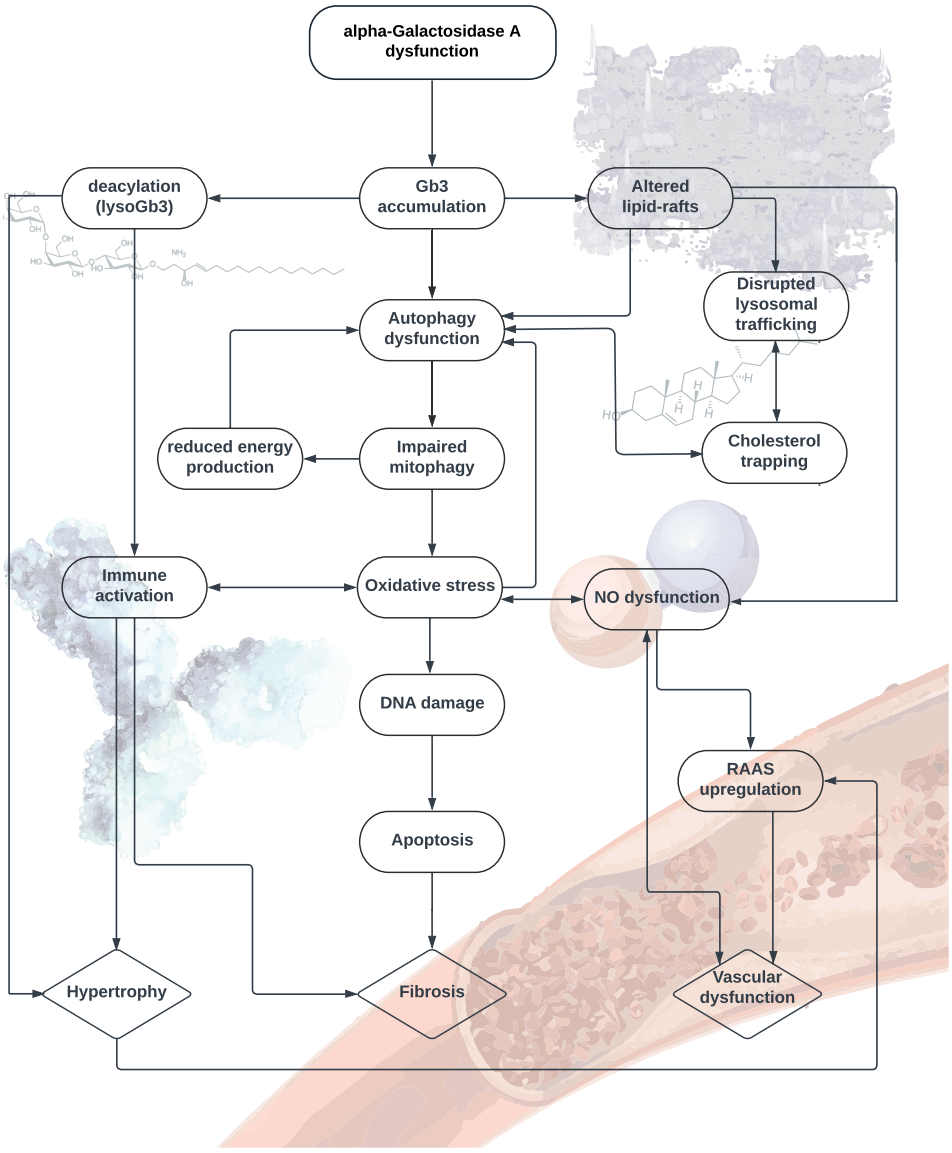


Figure 1: *Hypothesis of the interaction between pathophysiological pathways ultimately leading to the clinical manifestations observed in FD.*

In conclusion –the future of Fabry disease may well lay in the past

Despite its uncommon occurrence, a lot of in depth knowledge has been obtained regarding FD in the last few decades, resulting in some amazing scientific advances. Looking back, however, we can identify some mistakes that have complicated interpretation of scientific studies. Firstly, though driven by the desire to offer

patients a treatment as soon as possible, the hasty legislation of ERT on the market contradictory resulted in a delay in the understanding of its clinical efficacy and optimal usage. Our group previously published suggestions for the use of adaptive pathways in drug development that might have benefitted the evaluation on effectiveness of ERT. The first suggestion was to include –among others- healthcare professionals and patients in the development process of new treatment options; the second suggestion was to start with initial authorization in classical male patients, as their disease course is more evident, effect of treatment can be established sooner in this group and could provide 'proof of concept' for subsequent authorization for the milder FD phenotypes. Finally they advocated for the development of independent disease registries and a transparent real-world data collection plan. These recommendation are still relevant when looking at newly emerging treatment options for FD[53]. Secondly, the initial dismissal of ADAs as a relevant complication of treatment, despite increasing evidence, slowed down the pursuit of a proper solution. And finally, the lack of a global registry of FD patients made that we were dependent on data and results published in medical literature (often sponsored by the pharmaceutical companies). However, we should remember to be aware of publication bias, as selective publication is a well-known pitfall leading to unrealistic estimates of the efficacy of a drug. A 2008 paper in the new England journal of medicine evaluated 74 FDA registered trials on antidepressants and warned against the so-called selective publication bias. The study demonstrated that 37 out of 38 clinical trials with a positive outcome were published whereas only 3 out of 36 trials with a neutral or negative outcome were published as such[54]. In my opinion the best way forward is to learn from past mistakes and avoid rushing the admittance of new compounds for the treatment of FD based on minimal evidence, to register and monitor all factors that might hamper treatment effect and to start/continue collecting unbiased data on FD patients in independent patient registries. Finally, we should not only focus on new experimental treatment options but use our increasing knowledge of the working mechanisms of readily available compounds to find targeted approaches to intervene in later stages of FD.

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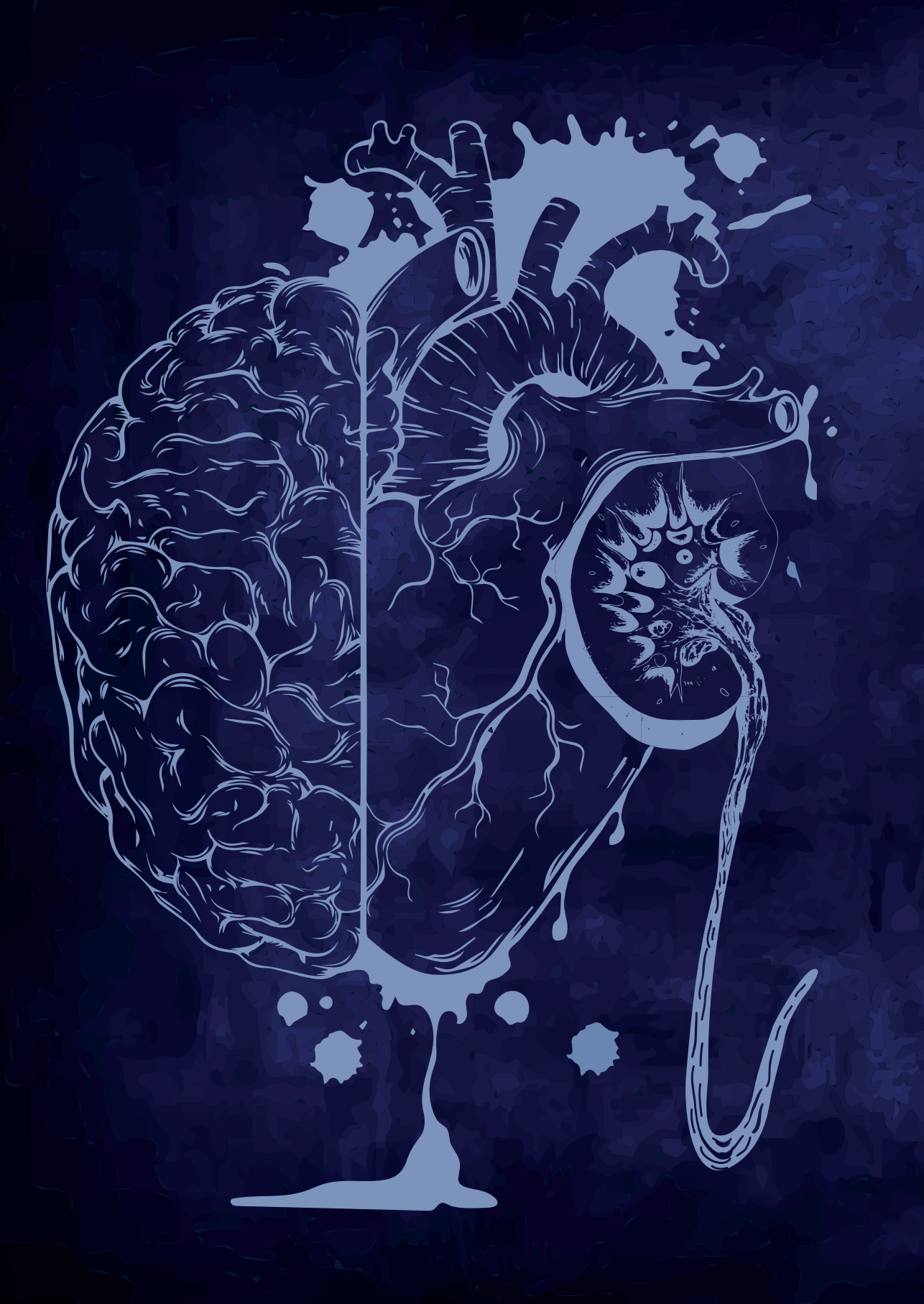


Appendices

Nederlandse samenvatting

Ondanks het zeldzame voorkomen van de ziekte van Fabry, is er in de laatste jaren veel aandacht, tijd en geld besteed aan onderzoek naar deze ziekte. Dit heeft 2 decaden geleden reeds geleid tot het ontwikkelen van 2 vormen van enzym vervangende therapie (enzyme replacement therapy, ERT), in de vorm van agalsidase alfa en agalsidase-beta. In de jaren erna zijn er honderden studies gedaan naar de effectiviteit van deze middelen, maar hoewel er steeds meer bewijs kwam dat de behandeling het ziekte proces iets kon vertragen, werd ook duidelijk dat deze niet geheel stop gezet kon worden en dat patiënten, ondanks behandeling, progressie hadden van ziekte. De laatste jaren heeft het onderzoek naar nieuwe behandelmethoden een grote vlucht genomen en zijn er tientallen nieuwe middelen waarnaar op dit moment klinisch onderzoek wordt verricht. Tegelijkertijd heeft de toegenomen aandacht voor de ziekte van Fabry geleid tot een toename van (genetische) diagnostiek naar dit ziektebeeld en zijn er steeds meer mutaties gevonden die een milder ziektebeeld geven of waarvan onduidelijk is of ze de ziekte van Fabry veroorzaken. De laatste jaren wordt er daarom steeds vaker onderscheid gemaakt tussen 'klassieke' ziekte (ernstigere vorm) van Fabry en 'niet-klassieke' ziekte (mildere vorm) van Fabry. Deze tweedeling heeft veel geholpen bij het maken van prognoses en behandelkeuzes voor individuele patiënten. In werkelijkheid echter lijkt de ziekte van Fabry meer een spectrum, lopend van een mild verhoogd cardiovasculair risico tot ernstige Multi-orgaan ziekte. Ten slotte is de meest voorkomende complicatie van de behandeling van Fabry met ERT, namelijk het ontstaan van antistoffen tegen het recombinante enzym, lange tijd onderbelicht geweest.

Het doel van dit proefschrift is daarom drievoudig, I. het verbeteren van prognostische informatie bij nieuw gediagnosticeerde patiënten met betrekking tot hun verwachte ziekteverloop met als doel betere keuzes te maken met betrekking tot de benodigde follow-up en eventuele behandeling. II. Het in kaart brengen van de klinische effecten van antistoffen tegen ERT en welke patiënten hierop het meeste risico lopen. III. Aanknopingspunten vinden om de behandeling van Fabry effectiever te maken door de timing van behandeling te optimaliseren en door antistof vorming tegen te gaan.

In **hoofdstuk 2** onderzochten we de niveaus van globotriaosylsphingosine (lysoGb3) in het plasma van 237 onbehandelde FD-patiënten in de loop van de tijd en bepaalden we de associatie met ziekte uitingen en –beloop. We laten zien dat in onbehandelde FD patiënten de plasma spiegel van de FD-biomarker lysoGb3 stabiel blijft gedurende tientallen jaren. Bovendien waren de plasma-

lysoGb3-spiegels voorafgaand aan de behandeling geassocieerd met de snelheid van progressie en/of ernst van bijna alle klinische FD ziekte uitingen (waaronder nierfunctie, albuminurie, cardiale morfologie en functie en cerebrale uitingen). Het feit dat deze biomarker al verhoogd is lang voordat klinische ziekte uitingen optreden, suggereert dat het meten van lysoGb3 bij nieuw gediagnosticeerde FD patiënten inzicht geeft in het te verwachtte natuurlijke ziekte beloop. Deze kennis vergemakkelijkt de klinische besluitvorming omdat het richting geeft bij het bepalen van de benodigde frequentie van follow-up visites bij asymptomatische patiënten en omdat het bij kan helpen in de besluitvorming of en wanneer een patiënt behandeld zou moeten worden met FD-specifieke behandeling.

In **hoofdstuk 3** laten we zien dat het starten van behandeling met ERT voor de leeftijd van 16 jaar bij mannelijke patiënten met het meest ernstige fenotype - klassieke ziekte - geassocieerd is met een gunstiger renaal en cardiaal beloop van de ziekte. We vergeleken zeven mannelijke patiënten met klassieke ziekte die in de kindertijd met behandeling begonnen en gedurende 10 jaar met ERT werden behandeld met 23 mannelijke patiënten van dezelfde leeftijd eveneens met klassieke FD die onbehandeld bleven. Vergeleken met onbehandelde patiënten kwam eiwit lekkage van de nieren (albuminurie) minder vaak voor en als deze aanwezig was in mindere mate dan bij behandelde patiënten. De gemiddelde massa van het hart was eveneens lager in de behandelde groep in vergelijking met de onbehandelde groep.

In **hoofdstuk 4** evalueerden we zowel de reeds beschikbare behandelingopties voor FD: ERT en chaperonnetherapie, alsook de nieuwe behandelingsmodaliteiten die nog in ontwikkeling zijn, zoals tweede generatie ERT, substraatreductietherapie, mRNA- en gen- therapie. We bespreken hierin het beschikbare bewijsmateriaal over werkzaamheid, de werkingsmechanismen en de mogelijke valkuilen van de verschillende behandeling opties.

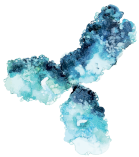
De prijs van de behandeling van Fabry, meer dan 200.000 euro per patiënt per jaar, blijft een maatschappelijke discussie. Aangezien het patent op de beschikbare ERT-preparaten reeds verlopen is, staat de markt open voor meer betaalbare biosimilars. In **hoofdstuk 5** beschrijven we de succesvolle ontwikkeling van een biosimilar van agalsidase-beta, aGalA biosidus (AGABIO) en tonen we de volledige gelijkenis van AGABIO met agalsidase-beta.

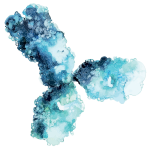
Een van de belangrijkste complicaties van de behandeling met ERT is de ontwikkeling van remmende anti-drug-antilichamen (iADA's) tegen recombinant αGalA, met name in mannen met het klassieke fenotype van de ziekte van Fabry.

In **hoofdstuk 6** analyseerden we het effect van iADA's op de effectiviteit van de behandeling met ERT in 39 mannelijke patiënten met klassieke ziekte van Fabry. We hebben aangetoond dat de gemeten remmende capaciteit van de antistoffen sterk negatief gecorreleerd was met de biochemische response op behandeling. Tevens hadden patiënten met iADA's een snellere achteruitgang van de nierfunctie in vergelijking met patiënten zonder iADAs. Verdere karakterisatie van de iADAs leerde ons dat deze voornamelijk van de IgG1- (in patienten met lage mate van inhibitie) en IgG4- subklassen (in patienten met hogere mate van inhibitie) waren, wat een sterkere inhiberende capaciteit van IgG4 ten opzichte van IgG1 suggereerd. De uitkomst van deze studie benadrukt het belang om de immuunrespons na het starten van behandeling met ERT in patiënten te monitoren en laat zien er behoefte is aan betere protocollen om met deze complicatie om te gaan.

Om het risico op het ontstaan van iADA te bepalen voordat de behandeling wordt gestart, hebben we in **hoofdstuk 7** een iADA-voorspellingsmodel gebouwd. Aangezien remmende ADA's zich bijna uitsluitend ontwikkelen bij mannelijke patiënten met het klassieke fenotype, hebben we alleen deze subgroep van patiënten opgenomen in het model. We hebben vastgesteld dat het hebben van een nonsens-mutatie in het GLA gen en hoge waarden van de biomarker lysoGb3 in het plasma bij aanvang van behandeling geassocieerd waren met een hoger risico op de ontwikkeling van iADAs. Het starten van de behandeling met de hoger gedoseerde agalsidase-bèta (vergeleken met agalsidase-alfa) was ook gerelateerd aan een verhoogd iADA-risico. Het gebouwde predictie model stelt ons in staat om het risico op het ontstaan van iADAs te voorspellen in nieuwe patiënten voor start van behandeling.

Eén van de nieuwe behandelingsmodaliteiten beschreven in **hoofdstuk 4** is een nieuwe gePEGyleerde vorm van αgalA, Pegunigalsidase-α (PRX-102). De verhoogde halfwaardetijd van PRX-102 en de verschillen in epitoop beschikbaarheid in vergelijking tot agalsidase- beta kunnen in theorie leiden tot verminderde immunogeniciteit. In **hoofdstuk 8** bespreken we een manuscript gepubliceerd door Lenders et al. En zoomen we in op de mogelijke valkuilen bij het vergelijken van de immunogeniciteit van verschillende enzym preparaten.





PhD Portfolio

student: Sanne Jolien van der Veen
period: June 2017 – July 2023
supervisor: Prof. Dr. C.E.M. Hollak
co-supervisors: Dr. M. Langeveld & Dr. A.B.P. van Kuilenburg

PhD training

General courses	**Year**	**ECTS**
BROK ('Basiscursus regelgeving Klinisch Onderzoek')	2017	1.0
biosafety en biosecurity in het algemeen (e-learning)	2017	0.1
Scientific writing in English for publication	2018	1.5
The AMC World of Science	2018	0.7
Endnote	2018	0.2
BROK re-accreditation	2022	0.**5**

Specific courses	**Year**	**ECTS**
Advanced immunology	2018	2.9

Seminars, workshops and master classes	**Year**	**ECTS**
21st ESGLD graduate course, Ecully, France	2017	0.5
ZonMW GGG-congres, Amsterdam, The Netherlands	2018	0.2
Refereeravond Cardiogenetica, Amsterdam, The Netherlands	2019	0.1
22nd ESGLD graduate course, Vic, Spain	2019	0.5

(Inter)national conferences	**Year**	**ECTS**
21st ESGLD workshop, Écully, France	2017	0.5
ESN najaarssymposium 2017, Utrecht, The Netherlands	2017	0.3
ESN Voorjaarssymposium 2018, Valkenburg a/d Geul, The Netherlands	2018	0.5
SSIEM annual symposium, Athens, Greece	2018	0.8
ESN Najaarssymposium 2018, Utrecht, The Netherlands	2018	0.1
6th Update on Fabry disease, Prague, Czech Republic	2019	1.3
ESN Voorjaarssymposium 2019, Doorn, The Netherlands	2019	0.3
SSIEM annual symposium, Rotterdam, The Netherlands	2019	0.5
22nd ESGLD workshop, Vic, Spain	2019	0.5
SSIEM virtual symposium day	2020	0.2
AGEM PhD student retreat, digital	2021	0.3
ESGLD 23rd summer meeting, digital	2021	0.2
AGEM PhD student retreat, Garderen, The Netherlands	2022	0.5
7th Update on Fabry disease, Wurzburg, Germany	2022	0.7
SSIEM annual symposium, Freiburg, Germany	2022	0.6

Presentations and Lectures	Year	ECTS
Oral presentation: "Een nieuwe vorm van ERT", Fabry patientendag 2017, Almere, The Netherlands	2017	0.5
Oral presentation: "Antibody formation against ERT in Fabry disease", The AMC World of Science, Amsterdam, The Netherlands	2018	0.3
Poster presentation: "Antibodies against recombinant alfa-galactosidase A in Fabry disease: subclass analysis and impact on response to treatment", SSIEM annual symposium, Athens, Greece	2018	0.5
Oral presentation: "Effect of anti-drug-antibodies on treatment efficacy in Fabry disease", Symposium EFWG meeting, Amsterdam, The Netherlands	2018	0.5
Poster presentation: "Predicting anti-drug-antibody development in male patients treated with agalsidase-alfa or –beta", 6th update on Fabry disease, Prague, Czech Republic	2019	0.5
Elevator pitch: "Predicting anti-drug-antibody development Fabry disease", ESN Voorjaarssymposium 2019, Doorn, The Netherlands	2019	0.1
Pitch + Poster presentation: "Predicting anti-drug-antibody development Fabry disease", 22nd ESGLD workshop, Vic, Spain	2019	0.2
Poster presentation: "Predicting anti-drug antibody development in male Fabry patients treated with agalsidase-alfa or –beta" SSIEM annual symposium, Rotterdam, The Netherlands	2019	-
Oral presentation: "Predicting anti-drug-antibody development Fabry disease", AGEM PhD student retreat, digital	2021	0.5
Digital presentation: "Effecten van vroege behandeling met enzymtherapie bij jongens met klassieke ziekte van Fabry", Fabry patientendag 2021	2021	0.5
Oral presentation: "Biomarker for Fabry disease, relation to disease severity", AGEM PhD student retreat, Garderen, The Netherlands	2022	0.5
Poster presentation: "Plasma globotriaosylsphingosine (lysoGb3) correlates strongly with disease severity in untreated Fabry disease patients: A tool to aid clinical decision making" 7th Update on Fabry disease, Wurzburg, Germany	2022	0.5
Oral presentation: "LysoGb3 and Fabry, relation to disease severity" SSIEM annual symposium, Freiburg, Germany	2022	0.5

Tutoring	Year	ECTS
Mentoring of 3rd year medical student: Supervision of statistical analyses and writing process of bachelor thesis.	2022	1.0

Other	Year	ECTS
Fabry patientendag 2017, Almere, The Netherlands	2017	0.1
Symposium EFWG meeting, Amsterdam, The Netherlands	2018	0.2
Fabry patientendag 2019, Almere, The Netherlands	2019	0.1

Awards and acknowledgements	Year
AGEM best publication award 2020, Amsterdam Gastroenterology Endcrinology Metabolism	2021
Top cited article 2020-2021, Journal of Inherited Metabolic Disease	2021
Top downloaded article 2020-2021, Journal of Inherited Metabolic Disease	2021

List of publications

van der Veen SJ, van Kuilenburg ABP, Hollak CEM, Kaijen PHP, Voorberg J, Langeveld M. Antibodies against recombinant alpha-galactosidase A in Fabry disease: Subclass analysis and impact on response to treatment. Mol Genet Metab. 2019. doi: 10.1016/j.ymgme.2018.11.008

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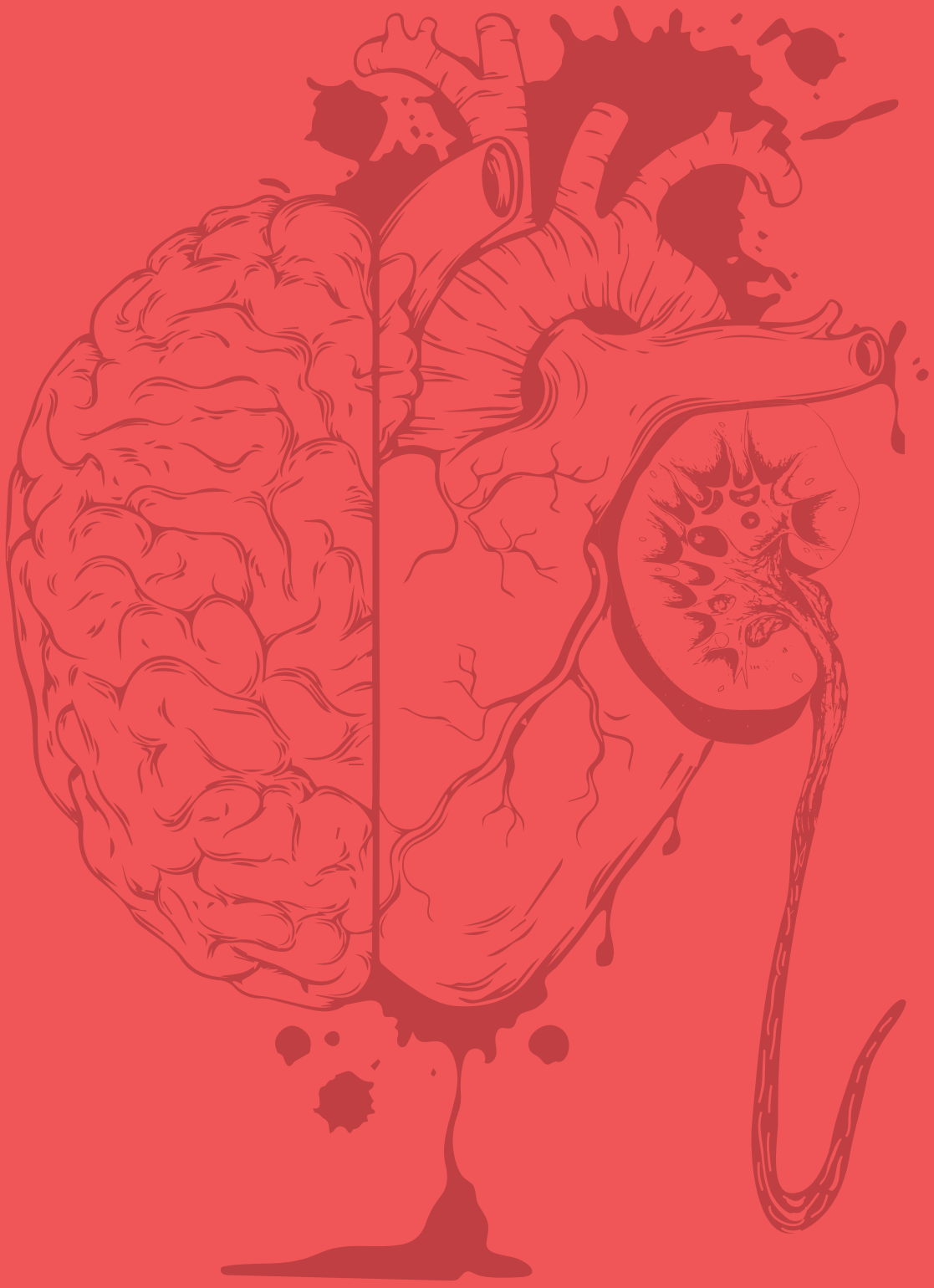

Dankwoord

Dankwoord

En dan eindelijk zijn we toegekomen aan het slotstuk van dit boekje. Tevens het belangrijkste (e.g. meest gelezen) onderdeel van dit boekje. Toen ik mijn semi-arts stage liep in het Spaarne ziekenhuis en **Maarten** voorstelde dat ik bij zijn promotoren in het AMC zou solliciteren voor deze PhD functie, had ik nooit kunnen bedenken dat ik een paar jaar later met zoveel nostalgische, weemoedige gevoelens afscheid zou nemen van diezelfde groep. Ik kan met recht zeggen dat de Sphinx en de Flexitarians voelden als een extended family en dat ik me bevoorrecht voel dat ik van deze groep deel uit heb mogen maken.

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About the author

Born and raised in Heemstede, the Netherlands and Boliqueme, Portugal, Sanne Jolien van der Veen spent her formative years alongside her parents (a doctor and an artist), two brothers, a dog, multiple rabbits and a horse.

Her educational journey began at the Stedelijk Gymnasium Haarlem, where she completed her grammar school education and earned her diploma in 2006. With a keen interest in the human body and a drive to make a meaningful impact on people's lives, she embarked on a path towards a career in medicine.

In pursuit of her goals, Sanne finished her third year of Biomedical science, delving into the intricacies of the human body at a molecular level. During these years, she developed a strong interest in pathophysiological processes, specifically within the field of metabolism, (auto)immunity and endocrinology, which drove her to further her studies in medicine. Following the completion of her master's degree in medicine in 2017, Sanne embarked on an exciting and challenging journey as a doctoral candidate, focusing her research on Fabry disease. Initially intending to focus mainly on antibody development in response to treatment with enzyme replacement therapy, her inquisitive and slightly chaotic nature led her to venture into several additional areas while also gaining a strong appreciation for coding, statistics and methodology. Her publication on predicting anti-drug antibodies development against enzyme replacement therapy in new Fabry disease patients earned her a best publication award from the Amsterdam Gastroenterology Endocrinology Metabolism (AGEM) research institute in 2020.

Beyond her academic pursuits, Sanne finds solace and inspiration in painting and drawing, serving as an outlet for her creativity and a means of visualizing complex ideas. Blending her two passions: art and science, she incorporates art into her work to illustrate intricate biological processes and make them accessible to a wider audience. One of the illustrations in her papers was used on the cover of the Journal of Inherited Metabolic Disease (JIMD) resulting in both a most cited and most downloaded award from the same journal. Several illustrations were also made for the Fabry patient association (FSIGN).

Sanne currently lives in Haarlem with her two sons Jasper (11) and Levi (9), sharing her house with Vera and her daughter Sophia (refugees from Ukraine), 3 cats and 2 rabbits. She works as a resident internal medicine at Spaarne Gasthuis.

Sanne loves:

- (her) Kids
- Lions > Snickers > M&Ms
- Cooking curries
- Baking fancy cakes
- Painting on everything (shoes, clothes, walls, cakes… anything really)
- Spending most of her free time in nature
- All animals, especially the fluffy ones
- Starting new hobbies
- Lying in the snow on top of a mountain with the sun shining, right before getting up and boarding/skiing down.