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Functional and Clinical Consequences of Novel α-Galactosidase A Mutations in Fabry Disease

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ABSTRACT: Fabry disease (FD) is a rare metabolic disorder of glycosphingolipid storage caused by mutations in the *GLA* gene encoding lysosomal hydrolase α-galactosidase A (α-gal A). Recently, the diagnostic procedure for FD has advanced in several ways, through the development of a specific biomarker (lyso-Gb3) and the implementation of newborn screenings, which acted as a catalyst to augment general awareness of the disease. Heterologous over-expression of α-gal A variants and subsequent in vitro measurement of enzyme activity provided molecular data to elucidate the relationship between mutation, enzyme damage, lyso-Gb3 biomarker levels, and clinical phenotype. This knowledge is the foundation for improved counseling with regard to prognosis and therapeutic decisions. Herein, we resume the approach of in vitro characterization, with a further 73 mainly novel *GLA* gene mutations. Patient lyso-Gb3 data were available for most of the mutations. All mutations were tested for responsiveness to pharmacological chaperone treatment and phenotypic data for 61 hemizygous male and 116 heterozygous female patients carrying a mutation associated with ≥20% residual activity, formerly classified as "mild" variant, were collected in order to evaluate the pathogenicity. We conclude that a mild *GLA* variant is typically characterized by high residual enzyme activity and normal biomarker levels. We found evidence that these variants can still be classified as a distinctive, but milder, sub-type of FD.
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KEY WORDS: Fabry disease; GLA; variants of unknown significance; GVUS; pharmacological chaperone therapy

Introduction

Fabry disease (FD; MIM# 301500) is a lysosomal storage disorder caused by mutations in the X-chromosomal *GLA* gene (Xq22), encoding α-galactosidase A (α-gal A; MIM# 300644). The estimated number of *GLA* gene variations is approaching four-figure number (Supp. Table S1; HGMD® Professional 2015.1 [http://www.hgmd.cf.ac.uk/]; and Fabry-database [http://fabry-database.org], accessed on 01-15-2015). Pathological changes in *GLA*—both the gene and its encoded protein—result in storage of complex sphingolipids in the lysosome, mainly Globotriaosylceramide (Gb3), which in turn causes FD, a cellular and microvascular dysfunction with multiple organ involvement.

In the classical form of FD, symptoms are typically first experienced in early childhood, consisting of acroparesthesia, abdominal pain and fever. During adolescence, the affected subjects exhibit angiokeratomas, decreased ability to perspire, proteinuria and progressive renal insufficiency and cornea verticillata. Progressing with age, the patients manifest cardiomyopathy and arrhythmia and cerebrovascular complications [Germain, 2010]. Formerly, the estimated prevalence of FD has been reported as one out of 40,000 to one out of 117,000 in males [Meikle et al., 1999, Desnick et al., 2001]. However, newborn screening initiatives suggest that the rate of *GLA* gene alterations exceeds this number by an order of magnitude. Indicated incidences range from about 1:1,500 in the Taiwanese [Hwu et al., 2009] to 1:13,341 in the Hungarian population [Wittmann et al., 2012] considering exonic changes only. Evidently, the elevated incidence of *GLA* mutations is due to the fact that many patients who present with a mild, late-onset symptomatology or with single organ involvement—as observed in cardiac, renal, and cerebrovascular variants of FD [Nakao et al., 1995; Nance et al., 2006; Rolfs et al., 2005; Brouns et al., 2010]—are screened for variations in the *GLA* gene to determine the etiology. This has led to the discovery of many novel mutations within the last two decades (Supp. Table S1). Moreover, adaptation of clinical management led to the inclusion of female heterozygous mutation carriers affected by FD [Guffon, 2003]. A study in Italy, however, reported an unchanged incidence of classic FD [Spada et al., 2006].

If a previously undescribed novel *GLA* variant is detected in a patient who lacks typical clinical features of FD, prognosis is often difficult, especially in females. Information gleaned from familial testing may be limited because members can remain symptom-free until the fifth or sixth decade or even throughout their entire life. Incorrect interpretation of the clinical significance of a *GLA* variant can cause inappropriate disease counseling. In order to improve the interpretation of uncertain *GLA* gene variants, we developed a cell-culture based system to investigate the downstream enzyme damage [Lukas et al., 2013]. A high proportion of mutations (60%–70%) lead to complete abolishment of, or very low, enzyme activity. In such cases, the prognosis is clear and respective patients are predicted to develop classic FD with all its phenotypic consequences. Enzyme replacement therapy (ERT) can be initiated immediately thus reaping the benefits of earlier treatment which is the most efficacious.

Additional Supporting Information may be found in the online version of this article.

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However, the number of *GLA* mutations known to result in α-gal A retaining a remarkable residual activity of about 20% or more of the normal level has increased dramatically. These so-called "mild" mutations generally occur in mono-, bi-, or oligo-symptomatic patients and are partially referred to as gene variants of unclear significance (GVUS) [van der Tol et al., 2014]. As all symptoms are not exclusive to FD (e.g., stroke, cardiomyopathy), but rather represent a differential diagnosis for a series of other disorders, the pathogenic nature of some mutations requires further investigation.

The present article describes 73 mutations, which underwent in vitro analysis. The aim of the study was to analyze *GLA* mutations in-depth on the basis of a holistic approach involving in vitro enzyme activity measurement of the mutant α-gal A enzyme in cell culture (over-expression) as well as determination of the biomarker globotriaosylsphingosine (lyso-Gb3) from either plasma or dried blood spots (DBS). Lyso-Gb3 is a deacylated metabolite of Gb3 with a higher sensitivity than Gb3 and a good correlation to the FD phenotype [Tsukimura et al., 2014, Smid et al., 2015(II)]. Furthermore, we introduce outline data for 61 male and 116 female patients with atypical FD (including age of diagnosis and symptomatic spectrum) in concurrence with one of 26 GVUS described in this study, in an attempt to elucidate the pathogenesis of these specific cases.

As mentioned, FD is currently treated with ERT. A paradigm shift in the approach to therapy was inspired by the fact that many of the mutations cause the protein to misfold, with the consequence of early ER-associated proteasomal degradation [Fan et al., 2007]. We therefore advocate that a great fraction of mutations can potentially be treated by an alternative therapeutic approach involving genotype-dependent pharmacological chaperone therapy (PCT). Consequently, every α-gal A mutant was tested for responsiveness to the pharmacological chaperone (PC) deoxygalactonojirimycin (DGJ; AT1001, Migalastat) in our cell culture system in order to establish whether PCT is a potentially suitable treatment option for patients with these genotypes. This experimental step provides preclinical evidence as to whether a mutation is a potential target for PCT with DGJ [Wu et al., 2011] which is currently investigated in a phase 3 clinical trial (ClinicalTrials.gov Identifier: NCT01458119).

Materials and Methods

Patients and Blood Samples

Blood samples were obtained from patients undergoing biochemical analysis or genetic testing for FD by Centogene AG (Rostock, Germany). All patients agreed for testing of their blood samples. The project was in concordance with the regulations of the local Ethical Committee of the University Rostock.

Cell Culture

HEK-293H cells were maintained in DMEM (Dulbecco's Modified Eagle Medium; Invitrogen, Karlsruhe, Germany) supplemented with 10% FBS (fetal bovine serum; PAA Laboratories, Pasching, Austria) and 1% penicillin/streptomycin (Invitrogen). All cells were incubated in a water-jacket incubator (Binder, Tuttlingen, Germany) under standard cultivation conditions (37°C, 5% CO_2).

Site-Directed Mutagenesis of α-Gal A

Expression vectors harboring *GLA* mutations were generated by site-directed PCR mutagenesis using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs, Ipswich, MA). The expression vector harboring the wild-type cDNA of *GLA* has been introduced before [Lukas et al., 2013]. Nucleotide exchanges or deletions were individually introduced by PCR amplification using sense and antisense primers designed according to the exponential amplification principle. Each mutant plasmid was sequenced at Source Bioscience (Berlin, Germany) to ensure sequence integrity. Throughout the manuscript, we refer to the *GLA* reference sequence: GenBank NM_000169.2. In accordance with HGVS recommendations (v 2.0), nucleotide numbering uses +1 as the A of the ATG translation initiation codon in the reference sequence, with the initiation codon as codon 1. Variants were submitted to the ClinVar (NCBI) public database (http://www.ncbi.nlm.nih.gov/clinvar/).

Transient Expression and Enzymatic Measurement of Mutant α-Gal A in HEK-293H Cells

The method for transient expression of mutant α-gal A in HEK-293H cells and subsequent determination of enzymatic activity has been carried out as described previously [Lukas et al., 2013]. In brief, we seeded HEK-293H cells on the day before transfection using 24-well culture plates (Sarstedt, Nümbrecht, Germany) and antibiotic-free high glucose DMEM supplemented with 10% FBS. On the day of transfection, the cells reached 70%–80% confluency. Lipofectamin 2000 (Invitrogen, Carlsbad, CA) and the *GLA* cDNA containing plasmid vector was added according to the instruction manual. After 4 hr incubation, the antibiotic-free medium was removed and 500 μl fresh DMEM supplemented with 10% FBS and 1% penicillin/streptomycin was added. In this step, DGJ (BIOZOL Diagnostica, Eching, Germany) was added to the culture medium from an aqueous stock solution (10 mM) where intended. The cells were incubated for another 60 hr before being harvested. After this period, the cells were homogenized in 200 μl deionized water and subjected to five freeze/thaw cycles using liquid nitrogen. The homogenate was centrifuged at 10,000*g* for 5 min in order to obtain the supernatant for the enzyme activity assay. The artificial substrate 4-MU-α-D-galactopyranoside (2 mM; Sigma–Aldrich, Munich, Germany) in 0.06 M phosphate citrate buffer (pH 4.7) was added to 50 ng of total protein. Protein measurement was carried out using BCA assay reagent (Thermo Fisher, Braunschweig, Germany). Enzyme reactions were terminated by the addition of 0.2 ml of 1.0 M glycine buffer (pH 10.5). The released 4-MU was determined by fluorescence measurement in a microplate fluorescence reader (Tecan, Männedorf, Switzerland). The measured enzyme activity was calculated as nmol 4-MU/mg protein and normalized to one hundred percent wild-type activity.

Lyso-Gb3 Level Determination from Plasma and DBS

For detailed methods, see the Supporting Information.

Statistical Tests

All statistical tests were carried out using GraphPad Prism5 software. To study the correlation of lyso-Gb3 with in vitro enzyme activity and age non-parametric rank correlation test to calculate spearman's *rho* was used.

Results

In Vitro Enzyme Activity Measurement of α-Gal A Mutants

Seventy-three mutations were tested for residual enzyme activity, and responsiveness to the PC DGJ (see Table 1). The majority of mutations have not previously been reported (45 out of 73) or have not been tested in a comparable overexpression system (63 out of 73). The remainder of the mutations (c.58G>C (p.A20P), c.118C>T (p.P40S), c.272T>C (p.I91T), c.281G>C (p.C94S), c.605G>A (p.C202Y), c.776C>G (p.P259R), c.826A>G (p.S276G), c.958A>T (p.N320Y), c.982G>C (p.G328R), c.1072G>A (p.E358K), RNA not analyzed) showed similar behavior to that observed by other groups, with regard to residual activity and responsiveness towards DGJ (Supp. Table S2). Altogether, 34 out of 73 of the mutant enzymes responded to DGJ treatment with a resulting elevation in activity according to the criteria established by our previous work [Lukas et al., 2013, also summarized in Table 1].

The data set was opposed to the DGJ-responsiveness prediction algorithm "Fabry-cep" (available at http://www.icb.cnr.it/project/fabry_cep/). "Fabry-cep" rated 19 out of 70 as "unclear". Mutations affecting the N-terminal signal peptide ($N = 5$) and nonsense mutations ($N = 3$) were excluded from the analysis. Comparison with the experimental data revealed 19 true negatives (in vitro non-responders), 13 true positives (in vitro responders), but also 11 false negatives and three false positives. Supp. Table S3 summarizes the results for all 46 predicted mutations indicating a positive predictive value (0.81) comparable to the former report [Cammisa et al., 2013].

Biomarker Globotriaosylsphingosine

Globotriaosylsphingosine (lyso-Gb3) is an effective biomarker for the classification of FD severity [Smid et al., 2015(II)]. In the present study, we measured this marker in plasma or DBS (for details see Supp. Methods S1). Records from 207 (132w/75m) patients with a *GLA* variant were collected (Table 1). There is a strong negative correlation between residual enzyme activity and height of the biomarker in the male portion of the cohort (Spearman's *rho*: –0.73, $P < 0.0001$, compare Fig. 1) and a moderate correlation for the females (Spearman's *rho*: –0.44, $P < 0.0001$). Most male individuals, even these with mutations with high residual in vitro enzyme activity (≥20%), showed increased biomarker values. Only few mutations caused inconspicuous biomarker levels in males, that is, lower than the estimated pathological cut-off of 0.9 ng/ml (c.7C>G (p.L3V), c.8T>C (p.L3P), c.683A>G (p.N228S), c.926C>T (p.A309V), c.968C>G (p.P323R), c.989A>G (p.Q330R), c.1055C>G (p.A352G), RNA not analyzed; in vitro enzyme activity range: 48.0%–117.7%). Lyso-Gb3 levels were only slightly elevated by mean (<5 ng/ml) in male patients with mutations c.179C>T (p.P60L), c.239G>A (p.G80D), c.337T>A (p.F113I), c.593T>C (p.I198T), c.641C>T (p.P214L), c.724A>G (p.I242V), c.1196G>C (p.W399S), RNA not analyzed (in vitro enzyme activity range: 15.6%–70.6%), whereas single individuals even had normal lyso-Gb3.

Mutations resulting in less than 1% residual enzyme activity were mostly associated with classic FD [Lukas et al., 2013]. Among these, all male patients had pathologically elevated biomarker levels. For the females, there appear to be factors other than *GLA* mutation type that determine biomarker elevation. Analyzing two extensive families with a classic FD-related mutation, p.C94Y [Eng et al., 1997] and p.E358K [Germain et al., 2002], respectively, we set out to test whether biomarker level is correlated with age (Fig. 2). Both mutant enzymes did not display any residual activity in the over-expression system. In the family with the p.E358K genotype, two out of four individuals between 3 and 22 years of age show normal lyso-Gb3, and other two patients show only a mild elevation. Individuals aged 25–63 years show modest to strong biomarker elevation. However, the relationship between elevated biomarker and age is not significant as determined by Mann–Whitney *U* test (data not shown). The same holds true for the 21 female patients with the c.281G>A (p.C94Y, RNA not analyzed) genotype. Even though it can be assumed that lyso-Gb3 increases progressively with age in a single patient, the variation between family members with the same genotype implies that factors other than age determine the biomarker level. This finding highlights the clinical heterogeneity of the disease even in patients with the same genotype.

"Mild" Mutations can Provoke FD Symptoms

Patients carrying mild mutations (≥20% residual in vitro activity) usually present with later-onset disease and a symptom spectrum different from the classically affected patients. In order to investigate disease severity in patients with mild mutations, we collected the clinical records from 61 male and 116 female patients. The clinical spectrum for this sub-fraction of mutations encompasses the whole spectrum of typical FD symptoms (Fig. 3A). However, the frequency of mono- or bi-symptomatic cases is high, 59.0% in males and 43.1% in females, respectively and in a small proportion of patients (seven out of 61 males and 15 out of 116 females) no apparent FD symptoms were detected; these patients were recruited as family members of affected patients. The median age of the asymptomatic (24.0/m and 24.4/f yrs) versus affected (51.6/m and 44.7/f yrs) individuals differed significantly ($P = 0.0276$/m and $P = 0.001$/f), using Mann–Whitney *U* test. This may argue for a potential very late onset pathogenicity of the mutations. In addition, 18 out of 61(m) and 51 out of 116(f) had a more severe clinical record with three or more symptoms. Interestingly, there is apparently no sex-dependent difference in disease severity, unlike patients with classic mutations [Vedder et al., 2007].

Discussion

In order to accurately interpret the various *GLA* gene mutations potentially causing FD, systematic biochemical in vitro characterization with a clear and comprehensive read-out is imperative for good clinical counseling. Cell culture-based over-expression and in vitro enzyme activity measurement was carried out for 73, partially novel, α-gal A variants.

In accordance with our preceding study we found good correlation between in vitro activity of the mutants and the patient's biomarker level (Fig. 1) which illustrates that this method delivers solid and clinically relevant data regardless of the sub-fraction of mutations under investigation. Even though, not surprisingly, the correlation is better in male patients, females tend to display genotype-dependent biomarker elevation related to the biochemical damage of the α-gal A enzyme. Despite the fact that the wide spectrum of clinical manifestations in FD is presumably influenced by the effects of a great number of modifier genes [Altarescu et al., 2005] and epigenetic factors [Barba-Romero et al., 2010], this study provides further evidence that reduced enzyme activity is actually the main determinant for biomarker elevation and, hence, phenotypic severity.

Table 1. Summary of Tested *GLA* Variants

N	Amino acid	cDNA	In vitro enzyme activity [% WT] in mean ± SEM (N)		Responder[a]	Responder prediction (Fabry_cep)[b]	Blood lyso-Gb3 [mean (N)][c]		PolyPhen-2[d]
			–DGJ	+DGJ			Male	Female	
1	**p.L3V**	**c.7C>G**	81.5 ± 9.2 (5)	88.0 ± 12.4 (5)	Yes	–	0.5 (1)	0.5 (1)	Benign
2	**p.L3P**	**c.8T>C**	117.7 ± 13.7 (4)	129.4 ± 12.6 (4)	Yes	–	0.5 (1)	0.5 (1)	Benign
3	p.A20P	c.58G>C	2.5 ± 0.4 (11)	4.9 ± 0.8 (12)	No	–	8.5 (1)	0.7 (1)	Benign
4	**p.A20D**	**c.59C>A**	2.8 ± 0.1 (3)	4.5 ± 0.2 (3)	No	–		3.7 (1)	Possibly damaging
5	**p.L21P**	**c.62T>C**	0.6 ± 0.1 (3)	1.7 ± 0.3 (3)	No	–	70.9 (1)		Probably damaging
6	**p.D33G**	**c.98A>G**	37.4 ± 5.1 (3)	62.0 ± 4.8 (3)	Yes	Unclear	17.6 (1)	1.0 (2)	Possibly damaging
7	**p.G35E**	**c.104G>A**	38.0 ± 7.8 (4)	70.8 ± 14.6 (4)	Yes	No	45.3 (1)	0.7 (1)	Probably damaging
8	**p.L36W**	**c.107T>G**	2.3 ± 0.6 (5)	22.3 ± 1.5 (5)	Yes	No	31.7 (1)	0.5 (1)	Probably damaging
9	p.P40S	c.118C>T	0 (4)	1.4 ± 0.5 (3)	No	No	11.8 (2)	1.6 (2)	Probably damaging
10	p.M42T	c.125T>C	2.9 ± 0.3 (4)	21.4 ± 5.0 (4)	Yes	No		0.5 (1)	Probably damaging
11	p.L45P	c.134T>C	0 (4)	0 (4)	No	Unclear	42.7 (2)	9.8 (1)	Probably damaging
12	p.E48D	c.144G>C	0 (4)	0 (4)	No	Unclear	82.2 (2)	11.4 (1)	Probably damaging
13	p.C56Y	c.167G>A	0 (4)	3.3 ± 1.1 (3)	No	No	28.4 (1)	2.5 (3)	Probably damaging
14	p.P60L	c.179C>T	15.6 ± 0.2 (3)	33.1 ± 1.0 (3)	Yes	No	1.4 (3)	2.2 (4)	Probably damaging
15	**p.I64F**	**c.190A>T**	0 (3)	0 (3)	No	Unclear	41.4 (1)	1.2 (2)	Probably damaging
16	**p.E71G**	**c.212A>G**	87.0 ± 4.8 (4)	104.6 ± 7.4 (4)	Yes	Unclear		0.5 (1)	Benign
17	**p.G80D**	**c.239G>A**	29.3 ± 5.7 (4)	30.4 ± 5.4 (4)	No	No	1.6 (3)	1.4 (5)	Probably damaging
18	**p.Y86H**	**c.256T>C**	0 (3)	0.7 ± 0.1 (3)	No	Unclear	23.7 (1)	0.8 (4)	Probably damaging
19	**p.I91N**	**c.272T>A**	0 (3)	0 (3)	No	No	104.0 (1)		Probably damaging
20	p.I91T	c.272T>C	0.7 ± 0.3 (7)	7.0 ± 1.0 (7)	Yes	No	12.9 (1)	0.6 (2)	Probably damaging
21	p.C94Y	c.281G>A	0 (6)	0 (6)	No	No	55.8 (7)	18.2 (20)	Probably damaging
22	p.C94S	c.281G>C	0 (6)	0 (6)	No	No	32.9 (2)	5.7 (1)	Probably damaging
23	**p.F113I**	**c.337T>A**	15.6 ± 1.9 (7)	34.0 ± 5.5 (3)	Yes	No	1.3 (1)	0.5 (3)	Probably damaging
24	**p.A121T**	**c.361G>A**	50.0 ± 8.4 (7)	55.5 ± 6.2 (7)	Yes	Unclear		0.5 (2)	Probably damaging
25	**p.I154T**	**c.461T>C**	98.0 ± 16.4 (8)	108.0 ± 18.6 (8)	Yes	Yes		0.5 (1)	Benign
26	p.W162*	c.485G>A	0 (5)	0 (5)	No	–			–
27	**p. V164L**	**c.490G>T**	43.1 ± 1.0 (5)	47.8 ± 1.1 (5)	No	Unclear			Possibly damaging
28	p.V164G	c.491T>G	1.4 ± 0.1 (6)	2.8 ± 0.5 (6)	No	No			Probably damaging
29	p.L167Q	c.500T>A	0 (6)	0.7 ± 0.3 (6)	No	No		10.7 (1)	Probably damaging
30	**p.L180F**	**c.540G>T**	32.4 ± 9.3 (8)	80.7 ± 15.6 (8)	Yes	Yes	12.2 (2)	1.0 (4)	Possibly damaging
31	p.M187V	c.599A>G	22.8 ± 5.0 (7)	67.0 ± 7.8 (7)	Yes	Unclear			Probably damaging
32	**p.M187I**	**c.561G>A**	3.1 ± 0.6 (5)	31.2 ± 4.5 (5)	Yes	Unclear			Probably damaging
33	**p.R196S**	**c.588A>C**	42.1 ± 2.5 (4)	67.8 ± 6.6 (4)	Yes	No		0.5 (1)	Probably damaging
34	**p.I198T**	**c.593T>C**	38.7 ± 3.1 (3)	50.4 ± 3.2 (3)	Yes	No	4.7 (1)		Probably damaging
35	p.C202Y	c.605G>A	0 (5)	1.4 ± 0.5 (5)	No	No	31.8 (1)	5.5 (1)	Probably damaging
36	**p.W204R**	**c.610T>C**	0 (3)	0 (3)	No	No	101 (1)		Probably damaging
37	**p.K213R**	**c.638A>G**[e]	68.1 ± 8.5 (4)	65.3 ± 11.4 (4)	No	Yes	30.4 (1)	0.9 (1)	Benign
38	**p.P214L**	**c.641C>T**	19.4 ± 1.4 (3)	64.1 ± 9.8 (3)	Yes	Unclear	4.6 (1)	1.0 (7)	Probably damaging
39	**p.I219M**	**c.657C>G**	15.2 ± 2.2 (5)	56.5 ± 9.4 (5)	Yes	Yes			Probably damaging
40	p.R220*	c.658C>T	0 (5)	0 (5)	No	–	62.8 (3)	7.1 (2)	–
41	**p.R227P**	**c.680G>C**	0 (7)	0 (7)	No	No		4.9 (1)	Probably damaging
42	**p.N228S**	**c.683A>G**	59.5 ± 9.8 (5)	70.6 ± 13.1 (5)	Yes	Yes	0.5 (1)		Probably damaging
43	**p.I242V**	**c.724A>G**	70.6 ± 15.7 (9)	89.4 ± 21.2 (9)	Yes	Yes	2.0 (2)		Benign

(*Continued*)

Table 1. Continued

N	Amino acid	cDNA	In vitro enzyme activity [% WT] in mean ± SEM (N)		Responder[a]	Responder prediction (Fabry_cep)[b]	Blood lyso-Gb3 [mean (N)][c]		PolyPhen-2[d]
			–DGJ	+DGJ			Male	Female	
44	p.L243F	c.729G>C	11.4 ± 2.0 (10)	70.8 ± 10.5 (10)	Yes	Yes		0.7 (2)	Probably damaging
45	p.S247P	c.739T>C	0 (6)	5.8 ± 1.5 (6)	Yes	Yes	81.9 (1)		Probably damaging
46	p.N249K	c.747C>A	23.7 ± 1.7 (4)	54.6 ± 3.4 (4)	Yes	Yes			Benign
47	**p.I253T**	**c.758T>C**	73.0 ± 4.8 (3)	115.8 ± 6.9 (3)	Yes	Unclear	8.45 (2)	1.4 (3)	Probably damaging
48	**p.V254A**	**c.761T>C**	26.4 ± 2.4 (3)	39.3 ± 3.2 (3)	Yes	Yes		2.5 (1)	Probably damaging
49	p.P259R	c.776C>G	20.5 ± 2.6 (12)	40.0 ± 4.5 (12)	Yes	No	44.8 (1)		Probably damaging
50	**p.W262R**	**c.784T>C**	0 (3)	0 (3)	No	No		12.3 (1)	Probably damaging
51	**p.V269G**	**c.806T>G**	0 (5)	0 (5)	No	Unclear	15.1 (3)	27.8 (2)	Probably damaging
52	p.S276G	c.826A>G	0 (5)	5.6 ± 1.4 (5)	Yes	Unclear	11.8 (2)	0.9 (4)	Probably damaging
53	**p.I289V**	**c.865A>G**	79.9 ± 6.4 (4)	95.0 ± 4.3(3)	Yes	Yes		0.5 (4)	Probably damaging
54	**p.A309V**	**c.926C>T**	48.0 ± 0.5 (5)	46.6 ± 1.6 (5)	No	Yes	0.5 (1)		Possibly damaging
55	**p.D313N**	**c.937G>A**	90.1 ± 23.0 (4)	95.2 ± 18.7 (3)	Yes	Yes			Benign
56	**p.D315N**	**c.943G>A**	65.3 ± 7.4 (7)	72.4 ± 12.4 (7)	Yes	Yes			Possibly damaging
57	**p.V316A**	**c.947T>C**	49.1 ± 5.5 (3)	58.3 ± 7.7 (3)	Yes	No		0.5 (1)	Probably damaging
58	**p.I317S**	**c.950T>G**	0 (4)	2.7 ± 0.7 (4)	No	No		0.8 (4)	Probably damaging
59	p.N320Y	c.958A>T	0 (6)	0 (6)	No	No		7.0 (2)	Probably damaging
60	**p.P323R**	**c.968C>G**	62.7 ± 4.4 (4)	63.7 ± 3.1 (4)	No	No	0.8 (1)	1.1 (2)	Benign
61	**p.Q327R**	**c.980A>G**	0 (5)	3.9 ± 0.1 (5)	No	Unclear		11.1 (1)	Probably damaging
62	**p.Q327L**	**c.980A>T**	0.6 ± 0.1 (5)	20.0 ± 3.0 (5)	Yes	No	24.0 (1)	5.5 (1)	Probably damaging
63	p.G328R	c.982G>C	0 (8)	0 (4)	No	No		6.8 (5)	Probably damaging
64	**p.Q330R**	**c.989A>G**	54.9 ± 5.4 (5)	62.8 ± 11.9 (5)	Yes	Yes	0.6 (1)		Benign
65	p.R342P	c.1025G>C	0 (7)	0 (7)	No	No	38.6 (4)	2.8 (6)	Probably damaging
66	**p.A352G**	**c.1055C>G**	53.7 ± 4.2 (4)	56.4 ± 2.9 (4)	No	Unclear	0.5 (1)	0.5 (1)	Probably damaging
67	**p.R356P**	**c.1067G>C**	2.1 ± 0.6 (6)	6.7 ± 2.3 (6)	No	No		0.5 (2)	Benign
68	p.E358K	c.1072G>A	0 (3)	0 (3)	No	Yes	45.8 (8)	27.1 (10)	Probably damaging
69	p.G360S	c.1078G>A	0 (3)	0.4 ± 0.2 (3)	No	Unclear			Probably damaging
70	**p.G375A**	**c.1124G>C**	44.1 ± 10.1 (5)	48.8 ± 12.6 (5)	No	Unclear		2.5 (2)	Benign
71	**p.R392S**	**c.1176G>T**	44.3 ± 1.6 (4)	46.2 ± 1.7 (4)	No	Unclear		4.3 (1)	Benign
72	**p.W399S**	**c.1196G>C**	53.0 ± 4.3 (3)	51.5 ± 5.1 (3)	No	No	1.2 (2)		Possibly damaging
73	p.R404del	*c.1212-14 del AAG*	0 (3)	0 (3)	No	–			–

Bold mutations are novel.

[a]According to responder criteria introduced in Lukas et al. (2013); increased enzyme activity of 1.5-fold or >5% compared with the untreated value.

[b]Fabry_cep estimates the likelihood of a mutants' responsiveness to DGJ. We examined a PSSM score of ≥–1 as presumed responder and ≤–3 as presumed non-responder. A score of –2 was regarded unclear according to the basic principles of the algorithm introduced in Andreotti et al. (2011).

[c]lyso-Gb3 values below 0.9 can be considered normal.

[d]PolyPhen-2 (Polymorphism Phenotyping v2.2.2) URL: http://genetics.bwh.harvard.edu/pph2/ (requested during May 2014) (Adzhubei et al., 2010).

[e]Likely splice site mutation

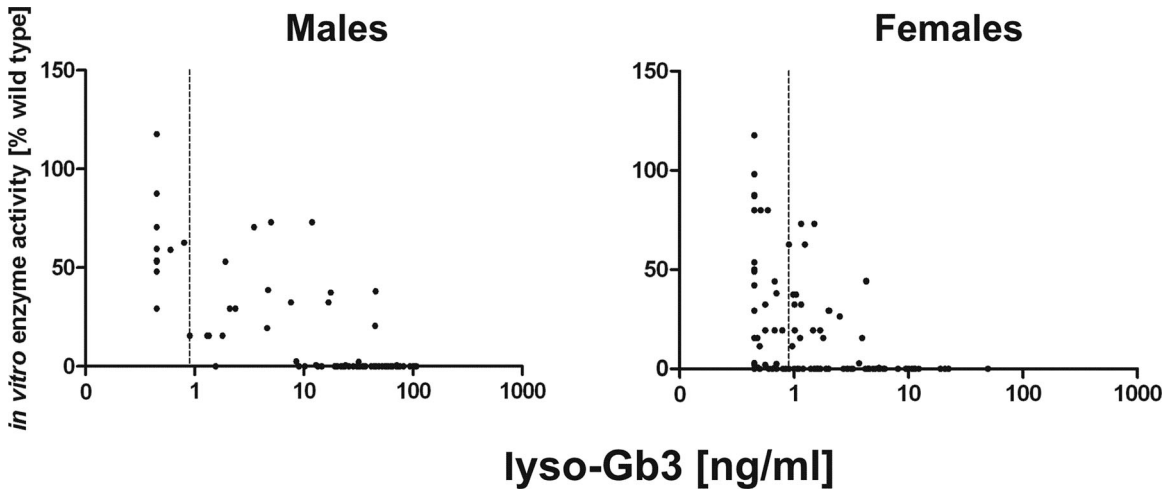


Figure 1. In vitro enzyme activity correlates with lyso-Gb3 level in males and females. The in vitro enzyme activity values of over-expressed α-gal A mutants measured in HEK-293H cell homogenates were plotted against the mean lyso-Gb3 of the patients with the corresponding mutation, that is, each dot represents one genotype (not one patient) (for details see Table 1). The dotted line indicates the pathological cut-off for lyso-Gb3. Individuals with lyso-Gb3 values below the limit of quantitation were calculated to display 50% of pathological cutoff value (0.45 ng/ml).

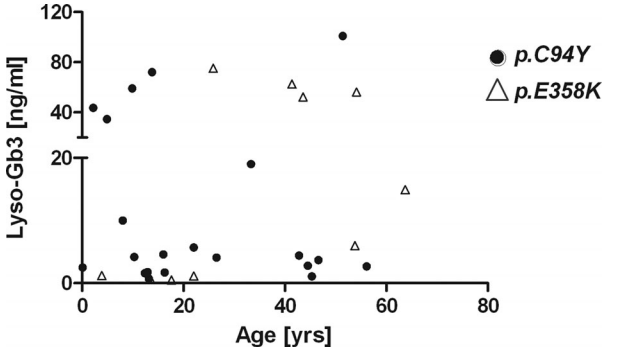
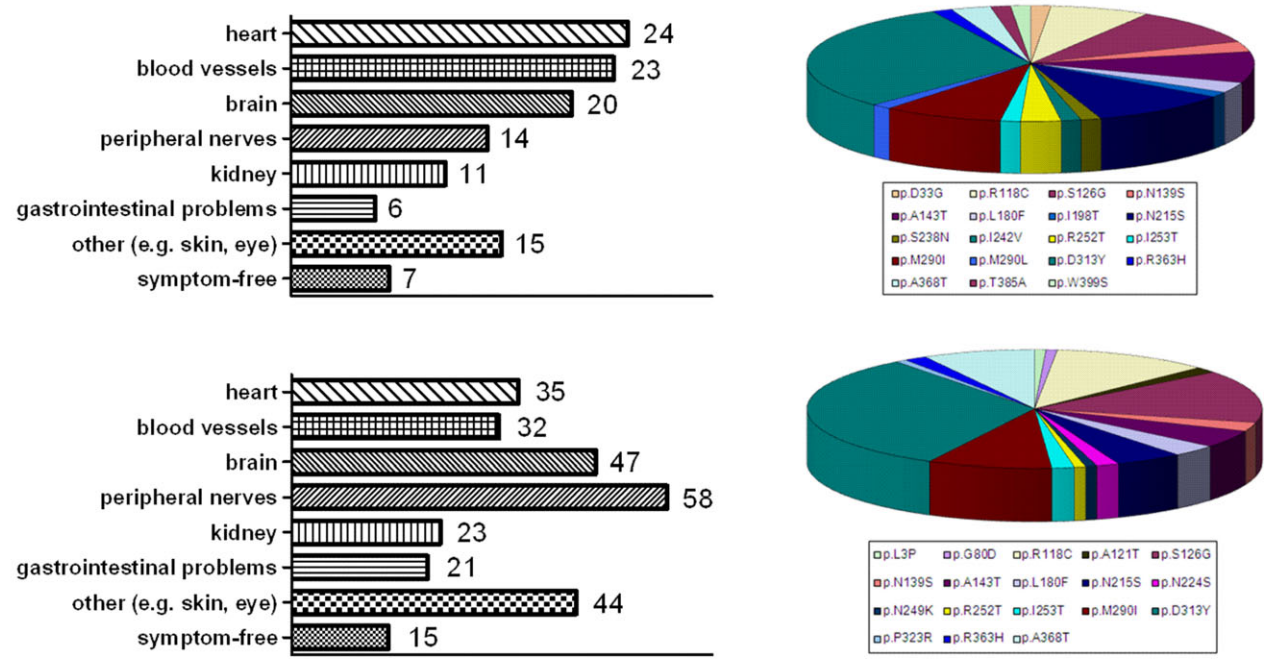


Figure 2. Lyso-Gb3 in female patients with classical FD mutations. The diagram shows lyso-Gb3 values as a function of age in female patients with p.C94Y (black circles) and p.E358K (open triangles). There is no age-dependence of biomarker level in female patients from both families.

However, even though we detected correlation between the enzyme damage (as can be interpolated from the enzyme activity assay) and patient lyso-Gb3 as reflected by Spearman's coefficients of –0.73(m) and –0.44(f), there are still a few specific cases of equivocal compliance between the two parameters. For example, the three mutants p.D33G, p.G35E, and p.G80D had a similar activity (29.3%–38.0% of normal) and were consequently labelled as "mild." Although two male patients with either p.D33G or p.G35E showed strong lyso-Gb3 elevation, the three hemizygous individuals carrying p.G80D only had normal to slightly elevated biomarker values (Table 1). One reasonable explanation is that the patients with unexpectedly high biomarker values had a greater chromosomal rearrangement not detectable by standard sequencing. There is also a certain chance that the assay conditions might not totally reflect the biochemical damage of these specific enzymes because excess amounts of an artificial fluorogenic α-gal A substrate have been used for the assay which may cause disparate kinetics than the natural substrate. However, it is interesting to note that the conversion from glycine to arginine at codon position 35 also leads to a significant residual enzyme activity, but three male patients had strongly elevated lyso-Gb3 of 63.0, 70.4, and 73.2 ng/ml (norm: <0.9 ng/ml) [Lukas et al., 2013]. Therefore, it can be argued that there is some unforeseen damage occurring to mutations at that particular site that cannot be resolved in the cell culture over-expression assay. For this reason, we carefully investigated the mutations that are situated in the vicinity of splice sites. We identified c.638A>G (→ p.K213R) as a potential splice site mutation (Alamut Visual version 2.7; Interactive Biosoftware, Rouen, France) which explains the high lyso-Gb3 despite of the high residual activity after over-expression. However, this analysis did not reveal splice site changes for c.540G>T (p.L180F) and c.641C>T (p.P214L).

In heterozygous female carriers, in addition to the enzyme activity the X-inactivation process plays a crucial role in determining the biomarker level by turning off either the normal or mutated allele. In terms of the *GLA* gene, balanced ratios of mutant:normal alleles reflecting random X-inactivation have been found, as well as evidence for skewed X-inactivation leading to ratios of 70:30 and higher which seems to firmly affect the phenotype [Echevarria et al., 2015]. Overall, lyso-Gb3-levels are highly variable across the whole spectrum of Fabry-related mutations and even intra-familial variation remains high. It can be speculated that biomarker levels are more deeply rooted in ontogenesis, rather than tied to a simple age-related progression. Further investigation is required to clarify how, and to what extent, lyso-Gb3 exposure time and level affect disease burden. Conceivably, long-term investigations on ERT (and substrate reduction therapy) effectiveness will facilitate the establishment and validation of biomarker-based therapeutic goals of FD.

Some genotypes are associated with normal lyso-Gb3, even in male patients. Moreover, these genotypes are characterized by absent classical FD symptoms with onset in the 5th to 7th decade of life. This holds true for many mutations with a residual activity ≥20% of normal. As an example, there is a controversial discussion about *GLA* mutation p.D313Y. Based on an earlier in vitro study, this mutation was labeled as a pseudodeficient variant [Yasuda et al., 2003], but is still deemed relevant in most recent literature [Bono et al., 2011] and screening programs [Baptista et al., 2010; Brouns et al., 2010] with evidence that this mutation leads to a particular CNS involvement [Brouns et al., 2010; Lenders et al., 2013]. However, the mutation is



B

	no. of individuals	median age at onset (IQR)	median lyso-Gb3 (IQR) [norm: <0.9]	Symptoms no/mono/bi/multiple	median *in vitro* enzyme activity (IQR)
male	61	51.6 yrs (22.0)	0.47 ng/ml (1.4)	7/22/14/18	51.3 (45.9)
female	116	44.7 yrs (21.4)	0.65 ng/ml (1.1)	15/27/23/51	51.3 (44.9)

Figure 3. Spectrum of symptoms in patients with mild mutations. The patient cohort harbors a total of 153 male and 246 female patients with mild mutations (in vitro enzyme activity > 20% of wild type). **A**: The figure illustrates the symptom spectrum for 61 male (upper) and 116 female (lower) individuals. The fractions of genotypes represented for each group is shown in the pie charts. **B**: Statistics of the patients represented above. Age at disease onset was equated with age at diagnosis and only symptomatic patients were included.

associated with normal plasma lyso-Gb3 in patients [Niemann et al., 2013]. Other recent reports questioned the pathology of mutations formerly regarded as pathogenic, for example, p.P60L, c.352C>T (p.R118C) and c.888G>A (p.M296I), RNA not analyzed [Mitobe et al., 2012; Smid et al., 2015(I); Ferreira et al., 2015].

A glance at the symptom spectrum of individuals harboring mutations with high residual enzyme activity in the over-expression system revealed evidence that the mutation was causative for the observed phenotype. (1) The number of patients with exonic *GLA* alterations in patients with FD symptoms is too high to be coincidental; (2) in the investigated cohort, median age at symptom onset in males and females was significantly higher than median age of the asymptomatic mild mutation carriers ($P = 0.0276$/m and $P = 0.001$/f); (3) in specific genotypes, for example, p.P60L, some individuals had slightly elevated biomarker, some did not, indicating that certain mutations may play the role of a genetic risk factor for common disease, that is, stroke or renal insufficiency, under certain genetic or extra-genetic conditions. However, genetic testing was typically carried out when the patient had a physical symptom. A large cohort of symptom-free and, reasonably, older individuals need to be screened for the presence or absence of mild *GLA* mutations in order to support the finding that these can nevertheless cause FD. Patients with early symptom onset (e.g., <40 years) are particularly good candidates for whole genome/exome analysis, in comparison with symptom-free mutation carriers.

To put it into perspective, some genotypes are probable targets for an alternative therapeutic approach with PCT. The question as to whether a given mutation can be addressed with a small molecule like DGJ has been approached by an in silico prediction algorithm "Fabry-cep" [Cammisa et al., 2013]. We examined PPV, sensitivity (true negatives) and specificity (true positives) for a total of 46 mutations (compare Supp. Table S3). While principally valuable (as indicated by a PPV of 0.81), "Fabry-cep" produced a high number of false-negative predictions leading to a low sensitivity of 0.54. However, "Fabry-cep" identified non-responders accurately leading to a specificity of 0.86 suggesting that this tool is capable of offering a deliberated prediction of possible responders in the absence of experimental data. Interestingly, our experimental analysis revealed that the fraction of responding mutants proved to be higher amongst mutations with residual activity in vitro. Mild mutations

with biochemical behaviour comparable to the wild type should also benefit from PCT. Even though the increase in enzyme activity is merely mild to moderate, the enzyme is stabilized and less vulnerable to heat denaturation and resistant to protease attack [Guce et al., 2011]. Therefore, an orally administered drug might be a preferable option, rendering the more torturous life-long intravenous therapy obsolete.

Of note, we have confirmed a positive genetic diagnosis by detection of a *GLA* mutation in about 1,205 patients with suspected FD, since 2010. The obtained numbers of 771 out of 434 (females/males) approach the expected Mendelian ratio of 2:1 for X-linked diseases, which indicates that female carriers are rarely asymptomatic throughout their entire lives.

Conclusions

We have characterized 73 GLA mutations (45 of which were novel) in a cell culture over-expression system. Most of the corresponding mutant enzymes display absent or strongly reduced enzyme activity. Hence the number of GLA mutations resulting in a residual activity ≥20% of normal level is increased (42.5%) relative to our previous study (26.9%, Lukas et al., 2013). Biomarker lyso-Gb3 is normal in most male and female patients with these mutations, so for this reason cannot serve as a surrogate for genetic diagnosis in these cases. There is, however, strong clinical evidence that mild mutations may lead to a mono- or oligo-symptomatic form of FD later in life. Based on in vitro testing, we predict a high proportion of responders to small molecule treatment (PCT based on the PC DGJ) among mildly affected patients.

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