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Original Article

Gene Mutations Versus Clinically Relevant Phenotypes
Lyso-Gb3 Defines Fabry Disease

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Background—Currently, no method is available to identify α-galactosidase A (agalA) mutations determining clinically relevant Fabry disease. In our largest European Fabry cohort, we investigated whether a biomarker, specific for the defect, could stratify persons at risk.

Methods and Results—A total of 124 individuals with agalA mutations were investigated with a comprehensive clinical workup, genetic analysis, and laboratory testing, including measurements of agalA activity and lyso-Gb3 (degradation product of the accumulating Gb3). Additionally, an extensive family screening with a clinical workup of relatives was performed. The patient population was divided into 2 samples: previously described mutations (n=72) and novel mutations (n=52). The patients with previously described mutations were subdivided into 2 groups: classical mutations, which were known to cause the classic type of Fabry disease with specific symptoms and a high risk for major events in all 3 main organs (heart, kidney, and central nervous system), and atypical mutations without the typical presentation. All patients with atypical mutations (n=17) had lower lyso-Gb3 levels than any of the patients with classical Fabry disease (n=55). A cutoff value of 2.7 ng/mL separated the 2 groups. Six out of 52 patients with novel mutations showed a lyso-Gb3 level <2.7 ng/mL. Clinical investigation, blinded to lyso-Gb3 results, revealed no classic organ involvement in these patients or their relatives. In contrast, the characterization of patients with lyso-Gb3≥2.7ng/mL suggested classical Fabry mutations in most of the patients (93%).

Conclusions—Our data show that the biomarker lyso-Gb3 may identify the clinically relevant agalA mutations leading to Fabry disease. **(*Circ Cardiovasc Genet*. 2014;7:8-16.)**

Key Words: biological markers ■ cardiomyopathies ■ Fabry disease ■ mutation

Fabry disease is an X-linked lysosomal storage disorder resulting from a deficiency of α-galactosidase A (agalA). The defect leads to substrate accumulation (globotriaosylceramides, Gb3) in all types of tissues containing lysosomes. Fabry disease is not only heterogenetic (private mutations) but also heterophenotypic. Typically, patients show a predominant involvement of 3 organs: heart, kidney, and nervous system. Progression of the disease ultimately leads to heart failure, dialysis, or stroke. Apart from the classical phenotype, some patients develop complications only in a single organ. These are referred to as cardiac or renal variants, often presenting as late onset disease.[1,2]

Editorial see p 2
Clinical Perspective on p 16

Additionally, in recent years, several α-galactosidase A (agalA) mutations, such as intronic polymorphisms or the exogenous mutation D313Y, have been described, but their exact pathogenetic importance is still unclear.[3,4] The identification of an agalA mutation associated with a clinically relevant phenotype is crucial for patient management and risk stratification. However, the still unresolved issue of how to appropriately match complementary genotypes and phenotypes remains a major impediment for the clinical application of such diagnostic information.

Plasma or urinary Gb3 (the accumulating enzyme substrate) are not helpful to identify clinical manifestation or disease severity in Fabry disease, neither in hemizygotes nor in heterozygotes. Furthermore, heterozygote female patients may exhibit the same symptoms and severe organ involvement as hemizygote males, although agalA activity in female patients is often only slightly reduced.

We hypothesized that assessing a degradation product of the accumulating protein Gb3, lyso-Gb3, can help in the definition of clinically relevant Fabry disease. We made use of a comprehensive diagnostic setup including detailed clinical, biochemical, and molecular genetic analyses in 1 of the

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largest European single center cohorts of patients with agalA mutations.

Methods

Study Protocol

Between March 2001 and October 2010, a total of 124 consecutive patients with an agalA mutation (51 males), referred to our Fabry center in Würzburg, were included. The study consisted of 2 parts: an evaluation study and a validation study. Patients with previously described mutations (listed in OMIM database [http://www.ncbi.nlm.nih.gov/omim/], PubMed [http://www.ncbi.nlm.nih.gov/sites/entrez?db=pubmed], or the mutation database by Sakuraba [www.fabry-database.org]) were included in the evaluation study, and novel mutations were included in the validation study. The assessment was always performed when the patients first presented at our third-level center for further evaluation and before the decision was made whether enzyme replacement therapy had to be initiated. Thus, none of the patients had received enzyme replacement therapy before study entry.

The study conformed to the principles outlined in the Declaration of Helsinki, and the Ethics Committee of the University Hospital had approved the research protocol. Informed consent was obtained from all patients or their guardians.

Clinical Workup

A comprehensive diagnostic workup as detailed in Figure 1 was performed on all patients of both substudies, including medical history and cardiac, renal, and neurological evaluation. Cardiac assessment included echocardiography and magnetic resonance imaging (MRI), as suggested in recent articles on Fabry cardiomyopathy and explained in more detail in the supplemental material.[5,6] Renal function was quantified by diethylene triamine penta-acetic acid clearance measurement, and proteinuria was assessed by 24-hour urine collection (Figure 1). In addition, a clinical disease severity score was calculated according to Giannini et al.[7]

Evaluation Study

All 72 patients with a previously described mutation were included. The patient's agalA mutation was categorized into 2 groups: classical mutation or atypical mutation, based on the findings from the clinical workup, along with information from the family pedigree evaluation, and also considering previous reports on patients with the same mutation.

A mutation was staged as classical when it resulted in a Fabry phenotype and had caused either of the following: (1) major events in at least 2 of the 3 main organs (heart, kidney, and central nervous system); or (2) specific Fabry symptoms plus 1 major event in 1 main organ plus 1 typical disease manifestation in another organ. This classification was chosen to ensure that no patients with polymorphisms or so-called organ variants were assigned to the classical Fabry group. Major events included end-stage renal disease (ie, glomerular filtration rate <30 mL/min or dialysis or renal transplant), advanced cardiomyopathy (left ventricular wall thickness >15 mm or left ventricular myocardial fibrosis), stroke, and transient ischemic attacks. All other mutations were staged atypical because they were associated with equivocal phenotypes or organ involvements.

Examinations performed at baseline

Organ/Domain	Investigation/Parameter	Performed in
medical history	symptoms, onset of symptoms, family history	100%
physical examination	routine examination, height, weight, blood pressure, heart rate	100%
cardiology		
electrocardiography	rest-ECG, bicycle exercise ECG, 24holter-ECG	100%
echocardiography	standard, strain rate imaging, speckle tracking	100%
cardiac MRI	standard, late enhancement imaging	80%
spiroergometry	exercise capacity, VO2	20%
neurology		
standard	examination, history, polyneuropathy tests, pain severity, quantitative sensoneurological testing,	100%
advanced	biopsies	50%
ultrasound	brain arteries	100%
kidney		
standard	proteinuria, urine analysis, DTPA-clearance	100%
advanced	kidney biopsies	20%
laboratory		
complete setting	including BNP, cystatin C	100%
pulmonary system	bodyplethysmography, blood analysis	90%
disease severity score	DS3	100%
other disciplines	eear-nose-throat, dermatology, ophthalmology	50-60%

Figure 1. Clinical investigation program performed at baseline. In the last column, the percentage of patients in whom the investigation was performed is given. BNP indicates brain-natriuretic peptide; DS3, disease severity score system; DTPA, diethylene triamine penta-acetic acid; MRI, magnetic resonance imaging; and Vo_2, oxygen uptake per time.

After mutation staging, lyso-Gb3 was determined in every single patient of the 2 groups (atypical and classical), and a cutoff value of lyso-Gb3 levels for identifying the clinical relevant classical Fabry mutation was determined.

Validation Study

All 52 patients with novel mutations, that is, previously undescribed, were included. According to the lyso-Gb3 cutoff value determined in the evaluation study (see Data Analysis), patients were categorized into 2 groups: classical and atypical. The diagnostic accuracy according to lyso-Gb3 was then tested by ascertaining the clinical phenotype using the same comprehensive investigation protocol as in the evaluation study (Figure 1).

Genetic Analysis and Enzymatic Testing

A complete analysis of the agalA gene, including all exon parts, the exon–intron boundaries, and all intron regions, was performed in all patients. The sequences were aligned to the published genomic sequence of the agalA gene. AgalA enzyme activity (reference level 0.4–1.0 nmol/min per mg protein) was assessed in white blood cells as previously described.[8]

Lyso-Gb3

For lyso-Gb3, lyso-ceramide had been used as a reference (Matreya LLC, Pleasant Gap, PA), and D5-fluticasone propionate (EJY Tech, Inc, Rockville, MD) was used as internal standard as described before.[9] Fifty microliter aliquots of all samples were used for sample preparation.

Data Analysis

Data are presented as count (percent), mean±standard deviation, and median (quartiles), as appropriate. Differences among groups were compared using appropriate tests according to the nature of the data (Student *t* test, Mann–Whitney *U* test, χ^2 test, Fishers exact test). A logistic regression model with lyso-Gb3 and sex showed that Lyso-Gb3 but not sex predicted typical versus atypical mutations (*P* for sex 0.10; *P* for lyso-Gb3 <0.0001). Receiver-operating characteristics (not shown) yielded a perfect specificity and sensitivity (ie, 100%) for the lyso-Gb3 cutoff value of 2.7 ng/mL in the evaluation study. This cutoff was therefore applied in the validation study. A *P* value <0.05 was considered statistically significant. Statistica Version 8 and SPSS Version 20 were used for statistical analyses.

Results

The clinical data of the 124 patients of the complete cohort are summarized in Table 1. The mutations determined in this study are shown in Figure 2. AgalA activity was significantly lower and lyso-Gb3 significantly higher in men compared with women. In general, men were more severely affected than women concerning Fabry symptoms. In addition, severe organ involvement and end-stage disease (eg, dialysis, kidney transplantation) were more often present in men (Table 1). Clinical manifestations were heterogeneous, even among patients originating from 1 family, ranging from presence or absence of typical symptoms and from no organ involvement to severe manifestations in both organ domains.

In total, 13 patients had a history of stroke, and 8 patients had a history of transient ischemic attacks. Advanced cardiomyopathy was present in 49 patients. Forty-seven patients presented with advanced renal impairment.

Evaluation Study

Out of 72 patients, 55 patients exhibited classical mutations according to the classification scheme described in Methods; accordingly, 17 out of 72 patients were categorized as atypical mutations. The lyso-Gb3 in classical and atypical patients was significantly different (43.9±41.7 versus 0.9±0.8 ng/mL, *P*<0.0001). Interestingly, all individuals with an atypical mutation had lower lyso-Gb3 levels than any of the ones with a clinical relevant mutation leading to Fabry disease. The lowest lyso-Gb3 level of a patient with a classical mutation was 2.8 ng/mL; the highest lyso-Gb3 of a patient with an atypical mutation was 2.5 ng/mL. Thus, a data-derived, sex-adjusted cutoff value of 2.7 ng/mL (see Data Analysis) perfectly separated both groups.

Validation Study

Fifty-two patients showed novel mutations and were included in the validation study sample cohort.

Applying the lyso-Gb3 cutoff level of 2.7 ng/mL yielded a low lyso-Gb3 group (n=6) and a high lyso-Gb3 group (n=46). The diagnostic workup blinded to the lyso-Gb3 revealed that among subjects of the low lyso-Gb3 group, indeed none of the patients or their relatives showed classic Fabry disease (Figure 3). The identified genetic mutations were the following: N139S, and different combinations of the 3 intron mutations, IVS0-10 C>T, IVS4-16 A>G, and IVS6-22C>T.

Importantly, in the high lyso-Gb3 group, clinical characterization and the additional information from relatives suggested a classical Fabry mutation in all but 3 patients (Figure 3). In these 3 patients, only scarce information on family history was available. Thus, the classification by lyso-Gb3 levels was able to describe the clinical relevance of the novel agalA mutations.

Lyso-Gb3 Analysis of the Complete Cohort

In total, 23 out of 124 patients (6 men and 17 women) showed a lyso-Gb3 level <2.7 ng/mL. Their genotypes and phenotypes, based on the clinical manifestation, are listed in Table 2. Five out of 23 patients with lyso-Gb3 <2.7 ng/mL exhibited polymorphisms. D313Y and A143T dominated among the exogenous mutations.

Only 1 out of 23 patients showed small late enhancement on CMR but with a pattern (basal septum) compatible with hypertensive heart disease and not with Fabry disease. Moreover, none of the 23 patients showed a hypertrophic cardiomyopathy. The 2 patients with reduced diethylene triamine pentaacetic acid clearance (patients 5 and 15) had long-standing and poorly controlled hypertension that explained the borderline left ventricular wall thickness with the septal bulge as typically found in hypertensive heart disease. When performing left ventricular mass calculation from cardiac magnetic resonance (indexed to sex- and age-specific body surface area), no left ventricular hypertrophy was detected in these patients. Patient 15 additionally had renal sarcoidosis, thus explaining the renal insufficiency. The stroke in patient 5 was embolic, and the 2 other patients with cryptogenic stroke showed no evidence of Fabry disease. An overview on patient characteristics stratified by atypical and classical mutations is shown in Table 3.

In addition, lyso-Gb3 was assessed in all women patients with a borderline agalA activity between 0.3 and 0.5 nmol/min per mg protein (n=25, normal value for agalA activity ≥0.4 nmol/min per mg protein). Of those, 11 patients had

Table 1. Clinical Baseline Characteristics of the Patients With agalA Mutations Subdivided by Sex

	Men n=51	Women n=73	*P* Value
Age, y	39±13	41±14	0.30
Alpha-galactosidase activity, nmol/min per mg protein	0.06±0.11	0.32±0.36	<0.0001
Lyso-Gb3, ng/mL	67.8±35.9	6.3±4.7	<0.0001
BMI, kg/m^2	23.1±3.5	23.8±4.9	0.37
HR, min^{-1}	62±12	67±11	0.008
SBP, mm Hg	124±16	123±19	0.72
DBP, mm Hg	81±11	81±11	0.93
AHT* (n)	15 (29%)	18 (25%)	0.54
General symptoms			
Abnormal sweating (n)	37 (73%)	16 (22%)	<0.0001
Heat or cold intolerance (n)	37 (73%)	25 (34%)	<0.0001
Chronic diarrhea (n)	20 (39%)	16 (22%)	0.04
Sudden deafness (n)	13 (25%)	10 (14%)	0.10
Angiokeratomata (n)	29 (57%)	12 (17%)	<0.0001
Neurology			
Stroke (n)	8 (16%)	5 (7%)	0.15
Chronic pain syndrome (n)	21 (41%)	14 (20%)	0.008
Acroparaesthesia (n)	36 (71%)	36 (51%)	0.13
Heart			
Cardiomyopathy (n)	33 (65%)	16 (22%)	<0.0001
LV mass, g	182±56	119±45	<0.0001
(assessed by MRI)			
AP (n)	5 (10%)	6 (8%)	0.76
LVEDD, mm	51±5	46±5	<0.0001
LVESD, mm	33±5	32±23	0.88
IVSd, mm	13.1±3.2	10.5±2.8	<0.0001
LVPWd, mm	12.1±2.7	10.2±2.7	0.0003
EF, %	62±7	64±5	0.08
E/A	1.4±0.4	1.4±0.5	0.98
DT, ms	210±63	207±55	0.73
Kidney			
Kidney transplant (n)	6 (12%)	1 (1%)	0.02
Dialysis (n)	6 (12%)	0 (0%)	0.004
Serum creatinin, mg/dL	1.5±1.8	0.8±0.2	0.002
Serum urea, mg/dL	33±17	28±17	0.18
DTPA clearance, ml/min	82±37	104±31	0.0004
Proteinuria, mg/day	169 (25,1293)	67 (0,178)	0.05
Proteinuria (n)	25 (41%)	28 (38%)	0.27
Medication			
Beta blocker (n)	8 (16%)	12 (17%)	1.00
Calcium channel blocker (n)	3 (6%)	1 (1%)	0.30
ACE inhibitor (n)	17 (33%)	21 (30%)	0.56
Aspirin (n)	15 (29%)	11 (15%)	0.07
Disease severity score*	12±7	6±5	<0.0001

Data are n (%), mean±standard deviation, and median (25th quartile, 75th quartile). ACE indicates angiotensin-converting enzyme; AHT, isolated arterial hypertension; AP, angina pectoris; BMI, body mass index; DBP, diastolic blood pressure; DT, deceleration time; DTPA, diethylene triamine penta-acetic acid; EF, ejection fraction; Gb3, globotriaosylceramide; GFR, glomerular filtration rate; HR, heart rate; IVSd, diastolic intraventricular septal wall thickness; LV, left ventricular; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; LVPWd, diastolic left ventricular posterior wall thickness; MRI, magnetic resonance imaging; and SBP, systolic blood pressure.

*Calculated according to (Giannini et al[7]): peripheral nervous, renal, cardiac, central nervous, and patient-reported domains were used to calculate the overall disease severity score.

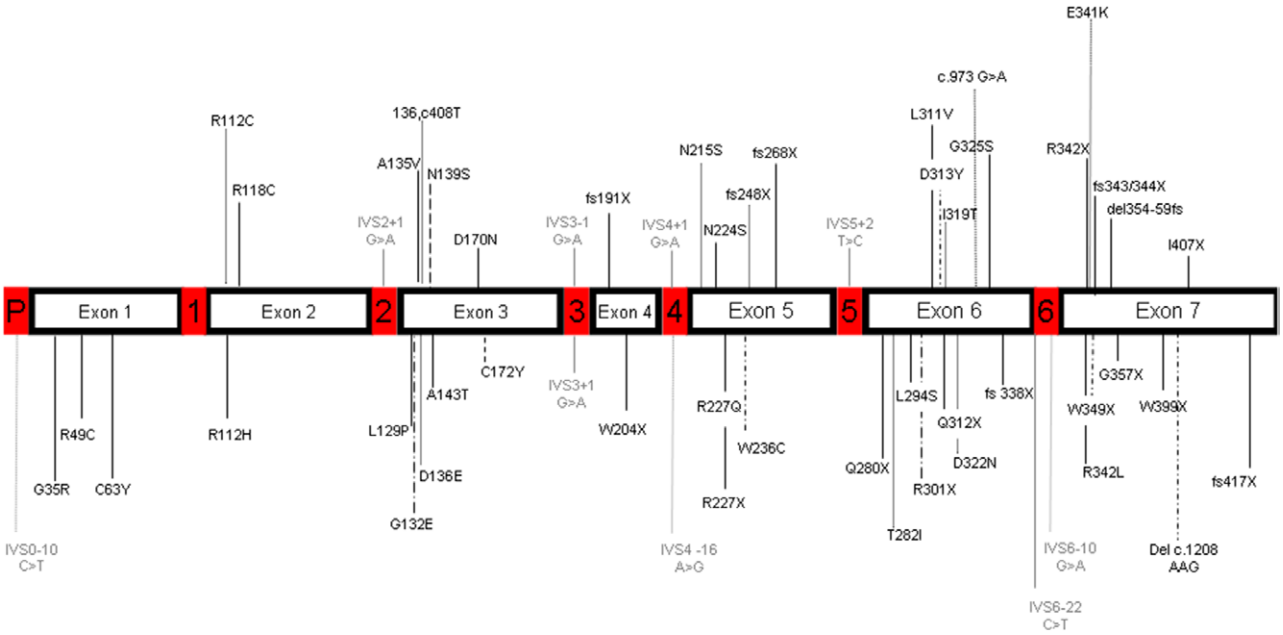

Figure 2. Scheme of an α-galactosidase A gen with exon and intron layers showing all mutations detected in the present study. Intron mutations are marked in gray, exon mutations in black. P indicates promoter.

lyso-Gb3 levels <2.7 ng/mL. None of these women had a clinically relevant agalA mutation. In contrast, all the mutations of the 14 other women with a lyso-Gb3 level >2.7 ng/mL had been staged as clinically relevant Fabry mutations. In contrast to the lyso-Gb3 stratification, agalA activity failed to differentiate clinically relevant from not relevant mutations. In the group of the 11 patients with decreased agalA activity (0.3–0.39 nmol/min per mg protein), 7 patients showed a clinically relevant and 4 patients a not relevant mutation. In the 14 patients with normal agalA activity (0.4–0.5 nmol/min per mg protein), 7 showed a clinically relevant agalA mutation, whereas 7 did not.

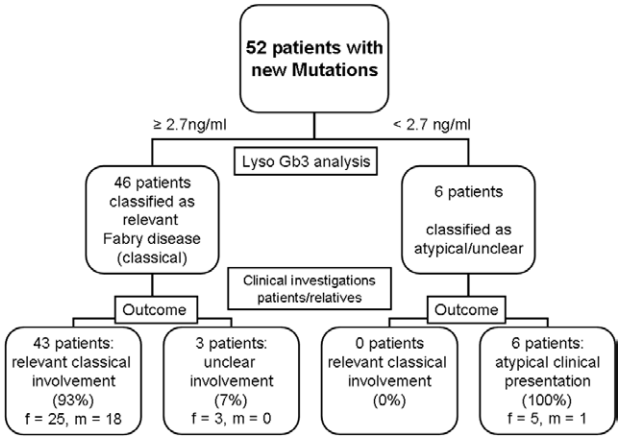

Figure 3. Flow chart of the 52 patients with new α-galactosidase A mutations. First, patients were grouped according to their lyso-Gb3 results (threshold 2.7 ng/mL), and then this classification was ascertained by clinical investigations. Please note that the classification by lyso-Gb3 almost perfectly matches the findings from the clinical workup. Features of classical involvement: (1) major events in at least 2 of the 3 main organs (heart, kidney, and central nervous system); or (2) specific Fabry symptoms plus 1 major event in 1 main organ plus 1 typical disease manifestation in another organ. Major events included end-stage renal disease (ie, glomerular filtration rate <30 mL/min or dialysis or renal transplantation), advanced cardiomyopathy (left ventricular wall thickness >15 mm or left ventricular myocardial fibrosis), stroke, and transient ischemic attacks. All patients with unclear involvement out of the 46 patients (men=18, women=28) classified as relevant Fabry disease were women. Five out of the six patients in the group with a lyso-Gb3 <2.7 ng/mL and atypical presentation were women. agalA indicates α-galactosidase A; f, female; Gb3, globotriaosylceramide; and m, male.

Discussion

Our data show that the new biomarker lyso-Gb3, a degradation product of the accumulating protein Gb3, allows detecting clinically relevant Fabry phenotypes associated with agalA mutations. This innovative diagnostic marker may pave the way for improved detection and management of clinically relevant Fabry disease.

Classical Fabry Disease and Variant Mutations

Since the discovery of the agalA genome,[10–12] classical Fabry disease and so-called variant forms have been discussed intensively.[12,13] Classical Fabry disease can affect all 3 major organs (ie, the heart, the kidney, and the nervous system) and, in end-stage disease, frequently triggers life-threatening events. In contrast, variant agalA mutations may result in less aggressive clinical phenotypes, that is, leading to single organ involvement and late onset disease. Furthermore, several polymorphisms have been described that may not lead to organ involvement at all.[14–16] This phenotypic and genotypic heterogeneity complicates the diagnosis of true Fabry disease. The currently available diagnostic strategies mainly rely on agalA activity and do not allow predicting which organ system and how severely it will be affected in an individual with a particular agalA mutation during lifetime. To complicate matters, women patients may show normal or only slightly decreased agalA activity levels attributable to random X inactivation.

Table 2. Characteristics of the α-Galactosidase A Mutation Patients With lyso Gb3 <2.7 ng/mL

Patient	Sex	Age	Mutation	Type	LVWT, mm	LE	DTPA-Cl	Proteinuria	Stroke	Symptoms	Lyso-Gb3	DS3
1	m	51	D313Y	aty	8.5	no	124	no	no	no	0.00	3
2	f	28		poly	7	no	99	no	no	+	0.00	7
3	f	57		poly	10	no	110	no	no	++	0.00	0
4	m	34		poly	11	no	119	no	yes	no	0.00	9
5	f	52	R118C	aty	12	no	73	no	yes	no	0.00	16
6	f	22	D313Y	aty	8	no	123	no	no	no	0.23	1
7	f	58	A143T	aty	8	no	102	no	no	no	0.23	1
8	f	48	W399X	aty	8.5	no	113	no	no	no	0.31	1
9	f	34	A143T	aty	8	no	149	no	no	+	0.32	4
10	f	37	N215S	aty	8	no	116	no	no	+	0.33	1
11	f	31	A143T	aty	8	no	93	no	no	+	0.40	4
12	f	50		poly	9	no	155	no	no	+	0.43	3
13	f	47	A143T	aty	9	no	110	no	yes	no	0.48	10
14	m	34	A143T	aty	9	no	124	no	no	no	0.55	0
15	f	44		poly	12	no	*50*	yes	no	no	0.65	11
16	f	25	N215S	aty	8	no	113	no	no	no	0.79	3
17	f	28	R112H	aty	6	no	102	yes	no	+	1.02	8
18	f	15	N215S	aty	8	no	119	no	no	+	1.37	4
19	f	50	N215S	aty	10	no	97	no	no	no	1.69	0
20	m	59	R112H	aty	11	no	104	no	no	+	2.07	7
21	f	44	N139S	aty	11	*yes*	110	no	no	no	2.46	1
22	m	63	R112H	aty	12	no	120	yes	no	no	2.49	5
23	m	22	N215S	aty	11·5	no	111	no	no	no	2.53	4

Aty indicates atypical; DS3, disease severity score system; DTPA-Cl, diethylene triamine penta-acetic acid clearance; f, female; LE, late enhancement; LVWT, left ventricular wall thickness; m, male; and poly, polymorphism.

+mild pain; ++severe pain.

Although genotyping is a powerful diagnostic tool, it has serious drawbacks in the setting of agalA mutations because various new forms still arise and several others have not been well characterized (because of the private character of agalA mutations or the frequently lacking family history). Therefore, information about future clinically relevant manifestations in a single patient with a particular mutation is limited.

Several approaches to define a genotype/phenotype relationship in the past have failed, as discussed in recent publications.[17–19] Thus, extensive clinical characterization using advanced investigations combined with detailed information on family history and screening of previously reported clinical descriptions of the same mutation are needed for the identification of so-called classical mutations leading to Fabry disease and atypical mutations not leading to classical Fabry disease.

Our data show that lyso-Gb3 may fill this highly relevant diagnostic gap.

Lyso-Gb3

Lyso-Gb3 has been known as a verotoxin receptor for 25 years. First described by Basta et al and later confirmed by others, it was initially used to evaluate a receptor-specific enzyme-linked immunoadsorbent assay for an Escherichia coli verocytotoxin.[20–22] Aerts and coworkers[23] were the first to show that lyso-Gb3 is a hallmark for Fabry disease in 2008. They could prove that lyso-Gb3 is increased in the plasma of affected women Fabry patients and also in the plasma and tissues of Fabry mice. They further showed that lyso-Gb3 levels are reduced by enzyme replacement therapy and that lyso-Gb3 is an inhibitor of agalA activity.[23] Togawa et al[24] investigated a small sample consisting of 6 men Fabry patients with classic mutations, 4 men with variant mutations, and 8 heterozygote women with Fabry disease. They found that lyso-Gb3 was increased in both symptomatic and asymptomatic subjects. Furthermore, a negative correlation was observed between increased lyso-Gb3 and decreased agalA activity,[24] Interestingly, the lyso-Gb3 levels from the variant mutations could not be distinguished from those of controls. In another study by Rombach et al,[25] the 2 mutations, R112H and P60L, had normal levels for lyso-Gb3. This is concordant with data from our study, where 3 patients with the R112H mutation exhibited a lyso-Gb3 level <2.7 ng/mL and no Fabry-related organ involvement. None of the studies mentioned above focused primarily on the differentiation between classical, clinically relevant and atypical, not Fabry disease-causing mutations.

Clinical Impact

Our thorough clinical investigation program combined with the evaluation of the index patient's relatives allowed us to define classical and atypical agalA mutations in the evaluation study. Measuring lyso-Gb3 in these 2 groups allowed us to define a cutoff value that reliably discerned classical from atypical

Table 3. Clinical Baseline Characteristics of All Patients With Typical and Atypical agalA Mutations

	Typical n=101	Atypical n=23	*P* Value
Men/women	45/56 (45%,55%)	6/17 (24%,76%)	0.08
Age, y	40±14	40±14	0.95
Alpha-galactosidase activity, nmol/min per mg protein	0.2±0.1	0.5±0.6	0.02
Lyso-Gb3, ng/mL	38.7±39.1	0.8±0.9	<0.001
BMI, kg/m^2	23.2±4.2	25.0±4.9	0.08
HR, min^{-1}	64±11	70±14	0.04
SBP, mm Hg	123±18	123±16	0.88
DBP, mm Hg	81±11	79±10	0.46
AHT* (n)	25 (25%)	5 (22%)	0.84
General symptoms			
Abnormal sweating (n)	51 (51%)	2 (9%)	<0.001
Heat or cold intolerance (n)	58 (58%)	4 (17%)	<0.001
Chronic diarrhea (n)	32 (32%)	4 (17%)	0.13
Sudden deafness (n)	21 (21%)	2 (9%)	0.15
Angiokeratomata (n)	41 (41%)	0	<0.001
Neurology			
Stroke (n)	11 (11%)	3 (13%)	0.50
Chronic pain syndrome (n)	33 (33%)	1 (4%)	0.003
Acroparaesthesia (n)	68 (68%)	4 (17%)	<0.001
Heart			
Cardiomyopathy (n)	49 (49%)	0 (0%)	<0.001
LV mass, g	153±59	100±28	<0.001
(assessed by MRI)			
AP (n)	10 (10%)	1 (4%)	0.36
LVEDD, mm	49±5	47±5	0.13
LVESD, mm	33±20	31±5	0.78
IVSd, mm	12.0±3.3	9.3±1.8	<0.001
LVPWd, mm	11.4±2.8	8.9±2.1	<0.001
EF, %	63±6	64±4	0.31
E/A	1.7±3.2	1.1±0.3	0.40
DT, ms	207±56	212±67	0.70
Kidney			
Kidney transplantation (n)	7 (7%)	0 (0%)	0.22
Dialysis (n)	6 (6%)	0 (0%)	0.28
Serum creatinin, mg/dL	1.1±1.3	0.8±0.2	0.28
Serum urea, mg/dL	31±19	27±7	0.33
DTPA clearance, ml/min	91±36	111±23	0.003
Proteinuria, mg/day	90 (0,649)	88 (0,169)	0.85
Proteinuria (n)	49 (49%)	3 (13%)	0.74
Medication			
Beta blocker (n)	16 (16%)	4 (17%)	0.53
Calcium channel blocker (n)	3 (3%)	1 (4%)	0.57
ACE inhibitor (n)	33 (33%)	5 (22%)	0.22
Aspirin (n)	22 (22%)	4 (17%)	0.44
Disease severity score	9±6	4±4	<0.001

Data are n (%), mean±standard deviation, and median (25th quartile, 75th quartile). ACE indicates angiotensin-converting enzyme; AHT, isolated arterial hypertension; AP, angina pectoris; BMI, body mass index; DBP, diastolic blood pressure; DT, deceleration time; DTPA, diethylene triamine penta-acetic acid; EF, ejection fraction; Gb3, globotriaosylceramide; GFR, glomerular filtration rate; HR, heart rate; IVSd, diastolic intraventricular septal wall thickness; LV, left ventricular; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; LVPWd, diastolic left ventricular posterior wall thickness; MRI, magnetic resonance imaging; and SBP, systolic blood pressure.

*Calculated according to (Giannini et al[7]): peripheral nervous, renal, cardiac, central nervous, and patient-reported domains were used to calculate the overall disease severity score.

mutations. Data of the validation study confirmed these findings and showed that lyso-Gb3 levels indicate surprisingly well, regardless of whether a newly found agalA mutation will be classically pathogenetic. This is especially helpful when the clinical information about the new mutation is limited and when family information is not available. In addition, our data show that the biomarker lyso-Gb3 is clinically helpful in patients with agalA activity levels in the gray zone. This is often the case in women patients because of the random X inactivation.

Our data challenge the current practice to establish the diagnosis of Fabry disease. When men patients show decreased agalA activity, the diagnosis is regarded proven. In women patients with borderline agalA activity, genotyping with the search for an agalA mutation is mandatory.[26] However, we found both decreased agalA activity and agalA gene mutations in several individuals in our study sample without concomitant Fabry disease–related organ involvement (of note: the family history was also free of any Fabry disease). Thus, the measurement of agalA activity in combination with genotyping is not sufficient for diagnosing Fabry disease. Even when the diagnostic approach to define a classical Fabry patient includes symptoms and organ involvement, the differentiation between a clinically relevant and an atypical mutation cannot always be made, for example, in young patients with newly found private mutations. In contrast, the sole measurement of lyso-Gb3 levels seems to be the biochemical key for an unclear agalA mutation pertaining to the manifestation of Fabry disease. Therefore, we suggest redefining Fabry disease based on 3 criteria: (1) information about the agalA mutation, (2) the level of lyso-Gb3, and (3) the typical Fabry symptoms and organ involvement. If a patient has an agalA mutation and an elevated lyso-Gb3 level and typical symptoms or organ involvement, this combination may be labeled classical Fabry disease. In patients exhibiting an agalA mutation plus elevated lyso-Gb3 levels who are still asymptomatic and free of organ involvement, this constellation may be labeled classical Fabry mutation. Finally, in patients with an agalA mutation but a normal lyso-Gb3 level, it may be labeled atypical α-galactosidase A mutation. Of note, the magnitude of agalA activity should play a secondary role, especially when the activity is in the gray zone ≈0.4 nmol/min per mg protein.

Limitations

It can be speculated that assessing patients with agalA mutations by lyso-Gb3 also might have an impact on the decision of whether enzyme replacement therapy should be initiated. However, the current study did not focus on that issue, necessitating further studies into this important research question. Lyso-Gb3 might not only be a marker of disease involvement but may also play a role in the pathophysiology of the disease.[23] As proven by this study, lyso-Gb3 seems to be of major value to predict the pathogenicity of a mutation. However, it has to be pointed out that lyso-Gb3 seems to be of minor value to describe the grade of disease severity in classically affected patients (because some young men patients with a classic mutation may exhibit higher lyso-Gb3 levels than older men patients with a classic mutation and severely affected organs).

We cannot exclude that some patients with so-called single organ late onset disease, being atypical variants, might develop severe involvement in this single organ later in their lives (≈60 years of age) although they present with low lyso-Gb3. This might be because of the fact that the clinical spectrum of resulting consequences in some mutations with a lyso-Gb3 around the threshold of 2.7 ng/mL reflects a continuum instead of a yes/no spectrum. Vice versa, we cannot exclude that the organ variants in other cohorts might be distinguishable from polymorphisms, possibly with a mean value of the organ variant group just below the lyso-Gb3 threshold. Epigenetic and environmental modifiers may contribute to the phenotypic variability but were not addressed by the current study.

Conclusion

The current study suggests that the biomarker lyso-Gb3 reliably predicts clinically relevant Fabry disease in patients with agalA mutations. This is especially helpful in novel mutations and in women with an agalA activity around the normal threshold.

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CLINICAL PERSPECTIVE

The current study suggests that the biomarker lyso-Gb3 can differentiate classical Fabry disease from atypical agalA mutations. This is especially helpful when the clinical information about a new mutation is limited and in women patients with an agalA activity level in the gray zone. The data question the current practice to establish the diagnosis of Fabry disease: it seems that the measurement of agalA activity in combination with genotyping is not sufficient for diagnosing Fabry disease. Fabry disease might be redefined based on three criteria: (1) information about the agalA mutation, (2) the level of lyso-Gb3, and (3) the typical Fabry symptoms and organ involvement. If a patient has an agalA mutation and an elevated lyso-Gb3 level and typical symptoms or organ involvement, this combination may be labeled classical Fabry disease. In patients exhibiting an agalA mutation plus elevated lyso-Gb3 levels who are still asymptomatic and free of organ involvement, this constellation may be labeled classical Fabry mutation. Finally, in patients with an agalA mutation but a normal lyso-Gb3 level, it may be labeled atypical α-galactosidase A mutation.

Supplemental Material

ECHOCARDIOGRAPHY. Left ventricular end-diastolic dimension (LVEDD) as well as end-diastolic thickness of the posterior wall (LVPWd) and the septum (IVSD) were measured using standard M-mode echocardiographic methods in parasternal long axis images (GE Vingmed Vivid 7, Horten, Norway; 3.5 MHz). Left ventricular (LV) myocardial mass was calculated using the Devereux formula. Ejection fraction (EF) was calculated using the modified Simpson method. Blood-pool pulsed Doppler of the mitral valve inflow was used to extract the ratio of early to late diastolic flow velocity (E/A) and the deceleration time (DT).

MAGNETIC RESONANCE IMAGING. Routine cine MRI with Gadolinium was carried out. The late enhancement (LE) technique (8 mm slice thickness, breathhold, short heart axis) was applied to detect changes of tissue integrity in the LV myocardium.[27] Images were acquired using an inversion recovery sequence (field of view 240x320 mm2; matrix 165x256). Short axis views at the basal, mid and apical segments, covering the entire ventricle, were used. Using this LE technique, every LV segment, using the standard 17-segment model, was evaluated for the occurrence of myocardial replacement fibrosis. All MRI data were analyzed blinded to echocardiographic results.