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Serum-Mediated Inhibition of Enzyme Replacement Therapy in Fabry Disease

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ABSTRACT

Fabry disease (FD) is a progressive multisystemic disorder, treatable with recombinant enzyme replacement therapy (agalsidase). However, recent studies suggest an endogenous inhibition of agalsidase in patients with FD, as reported for other lysosomal storage diseases. To assess the clinical consequences of serum-mediated agalsidase inhibition in affected patients, we determined the agalsidase inhibition status of 168 patients (68 male) with FD and compared outcomes of inhibition-positive patients with those of inhibition-negative patients. The assessment included clinical events during time on agalsidase, determination of renal and cardiac function, and evaluation of FD-related symptoms. The frequency of serum-mediated agalsidase inhibition was 40% in agalsidase-treated males. Inhibition did not depend on the compound initially used (agalsidase-α or -β). Agalsidase inhibition was associated with higher lyso-globotriaosylceramide levels and worse disease severity scores in patients. Compared with agalsidase inhibition-negative men, agalsidase inhibition-positive men showed greater left ventricular mass (P=0.02) and substantially lower renal function (difference in eGFR of about –30 ml/min per 1.73 m^2; P=0.04), which was confirmed by a longitudinal 5-year retrospective analysis. Additionally, affected patients presented more often with FD-typical symptoms, such as diarrhea, fatigue, and neuropathic pain, among others. Therefore, patients with poor clinical outcome on agalsidase should be tested for agalsidase inhibition. Future studies are warranted to determine if affected patients with FD benefit from acute reduction of anti-agalsidase antibodies or long-term immune modulation therapies to suppress agalsidase inhibition and to identify mechanisms that minimize antibody generation against agalsidase.

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Fabry disease (FD; OMIM #301500) is an X-linked (Xq22.1) inherited disorder caused by a deficiency of lysosomal α-galactosidase A (GLA; 300644) activity. Progressive globotriaosylceramide (Gb3) accumulation in various cells results in a multisystemic disorder with first symptoms and manifestations in early childhood and a reduction in lifespan of 10–15 years without adequate therapy, with death due to stroke, myocardial infarction, life-threatening cardiac arrhythmia, and ESRD.[1] Since 2001, patients with FD have been treated with two different enzyme replacement therapies (ERT), based on infusion of recombinant enzymes (agalsidase-α and agalsidase-β).[2,3] Recent studies suggested that infusion of recombinant enzyme may lead to formation of antibodies, resulting in short-term acute complications,[2] as well as deleterious long-term effects by therapy inhibition, resulting in severely decreased Gb3 and

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lyso-Gb3 depletion.[4–7] Reduced lyso-Gb3 clearance, as a marker of disease progression, may be accompanied by a deterioration of clinical manifestations and symptoms in affected patients. Until now, only indirect associations between ERT inhibition and end-organ manifestations have been shown, in that elevated lyso-Gb3 levels of inhibition-positive patients were associated with left ventricular mass (LV_{mass}) and the formation of white-matter lesions.[7]

In the current study, we analyzed 168 patients with FD (68 male) for serum-mediated ERT inhibition and addressed the impact of ERT inhibition in these patients' clinical outcome.

RESULTS

ERT Inhibition Status in Patients with FD

To analyze serum-mediated ERT inhibition as previously reported,[4,7] we tested sera of 168 patients with FD using an *in vitro* GLA inhibition assay. Patients had been consecutively recruited at the Fabry center of the University Hospital Muenster (IFAZ) between 2001 and 2014. Serum-mediated ERT inhibition assays were performed using patients' serum samples of the last visit. Out of 68 male patients, 24 were naive and 44 had been treated for at least 9 months with ERT (*i.e.*, agalsidase-α, or -β). Treatment-naive male patients with FD had significantly lower mean ERT inhibition in comparison to male patients given ERT ($P<0.001$; Figure 1A). Out of 100 tested female patients with FD, 68 were naive and 32 had been treated for at least 9 months with ERT. In contrast to male patients with FD, the mean ERT inhibition did not differ significantly between women ($P=0.1385$; Figure 1B). To analyze whether ERT inhibition depends on the applied product and to confirm previous studies concerning the crossreactivity of antibodies,[4,7] inhibition assays were performed in a crossover design with agalsidase-α as well as agalsidase-β for male patients under ERT. Linear regression revealed that the value of mean ERT inhibition is product-independent, indicating no measurable specific inhibition for any product ($r^2=0.93$; $P<0.0001$). Further linear regression analyses revealed increased lyso-Gb3 levels ($r^2=0.18$, $P=0.01$; Figure 2A) and Mainz Severity Score Index (MSSI) values ($r^2=0.22$, $P=0.004$; Figure 2B) with increasing ERT inhibition. Linear regression analyses also identified increased creatinine levels ($r^2=0.11$, $P=0.03$; Figure 2C) with increasing ERT inhibition in males.

Clinical Impact of ERT Inhibition

To analyze a potential effect of serum-mediated ERT inhibition on clinical outcome of affected patients, we compared clinical data (current visit/data assessment) of inhibition-negative (ERT^{i-}) with inhibition-positive (ERT^{i+}) men with FD at ERT inhibition status assessment (Table 1; Table 2). A detailed overview of the analyzed men under ERT is provided in Supplemental Table 1. Patients with a mean ERT inhibition of agalsidase-α >50% cut-off were designated as inhibition-positive (Figure 1C). None of the patients had been switched less than 12 months before inclusion (Supplemental Table 1). Out of 44 men with FD under ERT, 19 (43.2%) were identified as ERT^{i+} (Figure 1C). For further analysis, 23 ERT^{i-} (mean age: 41.2±16.1 years) and 18 ERT^{i+} (mean age: 44.4±9.6 years) men with FD were compared (Table 1). Three patients were excluded from the following analyses because they did not meet the inclusion criteria, which was at least 12 months under ERT. In general, no significant differences in terms of age

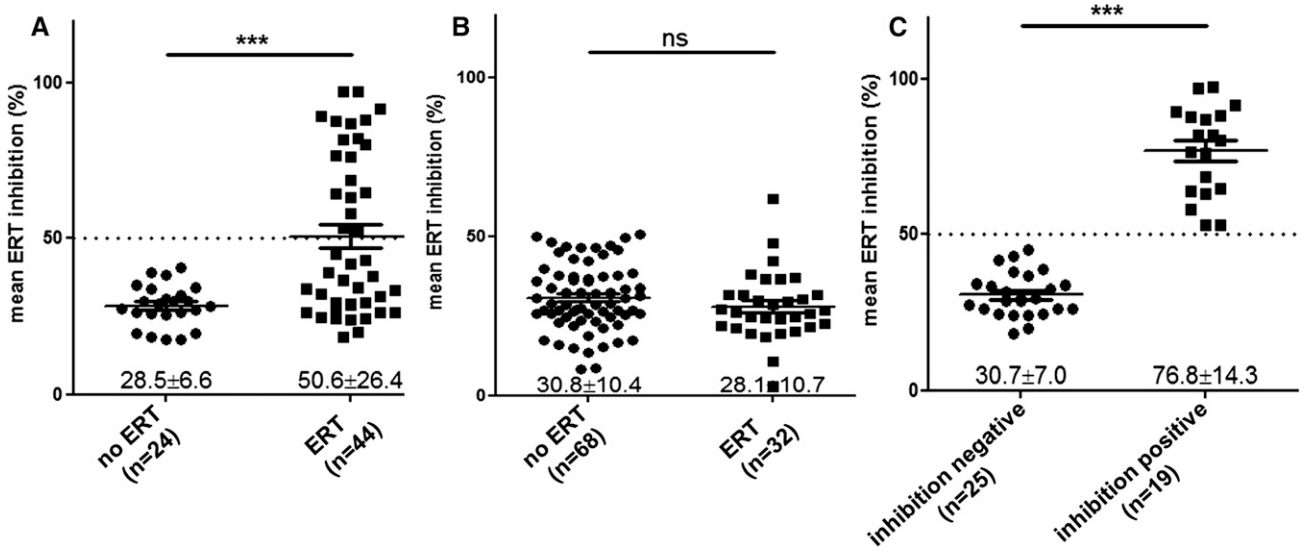

Figure 1. Measurements of serum-mediated ERT inhibition in Fabry patients. ERT inhibition in (A) men, (B) women, and (C) identification of ERT inhibition positive male patients with FD. The dotted line represents the cut-off value of 50% mean ERT inhibition. Error bars represent SEM. Values represent mean±SD. NS, not significant; ***$P<0.001$.

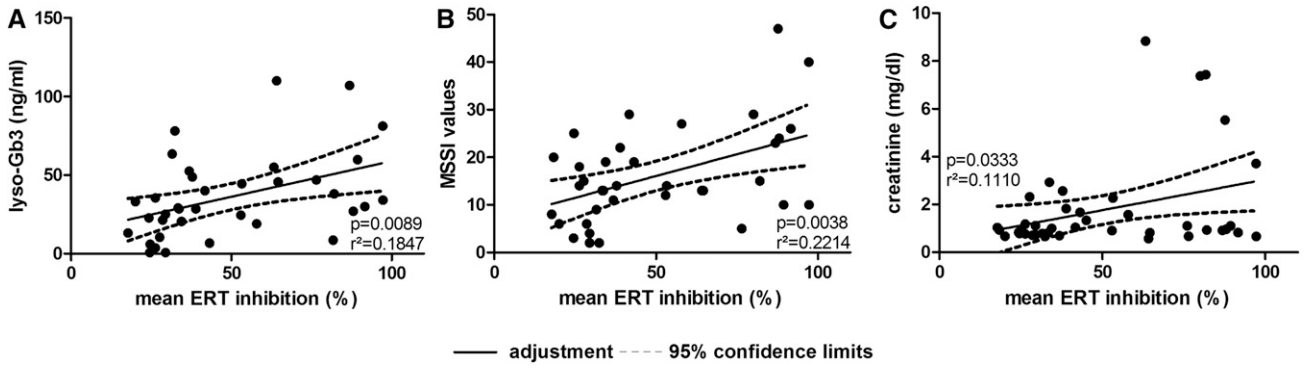

Figure 2. Association of serum-mediated ERT inhibition with clinical parameters. ERT inhibition is associated with (A) increasing lyso-Gb3 levels, (B) increasing MSSI values, and (C) increasing creatinine values in men with FD under ERT.

Table 1. General differences between ERT^{i-} and ERT^{i+} men

Measures	ERT^{i-} (n=23)	ERT^{i+} (n=18)	P Value
Age, years	41.2±16.1	44.4±9.6	0.46
Mean ERT inhibition, %	29.5 (17.8–45.1)	80.9 (53.0–97.3)	<0.001
Duration of ERT, months	58.7±43.1	86.2±45.4	0.05
Initial ERT with agalsidase-β, n	13 (56.5)	9 (50.0)	0.76
Ever required premedication, n	2 (8.7)	7 (38.9)	0.03
Ever switch of ERT, n	12 (52.2)	12 (66.7)	0.52
GLA activity, % of reference	16.0±13.8	13.5±8.3	0.52
Lyso-Gb3, ng/ml	27.0±20.9	48.7±30.1	0.02
Nonsense mutation, n	6 (26.1)	13 (72.2)	<0.01
RAAS blockers, n	14 (60.9)	9 (50.0)	0.54
Diuretics, n	4 (15.0)	3 (18.8)	0.99
Stroke/TIA patients, n[a]	4 (17.4)	4 (22.2)	0.99
MSSI score	13.0±7.7	20.5±11.8	0.03
DS3 score	18.0±10.8	25.2±10.2	0.04

ERT^{i+} was defined as ERT inhibition >50%. Values are given in mean±SD, median (range) or n (%).TIA, transient ischemic attack.
[a]Under ERT; switch of ERT includes a switch from agalsidase-β to -α and *vice versa*; premedication includes treatment with cortisone, antihistamines, *etc.*

(P=0.46), GLA activity (P=0.52), and prescription of renin-angiotensin-aldosterone system (RAAS) blockers or diuretics (both: P=0.99) were observed (Table 1). Time under ERT was slightly higher in ERT^{i+} patients (P=0.05), and those patients had significantly higher lyso-Gb3 levels in plasma (P=0.02) in comparison with ERT^{i-} patients (Table 1). ERT^{i+} patients presented with increased MSSI (P=0.03) and Disease Severity Scoring System (DS3) values (P=0.04) (Table 1). ERT^{i+} patients more often required antiallergic premedication (*i.e.*, antihistamines, cortisone) due to acute infusion reactions (P=0.03; Table 1).

In terms of clinical manifestations, none of the patients in either group developed more strokes/transient ischemic attacks during ERT per patient (P=0.99, Table 1) with no differences in recurrent events (P=0.99). Additional Cox regression analysis showed that no increased hazard ratios for stroke/transient ischemic attacks in ERT^{i+} patients (hazard ratio, 1.602; 95% confidence interval, 0.393 to 6.528; P=0.51) under ERT existed.

Cardiac measures for interventricular septum thickness in diastole and ejection fraction showed no differences between the groups (Table 2). While LV_{mass} did not significantly differ between both groups (P=0.22; Table 2), ERT^{i+} patients showed a trend toward higher relative wall thickness (P=0.06; Table 2). However, multivariate regression analysis with adjustment for age, duration of ERT in months, body weight, the prescription of RAAS blockers, nonsense mutations, and systolic and diastolic BP showed significantly increased LV_{mass} in ERT^{i+} patients (P=0.02; Table 3), which might be concentric (P=0.09, Table 2) according to Lang *et al.*[8]

N-terminal–pro brain natriuretic peptide (NT-proBNP) levels as a marker for cardiac failure seemed slightly, but not significantly increased in ERT^{i+} patients (P=0.29, Table 2). As NT-proBNP levels are influenced by renal function, additional multivariate regression analyses with adjustment for age, ERT duration, prescription of diuretics and RAAS blockers, and eGFR confirmed no difference in NT-proBNP levels between the two groups (ERT^{i-}: 423.2±118.0 pg/ml; ERT^{i+}: 696.8±149.1 pg/ml; P=0.15).

Renal measures revealed no increased risk for albuminuria (P=0.99) but a potential trend for decreased eGFR in ERT^{i+} patients (P=0.10; Table 2). Multivariate regression analysis with adjustment for age, duration of ERT, presence of nonsense mutations, and the prescription of diuretics and RAAS blockers confirmed that ERT^{i+} patients had severely decreased eGFR determined by creatinine-based Chronic Kidney Disease-Epidemiology Collaboration (CKD-EPI) formula compared with ERT^{i-} patients (P=0.04; Table 3). The same model without correction for nonsense mutations revealed similar results (Supplemental Table 2).

The observed eGFR difference of up to 30 ml/min per 1.73 m^2 indicates that ERT^{i+} patients were to be classified in lower CKD stages according to current Kidney Disease: Improving Global Outcomes guidelines[9] compared with ERT^{i-} patients.

Table 2. Clinical differences between ERT^{i-} and ERT^{i+} men

Measures	ERT^{i-} (*n*=23)	ERT^{i+} (*n*=18)	*P* Value
Cardiac measures			
IVSd, mm	12.7±4.7	13.6±4.9	0.48
LVH, *n*	9 (39.1)	9 (56.3)	0.34
LV_{mass}/BSA, g	77.9±30.8	99.8±54.8	0.22
RWT, cm	0.46±0.23	0.55±0.25	0.06
RWT ≥0.42 cm, *n*	7 (31.8)	9 (56.3)	0.19
Categorization of increase in LV_{mass}, *n*			
Normal geometry	15 (68.2)	5 (35.7)	0.09
Eccentric hypertrophy	1 (4.5)	1 (7.1)	0.99
Concentric remodeling	3 (13.6)	6 (42.9)	0.11
Concentric hypertrophy	2 (9.1)	2 (14.3)	0.63
Ejection fraction, %	59.6±7.2	61.3±7.0	0.46
NT-proBNP, pg/ml	787.9±2317.0	701.5±975.8	0.29
ICD/pacemaker, *n*	3 (13.6)	0 (0.0)	0.24
Renal measures			
ACR, mg/g	614.9±796.7	699.8±906.5	0.86
Albuminuria, *n*	14 (73.7)	9 (75.0)	0.99
Dialysis, *n*	1 (4.3)	4 (22.2)	0.15
Kidney transplantation, *n*	1 (4.3)	1 (5.6)	0.99
Hyperfiltration, *n*	4 (17.4)	3 (16.7)	0.99
Creatinine, mg/dl	1.19±0.64	2.57±2.76	0.28
eGFR, ml/min per 1.73 m^2	89.6±36.0	72.1±47.2	0.22
eGFR, ml/min per 1.73 m^{2a}	92.6±33.8	70.7±48.3	0.10
CKD stage, *n*			
1	7 (30.4)	6 (33.3)	0.99
2	10 (43.5)	5 (27.8)	0.35
3	4 (17.4)	2 (11.1)	0.68
4	2 (8.7)	2 (11.1)	0.99
5	0 (0.0)	3 (16.7)	0.08

ERT inhibition positive is defined as ERT inhibition >50%. Values are given in mean±SD, or *n* (%). Albuminuria is defined as an albumin-to-creatinine ratio (ACR)>30 mg/g. BSA, body surface area; IVSd, left ventricular septum thickness in diastole; LVH, left ventricular hypertrophy is defined as an IVSd >12 mm. RWT, relative wall thickness. Creatinine-based eGFR is calculated using the CKD-EPI] formula according to Levey *et al.* 2009. Hyperfiltration is defined as an eGFR (creatinine-based) >125 ml/min per 1.73 m^2.
[a]Without NTX patients.

Table 3. Multivariate regression analysis to assess the influence of serum-mediated ERT inhibition on cardiac and renal measures

Measures	ERT^{i-} (*n*=23)	ERT^{i+} (*n*=18)	*P* Value	Δestimate
LV_{mass}/BSA, g/m^2	71.5±8.1	105.8±9.4	0.02	34.4±13.8
RWT, cm	0.42±0.05	0.57±0.06	0.07	0.15±0.08
eGFR, ml/min per 1.73 m^2	93.3±8.9	64.1±9.9	0.04	−29.3±13.3

The mixed model approach for LV_{mass} and RWT calculations was adjusted for age, duration of ERT, prescription of angiotensin aldosterone system blockers, nonsense mutations, body weight, systolic and diastolic blood pressure. The mixed model approach for eGFR calculation was adjusted for age, duration of ERT, nonsense mutations, the prescription of renin angiotensin aldosterone system blockers and diuretics. Patients with renal transplantation and hyperfiltration were excluded from calculations. Creatinine-based eGFR was calculated via CKD-EPI formula according to Levey *et al.* 2009.

Evaluation of FD-typical symptoms revealed that ERT^{i+} patients with FD had increased risks for diarrhea (*P*=0.04), tinnitus (*P*=0.003), fatigue (*P*=0.03), and neuropathic pain (*P*=0.004) (Table 4). In addition, these patients had slightly increased risks for edema (*P*=0.08), hypacusis (*P*=0.05) as well as cornea verticillata (*P*=0.04; Table 4). Figure 3 provides an overview of the main outcomes in a Forest plot of relative risks and confidence intervals of ERT^{i+} in comparison with ERT^{i-} patients with FD.

To further analyze if the observed differences between ERT^{i-} and ERT^{i+} patients might result from a more disruptive mutation (*i.e.*, nonsense mutation), we performed subgroup analyses for nonsense and missense mutations. ERT^{i-} patients carrying nonsense mutations showed significantly lower lyso-Gb3 levels in comparison to ERT^{i+} patients (28.8±2.9 versus 57.3±29.8 ng/ml; *P*=0.03). In addition, ERT^{i-} patients carrying missense mutations had lower left ventricular masses and higher eGFR in comparison with ERT^{i+} patients (LV_{mass}/body surface area: 84.2±29.1 versus 143.4±76.3 g; *P*=0.02; eGFR: 90.0±32.2 versus 42.6±48.2 ml/min per 1.7 m^2; *P*=0.03).

The general observed high risk for ERT inhibition in patients with nonsense mutations could be confirmed by an analysis of seven patients carrying the R220× mutation, distributed to two non-related families (family one, four cousins; family two, three brothers). At ERT inhibition assessment seven patients had been on ERT for 109±40 months with a mean age of 46±5 years. Mean residual GLA activity differed between 6 and 16% of the reference value. Although only one of the three brothers was ERT^{i-}, all other R220× patients were ERT^{i+}. To analyze whether ERT inhibition, and therefore the formation of antibodies against recombinant ERT, is based on low or nearly absent residual enzymatic activities, we analyzed residual GLA activities of patients with missense and nonsense mutations separately. Residual GLA activities between ERT^{i-} and ERT^{i+} in patients with nonsense mutations did not significantly differ (ERT^{i-}: 11.5±10.2 versus ERT^{i+}: 13.3±9.2% of reference; *P*=0.63), which was also seen in patients with missense mutations (ERT^{i-}: 17.6± 14.8 versus ERTi+: 13.0±6.6% of reference; *P*=0.90). Additionally, we analyzed two brothers with the missense mutation p.C94S. Both were at the same age. One patient (only 9.4% of reference GLA activity) had received ERT for >120 months and was ERT^{i-}, whereas his brother (15.6% of reference GLA activity) started ERT 13 months before inhibition assessment and became ERT^{i+}.

Long-Term Effect of ERT Inhibition

To analyze the potential effect of ERT inhibition in more detail, we performed a retrospective analysis of male patients under ERT (Table 5). Inclusion criteria for this analysis were time under ERT of 4.5–5.5 years and documented values for creatinine, MSSI, and DS3. To control whether ERT inhibition status is stable over time,

Table 4. Differences between ERT^{i-} and ERT^{i+} men of FD typical symptoms

Symptoms	ERT^{i-} (n=23)	ERT^{i+} (n=18)	P Value	RR (95% CI)
Edema, *n*	4 (19.0)	8 (50.0)	0.08	2.04 (1.04 to 4.19)
Diarrhea, *n*	5 (26.3)	10 (66.7)	0.04	2.53 (1.10 to 5.83)
Diarrhea, d/mo	4.3±1.8	4.6±3.6	0.88	
Abdominal pain, *n*	6 (31.6)	9 (50.0)	0.32	1.47 (0.77 to 2.81)
Hypohidrosis, *n*	12 (54.5)	14 (77.8)	0.19	1.89 (0.76 to 4.64)
Tinnitus, *n*	3 (14.3)	11 (61.1)	0.003	2.81 (1.41 to 5.57)
Cornea verticillata, *n*	4 (22.2)	9 (60.0)	0.04	2.31 (1.08 to 4.94)
Hypacusis, *n*	2 (8.7)	6 (35.3)	0.05	2.18 (1.17 to 4.07)
Fabry crisis, *n*	3 (14.3)	5 (31.3)	0.25	1.65 (0.81 to 3.35)
Neuropathic pain, *n*	8 (34.8)	14 (82.4)	0.004	3.82 (1.30 to 11.25)
Fatigue, *n*	1 (5.0)	6 (35.3)	0.03	2.34 (1.34 to 4.09)

Values are given in mean±SD or *n* (%). RR, relative risk.

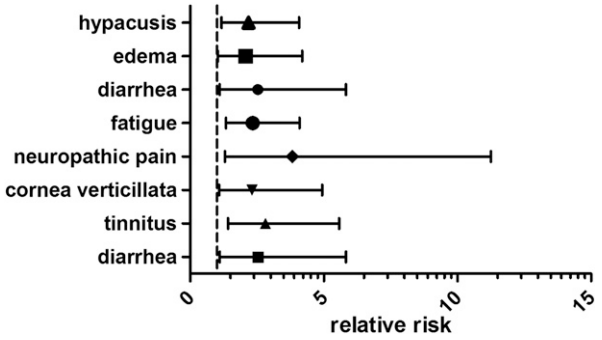

Figure 3. Forest plot of increased risks of ERT^{i+} compared with ERT^{i-} men with FD.

we performed additional inhibition measurements of previous blood samples of ten included ERT^{i+} patients (Supplemental Figure 1). Serum samples were collected 12–24 months before initial status inhibition assessment (inclusion). All analyzed samples showed stable inhibition over time. The mean age of both groups (ERT^{i-} and ERT^{i+}) at inhibition status assessment did not differ (43.4±17.9 versus 41.3±9.5 years; P=0.7148; Table 5). Retrospectively, ERT^{i-} and ERT^{i+} patients showed similar creatinine-based eGFR, MSSI, and DS3 values (Table 5). Five years later ERT^{i+} patients showed a severe decrease of renal function (P=0.03) and increase of MSSI and DS3 (P=0.01, P=0.02, respectively), whereas ERT^{i-} patients remained stable (Table 5, Figure 4).

DISCUSSION

In the current study, we retrospectively analyzed 168 patients with FD for serum-mediated ERT inhibition and determined the impact of ERT inhibition on clinical outcome.

Our main findings are: (*1*) The frequency for serum-mediated ERT inhibition was about 40% in ERT-treated men, (*2*) Inhibition did not depend on initial ERT compound (*i.e.*, agalsidase-α or -β), indicating a cross-reactivity of involved antibodies–this cross-reactivity of antibodies is a confirmation of previous studies,[4,7] (*3*) ERT inhibition was associated with increasing lyso-Gb3 levels and worse severity score values, and (*4*) ERT^{i+} males showed severely impaired cardiac structural disease burden and impaired renal outcome.

Initial studies addressing immune reactions to ERT in patients with FD suggested that up to 73% of newly ERT-treated patients with FD develop antibodies toward the infused enzyme over time,[2,10] resulting in short-term complications including acute allergic reactions, necessitating premedication with histamines, cortisone, *etc.* Two recent studies demonstrated that antibody formation results in serum-mediated ERT inhibition, affecting the Gb3 clearance in ERT^{i+} men with FD.[4,7] However, the long-term effect of serum-mediated ERT inhibition on clinical outcome remains elusive. By establishing a similar approach for *in vitro* serum-inhibition measurement, defining ERT^{i+} as >50% ERT inhibition, we confirm previous data[4,7] and provide new evidence for an association between ERT inhibition and more severe end-organ manifestations.

By analyzing a large well-characterized group of patients we were able to address clinical differences between ERT^{i+} and ERT^{i-} patients in detail. Besides a deterioration of Fabry-specific symptoms such as fatigue, neuropathic pain, also reflected by two different disease severity scores and elevated lyso-Gb3 levels, serum-mediated ERT^{i+} patients showed an increase of LV_{mass} and severe impairment of renal function, which is typical for FD with ongoing disease progression.[1]

Our additional analyses of patients with missense or nonsense mutations indicate that the observed differences at time of inhibition assessment between ERT^{i-} and ERT^{i+} patients are most likely independent of the type of mutation. The retrospective 5-year analysis revealed that ERT^{i+} patients showed a severe decline of renal function and increase of established disease severity scores over time. A potential effect of switch of product (*i.e.*, from agalsidase-β to agalsidase-α) as reported by Weidemann *et al.*, can be excluded because both groups showed similar frequencies of ERT "switched" patients. Of note, none of the patients was dose-reduced (agalsidase-β). Therefore, a general more severe disease progression in ERT^{i+} patients due to nonsense mutations, age, and switch of product can be excluded, indicating a direct effect of ERT inhibition on clinical outcome in our study cohort.

Our observations indicate impaired clinical effects of ERT in ERT^{i+} compared with ERT^{i-} patients. As demonstrated, endogenous anti-GLA antibodies inhibit ERT already during enzyme infusion, leading to a reduction of circulating enzyme titer.[4] This observation suggests that our results in terms of decreasing kidney function and increase of LV_{mass} with ERT inhibition are based on the inefficiency of low ERT doses for cellular Gb3 clearance in the kidney and heart.[11–13] As we identified increased LV_{mass}, but no impairment of cardiac output (*i.e.*, ejection fraction), we conclude that the affected patients are in an incipient stage of cardiac involvement in

Table 5. Longitudinal 5-year retrospective analysis of ERT^{i-} and ERT^{i+} men

Measures	5-year retrospective	Inhibition status assessment	*P* Value	Change (95% CI)
ERT^{i-} *n*=12, age: 43.4±17.9 yr				
eGFR, ml/min per 1.73 m^2	79.3±33.8	72.6±36.2	0.24	−6.7 (–18.7 to 5.3)
Hyperfiltration, *n*	3 (25.0)	3 (25.0)	0.99	
NTX, *n*	0 (0.0)	0 (0.0)	0.99	
MSSI	12.8±8.5	14.7±8.5	0.21	1.8 (–1.2 to 4.9)
DS3	16.4±9.2	17.1±9.3	0.77	0.7 (–4.2 to 5.5)
ERT^{i+} *n*=12, age: 41.3±9.5 yr				
eGFR, ml/min per 1.73 m^2	79.3±35.9	66.0±42.3	0.03	−13.3 (– 24.7 to 1.8)
Hyperfiltration, *n*	2 (16.7)	3 (25.0)	0.99	
NTX, *n*	0 (0.0)	1 (8.3)	0.99	
MSSI	14.6±9.5	19.0±12.2	0.01	4.4 (1.3 to 7.6)
DS3	16.0±7.7	22.2±12.3	0.02	6.2 (1.0 to 11.3)

ERT^{i-}, two patients received agalsidase-β, ten patients received agalsidase-α, ten patients switched ERT (two to agalsidase-β and eight to agalsidase-α); ERT^{i+}, five patients received agalsidase-β, seven patients received agalsidase-α, seven patients switched ERT (two to agalsidase-β and five to agalsidase-α). Patients with renal transplantation (NTX) or hyperfiltration (eGFR >125 ml/min per 1.73 m^2) were excluded from calculations. ERT medication was given in standard doses (agalsidase-α: 0.2 mg/kg body wt intravenous, every other week; agalsidase-β: 1.0 mg/kg body wt, intravenous, every other week).

terms of cardiac fibrosis, which might also be supported by unchanged NT-proBNP levels.

In comparison to females, approximately 40% of all men receiving ERT were identified as ERT^{i+}, which is in line with recent studies.[7] Most of these patients were nonsense mutation carriers and so antigen naive. The immune-mediated reaction to specific antigens (*i.e.*, drugs) is multifactorial. Patients with FD with absent or nearly absent endogenous GLA activity seem to have a high risk for antibody formation against recombinant ERT (drug hypersensitivity), which is common in patients with nonsense mutations. Out of 19 patients with nonsense mutations, 68% were ERT^{i+}. This observation is underlined with our results by the seven patients with the R220× mutation from whom six patients were ERT^{i+}. Similar observations have been reported previously.[4] However, our additional analysis of patients with missense or nonsense mutations further revealed that low enzymatic activities are not always associated with ERT inhibition and that even higher residual GLA activities do not protect against ERT inhibition. These results indicate that ERT inhibition is not only due to the absence or presence of significant residual enzyme activity, but might depend on other, as yet unknown, individual immune-modulating factors. As a consequence, especially male patients are under high risk for therapy escape or therapy resistance. Hence, the clinical parameters of these patients should be carefully followed-up, including measurements of serum-mediated ERT inhibition.[7,14] Interestingly, out of 32 women with FD, we observed only one carrying a missense mutation that was defined as ERT^{i+}. Her residual GLA activity was about 25% of the reference. Additional testing of larger female FD cohorts under ERT should be performed to assess if additional women are also ERT^{i+}. If so, this could also point to the possibility that ERT inhibition is not only based on low or absent GLA activities.

Of note, we neither observed any differences concerning the ERT inhibition associated with the initial ERT compound (*i.e.*, agalsidase-α or -β), nor did we detect drug-specific reactions (<7% variance between agalsidase-α and -β inhibition values), indicating that the applied dose of recombinant enzyme (agalsidase-α: 0.2 mg/kg body wt every other week; agalsidase-β: 1.0 mg/kg body wt every other week) may not influence antibody formation. Once antibody formation occurs, inhibition may be independent of ERT compound use. In addition, our data suggest that a switch of the ERT will not trigger further antibody formation.

Serum-mediated inhibition of ERT with deleterious effects on clinical outcome has also been reported for other lysosomal storage diseases such as Pompe disease, Gaucher disease, and Mucopolysaccharidosis type IV.[15–18] Future studies using an interventional design are warranted to analyze if affected patients with FD may benefit from acute reduction of anti-agalsidase antibodies or long-term immune modulation therapies to permanently suppress serum-mediated ERT inhibition and to improve their clinical outcome as reported for other diseases.[15,17] For the general prevention of the serum-mediated ERT inhibition, prospective studies with early hyposensibilization therapy for recombinant GLA especially in young male FD patients may be helpful. ERT^{i+} patients with a severe disease progression, who have reached the end of their options despite a weight-adapted ERT, should be considered for an immune-modulating therapy, such as immunoadsorption and/or treatment with immunosuppressive drugs, such as anti-CD20 monoclonal antibodies.

Clinical Impact

Forty percent of male patients with FD may develop permanent inhibition status against ERT once infusion is started. As inhibition-positive patients show a worsened cardiac and renal outcome, we strongly suggest treating these patients with the highest available dose of agalsidase besides the specific cardiac and renal medical treatment. Patients with poor clinical outcome under ERT have to be tested for ERT inhibition and may benefit

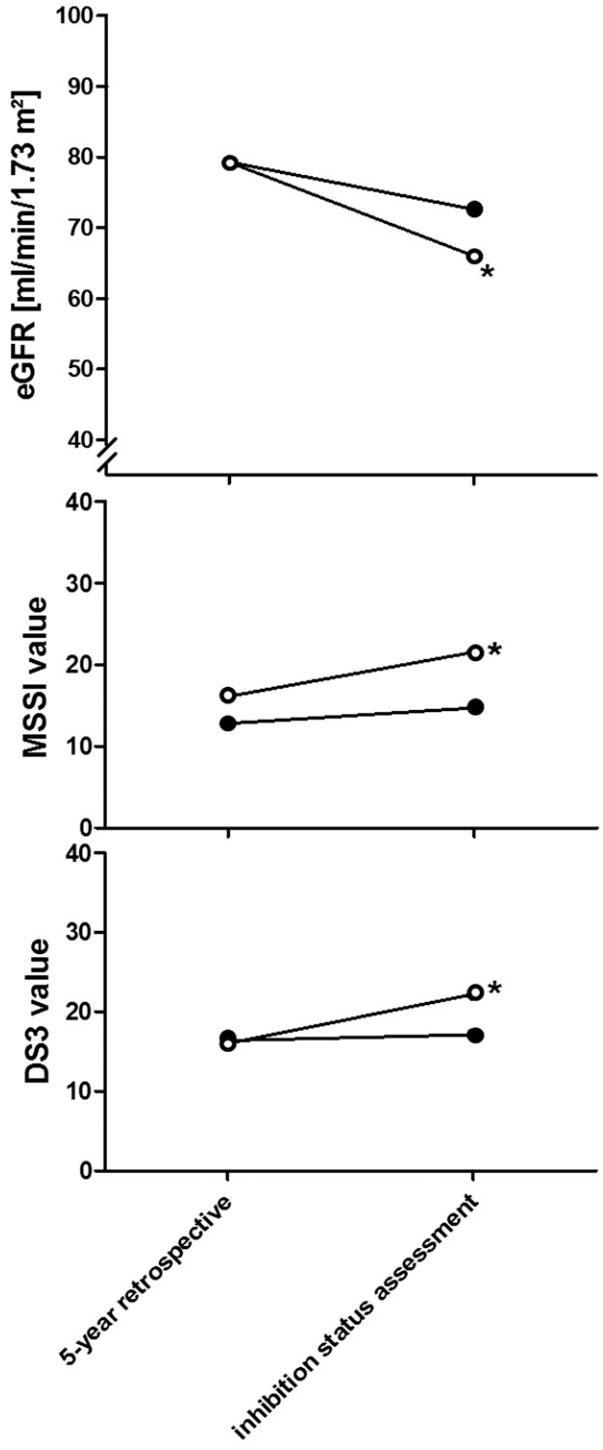

Figure 4. Longitudinal 5-year retrospective analysis of ERT inhibition negative (ERT^{i-}) and positive (ERT^{i+}) men. Filled circles represent ERT^{i-} patients, empty circles represent ERT^{i+} patients.

from immune modulation therapies. Future studies are warranted to analyze how antibody generation against ERT could be avoided or minimized. Follow-up longitudinal studies of individual patients over at least 5 years are now critically important to assess changes in lyso-Gb3, frequencies, and differences in organ complications and compare the effect of antibody-positive and -negative patients.

Limitations

The retrospective cross-sectional study design might be a limitation of the current study. Although previous blood samples were not available for all included patients, retrospective analysis of ten samples of ERT^{i+} patients indicated a stable inhibition status over time. In our 5-year retrospective analysis, ERT^{i+} patients showed a more severe renal decline, even if kidney biopsies are missing to evaluate the effect of ERT and assess Fabry-specific and additional factors for renal decline. The higher frequency of several classic FD symptoms may not be explained by ERT inhibition alone, as symptoms such as cornea verticillata are largely insensitive to ERT.

CONCLUSIONS

The prevalence of increased serum-mediated ERT inhibition is 40% in ERT-treated men and inhibition neither depends on the initial ERT compound, nor is it selective for any recombinant GLA product. ERT inhibition-positive men have significantly increased risks for FD-typical symptoms and showed severely impaired cardiac as well as renal function.

CONCISE METHODS

Patients and Study Design

In all, 168 patients were consecutively recruited at the Fabry center of the University Hospital Muenster (IFAZ) between 2001 and 2014. Patients were retrospectively analyzed in an open cohort study. Timepoint of data assessment and determination of serum-mediated ERT inhibition was the last visit. Patients under ERT had been treated either with agalsidase-α (0.2 mg/kg body wt, every other week) or agalsidase-β (1.0 mg/kg body wt, every other week). All investigations were performed after the approval of the Medical Association of Westfalian-Lippe and the Ethical Committee of the Medical Faculty of the University of Muenster (project-no. 2011–347-f, date of report: July 7, 2011). Written informed consent of patients was obtained for analysis and publication.

A comprehensive diagnostic work-up had been performed in all patients, including medical history and cardiac, renal, and neurologic evaluation.

Patients underwent standard echocardiographic examinations performed in accordance with the current guidelines of the American Society of Echocardiography.[19] Routines included measurement of conventional and Doppler-derived parameters: interventricular septum thickness in diastole and LV posterior wall diameter, LV end-diastolic and end-systolic dimensions and volumes were registered for systolic, early (E) and late (A) mitral inflow velocities, early myocardial relaxation velocity (e′) and deceleration time for diastolic function. Indices for fraction of shortening, stroke volume, ejection

fraction, and the quotients E/A and E/e′ were calculated automatically. Left ventricular hypertrophy (LVH) was defined as an interventricular septum thickness in diastole >12 mm and LV_{mass} was adjusted for body surface area according to recent recommendations.[8] Relative wall thickness calculations and subsequent classification of LV_{mass} increase were performed according to Lang *et al.*[8]

Renal function was quantified by eGFR using the CKD-EPI formula for creatinine, cystatin C, and cystatin C-creatinine.[20,21] None of the patients received dialysis before initial ERT. The albumin-to-creatinine ratio was calculated from spot urine. Albuminuria was defined as an albumin-to-creatinine ratio >30 mg/g protein.

CKD stages were classified according to Kidney Disease: Improving Global Outcomes guidelines with creatinine-based eGFR values.[8] Disease severity was assessed using the MSSI and the DS3.[22,23]

Since residual inhibition (basal) has been reported also in ERT naive and even healthy volunteers (data not shown), which is due to proteolytic effects of human blood samples, patients with a mean ERT inhibition of >50% cut-off, were designated as ERT^{i+} according to Rombach *et al.*[7]

A detailed overview of the analysis of men under ERT including detected *GLA* mutations, residual GLA activity, lyso-Gb3 levels, and ERT compound at inhibition assessment, information on switch of ERT compound before inhibition assessment and inclusion of patients in longitudinal 5-year retrospective analysis, as well as FD symptoms and manifestations, is provided in Supplemental Table 1.

Biochemical and Genetic Analyses

The ERT inhibition assays were performed as reported elsewhere.[4,7] Mean storage time of tested serum samples was 17±11 months at –80°C. For serum preparation, fresh blood samples were centrifuged for 10 min at 5000 rpm. Five microliters of serum were preincubated for 15 minutes at room temperature with 1 ng agalsidase-α or -β. Subsequently, GLA activity was determined using 4-methylumbelliferyl-α-D-galactopyranoside (Santa Cruz Biotechnology, Heidelberg, Germany), as described elswewhere.[24] *N*-Acetylgalactosamine (Santa Cruz Biotechnology) was used as specific inhibitor of endogenous α-galactosidase B activity.[25] To determine mean ERT inhibition in percent, absolute values were compared with non-serum–treated (preincubated with 5 μl 0.7% NaCl) GLA activity of 1 ng agalsidase-α, or -β, respectively. Each sample was measured at least three times in triplets. The intra-assay coefficient of variation was 3.1%.

For HPLC lyso-Gb3 determination, lyso-Ceramide was used as reference (Matreya, LLC, Pleasant Gap, PA) and D5-Fluticasone Propionate (EJY Tech, Inc., Rockville, MD) served as internal standard.

Statistical Analysis

If not stated otherwise, continuous variables were expressed as mean with SD, or as median (range). Categorical data were expressed as numbers and relative frequencies as percentages. Differences between groups were analyzed with the unpaired *t* test or Mann–Whitney *U* test for continuous data, and the Fisher's exact test for categorical data. Statistical significance was considered at a two-sided $P<0.05$.

Multivariate regression analyses for LV_{mass} and relative wall thickness calculations were adjusted for age, duration of ERT, prescription of RAAS blockers, body weight, and systolic and diastolic BP. Multivariate regression analyses for eGFR calculations were adjusted for age, duration of ERT, and the prescription of RAAS blockers and diuretics. Patients with renal transplantation after ERT initialization were excluded from eGFR calculations.

Results are reported with their respective 95% confidence intervals or ±SD. SAS version 9.3 (SAS Institute Inc., Cary, NC) and GraphPad PRISM V5.0 software (GraphPad Software Inc., La Jolla, CA) were used for statistical analyses.

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