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Characterisation of novel α-galactosidase A mutations in Fabry disease based on in vitro, in vivo and pharmacological data

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Jan Lukas

aus Rostock,

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Reviewers

1st: Prof. Dr. med. Arndt Rolfs	Albrecht-Kossel-Institut Universitätsmedizin der Universität Rostock
2nd: Prof. Dr. rer. nat. Reinhard Schröder	Institut für Biowissenschaften, Abt. Genetik Universität Rostock
3rd: Prof. Dr. med. Martin Dichgans	Institut für Schlaganfall-und Demenzforschung Klinikum der Universität München

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Content

Abbreviations

α-gal	α-galactosidase A (enzyme)
ABX	Ambroxol
BBB	blood-brain-barrier
BHX	Bromhexine
BiP/GRP78	immunoglobulin heavy chain binding protein/glucose-regulated protein 78
cDNA	complementary DNA
Da	dalton
DNA	deoxyribonucleic acid
DMEM	Dulbecco´s modified eagle medium
DMSO	dimethylsulfoxide
DTT	1,4 dithio-DL-threitol
EDTA	ethylendiamin-tetraacetate
ER	endoplasmic reticulum
ERAD	endoplasmic reticulum associated degradation machinery
et al.	et alii
EtOH	ethanol
FBS	fetal bovine serum
FD	Fabry disease
g	gram, acceleration of gravity
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
Gb3	globotriaosylceramide
GD	Gaucher disease
GLA	α-galactosidase A (gene)
GSL	glycosphingolipid
HDACi	histone deacetylase inhibitor
HEK293H	human embryonic kidney cells
HGMD	human gene mutation database
hr/hrs	hour/s
k	kilo
KIF	Kifunensin
LSD	lysosomal storage disease
lyso-Gb3	globotriaosylsphingosine
MeOH	methanol
MG-132	n-benzoyloxycarbonyl (Z)-Leu-Leu-leucinal
min	minute
MPR	mannose-6-phosphate receptor
MRI	magnetic resonance image
n, N	nano, number of experiments
PBS	phosphate buffered saline
PC(T)	pharmacological chaperone (therapy)
PCR	polymerase chain reaction
PD	Pompe disease
PDi	proteasomal degradation inhibitor
PDL	poly-D-lysine
PPAR	peroxisome proliferator-activated receptor

RNA	ribonucleic acid
Rosig	Rosiglitazone
RT	room temperature
SDS	sodium dodecyl sulfate
SNP	single nucleotide polymorphism
sol	solution
SRT	substrate reduction therapy
TBS	tris-buffered saline
Tg	Thapsigargin
TM	Tunicamycin
Tris	tris-(hydroxymethyl-)aminomethane
UPR	unfolded protein response
UPS	ubiquitin-proteasome system

1 Introduction

1.1 Lysosomal storage diseases

Lysosomal storage diseases (LSDs) are a heterogeneous family of metabolic disorders. In most of these diseases a single defective glycoside hydrolase causes the accumulation of its substrate, usually lipid deposits, leading to pathologic complications of various cell functions, which in turn implicates multisystemic failure for the patients. To date, about 50 distinct disorders have been described and classified for the chemical nature of the accumulating material (Table 1). Taken individually, LSDs are rare conditions with incidences of 1:40,000 – 1:4,200,000 according to an early study from Meikle and colleagues (1999). Altogether, the combined incidence of LSDs may range between 1:5,000 – 1:10,000 life births. Even though a standardised definition for rare diseases is not widely accepted, perspicuously not all that rare to suffer from an LSD which emphasises the need for scientific research to find new therapeutic strategies. Especially by taking into consideration that in certain risk populations some of the LSDs occur in much higher rates (Gaucher disease has an incidence of 1 in 855 births in the Ashkenazi Jewish population as one example) and newborn screenings recently suggest much higher patient numbers than originally assumed (Spada et al, 2006, Hwu et al., 2009, Wittmann et al., 2012). Moreover, only for a fraction of the diseases a causative treatment is available.

Lysosomal enzymes are involved in the catabolism of cellular macromolecules, thereby degrading them in a stepwise manner to provide the resources for a cellular reorganisation. In this recycling process, glycosphingolipids (GSLs) play an important role. GSLs consist of a ceramide (sphingosine + fatty acid) and a sugar. The kind of sugar on his part determines the core structure of the GSL (Varki et al., 2009). GSLs take over many functions. Being formed in the endoplasmic reticulum (ER) and Golgi apparatus, they are situated on the outer cell membrane. Due to their specific chemical behaviour they function as structural components (e.g. formation of lipid rafts), non-peptidic receptors (e.g. host-pathogen/toxin interaction) and are involved in signalling events. However, in many lysosomal storage diseases, GSLs are inappropriately accumulating (Table 1), because a failure to degrade the material is existent.

Table 1: List of LSDs classified according to accumulated material

Disease	OMIM #	Deficient enzyme
Mucopolysaccharidoses		
MPS I (Hurler syndrome)	607014, 607015, 607016	α-L-iduronidase
MPS II (Hunter syndrome)	309900	iduronate sulfatase
MPS IIIA (Sanfilippo syndrome A)	252900	heparan sulfamidase
MPS IIIB (Sanfilippo syndrome B)	252920	N-acetylglucosaminidase
MPS IIIC (Sanfilippo syndrome C)	252930	acetyl-CoA:alpha-glucosaminide
MPS IIID (Sanfilippo syndrome D)	252940	N-acetylglucosamine 6-sulfatase
MPS IVA (Morquio syndrome A)	253000	galactose-6-sulfate sulfatase
MPS IVB (Morquio syndrome B)	253010	Beta-galactosidase
MPS VI (Maroteaux-Lamy syndrome)	253200	N-acetylgalactosamine-4-sulfatase
MPS VII (Sly syndrome)	253220	β-glucuronidase
Glycoproteinoses		
Aspartylglucosaminuria	208400	aspartylglucosyminidase
Fucosidosis	230000	α-L-fucosidase
α-Mannosidosis	248500	α-D-mannosidase
β-Mannosidosis	248510	β-D-mannosidase
Mucolipidosis I (sialidosis)	256550	neuraminidase
Schindler disease	609241	α -N-acetylgalactosaminidase
Sphingolipidoses		
Fabry disease	301500	α-galactosidase A
Farber disease	228000	N-acylsphingosin-amindohydrolase 1
Gaucher disease	230800, 230900, 231000	glucocerebrosidase
GM1-gangliosidosis	230500	β-galactosidase-1
Tay–Sachs disease	272800	hexosaminidase A
Sandhoff disease	268800	hexosaminidase B
Krabbe disease	245200	galactosylceramidase
Metachromatic leukodystrophy	250100	arylsulfatase A
Niemann–Pick disease, types A and B	257200, 607616	acid Sphingomyelinase
Other lipidoses		
Niemann–Pick disease, type C	257220,	NPC1 protein, NPC2 protein
Wolman disease	278000	acid Lipase A
Neuronal ceroid lipofuscinosis	256730	palmitoyl protein thioesterase
Glycogen storage disease		
Glycogen storage disease, type II (Pompe disease)	232300	acid α –glucosidase
Multiple enzyme deficiencies		
Multiple sulphatase deficiency	272200	sulfatase-modifying factor 1
Galactosialidosis	256540	Cathepsin A
Mucolipidosis II/III	252500, 252600	N-acetylglucosamine-1-phosphotransferase
Mucolipidosis IV	252650	Mucolipin 1
Lysosomal transport defects		
Cystinosis	219800	Cystinosin
Sialic acid storage disease	269920	Solute carrier family 17, Member 5
Other disorders due to defects of lysosomal proteins		
Danon disease	309060	LAMP-2
Hyaluronidase deficiency	601492	hyaluronidase 1

modified from Vellodi, 2005

1.1.1 Fabry disease

Fabry disease (FD) is a rare X-chromosomal disorder caused by the absence or reduction of lysosomal α-galactosidase A (α-gal) activity. In 1898, dermatologist Johann Fabry first described the disease as "angiokeratoma corporis diffusum" in a 13 year old boy (Mehta, 2002). In 1970, the disease could be linked to a specific enzyme defect noticed by the patients' inability to process artificial α-galactose substrates (Kint et al., 1970). FD is a multisystemic and heterogeneously developing disorder of glycosphingolipid storage due to improper catabolism within the lysosomes. As mentioned, GSLs are integral parts of cellular membranes with structural and physiological function. Recently, evidence for a direct effect of globotriaosylceramide (Gb3 or ceramide trihexoside), the predominantly accumulated material was reported to be involved in the pathophysiology (Namdar et al., 2012) being excessively present in endothelial cells, vascular smooth muscle, erector pilori muscles in the skin, myocardium and corneal epithelial cells (Mehta, 2002).

A retrospective study indicated an incidence of 1:117,000 births (Meikle et al., 1999). However, on the one hand this number is not taking into account female heterozygosity, because only data of hemizygote males was obtained and, moreover, recent studies propose that the prevalence of FD could be by far higher than initially thought. This is due to a changed understanding of the disease progress. When genetic testing became available for Fabry disease in the late 80s, most of the detected cases presented with severe phenotypic signs, the so-called "classic" FD. And it was believed that the disease can only affect males. Nowadays, it is agreed that also atypical variants of the disease exist and that heterozygote females can develop disease symptoms, usually following milder disease courses. These are characterised by either a later onset, a milder progression, a mono- or oligosymptomatic manifestation (commonly cardiac, renal or CNS variants are reported, Frustaci et al., 2001; Rolfs et al., 2005; Nance et al., 2006; Shabbeer et al., 2006) or combinations thereof.

1.1.1.1 Symptoms and progress of the disease

FD "classically" presents with hypohidrosis, chronic pain, ocular opacities, liver and kidney impairment, skin lesions, gastrointestinal complications and cardio- as well as cerebrovascular degeneration (Moran et al., 2003; Hoffmann, 2009; Mehta et al., 2010) often showing the full-blown picture only in a minority of very severe cases. The most common symptoms are shown in Figure 1. However, symptoms develop over time, whereas some may never manifest in one patient.

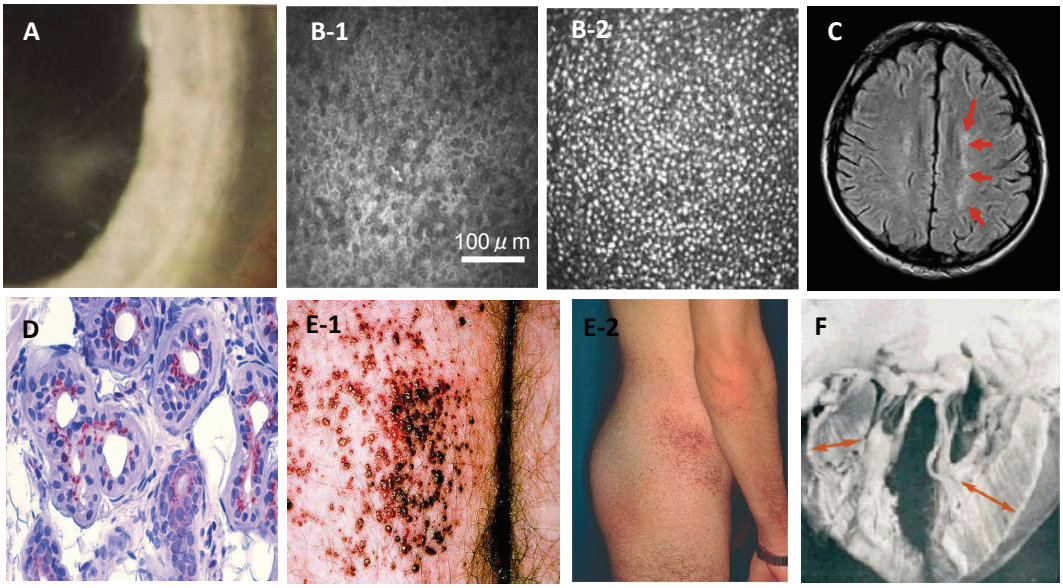


Figure 1: Clinical manifestations of Fabry disease. **A**: Cornea verticillata; courtesy of Prof. Dr. Rudolf Guthoff, Ophthamology, Rostock **B**: Confocal *in-vivo* microscopy: Normal cornea (left, B-1) compared to FD patient cornea that shows hyper-reflective globotriaosylceramide inclusions within corneal basal epithelial cells (right, B-2) **C**: Confluent white matter lesions in a patient with FD **D**: Globotriaosylceramide accumulation in a skin biopsy from a patient harbouring mutation A156V; courtesy of Thomas Jansen, M.D., Essen **E**: Angiokeratoma corporus diffusum; courtesy of Luis Requena, M.D., Madrid, and Heinz Kutzner, M.D., Friedrichshafen **F**: Distinctly thickened myocardium (adapted from: Desnick et al., 2001).

Typically, early-onset FD cases progress more severe symptoms usually attracting first attention in childhood, when the youths are less capable and resilient (Zarate and Hopkin, 2008). Early signs include pain, hypohidrosis, angiokeratoma and gastrointestinal disturbances (Table 2). A risk of premature mortality occurs as a result of renal, cardiovascular and cerebrovascular damage (Ramaswami et al., 2008).

Until recently, it was thought that female heterozygous carriers are not affected by FD, but pioneer work (Whybra et al, 2001, MacDermot et al., 2001) could convincingly show that, even though typically milder affected, females have a significant burden of the disease. Today it is believed that due to randomized X-linked inactivation of healthy alleles in females and the sometimes atypical course of the disease, FD is dramatically underdiagnosed. Lately, newborn screenings and epidemiologic studies of risk populations suggested incidences of 1:3,100 – 1:13,341 (Sachdev et al., 2002; Rolfs et al., 2005; Brouns et al., 2010; Wittmann et al., 2012; Rolfs et al., 2013) which would make FD the most frequent among the LSDs.

Table 2: Progression of Fabry disease

Progression of Fabry disease	
Childhood and adolescence (≤16 years)	Neuropathic pain Skin lesions (angiokeratomas) Ocular abnormalities Hearing impairment Sweating abnormalities Bowel disturbance Lethargy and fatigue
Early adulthood (17-30 years)	Extensive skin lesions Proteinuria, haematuria, lipiduria Oedema of the upper or lower limbs Fever, heat sensitivity, sweating abnormalities Diarrhoea, abdominal pain
Later adulthood (>30 years)	Cardiac disease Stroke, transient ischaemic attack Renal impairment

Table 2: Stages in Fabry disease. Early symptoms are a burden for the young patients leading to a significant decrease of QoL. Later symptoms are fatal leading to a decrease in life expectancy typically due to specific vasculopathy. http://www.focusonfabry.co.uk/healthcare_symptoms.aspx?mode=print (modified).

1.1.1.2 Diagnosis in Fabry disease

Diagnosis of the disease might be complicated and is often delayed. Fabry disease is an important differential diagnosis in stroke, kidney and cardiovascular diseases. Many symptoms are unspecific due to their occurrence in numerous genetic and non-hereditary diseases (Hoffmann et al., 2008). Once suspected, it is still difficult to diagnose a patient conclusively with FD, because so-called Fabry polymorphisms or mild mutations are found by genetic screening programmes of certain patient cohorts. The typical path to Fabry diagnosis besides extensive clinical care for the patients involving the relevant clinical diagnosis (clinical symptoms, detectable organ involvement, Weidemann and Niemann, 2010) can consider genealogical tree examination, enzyme activity measurement in patient leucocytes (only males), biomarker Gb3 investigation and *in silico* phenotype prediction tools (e.g. SIFT, Ng and Henikoff, 2003; PolyPhen, Ramensky et al., 2002). Moreover, we propose that the *in vitro* overexpression of mutant enzyme can be a precise determiner of the enzyme defect and should be utilised as well for mutations of uncertain biochemical consequence.

Recently, a novel tool to facilitate FD diagnosis was proposed by the discovery of the biomarker lyso-Gb3, the deacetylated form of Gb3. For the analysis of these compounds, plasma samples are easily obtained, rapidly and cost-effectively processed and tested. The sensitivity of this novel diagnostic tool was illustrated in many studies (Aerts et al., 2008, van Breemen et al., 2011). Like Gb3, Lyso-Gb3 is apparently involved in disease pathology playing a role in the development of

Fabry-related kidney disease (Sanchez-Niño et al., 2011). At a later time point this biomarker will be revisited as an important element for the study as well. However, only a combination of all this information can help therapeutic management for patients with disputed Fabry disease.

1.1.1.3 The α-galactosidase A gene (*GLA*)

The α-galactosidase A (EC 3.2.1.22) is a homodimeric lysosomal enzyme consisting of two 46 kDa sized monomers. It is synthesised as a precursor with an N-terminal signal peptide of 31 amino acids that is cleaved within the ER (Chen et al., 2000). The GLA gene is located at the long arm of the X chromosome (Xq22.1) and its deficiency is the trigger for FD. In 1989, the *GLA* gene was sequenced (Kornreich et al.) and since then, about 600 mutations of the gene of which about two third are missense mutations causing single amino acid substitutions were described in the context of Fabry disease (HGMD, http://fabry-database.org/mutants/). The enzyme belongs to the glycosyl hydrolase family 27 and shares strong sequence and structure similarity with α-N-acetylgalactosaminidase (Fujimoto et al., 2003), the enzyme deficient in Schindler disease. It catalyses the cleavage of the terminal α-galactose of Gb3 and other glycosphingolipids. Specific N-glycosylation at residues Asn139, Asn192 and Asn215 is important for the correct intracellular sorting and leads to the translocation of the enzyme to the Golgi apparatus and subsequent delivery to the lysosomes (Ioannou et al., 1998). Moreover, it allows for its transport *via* Mannose-6-Phosphate receptors (MPRs). However, a portion of the enzyme is secreted which has been tried to exploit for therapeutic strategies (Medin et al, 1996) since neighbouring cells could serve as a source of α-galactosidase A for activity-deficient cells. The crystal structure of the human enzyme was elucidated in 2004 (Garman and Garboczi, 2004) which increased the understanding of the enzymes´ structure and function by means of elucidating the importance of single amino acids for functionality and stability of the enzyme (Garman, 2007). In 2010, the Garman laboratory was able to demonstrate the enzymatic mechanism of the substrate binding revealing a relative unselective for the nature of molecule attached to the α-sited galactose (Guce et al., 2010). On the contrary, it was shown that the active site accommodates only α-anomeric galactose. In combination with the finding that the enzyme includes a second ligand-binding site preferring the β-anomer this holds exciting implications for the generation of improved drug design. Though it has little homology to other human hydrolases many glycosidases share the $(\beta/\alpha)_8$-barrel as a common structure element (Golubev et al., 2004).

There are currently two recombinant enzyme formulations, Replagal (Shire Human Genetic Therapeutics, Dublin, Ireland) and Fabrazyme (Genzyme Therapeutics, Cambridge, USA), available for enzyme replacement therapy. Besides this application in FD, the enzyme is known to convert donor blood type B into blood of type 0, the universal donor type. Therefore, a lot of effort had

been applied to synthesise and purify recombinant α-gal in many organisms such as *Escherichia coli* bacterial cells, COS monkey cells, baculovirus-infected Sf9 insect cells, *Pichia pastoris* yeast cells, transduced human bone marrow cells, and genetically engineered human fibroblasts (Replagal) and CHO chinese hamster ovary cells (Fabrazyme) (Garman and Garboczi, 2004), whereas the latter two are the named clinical formulations as indicated.

1.1.1.4 The mutations – a genotype/phenotype correlation?

Far more than 600 mutations have been reported in FD to date (HGMD). Most of those are private (restricted to a single or few families) and, as indicated, missense mutations resulting from single nucleotide substitutions (about 450) which poses the question for the biochemical severity of the enzyme defect on the one and the clinical outcome of stronger and less stronger affected enzymes for the patients on the other hand. It was demonstrated that many mutations were related to the classical phenotype, hence most severe form of the disease (Ishii et al., 1992, Eng et al., 1994, Eng et al., 1997). However, physicists were aware of milder kinds of the disease even at that time (Davies et al., 1993). Later on, when enzyme activity measurement became more applicable for clinical purposes it appeared that patients with "no α-Gal activity have the early-onset classic phenotype, whereas those with residual activity present with the later-onset subtype" (Chien et al., 2012). However, only a small number of mutants were initially tested *in vitro* and a systematic approach to merge clinical and "wet-lab" data is still missing even though the foundation of the Fabry Registry (Genzyme) and the Fabry Outcome Survey (FOS, Shire Human Genetic Therapeutics, Dublin, Ireland), created two observational databases that track phenotypic development of the patients (Hopkin et al. 2008, Mehta et al. 2002). This could be utilised to further stimulate interdisciplinary researchers to accelerate the progress of understanding the pathophysiology of the disease by means of the defective enzyme and initiate greater effort screenings to systematically investigate the damaged enzyme itself (Shin et al., 2007,2008) and relate the findings to the clinical situation. Investigations on the pharmacological chaperone 1-Deoxygalactonojirimycine (DGJ, AT1001, Migalastat), a compound with the capacity to stabilise mutant α-gal thereby increasing the portion to be harnessed by the cells (see section 1.2.2.2) initiated more laboratories to utilise *in vitro* enzyme activity measurement to characterise the mutations (Hamanaka et al., 2008; Benjamin et al., 2009; Filoni et al., 2010) which led to a meanwhile large knowledge base from a high number of indexed mutants.

Computational analysis revealed that clinical phenotype can be predicted to be variant or classic on the basis of conformational change analysis as a main discriminator, and hydropathy and size change information on the substituted amino acid as auxiliary factors increasing accuracy of the prediction method (Saito et al., 2010). To evaluate pathogeneceity of *GLA* mutations, it was

proposed that solvent accessibility of the substituted amino acid strongly correlates with enzyme damage (Garman and Garboczi, 2007). Combined with knowledge about the importance of residues within the molecule obtained from a structure based sequence alignment with members from the α-amylase superfamily this model could be validated and refined (Kuipers et al., 2010). Such analyses are important since a lot of novel Fabry mutations are found in a very early clinical stage when prognosis is insecure and a therapeutic schedule needs to be established on the basis of significant phenotype prediction models. For further characterisation of Fabry mutations, different paradigms were investigated including (1) aggregation behaviour as indicator for the gain in hydrophobicity (Siekierska et al., 2012), (2) kinetic parameters (Ishii et al., 2007), pH and thermostability (Yasuda et al., 2003; Ishii et al., 2007; Ishii, 2012). Typically, the experimental studies were based on overexpression (Ashley et al., 2002; Shimotori et al., 2008; Wu et al., 2011) or patient-derived cell systems (Benjamin et al., 2009) analysing either the residual GLA enzyme activity or focusing on enzyme trafficking and stability aspects (Yam et al., 2006; Ishii et al., 2007).

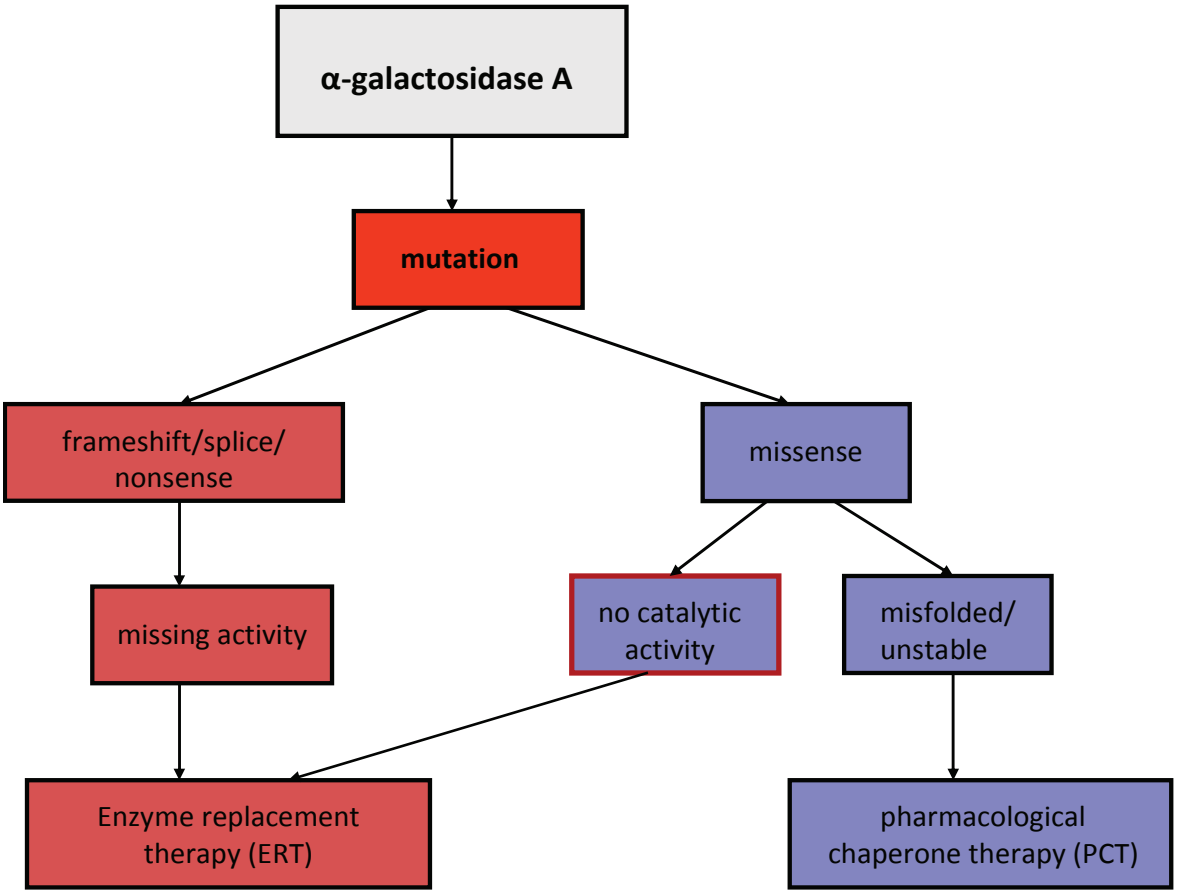


Figure 2: Scheme of the diverse nature of GLA mutations and potential therapeutic management strategies. GLA mutations can lead to a complete deficiency of α-gal activity (splice mutations, nucleotide deletions or insertions). Mutant enzyme can display catalytic defects usually leading to a loss of activity as well. Mutants that harbour residual enzyme activity commonly result in a milder disease phenotype and comprise implications for alternative disease management strategies.

Beyond that, the obtained biophysical and biochemical knowledge about the mutated enzyme has been applied in computational models in order to predict the prospective responsiveness of the enzyme to an alternative therapeutic approach utilising active-site-specific pharmacological chaperone DGJ (Andreotti et al., 2010,2011; Siekierska et al., 2012) with the purpose to address patients harbouring presumably mild mutations (see Figure 2).

Because of the broad variety of clinical spectra, no clear genotype/phenotype correlation has been described to date (Tsuboi et al., 2012). With intent to examine phenotypic outcome a limited number of Fabry mutations displaying a differential level of enzyme damage was taken as a basis. This led to the development of a computational prediction algorithm (Saito et al., 2010), however, not taking into account that the same mutation can result in variable phenotypic manifestations in the patients. For example, the mutation D313Y, still unknown for its clinical course was found in equivocal Fabry subjects. It had a high residual activity of about 60 % indeed, was found to be extremely pH sensitive with an unstable conformation at pH 7.4 (Yasuda et al., 2003) on the contrary which could be a possible explanation for a pathological effect. In another example, it was shown that mutation E59K (found only in classically affected patients) had a residual activity of 10 % in Cos 7 cells (Ishii et al., 2007). In addition to its degradation defect it had catalytically changed abilities with an increased K_M value (Ishii et al., 2007; Andreotti et al., 2011). This could cause the enzyme to function normal in an *in vitro* testing system utilising artificial substrate in excess, but under conditions with limited substrate availability lead to decreased substrate conversion.

1.1.1.5 Pathophysiology of Fabry disease

Globotriaosylceramide (Gb3 or ceramide trihexoside) is synthesised from Lactosylceramide and galactose (Figure 3A) by the enzyme 1,4-Galactosyltransferase (A4GALT) by forming an α-1,4 glycosidic linkage. Catabolism starts with the hydrolysis of the terminal alpha linkage to the galactose by α-gal (Figure 3B). Gb3 is composed of ceramide, whose compounds are sphingosine and a long-chain fatty acid bound to three sugar moieties. The carbon body of Gb3 can vary in length from 14 to 26 C-atoms. The globo series of the major GSLs are defined by their internal core carbohydrate (galα1-4gal) (Lingwood, 2011). Defective cleansing of Gb3 leads to its accumulation in endothelial cells of the smooth muscle vessels, in soft tissue, the skin, in diverse glomerular cell types in the kidney and hepatocytes (Mehta et al., 2010).

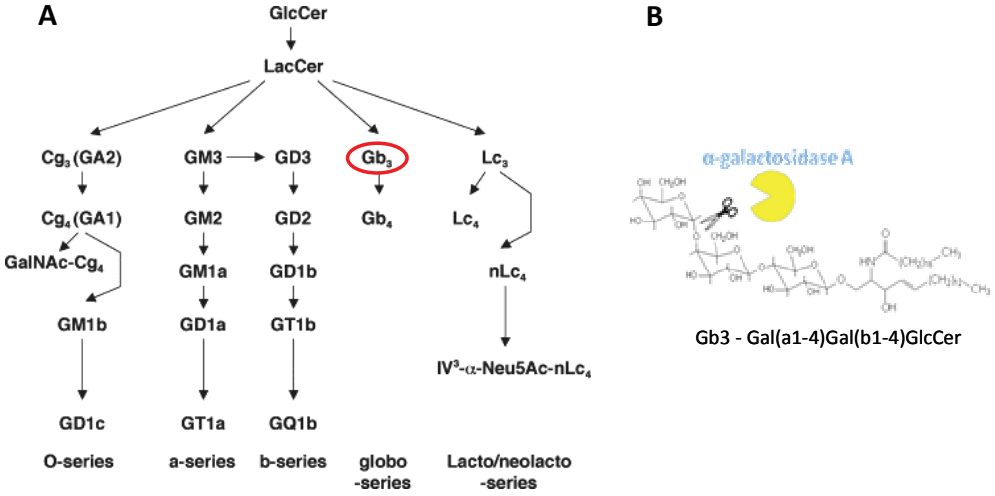


Figure 3: Synthetic pathway of the major GSL classes. **A**: Gb3 (red oval) is formed by α-1,4 glycosidic bonding between galactose and lactosylceramide catalyzed by the A4GALT gene product. SRT inhibits ceramide glycosyltransferase (CGT), the enzyme catalysing the generation of glycosylceramide (GlcCer), therefore preventing the initial step in GSL synthesis. Neville et al (2004), modified. **B**: Structure of Gb3 plus indication of α-gal cleavage site.

It is argued whether and/or to which extent Gb3 or its deacetylated metabolite lyso-Gb3 which also accumulates in urine and plasma (Auray-Blais et al., 2010) contribute to the pathophysiology of FD, especially taken into consideration that there are about 300 GSL structurally defined, but a much higher number can be assumed in the human "glycome" (Lingwood, 2011), which might only be present in small but yet toxic traces in the state of abnormal metabolism. As mentioned before, Gb3 as well as lyso-Gb3 have been demonstrated to play a role in cell damage, pro-inflammatory events (Namdar et al., 2012) and progressive kidney disease (Sanchez-Niño et al., 2011), respectively. Lyso-Gb3 was proven to be a dependable biomarker in hemizygote males in FD (Aerts et al., 2008, van Breemen et al., 2011). Therefore, we utilised its predictive power in the present study in order to fully characterise *GLA* mutations extensively.

1.2 Therapeutic strategies

1.2.1 Enzyme replacement therapy (ERT)

Enzyme replacement therapy (ERT) became available for FD in 2001. To date, ERT is the only approved treatment for FD. It is functional on the one hand, because the enzyme is a soluble and rather small protein and its sufficient production in several cellular systems is readily engineered and on the other, because the α-gal carries phosphorylated high mannose oligosaccharide chains (Matsuura et al., 2008) being recognised by plasma membrane bound MPRs mediating cellular

uptake and trafficking to the lysosomes. Whereas different in the N-glycosylation pattern, both medical formulations consist of identical polypeptide sequences. Although apparently well-tolerated by most patients, ERT still displays adverse events including immunogenecity (Eng et al., 2001) and infusion reactions. While direct evidence is missing, it is likely that neutralising effects of IgG antibodies towards the administered enzymes can decrease the efficaciousness of the therapy and, likewise, therapeutic success. Further shortcomings of ERT should be mentioned. It could be demonstrated that plasma half life (of Replagal in this case) is very short (Pastores, 2007) and even though a sustained effect of the enzyme can be postulated, regular infusions of the recombinant enzyme need to take place frequently. Strategic disease management involving specialist physicians of many fields such as paediatricians, nephrologists, cardiologists, neurologists, geneticists finally agreed on a bi-weekly treatment scheme (Mehta et al., 2010), which makes this therapy a costly and for the patients a significant burden prone procedure. Herein, the administered dosage varies between the two approved formulations (Replagal: 0.2 mg/kg Fabrazyme: 1.0 mg/kg).

However, ERT helps to slow down or even revert disease progression (Mehta et al., 2010). Left ventricular wall thickness is a reversible hallmark of the disease (Kampmann et al., 2009). Most importantly, patients reported an improvement of their condition starting with a facilitation of the neuropathic pain crisis, intestinal problems and so on (Germain, 2010). On the contrary, advanced heart and kidney disease such as presented by tissue fibrosis is an irreversible event (Weidemann et al., 2005; Germain, 2010), thus strengthening the demand for an early diagnosis and therapeutical schedule before the disease is manifested with late stage cardiac or renal symptoms.

Cerebrovascular disease is supposedly non-addressable by ERT, because the enzyme does not cross the blood-brain-barrier (BBB). Whether peripheral events can ameliorate central nervous system disease progress remains an issue of closer examination (Moore and colleagues found a possible mechanistic explanation in 2001 proposing that ERT reverted nitric oxide pathway dysregulation in Fabry patients), but the search for alternative treatment strategies is important and a relief for certain disease forms (as characterised by their genetic defects) might be imminent.

1.2.2 Small molecules and therapeutic potential

Advantages of small molecules are evident. Compared to an enzyme therapy, costs are presumably lower, many drugs are orally available, tissue penetration and drug distribution is

broader especially with regards to the BBB. Therefore, diseases with a significant CNS involvement can be addressed where an ERT is destined to fail.

Pharmacologic agents that are able to up- or downregulate proteostasis network capacity are a powerful tool to address protein folding diseases. In prevalent diseases like Alzheimer's, Huntington's and Parkinson's the bases of the disease are aggregating protein deposits. The gain of this toxic function could be addressed with small molecules manipulating expression or activity of ER-bound or cytosolic chaperones (Powers et al., 2009). In the case of Amyotrophic lateral sclerosis (ALS), a common form of motor neuron disease, approximately 20 % of the familial disease form is provoked by accumulation of the SOD1 gene. A proposed treatment strategy envisages a co-inducer of Hsp70 superfamily genes, Arimoclomol, to assist clearing of SOD1 (Phukan, 2010).

Loss-of–function mutations which are present in LSDs often exhibit premature degradation of unstable proteins and, therefore, call for a different approach. It was demonstrated that folding assistance provided by so-called pharmacological chaperones (PCs) had a beneficial outcome in many LSDs including Fabry disease, Gaucher disease, Pompe disease, Tay-Sachs and Sandhoff disease (Lieberman et al., 2009).

Particularly in diseases with a metabolic facet, where it comes to accumulation of no longer degradable storage material, the block of further synthesis of these impeded disease progression. While not specifically acting on the damaged protein, this *substrate reduction therapy* (SRT) adopts an exceptional position and will only be briefly referenced here.

Of note, most of all approved drugs are belonging to this class of small molecules (with a molecular weight <1000g*mol^{-1}), thus ERT is more of an exception than the compounds of this study. However, drugs displaying their effects on the macromolecular compounds of the complex proteostasis network are a quite novel route in medicine.

1.2.2.1 Substrate reduction therapy (SRT)

For a group of glycolipid disorders, SRT could be utilized to reduce GSL synthesis *via* the inhibition of the ceramide glucosyltransferase (CGT, glycosylceramide synthase) with iminosugars. This enzyme catalyses the first step in GSL synthesis (see Figure 3A). Among LSDs GM1 Gangliosidosis, Tay-Sachs/Sandhoff, Fabry , Niemann Pick Type C and Gaucher disease come into consideration as candidate diseases for a treatment (Butters et al., 2005) with the iminosugar N-Butyl-Deoxynojirimycine (NB-DNJ, Miglustat). For the latter two, NB-DNJ is already clinically approved in many countries. In recent years, eliglustat tartrate, a novel inhibitor of glycosylceramide synthase was identified and found effective in a mouse model of Fabry disease (Marshal et al., 2010). In

comparison to NB-DNJ, eliglustat tartrate does not cross the BBB (Ashe et al., 2011). For this reason, it was predominantly discussed in the context of diseases without neurologic involvement like Fabry, and it is currently in phase 3 clinical trials for the non-neuronopathic type 1 GD (Ashe et al., 2011).

1.2.2.2 Pharmacological chaperone therapy (PCT)

The term pharmacological chaperone is borrowed from endogenous protein chaperones present in living cells. In contrast to those protein chaperones PCs belong to the group of small molecules. The similarity to the mechanism of action is that PCs create an environment in which the protein can fold properly overcoming the difficulties of misfolding, destabilisation and aggregation. However, while helping the nascent polypeptide to fold, protein chaperones bind to folding intermediates maintaining free-energy balance favourable for the generation of sufficient product, hence, mature protein (Powers et al., 2009). Another difference to molecular or protein chaperones PCs comprise a certain specificity to their target enzyme. It is believed that they bind to the fully-synthesized protein. Evidently, most PCs are active-site specific functioning as competitive inhibitors (Asano et al., 2000; Sawkar et al., 2002). Therefore, PCs in lysosomal storage disorders overcome the instability defect of misfolded proteins and allow for their stable conformation at neutral pH in the ER and subsequent transport to the lysosomes.

Anyway, this somehow counterintuitive approach comprises certain dangers. The iminosugar isofagomine only had beneficial effect on cellular glucocerebrosidase (acid β-glucosidase, GCase, gene symbol: GBA) after a washout period of the drug was conducted to recover enzyme activity *in situ* (Steet et al., 2006). Steet and colleagues furthermore stated that small molecule inhibitors could potentially jeopardise therapeutical benefit of the PCs. Asano and colleagues believed that the more potent the inhibitor, the more efficacious the chaperone effect (Asano et al., 2000).

In a large-scale study of 20,000 compounds from chemical classes other than iminosugars could be found to be active on the GCase (Zheng et al., 2006). This illustrates that there are several candidate chaperones for Gaucher disease. To date, a significant number of chaperones have been identified also for other LSDs. Table 3 shows an overview of LSDs and their identified PCs.

Without exception, pharmacological chaperones persist from low-molecular weight molecules. Therefore, it can be assumed that these drugs can pass the BBB allowing central nervous benefit for the mutant enzyme. The treatment of Fabry disease cases involving cerebrovascular symptoms could be an addressable patient cohort. CNS involvement is not restricted to classic FD, but frequently found in patients with milderly affected enzyme (Brouns et al, 2010). A switch from ERT to PCT could cause a dramatic attenuation of disease burden for those patients.

Table 3: Lysosomal storage disorders and their pharmacological chaperones

Disease	Chaperone	Reference
Fabry disease	1-Deoxygalactonojirimycine	Fan et al. (1999)
	α-galacto-homonojirimycin	Asano et al. (2000)
	α-allo-homonojirimycin	
	β-1-C-butyl-deoxygalactonojirimycin	
Gaucher disease	N-nonyl-deoxynojirimycin (NN-DNJ)	Sawkar et al. (2002)
	N-octyl-2,5-anhydro-2,5-imino-D-glucitol	Lin et al. (2002)
	N-octyl-isofagomine	Sawkar et al. (2005)
	Isofagomine (IFG)	Steet et al. (2006), Khanna et al. (2010)
	N-octyl-β-valienamine (NOV)	Chang et al. (2006)
	calystegines A_3,B_1, B_2, C_1	Yu et al. (2006)
	1,5-dideoxy-1,5-iminoxylitol (DIX)	
	α-1-C-nonyl-DIX	
	α-1-C-octyl-DNJ	
	Ambroxol (ABX)	Maegawa et al. (2009)
	cis-(+)-5-(2-Dimethylaminoethyl)-2-(4-methoxy-phenyl)-4-oxo-2,3,4,5-tetrahydro-1,5- benzothiazepin-3-ylacetat (Diltiazem)	Rigat & Mahuran (2009)
Pompe disease	N-butyl-DNJ	Okumiya et al. (2007)
	DNJ	Parenti et al. (2007)
Tay-Sachs/Sandhoff	N-acetyl-glucosamine-thiazoline (NGT)	Tropak et al. (2004)
	6-acetamido-6-deoxycastanospermine (ACAS)	Maegawa et al. (2007)
	bisnaphthalimide nitro-indan-1-one	Tropak et al. (2007)
	pyrrolo[3,4-d]pyridazin-1-one	
	pyrimethamin (PYR)	
G_{M1}-Gangliosidosis	N-octyl-4-epi-β-valienamine (NOEV)	Matsuda et al. (2003)

Table 3: Modified from Fan et al. (2008)

1.2.2.2.1 The pharmacological chaperone Ambroxol

Enzyme inhibitors that allosterically bind their target proteins are rare among the lysosomal hydrolases. Ambroxol (ABX, Mucosolvan®, Boehringer Ingelheim) depicts one of those exceptional cases. It was found as an inhibitor of the GCase in GD (Maegawa et al., 2009). It was demonstrated that, in contrast to other known PCs in Gaucher disease (e.g. NN-DNJ), ABX is capable of restoring functionality of the severe neuronopathic Gaucher type 2 and 3-related mutation L444P (Bendikov-Bar et al., 2013) one of the three most common mutant alleles in GD (Wan et al., 2006). Ambroxol apparently stabilises GCase folding state and allows the intracellular transport to the target organelle. This function of Ambroxol can assumingly ameliorate the course of Gaucher disease and improve the patients´ quality of life. It should be pointed out that the binding affinity of Ambroxol to the GCase is expected to be low (K_a in the micromolar range). Since the quality of the chaperoning effect is related to an efficient binding and, particularly,

inhibition of the target enzyme (Asano et al., 2000), the stronger the inhibitory function *in vitro* the more likely the beneficial effect towards the target enzyme *in vivo*. The Ambroxol derivative Bromhexine is a less efficient inhibitor of GCase and was not demonstrated to have a beneficial effect on GCase activity yet.

In the same vein, sodium 4-phenylbutyrate (4-PBA), another non-iminosugar compound, a low molecular weight fatty acid that is known for its ability to stabilise proteins and assist trafficking deficient α-gal mutants (Yam et al., 2007). Like glycerol, polyols, and dimethylsulfoxide 4-PBA acts as general, hence less specific chaperone. Since a reversal of lysosomal Gb3 storage in mice fibroblasts harbouring the Fabry mutation R301Q could not be detected, it was concluded that this compound would display no benefit for FD patients. However, this finding demonstrated the different modes of action and the various pitfalls of drugs functioning on the one hand to release the ER retention blockade of unstable/misfolded enzyme, but being unable to eliminate the deficit of function.

An increasing body of evidence suggests a more general effectiveness of the presented inhibitors. For instance, NB-DNJ was shown to act as a chaperone for Pompe disease (Parenti et al., 2007; Okumiya et al., 2007) as well as Gaucher disease (Sánchez-Ollé et al., 2009). Moreover, it inhibits the enzyme CGT and can be used for SRT in glycosphingolipidoses (Butters et al., 2005). This beneficial outcome is already clinically exploited in Niemann-Pick Type C1 (NPC1 disease), GD and FD - diseases in which GSL production contributes to the pathophysiology. DNJ is a PC in Pompe disease stabilising mutant α-glucosidase. It is also found to inhibit ER-bound glycosidases I and II (possibly leading to strong gastrointestinal adverse effects).

This indicates that testing of compounds in FD that proved functionality in other LSDs are a worth venture due to potential unexpected cross-reactivities. Therefore, a possible ABX effect was studied in the present investigation.

1.2.2.3 Proteasomal degradation inhibitors

The proteasome system functions as a lysosomal-independent degradation machinery for proteins and is involved in cell cycle regulation, apoptosis and inflammation (Marfella et al., 2007). The proteasome is composed of a barrel shaped 20S core substructure providing the cavity with the proteolytic activity in which targeted proteins are degraded. The 19S complex is built of at least 19 different subunits. "Lid" and "base" of this 19S proteasome display regulatory function controlling the opening of the gate of the 20S proteasome and the recognition and unfolding of substrate proteins. These two particles assemble and build the functional 26S proteasome (Ciechanover et al., 2000). Recently, components blocking the activity of the proteasome were

proposed as cancer drugs and for the treatment of autoimmune, demyelinating and neurodegenerative diseases (Fierabracci, 2012). Lysosomal enzymes are synthesised, folded and processed in the ER. Usually the procession contains posttranslational modifications such as nitrogen-glycosylation at asparagine residues which are necessary for further sorting and transport. This action is dependent on many individual consecutive steps and an even higher number of responsible factors who are key in the maintenance of general cellular proteostasis. A mutation of a (lysosomal) protein, subsequent misfolding of the polypeptide and its accumulation causes the cell to initiate the unfolded protein response (UPR) in order to boost the folding capacity. There is evidence that whenever the UPR is exhausted, the cell "switches" to the system of endoplasmic reticulum associated degradation (ERAD) (Friedlander et al., 2000) which in turn leads to increased clearance of accumulated material in the ER by molecular factors assigning misfolded proteins to their proteasomal degradation. In the worst case, increased proteasomal activity leads to the apoptotic decline of the cell. At best, the cell is no longer able to achieve its requirement for the respective protein function. To date, proteasomal degradation inhibitors (PDi) were tested for a potential adjuvant function in disease models for LSDs other than FD. For instance, the proteasomal inhibitor MG-132, known to abolish the 20S proteolytic activity enhanced mutant GCase folding, trafficking and activity in GD patient fibroblasts (Mu et al., 2008; Wang et al., 2011). Lactacystine, a nonpeptidic proteasome inhibitor was isolated from *Streptomyces* sp. the active metabolite clasto-Lactacystine is formed by a spontaneous intramolecular rearrangement (Gulder & Moore, 2010). For a subset of investigated missense mutations an increase of cellular α-gal enzyme level could be detected (Ishii et al., 2007) indicating the stabilisation of the unstably folded mutant enzyme. However, Ishii et al. concluded that “final degradation of mutant α-gal A protein may involve different proteases”. However, the effect on the mutant enzymes´ activities was not analysed.

1.2.2.3.1 The glitazones

Another way to avoid proteasomal degradation is to spare the precedent ubiquitination process. The nuclear hormone receptor peroxisome proliferator-activated receptor gamma (PPARγ) agonist Rosiglitazone (Rosig, Avandia®, GlaxoSmithKline plc London, United Kingdom) and its derivatives (e.g. Troglitazone, Pioglitazone) serve as diabetes type 2 (T2DM) drugs by sensitising the cells to the body’s own insulin thereby stimulating glucose uptake and reducing blood glucose level. Rosig and the other glitazones were identified as agents diminishing cellular ubiquitination (Marfella et al., 2007). The mechanism by which the glitazones inhibit the ubiquitination process is not understood. Possibly, Rosig functions *via* PPARγ-regulated genes encoding members of the ubiquitination machinery or it could act *via* a direct binding to specific motifs of a subset of target

proteins as it is reported that binding to PPARγ instantly influences its ubiquitination (Kilroy et al., 2009) indicating (even though highly speculative) that it is involved in an action/immediate destruction cycling of a significant number of genes. Also a binding to specific E1, E2 or E3 proteins thereby inhibiting proper function is conceivable. The general actions of the ubiquitin-proteasome system (UPS) are illustrated in Figure 4. E1, E2 and E3 enzymes are activating ubiquitin followed by its fusion to target proteins. At least four ubiquitin molecules have to be attached to a protein to make it recognisable for the 26S proteasome and condemn a protein to final degradation. As for the treatment of T2DM this inhibiting function on the UPS may have a favourable anti-inflammatory impact since proteasomal over-activity is associated with enhanced inflammation and NFκb expression (Marfella et al., 2006). Diabetes patients treated with Rosiglitazone showed anti-inflammatory effects linked with the modulation of NFκB activity, lower levels of ubiquitin and proteasome 20S activity, cytokines and oxidative stress (Marfella et al., 2007). However, due to idiosyncratic liver toxicity (Troglitazone) or their association to cardiovascular events such as heart attack and stroke (Rosiglitazone and Pioglitazone), all three drugs are meanwhile withdrawn from the market. A couple of other compounds are reported to suppress the protein ubiquitination *via* diverse target proteins.

1.2.2.4 Endoplasmic Reticulum stress inducers

As mentioned under 1.2.2.3, the ER stress response, or topically referred to as unfolded protein response (UPR) contains a large amount of factors building a network to protect the cell against multiple threats. Originally discovered as a reaction on temperature stress, many members are still classified as heat shock proteins. The aim of the UPR is to activate the signalling pathways leading to upregulation of molecular chaperone expression. Those chaperones increase the cell's capacity to fold the proteins synthesised within the ER correctly. The pathway is tightly associated with the pathways for protein degradation and apoptosis in an event of prolonged stress. Several compounds have been identified to trigger the UPR by de-regulating proteostasis due to distinct causes. Kifunensin (KIF), a specific ER α-mannosidase inhibitor causes ER stress and leads to increased α-gal levels (Ishii et al., 2007) possibly due to an enhanced cellular protein processing capacity capable to stabilise mutants with an abnormal folding state and a simultaneous stall of the proteasomal degradation machinery. In 2011, Tunicamycine (TM) and Thapsigargin (Tg), two known ER stress inducing compounds were tested to enhance mutant GCase activity in Gaucher disease fibroblasts (Wang et al., 2011).

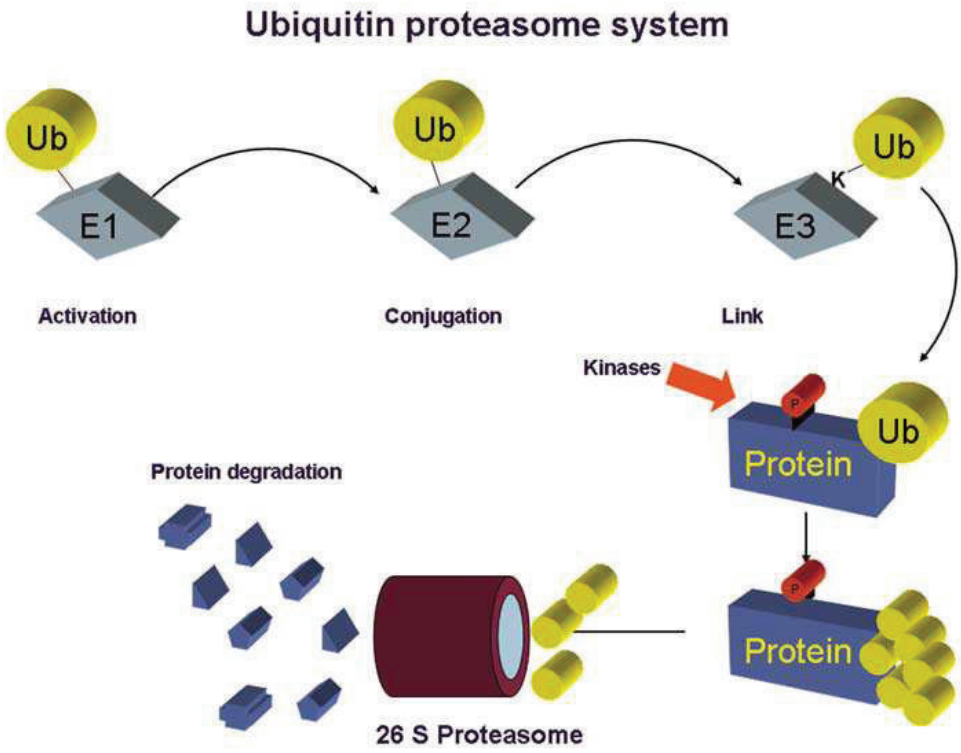


Figure 4: Protein modification by ubiquitination. Ubiquitination of a protein at its epsilon amine of the lysine (K) residues is the initial signal for degradation. Preceding this action, the molecule needs to be activated by E1 ubiquitin activating enzymes followed by the ubiquitin conjugation utilising E2 (ubiquitin conjugating enzymes) and E3 (ubiquitin ligases). The single ubiquitins are fused to each other *via* their K48 residues. Subsequently, the 19 S proteasomal subcomplex ("lid") recognises the ubiquitin chain, the ubiquitin is cut off the target protein and the 20 S proteasomal unit starts the degradation process (shown is the completely assembled 26 S proteasome complex consisting of two 19 S and one 20 S proteasomal subunit. Picture taken from Marfella et al., 2007.

1.2.2.4.1 Genetic modifier genes

The term genetic modifier stems from the finding that most hereditary diseases display a phenotypic variability not only allegeable by the monogenic cause of the disease. Those genes can exert influence on disease phenotype by their differential expression pattern in individual patients determining age of disease onset and severity of the course. In most diseases following Mendelian inheritance, a distinct genotype/phenotype correlation is not traceable, so for Fabry disease (Mehta et al., 2006). In the common sense, however, modifier genes describe genes whose alterations affect disease progression. In FD, the vitamin D receptor polymorphisms were found to modify the disease-specific severity score (Teitcher et al., 2008). Likely candidates with potential modifier gene function are interaction partners of the respective protein, thus α-gal. It was shown that mutant α-gal R301Q tended to have a stronger interaction with the molecular chaperone BiP/GRP78 than the normal enzyme (Yam et al., 2006). On the contrary, BiP/GRP78

was found in less contact with the GCase mutant L444P in Gaucher disease (Schmitz et al., 2005) compared to the normal enzyme allowing for two conclusions: (1) BiP/GRP78 assists the folding of both enzymes with different prosperity for the two illustrated cases or (2) BiP/GRP78 gives mutant enzymes over to their premature degradation. A recent study focused on candidate genes from this class of molecular chaperones to test their effectivity in therapeutic implementation (Wang et al., 2011). This approach was never assigned to FD. Moreover, other players influencing the processing and transport of α-gal are conceivable. The endocytic route (Figure 5) illustrates several bodies of intervention for genes heterologously expressed, or, in this case actuated *via* pharmacological agents assumed to affect expression regulation, that could beneficially influence lysosomal enzyme trafficking. After leaving the ER, further modulation of the sugar chains (e.g. phosphorylation), sorting and packaging in vesicles occurs in the trans golgi network (TGN). Mis-sorting and subsequent retention/secretion could be one cause for mutant α-gal to be prevented from proper cellular function. At last, the correct destination organelle must be accessed. Even inside the lysosome, α-gal depends on Saposin B for his part serving as a facility to make the cleavage point of high molecular hydrophobe material amenable (Kase et al., 2000).

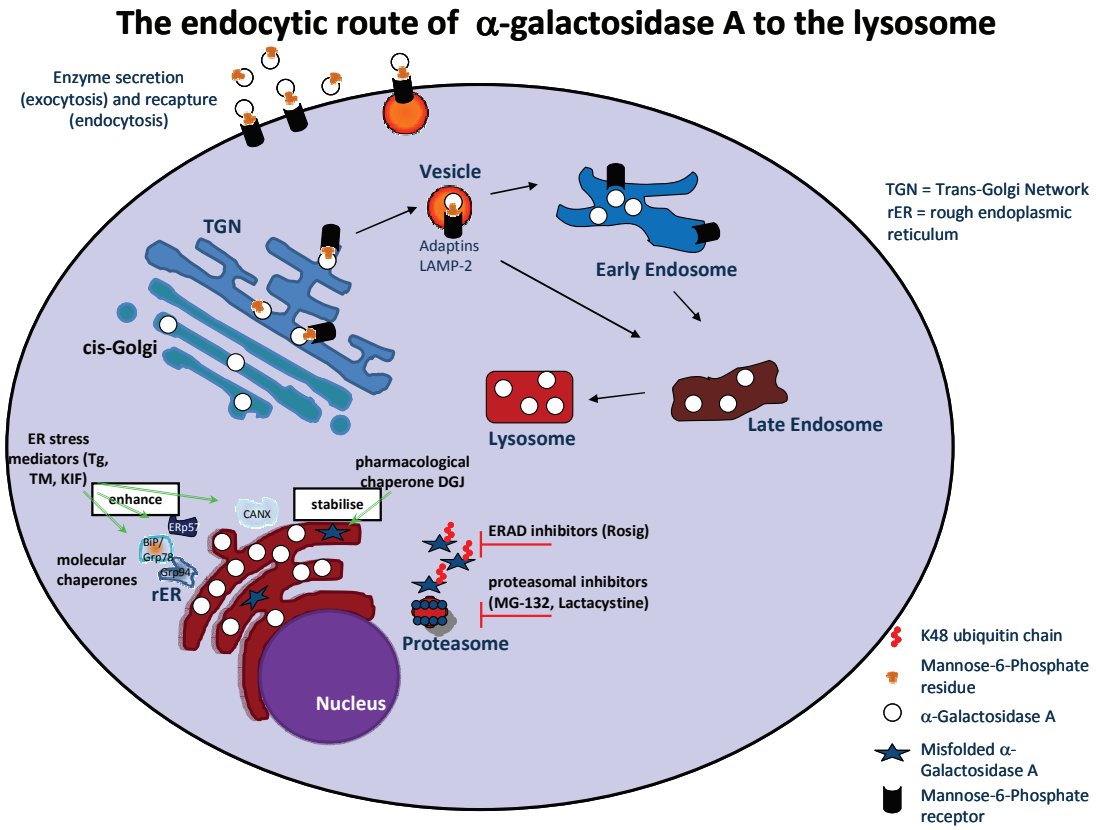


Figure 5: The endocytic route. Intracellular trafficking of α-galactosidase depends on a stable structure of the protein. Instable or wrongly folded enzyme is destined to be degraded by the Endoplasmic Reticulum-associated degradation (ERAD) machinery

1.3 Aim of the study

To our knowledge, all reported Fabry mutations leading to a complete abolishment of α-gal activity are related to the classic type of disease in males. However, when it comes to residual activity, the clinical picture diversifies and in light of the random inactivation of X-linked genes (Lyon, 1966) observed in females, clinical data are difficult to interpret. Herein, we aimed for a highly standardised *in vitro* overexpression system for the mutations to obtain a quantifiable model for the damage a given α-gal mutation bears. Furthermore, investigations were applied to examine whether enzyme activity is associated with the course of disease and whether a likely border of enzyme activity exists below which an onset of disease symptoms is inevitable and/or essentially related to the classical disease course. To do so, biomarker lyso-Gb3 was introduced as a clinical measure for a comparative analysis with the *in vitro* findings. The biomarker lyso-Gb3 believed to reflect clinical severity of the disease. Moreover, the model for α-gal mutations will be extended by computational prediction analysis (Polymorphism Phenotyping (PolyPhen) algorithm). Additionally, pharmacological agent (small molecule) treatment was supposed to reveal useful mechanisms that can be exploited to stimulate mutant α-gal activity above potential critical borders. The small molecule inhibitor DGJ is currently in clinical trials having been found to have pharmacological chaperone function and elevate the activity of degradation-prone mutations in FD while having no effect on catalytically damaged enzymes. However, about 60 % of the tested mutations were found to profit from the treatment. Mechanisms other than "chaperoning" mutant enzymes have been proposed in lysosomal storage disorders mostly aiming at an increased activity of the mutant enzyme of the specific disease. To test for such substances, the system of the present study delivers an ideal platform. Compiling the *in vitro*, *in vivo* and *in silico* knowledge about *GLA* mutations and the mechanistic properties of “hit” substances will add to the understanding of cellular enzyme procession and transport as potential therapeutic targeting and the chemical demands for chaperone constitution for future studies.

Moreover, the results of this study are of importance for clinicians to schedule disease management for the patients, because early diagnosis and correct therapeutic decisions (especially for pre-clinical and mild cases) can drastically ameliorate prognosis.

2 Materials and Methods

2.1 Materials

2.1.1 Technical equipment

Table 4: Technical equipment

Device	System	Company
aspiration pump	Laboport	KNF Neuberger, Freiburg im Breisgau, GER
balance	M-Pact AX224	Sartorius AG, Göttingen, GER
camera	DS2M	Nikon, Düsseldorf, GER
centrifuge	3K10	Sigma Zentrifugen, Osterode am Harz, GER
centrifuge	Microfuge 16	Backman Coulter GmbH, Krefeld, GER
centrifuge	Z 233 MK-2	Hermle Labortechnik,Wehingen, GER
cell counter	CASY®	Roche Diagnostics/Innovatis Bielefeld, GER
CO_2 incubator		Binder GmbH, Tuttlingen, GER
fluorescence microscope	Biozero	Keyence, Neu-Isenburg, GER
gel documentation	Argus X1 version 2.2.8	Biostep, Jahnsdorf, GER
gel electrophoresis system	Criterion™Cell	Bio-Rad, München, GER
Genetic Analyzer	3130 xl	Applied Biosystems, Darmstadt, GER
heating block	Thermomixer compact	Eppendorf, Hamburg, GER
ice maker	AF80	Scotsman Ice Systems, Mailand, ITA
incubation hood	IH 50	Noctua GmbH, Nössingen, GER
infrared imaging system	Odyssey	Li-Cor biosciences GmbH, Bad Homburg, GER
inverted microscope	Eclipse TS100	Nikon, Düsseldorf, GER
magnet stirrer	MSH-20A	Witeg Labortechnik GmbH, Wertheim, GER
microplate reader	GENios	Tecan, Mennedorf, SUI
multi channel pipet	Transferpette -8	Eppendorf, Hamburg, GER
multi step pipet	Multipette plus	Eppendorf, Hamburg, GER
orbital shaker	KH 2/500	Noctua GmbH, Nössingen, GER
orbital shaker	KM-2 Akku	Edmund Bühler GmbH, Hechingen, GER
PCR cycler	Mastercycler gradient	Eppendorf, Hamburg, GER
pH meter	Mettler	Toledo, Gießen, GER
pipets	research/reference	Eppendorf, Hamburg, GER
power supply	PowerPac™ HC	Bio-Rad, München, GER
power supply	PowerPac 200	Bio-Rad, München, GER
protein transfer apparatus	TransBlot® SD Cell	Bio-Rad, München, GER

real time PCR cycler	LightCycler 1.5	Roche Applied Sciences, Penzberg, GER
sterile working bench	Antares 48	Biohit, Köln, GER
touch vortex mixer	vortex-genie 1	Scientific industries, inc., Bohemia, USA
ultrapure water device	type HP5 UF	TKA Wasseraufbereitungssysteme GmbH, Niedererlberg, GER
UV transilluminator		Herolab GmbH Laborgeräte, Wiesloch, GER
water bath (+shaker function)	WNB14	Memmert GmbH & Co KG, Schwabach, GER

2.1.2 Chemicals

2.1.2.1 Buffers

SDS-Page gel electrophoresis buffer (10X)

30.3 g Tris

141 g glycine

10 g SDS

ad 1l H_2O dest.

Tris buffered saline (TBS) (10X)

1 M Tris pH 7.5

1.5 M NaCl

Laemmli sample buffer (5X)

3,03 g Tris

10 ml glycerol (87%)

10 g SDS or 5 ml 20 % sol.

2,5 ml 2-mercaptoethanol

1 % Bromophenol blue sodium salt

ad 50 ml H_2O dest.

Tris acetate EDTA (TAE) buffer (50 X)

242 g Tris base
57 ml acetic acid } Tris-acetate 2 M

100 ml 0,5 M EDTA (50 mM final), pH 8

ad 1l H_2O dest.

SDS-transfer buffer (1X)

5,82 g Tris

2,93 g glycine

3.75 ml 10 % SDS

200 ml Methanol

ad 1l H_2O dest.

RIPA buffer

20 mM Tris pH 7.4

137 mM NaCl

0.1 % SDS

0.5 % Sodium deoxycholic acid

1 % Triton X-100

10 % Glycerol

2 mM EDTA

Add 1 tablet complete, Mini, EDTA-free

Protease Inhibitor Cocktail tablets directly before use

Glycine-NaOH buffer

a) NaOH 1 M

b) Glycine (Glykokoll buffer substance acc. Sörensen)

Mix both solutions to obtain pH 10.5

Phosphate citrate buffer (for α-galactosidase A)

0.06 M pH 4.7

a) Na_2HPO_4 x $2H_2O$ buffer substance acc. Sörensen

b) acetic acid 0.03 M

mix solutions to obtain pH 4.7

add 0.02 % sodium azide

Sodium acetate buffer

200 mM sodium acetate

adjust pH with acetic acid to 4.0

add 0.02 % sodium azide

for substrate sol. add 10 mg 4-methylumbelliferyl-α-D-glucopyranoside in 7.7 ml buffer. Incubate at 60 °C until the substrate is completely dissolved

Phosphate citrate buffer (for glucocerebrosidase)

0.06 M pH 4.5 (cell-based assay)/5.8 (inhibition test)

a) Na_2HPO_4 x $2H_2O$ buffer substance acc. Sörensen

b) acetic acid 0.03 M

mix solutions to obtain pH 4.5/5.8

add 0.02 % sodium azide and 0,15 % Triton X-100

for substrate sol. add 17 mg 4-methylumbelliferyl-β-D-glucopyranoside in 10 ml buffer.

2.1.2.2 Biochemicals

1-Deoxygalactonojirimycine	Sigma Aldrich, St. Louis, USA
2-mercaptoethanol	Invitrogen, Carlsbad, USA
4-methylumbelliferone	Sigma Aldrich, St. Louis, USA
4-methylumbelliferyl α-D-galactopyranoside	Sigma Aldrich, St. Louis, USA
4-methylumbelliferyl α-D-glucopyranoside	Sigma Aldrich, St. Louis, USA
4-methylumbelliferyl β-D-glucopyranoside	Sigma Aldrich, St. Louis, USA
Agalsidase alfa	Shire Human Genetic Therapeutics, Dublin, Ireland
Ambroxol hydrochloride	Sigma Aldrich, St. Louis, USA
ampicillin sodium salt	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Bromhexine hydrochloride	Sigma Aldrich, St. Louis, USA
bromophenol blue sodium salt	Carl Roth GmbH & Co KG, Karlsruhe, Germany
complete, Mini, EDTA-free Protease Inhibitor Cocktail tablets	Roche Applied Sciences, Penzberg, Germany
D (+)- galactose cell culture grade	AppliChem GmbH, Darmstadt, Germany
DTT	Sigma Aldrich, St. Louis, USA
EDTA	Carl Roth GmbH & Co KG, Karlsruhe, Germany
glycerol (87 %)	Merck KGaA, Darmstadt, Germany

Imiglucerase	Genzyme Therapeutics, Cambridge, USA
kanamycin sulfate	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Kifunensin	Merck, KGaA, Darmstadt, Germany
LB agar (Luria/Miller)	Carl Roth GmbH & Co KG, Karlsruhe, Germany
LB medium (Luria/Miller)	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Lipofectamine 2000	Invitrogen, Carlsbad, USA
MassRuler DNA Ladder Mix	Fermentas, St. Leon-Rot, Germany
MG-132	Merck, KGaA, Darmstadt, Germany
Nonidet P-40 (NP-40)	Merck, KGaA, Darmstadt, Germany
peqGOLD Protein-Marker IV	Peqlab Biotechnologie GmbH, Erlangen, Germany
protein G PLUS-Agarose	Santa Cruz biotechnology, inc., SC, USA
SDS	Sigma Aldrich, St. Louis, USA
skim dry milk	Sigma Aldrich, St. Louis, USA
sodium deoxycholic acid	Sigma Aldrich, St. Louis, USA
Thapsigargin	Sigma Aldrich, St. Louis, USA
Triton X-100	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Tunicamycin	Sigma Aldrich, St. Louis, USA
Tween-20	Serva, Heidelberg, Germany
Vectashield® mounting medium with DAPI	Vector Laboratories Ltd., Peterborough, UK
zeocin	Invitrogen, Carlsbad, USA

Other routinely used chemicals were purchased from Merck, Sigma and Roth unless indicated otherwise

2.1.2.3 Cell culture solutions and media

DMEM High Glucose (4.5 g/l)	+ L-Glutamine, + sodium	PAA, Cölbe, Germany
fetal bovine serum (FBS)		PAA, Cölbe, Germany
Opti-MEM		Invitrogen, Carlsbad, USA
PBS		Biochrom AG, Berlin,
PDL		Sigma Aldrich, St. Louis, USA
penicillin/streptomycine (100X)		Invitrogen, Carlsbad, USA
trypsin/EDTA 0.05 %		PAA, Cölbe, Germany
trypsin/EDTA 0.25 %		PAA, Cölbe, Germany

2.1.3 Life Science Kits

BCA protein assay kit	Thermo Scientific, Braunschweig, Germany
CopyCutter™ EPI400™ Chemically Competent *E. coli*	Illumina, Inc., San Diego, USA
Criterion precast 4-15 % Tris-HCl gels	Bio-Rad, München, Germany
DNA clean & concentrator™-25 kit	Zymo Research, Freiburg im Breisgau, Germany
FastLane cell cDNA kit	Qiagen GmbH, Hilden, Germany
LightCycler® DNA Master SYBR Green I	Roche Applied Sciences, Penzberg, Germany
QiaFilter plasmid Maxi kit	Qiagen GmbH, Hilden, Germany
QiaFilter plasmid Midi kit	Qiagen GmbH, Hilden, Germany
QuantiTect Primer Assay	Qiagen GmbH, Hilden, Germany
S.N.A.P. UV-free gel purification kit	Invitrogen, Carlsbad, USA

TOPO® TA cloning® Kit Dual Promotor	Invitrogen, Carlsbad, USA
Zyppy Plasmid Miniprep Kit	Zymo Research, Freiburg im Breisgau, Germany

2.1.4 Antibodies

Table 5: Primary and secondary antibodies.

	primary antibodies		
anti-α-Galactosidase A (polyclonal)	rabbit		Eurogentec, Liège, Belgium
anti-α-Galactosidase A (polyclonal)	rabbit	[H-104]	Santa Cruz biotechnology inc., SC, USA
GAPDH	mouse	[6C5]	abcam, Cambridge, UK
K48-linkage Specific Polyubiquitin	rabbit	4289 S	New England Biolabs, Ipswich, USA
anti-V5 epitope tag	mouse		Invitrogen, Carlsbad, USA
	secondary	**Antibodies***	
IRDye800 anti mouse IgG (H+L)	goat		Rockland Immunochemicals, Gilbertsville, USA
Alexa Fluor 680® anti mouse IgG (H+L)	goat		Molecular Probes, Carlsbad, USA
IRDye800 conjugated affinity purified rabbit IgG (H+L)	goat		Rockland Immunochemicals, Gilbertsville, USA
Alexa Fluor® 680 anti rabbit IgG (H+L)	goat		Molecular Probes, Carlsbad, USA
IRDye 680LT anti rabbit	goat		Li-Cor, biosciences GmbH, Bad Homburg, Germany
Alexa Fluor® 488 anti rabbit IgG (H+L)	goat		Molecular Probes, Carlsbad, USA
Alexa Fluor® 568 anti mouse IgG (H+L)	goat		Molecular Probes, Carlsbad, USA

*** secondary antibodies were applied 1:1,000 for immunocytochemistry and 1:10,000 for Western blot unless stated otherwise**

2.1.5 Cell lines

Table 6: Cell lines

cell line	**origin**	**provider**
Fabry B-lymphocyte GLA[A143T]	patient blood	prepared in-house
Fabry B-lymphocyte GLA[N215S]	patient blood	prepared in-house
HEK293H	human embryonic kidney	Invitrogen, Carlsbad, USA
GM00882	human fibroblast	Coriell cell repository, Camden, USA
GM00852	human fibroblast	Coriell cell repository, Camden, USA

2.1.6 Plasmid vectors

See method: **Cloning of genes in mammalian expression vectors** below

2.1.7 Enzymes

Antarctic Phosphatase	New England Biolabs, Ipswich, USA
T4 DNA Ligase	New England Biolabs, Ipswich, USA
iProof High Fidelity DNA Polymerase	Bio-Rad, München, Germany
Klenow Large Fragment (3´- 5´exo) DNA Polymerase I	New England Biolabs, Ipswich, USA
PfuUltra DNA Polymerase	Stratagene, La Jolla, USA
Taq DNA Polymerase	Qiagen GmbH, Hilden, Germany

2.1.7.1 Restriction enzymes

Table 7: Restriction enzymes.

Enzyme name	restriction/recognition site	company
Apa I	5´...GGGCC▼C...3´ 3´...C▲CCGGG...5´	New England Biolabs, Ipswich, USA
*Bam*H I	5´...G▼GATCC...3´ 3´...CCTAG▲G...5´	New England Biolabs, Ipswich, USA
*Bst*B I	5´...TT▼CGAA...3´ 3´...AAGC▲TT...5´	New England Biolabs, Ipswich, USA
Hind III	5´...A▼AGCTT...3´ 3´...TTCGA▲A...5´	New England Biolabs, Ipswich, USA
Not I	5´...GC▼GGCCGC...3´ 3´...CGCCGG▲CG...5´	New England Biolabs, Ipswich, USA
Xba I	5´...T▼CTAGA...3´ 3´...AGATC▲T...5´	New England Biolabs, Ipswich, USA
Xho I	5´...C▼TCGAG...3´ 3´...GAGCT▲C...5´	New England Biolabs, Ipswich, USA
Dpn I	CH_3 5´...GA▼TC...3´ 3´...CT▲AG...5´ CH_3	Stratagene, La Jolla, USA

2.2 Methods

2.2.1 Assay setup and selection of mutations

The determination of mutant enzyme activity was carried out using a heterologous overexpression system in cultured HEK293H cells. The cells are easily to transfect with foreign DNA and were utilised (among other cells) in similar studies (Parenti et al., 2007; Wu et al., 2011, Valenzano et al., 2011). The plasmid vector employs the commonly used cytomegalovirus (CMV) promoter for strong expression in HEK293H cells (Qin et al., 2010). 171 mutations were investigated with regard to residual enzyme activity and degradation status (see next paragraph) at first in order to evaluate the degree and the nature of the damage, respectively. Among the mutations tested, 50 had not been described before. 158 missense mutations, 6 nonsense mutations causing immediate polypeptide abort, 4 small deletions (1-2 nucleotides) and 3 small insertions (1-4 nucleotides) were selected for the present study (Table 10). Selection criteria contained:

(1) The mutation was novel or not well described (no biochemical activity data were available; N=138)

(2) The mutation led to a single amino acid substitution (N=158/171)

(3) Patient-derived data (phenotype severity, biomarker lyso-Gb3) were available (N=105/171)

(4) Three sites (Arg118, Ser126, Asp264) were selected for “permuting” the native amino acid into every possible residue resulting from single nucleotide exchanges. R118C and S126G are involved in variant forms of the FD phenotype and found to be more prevalent (Spada et al., 2006, own data). D264V/Y are involved in classical FD (N=15/171)

Measures for residual enzyme activity were connected with a test for responsiveness to the PC 1-deoxygalactonojirimycine since this analysis has important implications for the clinical feasibility of PCT in patients harbouring the respective mutations.

2.2.2 Cloning of genes in mammalian expression vectors

For the present study overexpression plasmids for the following genes had to be generated:

Table 8: Commercial cDNA clones used for cloning in mammalian overexpression plasmids.

gene symbol	RefSeq (GeneBank)	protein name	origin cDNA clone (where applicable)
GAA	NM_000152.3	acid glucosidase	IRATp970C0971D
GLA	NM_000169.2	α-galactosidase A	IRAUp969H0320D

Typically, a full open reading frame (ORF) cDNA clone was obtained from ImaGenes (Berlin, Germany) or Origene (Rockville, USA). Genes were amplified using ORF-specific primers introducing unique restriction sites for subsequent cloning (see Appendix) *via* polymerase chain reaction (PCR). PCR was carried out with iProof DNA polymerase (Bio-Rad, München, Germany). The amplified fragments were incubated with Taq DNA polymerase to generate terminal Adenosin overhangs for TOPO TA cloning with the pCR II TOPO vector Kit (Invitrogen, Carlsbad, USA) according to the manufacturer's manual. After ligation, chemically competent *E. coli* cells from strain XL10 Gold were transformed with vector suspensions and plated on antibiotica containing selective agar dishes. Post-incubation overnight at 37°C, clones were picked, DNA was isolated and plasmids were analysed by restriction digestion for the desired gene insertion. If the expected band pattern was visualised on a 1 % TAE agarose gel, the clone(s) was/were subjected to sequence analysis on a 3130 xl Genetic Analyzer in order to adjust with the respective RefSeq (see table). For this purpose, the Vector NTI software 10.3 was used (Invitrogen, Carlsbad, USA). A clone harbouring sequence identity was used to subclone in a mammalian expression vector using standard protocols.

Table 9: Mammalian vector system used for heterologous expression.

vector name	**supplier**	**expression of…**
pcDNA3.1/V5-His_6	Invitrogen	GLA, GAA

2.2.3 Site-directed mutagenesis of α-galactosidase A and acid α-glucosidase

Expression vectors harbouring α-galactosidase A or acid α-glucosidase mutations were generated by site-directed PCR mutagenesis using the QuikChange® II XL Site-Directed Mutagenesis Kit according to the manufacturer's instructions. Sequence modifications were individually introduced by PCR amplification with *Pfu*Ultra DNA polymerase; the pcDNA3.1/GLA or pcDNA3.1/GAA plasmid vectors containing the wild type sequences of the two genes served as templates. Primer sets contained 25-37-mers with sense and antisense primers carrying one of the respective mutations central to their length (a complete list of primers can be seen in the Appendix section). Each mutant plasmid was sequenced on a 3130 xl Genetic Analyzer.

2.2.4 Transient transfection of HEK293H cells

1.5×10^5 cells were seeded 24 hours before transfection in each well of a 24 well culture plate using 500 µl DMEM medium supplemented with 10 % FBS. Transient expression of mutant enzymes in HEK293H cells was carried out using Lipofectamine 2000 transfection reagent according to the manufacturer's protocol. Typically, prior to transfection, a mixture of plasmid

DNA (0.8 µg) and Lipofectamine 2000 transfection reagent (2 µl) in 100 µl of serum-free DMEM or Opti-MEM medium was incubated at room temperature for 20 min and applied to the cells thereafter. The cells were subsequently incubated for 6 hours at 37 °C, the medium containing the transfection reagent was removed and 500 µl fresh DMEM was added. In this step, the respective treatment was added where intended. One additional medium (including treatment) removal was performed another 48 hours later. Overall, cells were incubated for 66 hours post transfection and 60 hours post treatment start unless indicated differently (see scheme below).

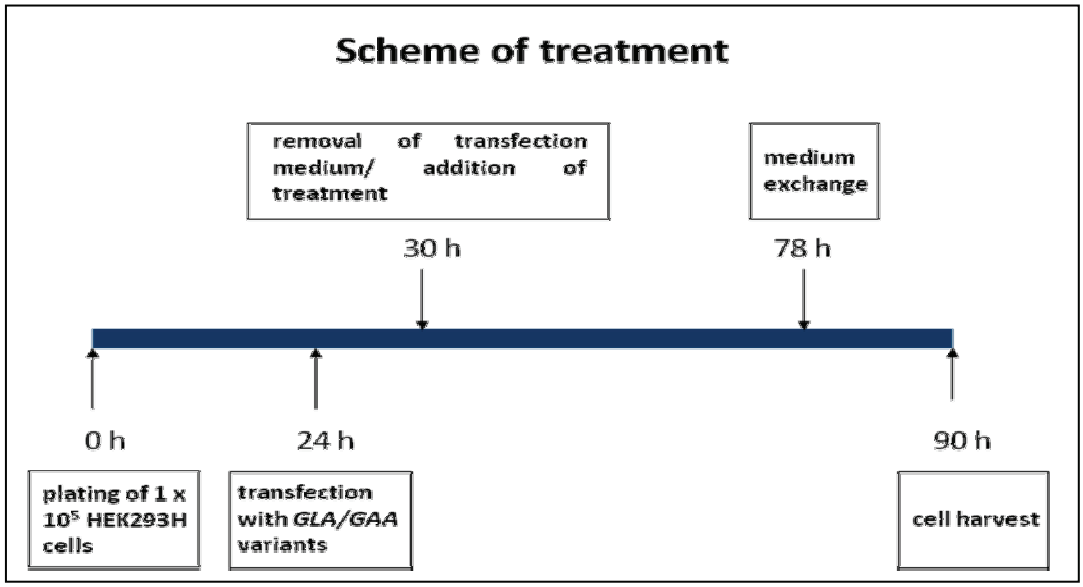


Figure 7: Scheme of treatment for the transient transfection experiments

2.2.5 Enzymatic measurement of overexpressed α-galactosidase A/acid α-glucosidase

Cell pellets obtained from confluently grown 24 well cell culture plates were homogenised in 200 µl water and subjected to 5 freeze-thaw cycles using liquid nitrogen. The supernatant collected after centrifugation of the homogenate at 10000 x g for 5 min was used in enzyme assays. Protein concentration was measured with the BCA protein assay kit according to the manufacturer´s manual. 10 µl of the cell lysates at a concentration of 50 µg/ml (α-gal) and 500 µg/ml (α-gluc) were assayed with 20 µl of 4-methylumbelliferyl-α-D-galactopyranoside (4-methylumbelliferyl-α-D-glucopyranoside) in 0.06 M phosphate citrate buffer, pH 4.7 (0.025 M sodium acetate, pH 4.0). Enzyme reactions were terminated by the addition of 0.2 ml of 1.0 M glycine buffer (pH 10.5), prepared by adjusting the pH using 1.0 M NaOH. Released 4-methylumbelliferone (4-MU) was determined by fluorescence measurement at 360 and 465 nm as the excitation and emission wavelengths respectively, using a microplate fluorescence reader (Tecan, Männedorf, Switzerland). The measured enzyme activity was analysed using the Magellan™ data analysis

software v6.6 calculated as nmol 4-MU/mg protein and normalised to one hundred percent wild type activity. The α-gal/α-gluc activity limit of quantification (LOQ, "0") in HEK293H cells was defined by a 95 percentile calculation from the empty vector transfections.

2.2.6 Enzymatic measurement of glucocerebrosidase in patient-derived fibroblasts

Patient fibroblasts (GM00852, allele 1: N370S, allele 2: 1 bp Ins, 84GG) were seeded at a density of 5 x 10^4 per well of a 6 well cell culture plate. After allowing the cells to adhere, treatment started the following day for 3 continuing days. Cells were harvested in Trypsin/EDTA (0,25%) and pelleted for homogenisation in 100 µl water with 0.25 % Triton X-100 and mechanically destroyed as described above. Protein content of the samples was determined and 9 µg whole protein was assayed in 0.06 M phosphate citrate buffer, pH 4.5 containing 1 mM 4-methylumbelliferyl-ß-D-glucopyranosid analogous to the α-gal activity measurement. Reactions were also stopped and the fluorescence determined, accordingly.

2.2.7 Glucocerebrosidase inhibition assay

40 munits of recombinant GCase (Imiglucerase, Cerezyme®, Genzyme Corporation, Cambridge, MA, USA) were dissolved in 0.06 M citrate phosphate puffer (pH 5.8). Increasing compound concentrations were added and reactions were started by the addition of 4-methylumbelliferyl-β-D-glucopyranoside (5 mM final concentration). The mixture was incubated for 15 minutes in a 37°C water bath under slight agitation. The reaction was stopped using 0.2 ml of 1.0 M glycine buffer (pH 10.5). The activity was determined as described above.

2.2.8 Western blot analysis

Western blot analysis for the detection of α-Gal A protein was performed using a custom made rabbit anti-α-Gal A polyclonal antibody from Eurogentec, Liège, Belgium (animals were immunized against peptides QRDSEGRLQADPQRFP (corresponding to amino acids 99-114) and KQGYQLRQGDNFEVWE (corresponding to amino acids 326-341). Either serum or IgG purified antibody samples from this production were taken. Furthermore, a mouse GAPDH monoclonal antibody 6C5 was used to visualise GAPDH as an internal loading control. Other target proteins are shown under **2.1.3**.

HEK293H cells were grown as described above and lysates were generated by aspirating the media from the 24 well culture plates, washing the cells once with 1 x PBS and directly applying 200 µl ice-cold RIPA buffer supplemented with protease inhibitor cocktail tablets prior to a 20 minute incubation on ice. Cells were then rinsed from the wells, transferred to microcentrifuge tubes and spun at 14000 x g for 10 minutes at 4°C to pellet debris. The supernatant was used for

the analysis. 50 µg protein were mixed with a suitable volume of 5 x Laemmli loading buffer, boiled for 5 minutes on a thermo shaker, centrifuged at 14000 x g for 10 minutes at 4°C and loaded on a Criterion precast 4-15 % Tris-HCl gel. Proteins were transferred to a nitrocellulose membrane (Amersham Hybond ECL, GE Healthcare, München, Germany). The membrane was blocked with 5 % (w/v) non-fat dried skimmed milk in TBS-Tween 20 at room temperature for 1 hour, and then treated with primary antibody in a milk/blot solution [3 % (w/v) non-fat dried skimmed milk in TBS-Tween 20] at 4°C overnight. The blot was then washed three times with excess TBS-Tween 20 and treated with a secondary antibody fluorescence label conjugated. Following extensive washing with TBS-Tween 20, protein bands were visualised by an Odyssey Infrared Imager. Quantification and protein size determination were performed using the Odyssey software.

2.2.9 Immunocytochemistry

$7x10^4$ HEK293H cells were plated on PDL coated coverslips. The next day, cells were transfected with the respective pcDNA3.1 vector carrying V5-epitope tagged wild type or mutant *GLA* cDNA. Immediately where intended, DGJ was added and the cells were grown for an additional 48 hours. After cultivation, cells were washed in PBS 3 times, and fixed in -20°C methanol for 5 minutes. After rinsing the cells with PBS again, cells are blocked and perforated with 5% normal goat serum, 0,3% Triton X-100 in PBS over night. Primary mouse monoclonal anti V5 antibody was added in 62.5 ng/µl for 1 hour. Cells were washed 3 times in PBS and secondary goat anti-mouse (Alexa Fluor 568) was added 1:1000. After that, mouse monoclonal antibody against LAMP2 (abcam) was added in the concentration 1:200. 1 additional hour of incubation was followed by another three-step wash procedure with PBS and secondary goat anti mouse antibody (Alexa Fluor 488) for the detection of lysosomal LAMP2 protein was added 1:1000. After washing again, mounting was taken out using DAPI-containing medium and analysed using fluorescence microscopy (Keyence BZ-8000K and the provided software).

2.2.10 Determination of kinetic properties of α-galactosidase A mutants

α-gal mutants were overexpressed in HEK293H cells. 60 hours after transfection in 24 well plates, the cells were harvested in 100 µl H_2O and the contents of two wells were pooled. Cells were lysed by 5 freeze-thaw cycles and subsequent centrifugation to obtain a cell-debris free lysate. Quantitative Western blot analysis was carried out to reveal the wild type : mutant ratio for each mutant. The amount of lysate required to obtain equal amounts of enzyme was calculated. Respective quantities of α-gal enzyme were subjected to the kinetic evaluation (Beutler et al., 1972). Substrate concentrations of 0.5, 1, 2, 4, 8 and 28 mM were assayed with constant amount

of enzyme in a Lineweaver Burk plot in order to obtain kinetic parameters. To prove that equal amounts of input enzyme (mutants and wild type) were employed for the assay, α-Gal content of the lysates was controlled with another Western blot (figure 8A). For standard curve, 5, 10, 20, 50 and 100 ng of agalsidase alfa (Replagal™, Shire Human Genetic Therapeutics, Dublin, Ireland) were subjected to Western blot analysis and fluorescence values were plotted (figure 8B). In the individual experiments between 2 and 6 ng of enzyme were employed.

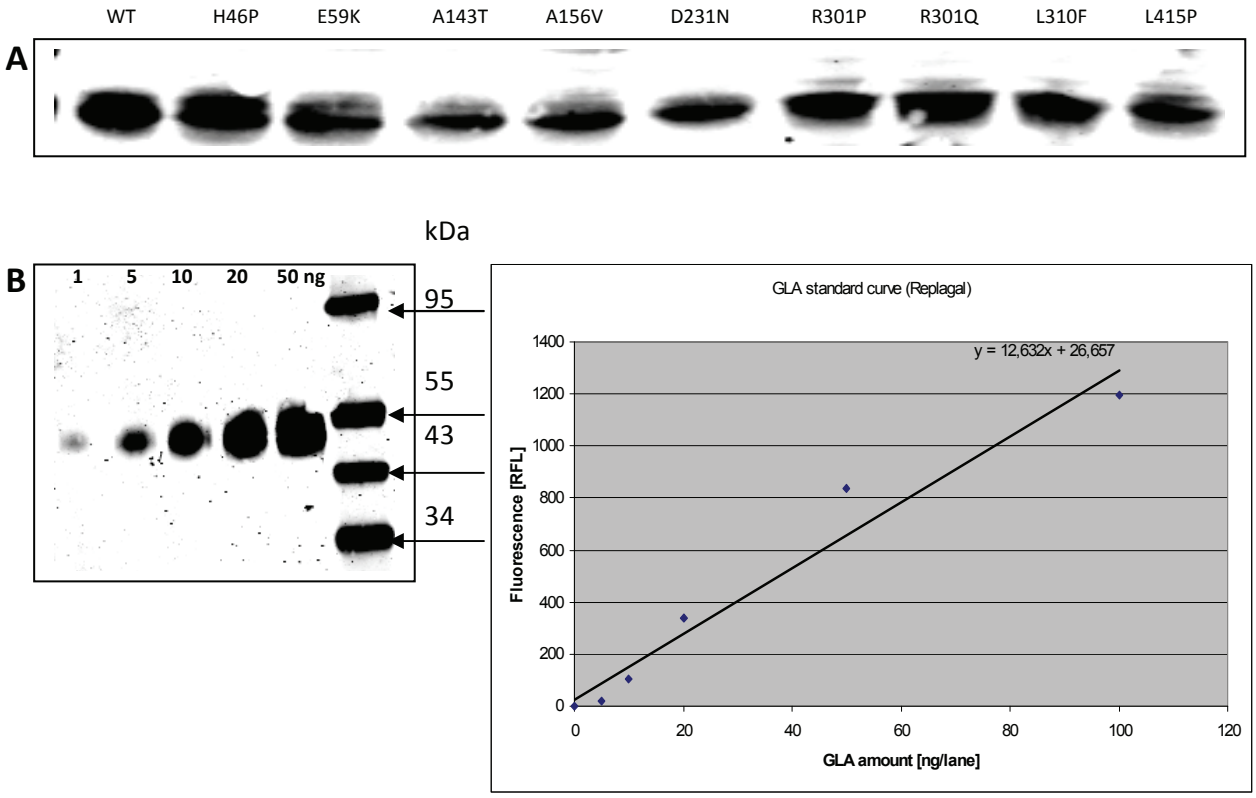


Figure 8: A. Control Western Blot. Semiquantitative analysis of α-Gal level determined by Western blot was used to assure similar amounts of enzyme was applied for examination of kinetic properties of the mutant enzymes. **B**. Recombinant enzyme agalsidase alfa used for enzyme replacement therapy (Shire Human Genetics Therapies, Dublin, Ireland) was used to estimate total enzyme amount subjected to the kinetic assay.

2.2.11 Statistical evaluation

All results are shown as mean ± SD/SEM of data as indicated in each diagram. N indicates the number of independent repeats. Statistical analysis was performed with a Student's t-test using Excel (Microsoft, USA) and Prism5 (GraphPad Prism Inc., USA).

The following analyses were applied with statistical support from Ulrike Grittner, PhD, Charité - University Medicine, Berlin, Department of Biometrics and Clinical Epidemiology / Centre for Stroke Research:

(1) Sperman's rho (r_s) rank correlation coefficient to test associations between α-gal level and enzyme activity

(2) linear trend tests to test associations between two ordinal variables, (e.g. *in vitro* enzyme activity and lyso-Gb3 (female/male) classes) and

(3) ordinal regression with only one of the four different prediction measures as the independent variable and with 'clinical phenotype' as the outcome for determination of the predictive values of *in vitro* enzyme activity, female lyso-Gb3 values, male lyso-Gb3 values, PolyPhen-scores and DGJ responsiveness. All analyses were done using IBM SPSS Statistics, Release 20.0.0 (© SPSS, Inc., 2011, Chicago, IL, www.spss.com).

3 Results

3.1 Characterisation of α-galactosidase A mutations

3.1.1 Residual *GLA* activity determination

α-gal activity was determined for 171 mutations. Virtually, the Fabry causing mutations are located along the whole molecule, no particular hot spot could be detected. Therefore, the mutations were picked correspondently representing the functional domains of the enzyme (e.g. ligand binding site, homodimerisation site). 158 were missense mutations, 6 nonsense mutations causing immediate polypeptide abort, 4 small deletions (1-2 nucleotides) and 3 small insertions (1-4 nucleotides) were examined. Among the tested mutations, 50 (29.2 %) had not been previously described (note: except for R118C, S126G and D264V/Y, the p.Arg118, p.Ser126 and p.Asp264 substitutions had not been found in patients yet). The whole spectrum of residual activity was detected. About 52.5 % of the missense mutations had no residual enzyme activity, a further 15.8 % had less than 20 % activity covering about 2/3 of all mutations tested. Table 10 summarises parameters of the analysed mutations. As stated (**2.2.1**), DGJ responsiveness was tested alongside identifying 42.8 % responders among the missense mutants under the directive that a responsive mutation had to gain a 1.5-fold increase or at least 5 % above the untreated state. Mutations in sites Arg118, Ser126 and Asp264 gave rise to enzymes that displayed an overall minor (if any) increase of activity through DGJ treatment indicating that the moieties are generally situated in sites where treatment with the PC does not result in significant α-gal activity elevation and that the type of amino acid substitution is less important (exception: D264N).

3.1.2 Introduction of clinical and computational parameters

For a full-blown analysis of the mutations, available data was implemented into the study involving clinical as well as computational parameters. Plasma lyso-Gb3 values from female and male patients were assembled and literature enquiry revealed the clinical presentation of the mutations. PolyPhen (polymorphism phenotyping) analysis (Ramensky et al., 2002) was run to consider the predictive power of a computational algorithm compared to the *in vitro* enzyme activity measurement. The applied algorithm incorporates three components in its scoring:

1) assessing level of sequence conservation over evolutionary time

2) physico-chemical properties of changed amino acid

3) proximity of changed amino acid to predicted structural and functional domains

To make sure that lyso-Gb3 was an adequate FD measure the pathological cut-off was statistically set to a value considerably higher than in healthy individuals (95th percentile of healthy individuals, fig. 9). The median values of the patient/proband cohorts showed values above the pathological cut-off of 0.9 ng/ml (determined for both genders), in particular 4.0 ng/ml for females and 26.9 ng/ml for males. The cut-off was calculated by 95th percentile determination of healthy individuals. It is noteworthy that lyso-Gb3 levels in males are approximately 10 times higher than in females on average. The gender-dependence of lyso-Gb3 values in hemizygote males and heterozygote females carrying the same mutation did not seem to affect the virtue of statistical biomarker investigation. However, there seem to be mutations displaying an overall inconspicuousness of some mutations (e.g. S126G, A143T, D313Y) on the biomarker level as indicated by the coloured dots in figure 9. For those mutations, the lyso-Gb3 values were gender-independently non-pathogenic except for two individuals harbouring mutation D313Y (female 3.4 ng/ml; male, 3.6 ng/ml) and one individual harbouring mutation A143T (male, 1.6 ng/ml). The mean value of 59 individuals harbouring mutation D313Y and 19 individuals harbouring mutation A143T was below pathological cut-off. The sensitivity, specificity and accuracy to identify the assigned clinical phenotype (classic, variant, classic/variant) for each mutation was 1.00, 0.5 and 0.85 for female and 1.0, 0.5 and 0.86 for male biomarker, respectively.

Table 10: Overview of mutations tested *in vitro* for enzyme activity.

Amino acid	cDNA	*in vitro* enzyme activity [% WT] in mean ± SEM (N)		Ratio DGJ/unt reated	Lyso-Gb3 [mean (N)]		clinical phenotype	PolyPhen‡	Reference
		-DGJ	+DGJ		male	female			
E7X	c19G>T	0(3)	0(3)	n.d.					Dobrovolny (2008) Am Soc Hum Genet Meet Abs . 150
A15E	c44C>A	0(3)	0(3)	n.d.	40.7 (2)			benign	Terryn (2012) Int J Cardiol epub
W24C	c72G>T	45.6±11.2(3)	89.8±21.0(3)	2.0				benign	own data
G35R	c103G>C	17.9±2.1(3)	72.3±4.7(3)	4.0	68.9 (3)	6.8 (4)	variant	probably damaging	Davies (1994) Hum Mol Genet 3:667
L36S	c107T>C	0(3)	0(3)	n.d.				probably damaging	Erdos (2008) Mol Genet Metab 95:224
A37T	c109G>A	69.6±13.6(3)	132.9±27.8(3)	1.9	1.6 (1)			probably damaging	Sadick (2007) Heart Lung Circ 16, 200
M42V	c124A>G	0(3)	11.9±2.0(3)	n.d.	111.0 (1)		classic	probably damaging	Davies (1996) Eur J Hum Genet 4:219
G43S	c127G>A	0(3)	0(4)	n.d.				probably damaging	Kwan (2007) Am Soc Hum Genet Meet Abs . 1537
H46P	c137A>C	40.1±1.2(3)	98.8±2.1(3)	2.5				benign	Hwu (2009) Hum Mutat 30:1397
R49G	c145C>G	0(3)	0(3)	n.d.	75.8 (1)	7.3 (3)	classic	probably damaging	Germain (2002) Mol Med 8:306
R49C	c145C>T	0(3)	5.1±0.5(3)	n.d.	89.9 (1)	21.5 (1)		probably damaging	Shin (2008) Pharmacogenet Genomics 18, 773
M51K	c152T>A	0(4)	8.7±4.4(4)	n.d.			classic	benign	Ashley (2001) J Hum Genet 46:192
M51I	c153G>A	37.4±5.9(7)	62.0±16.2(3)	1.7			variant	benign	Spada (2006) Am J Hum Genet 79:31
C52W	c156C>G	0(3)	0(3)	n.d.				probably damaging	own data
E59K	c175G>A	2.2± 0.5(8)	18.5±2.9(4)	14.2			classic	benign	Eng (1994) Hum Mol Genet 3:1795
C63Y	c188G>A	0(3)	0(3)	n.d.	77.3 (1)	5.4 (2)		probably damaging	Schafer (2005) Hum Mutat 25:412
S65I	c194G>T	0(4)	11.2±1.2(4)	n.d.				probably damaging	own data
E66K	c196G>A	6.8±0.3(3)	18.3±2.3(3)	2.7	6.1 (2)		classic	probably damaging	Schafer (2005) Hum Mutat 25:412
L68F	c202C>T	0(3)	4.5±1.8(3)	n.d.	68.4 (1)		classic	probably damaging	Shabbeer (2002) Mol Genet Metab 76:23
A73V	c218C>T	44.0±8.0(8)	64.7±8.3(3)	1.5			variant	probably damaging	Spada (2006) Am J Hum Genet 79:31
K82X	c244A>T	0(3)	0(4)	n.d.					Dobrovolny (2008) Am Soc Hum Genet Meet Abs . 150
D83N	247G>A	62.9±12.8(4)	71.6±9.7(4)	1.1		0.5 (1)	variant	probably damaging	own data
Y86D	c256T>G	0(3)	0(4)	n.d.				probably damaging	Dobrovolny (2008) Am Soc Hum Genet Meet Abs . 150

L89H	c266T>A	0(3)	0(3)	n.d.				possibly damaging	own data
p.D93fsX93	c276InsTGAT	0(3)	0(4)	n.d.					own data
D93Y	c277G>T	0(3)	0(3)	n.d.				probably damaging	Ebrahim (2012) J Inherit Metab Dis 35, 325
D93E	c279C>G	0(3)	0(3)	n.d.				possibly damaging	Burlina (2008) J Neurol 255, 738
W95L	c284G>T	0(3)	0(3)	n.d.				probably damaging	Dobrovolny (2008) Am Soc Hum Genet Meet Abs . 150
W95C	c285G>T	0(3)	0(3)	n.d.				probably damaging	own data
R100T	c299G>C	0(4)	0(4)	n.d.				probably damaging	Eng (1997) Mol Med 3:174
S102L	c305C>T	71.6±4.4(4)	78.9±8.3(4)	1.1		0,5 (1)		benign	own data
E103X	c307G>T	0(3)	0(4)	n.d.					Dobrovolny (2008) Am Soc Hum Genet Meet Abs . 150
R112C	c334C>T	0(5)	0(3)	n.d.	20.2 (1)	3,6 (5)	classic	probably damaging	Ishii (1992) Hum Genet 89:29
R112H	c335G>A	1.6±0.6(3)	19.4±2.3(3)	12.1	2.2 (2)	1.0 (1)	variant	probably damaging	Eng (1994) Hum Mol Genet 3:1795
R118S	c352C>A	76.2±9.5(3)	70.9±2.6(3)	n.d.				benign	*
R118G	c352C>G	37.6±4.1(3)	44.0±5.0(3)	1.2				benign	*
R118C	352C>T	20.0±1.3(3)	23.7±2.9(4)	1.2	0.6 (1)	0.5 (1)	variant	possibly damaging	Spada (2006) Am J Hum Genet 79:31
R118H	c353G>A	67.4±3.4(3)	66.9±3.1(3)	n.d.				benign	*
R118P	c353G>C	49.0±4.9(3)	47.7±2.0(3)	n.d.				benign	*
R118L	c353G>T	51.7±3.8(3)	51.4±4.0(3)	n.d.				benign	*
L120V	c358C>G	50.1±5.0(3)	62.0±2.6(3)	1.2				probably damaging	Chien (2009) Hum Genet 125:336 [2]
H125P	c374A>C	0(3)	0(3)	n.d.				probably damaging	Auray-Blais (2008) Mol Genet Metab 93:331
S126R	c376A>C	19.7±1.2(6)	23.5±2.3(4)	1.2				possibly damaging	*
S126G	c376A>G	51.3±9.6(4)	67.4±11.9(4)	1.3	0.5 (4)	0.5 (8)	variant	benign	Branton (2002) Medicine (Baltimore) 81:122
S126C	c376A>T	2.1± 0.4(3)	2.7±0.2(3)	n.d.				possibly damaging	*
S126N	c377G>A	14.2±2.4(3)	17.0±2.3(3)	1.2				benign	*
S126T	c377G>C	48.9±11.4(3)	42.8±7.9(4)	n.d.				benign	*
S126I	c377G>T	40.5±8.1(4)	43.2±7.7(4)	1.1				possibly damaging	*
L129P	c386T>C	0(3)	0(3)	n.d.	72.1 (2)	7.9 (2)	classic/variant	probably damaging	Whybra (2001) J Inherit Metab Dis 24:715
L131P	c392T>C	0(3)	0(3)	n.d.	68.6 (4)	7.0 (2)	classic	probably damaging	Eng (1994) Hum Mol Genet 3:1795
G132R	c394G>A	0(3)	0(3)	n.d.			classic	probably damaging	Shabbeer (2002) Mol Genet Metab 76:23
G132E	c395G>A	0(3)	0(3)	n.d.	119.0 (1)	12.3 (1)		probably damaging	Kwan (2007) Am Soc Hum Genet Meet Abs .:1537
A135V	c404C>T	0(3)	6.9±2.4(3)	n.d.	104.2 (2)		classic	benign	Shabbeer (2005) Hum Mutat 25:299

D136E	c408T>A	0(3)	31.3±3.2(3)	n.d.	42.3 (1)	6.6 (2)		probably damaging	own data
G138R	c412G>A	0(3)	0(3)	n.d.		3.6 (1)	classic	probably damaging	Eng (1997) Mol Med 3:174
N139S	c416A>G	147.8±28.3(3)	176.4±36.4(3)	1.2	88.6 (1)	3.0 (4)	variant	benign	Havndrup (2010) Eur J Heart Fail 12:535
T141I	c422C>T	0(3)	0(3)	n.d.			classic	probably damaging	Shabbeer (2002) Mol Genet Metab 76:23
C142R	c424T>C	0(3)	0(3)	n.d.			classic	probably damaging	Topaloglu (1999) Mol Med 5:806
A143T	c427G>A	31.3±5.6(7)	49.4±4.8(6)	1.6	0.5 (8)	0.5 (12)	classic/variant	benign	Eng (1997) Mol Med 3:174
A143P	c427G>C	0(3)	0(5)	n.d.			classic	possibly damaging	Eng (1994) Hum Mol Genet 3:1795
G147R	c439G>A	0(3)	0(3)	n.d.		16.2 (1)		probably damaging	Schafer (2005) Hum Mutat 25:412
A156D	c467C>A	0(3)	0(3)	n.d.				probably damaging	own data
A156V	c467C>T	4.3±1.0(9)	16.8±2.3(5)	3.9			classic	probably damaging	Okumiya (1995) Hum Genet 95:557
W162G	c484T>G	0(3)	5.2±1.2(3)	n.d.	42.5 (3)	6.8 (3)		probably damaging	own data
D165H	c493G>C	3.4±1.1(3)	11.9±1.1(3)	3.5		3.7 (1)		probably damaging	Rakoczi (2007) Orv Hetil 148:1087
D165Y	c493G>T	0(3)	0(3)	n.d.				probably damaging	own data
D165V	c494A>T	0(4)	0(4)	n.d.	9.1 (1)		classic	probably damaging	Davies (1994) Hum Mol Genet 3:667
D170N	c508G>A	0(3)	0(3)	n.d.	72.1 (1)	4.8 (1)		probably damaging	own data
C172G	c514T>G	0(3)	4.4±2.0(3)	n.d.	59.3 (1)	21.7 (1)	classic	probably damaging	Yasuda (2003) Hum Mutat 22:486
C172Y	c515G>A	0(3)	0(3)	n.d.	80.2 (1)	5.6 (1)	classic	probably damaging	Eng (1994) Hum Mol Genet 3:1795
p.C174fsX 175	c521InsT	0(3)	0(3)	n.d.		5.9 (2)			own data
D175N	c523G>A	70.0±2.1(3)	84.3±8.5(3)	1.2				benign	own data
D175E	c525C>G	89.8±2.2(3)	89.0±7.3(3)	n.d.		0.5 (1)		benign	own data
p.D175fsX 184	c525 526delCA	0(3)	0(3)	n.d.					own data
G183V	c548G>T	0(3)	6.7±2.1(4)	n.d.				probably damaging	Dobrovolny (2008) Am Soc Hum Genet Meet Abs . 150
M187T	c561G>C	0(3)	0(4)	n.d.		2.9 (1)	classic	possibly damaging	Shabbeer (2006) Hum Genomics 2:297
L191P	c572T>C	0(3)	0(3)	n.d.		4.9 (1)	classic	probably damaging	Cooper (2000) Hum Genet 107:535
W204C	c612G>C	0(3)	4.4±0.9(3)	n.d.		8.9 (1)		probably damaging	own data
K213M	c638A>T	83.4±29.6(5)	82.5±15.5(5)	n.d.	9.3 (1)			Possibly damaging	own data
N215S	c644A>G	39.5±1.5(6)	63.9±3.3(6)	1.6	4.2 (7)	1.1 (6)	variant	benign	Davies (1993) Hum Mol Genet 2:1051
I219T	c656T>C	53.3±2.2(3)	85.3±10.0(3)	1.6				benign	Hwu (2009) Hum Mutat 30:1397
R220Q	c659G>A	104.5±11.3(4)	144.0±11.9(4)	1.4				benign	own data

N224S	c671A>G	31.1±2.4(3)	82.2±2.2(3)	2.6		5.3 (3)	classic	probably damaging	Ashton-Prolla (2000) J Investig Med 48:227
H225D	c673C>G	32.2±8.1(3)	60.5±19.1(3)	1.9	2.8 (1)			possibly damaging	own data
H225R	c674A>G	0(7)	3.0±0.8(4)	n.d.			classic	possibly damaging	Politei (2005) Rev Neurol 41:506
R227Q	c680G>A	0(3)	0(3)	n.d.	53.7 (5)	11.2 (3)	classic	probably damaging	Eng (1993) Am J Hum Genet 53:1186
D231N	c691G>A	0(3)	0(3)	n.d		5.9 (1)	classic	probably damaging	Redonnet-Vernhet (1996) J Med Genet 33:682
I232T	c695T>C	11.5±2.8(3)	61.6±7.2(5)	5.1				possibly damaging	Tai (2012) Clin Chim Acta 413, 422
W236C	c708G>C	0(3)	0(3)	n.d.	95.8 (1)	7.8 (2)	classic	probably damaging	Davies (1996) Eur J Hum Genet 4:219
S238N	c713G>A	36.0±6.7(4)	94.3±24.5(4)	2.6	6.5 (1)		variant	probably damaging	Monserrat (2007) J Am Coll Cardiol 50:2399
I242N	c725T>A	3.1±1.4(4)	49.8±13.9(4)	16.1			classic	probably damaging	Takata (1997) Brain Dev 19:111
R252T	c755G>C	117.0±10.0(4)	134.3±22.5(4)	1.1		0.5 (4)		benign	own data
I253S	c758T>G	4.4±1.6(5)	53.4±7.1(5)	10.3				probably damaging	own data
G261V	c782G>T	0.2±1.4(5)	3.5±1.1(5)	n.d.	84.4 (2)			probably damaging	own data
W262C	c786G>C	0(3)	0(3)	n.d.	9.4 (1)	7.7 (4)	classic/variant	probably damaging	Schafer (2005) Hum Mutat 25:412
D264N	c790G>A	37.9±3.7(3)	84.5±6.4(3)	2.2				probably damaging	*
D264H	c790G>C	1.7±0.9(3)	10.2±2.9(3)	6.0				probably damaging	*
D264Y	c790G>T	0(3)	5.4±2.9(3)	n.d.	98.9 (1)	13.7 (3)	classic	probably damaging	Shabbeer (2005) Hum Mutat 25:299
D264A	c791A>C	0(3)	0(3)	n.d.				probably damaging	*
D264G	c791A>G	0(3)	0(3)	n.d.				probably damaging	*
D264V	c791A>T	0(3)	0(3)	n.d.	59.3 (1)		classic	probably damaging	Eng (1993) Am J Hum Genet 53:1186
D264E	c792C>A	0(3)	0(3)	n.d.				probably damaging	*
M267T	c800T>C	27.5±4.4(4)	30.5±1.8(4)	1.2	2.7 (1)			probably damaging	own data
L268S	c803T>C	0(3)	10.8±0.7(3)	n.d.	18.0 (1)	3.0 (1)	classic	probably damaging	Schafer (2005) Hum Mutat 25:412
V269M	c805G>A	0(4)	17.3±1.4(4)	n.d.			classic	probably damaging	Shabbeer (2006) Hum Genomics 2:297
V269A	c806T>C	9.0±1.4(9)	45.0±4.3(4)	5.0			classic	possibly damaging	Davies (1993) Hum Mol Genet 2:1051
p.V269fsX 281	c807delG	0(3)	0(3)	n.d.					own data
I270M	c810T>G	2.3±1.7(4)	33.7±16.8(4)	14.7					own data
N272S	c815A>G	0(3)	0(3)	n.d.			classic	probably damaging	Verovnik (2004) Eur J Hum Genet 12:678
F273L	c819T>A	0(6)	0(3)	n.d.			variant	probably damaging	Zhang. SH et al.. 2007
L275F	c823C>T	0(4)	0(3)	n.d.		3.9 (1)	classic/variant	probably damaging	Dobrovolny (2008) Am Soc Hum Genet Meet Abs . 150

L275H	c824T>A	0(5)	0(5)	n.d.				probably damaging	own data
T282I	c845C>T	5.0±0.5(3)	47.7±0.7(3)	9.5	22.6 (2)			possibly damaging	own data
Q283P	c848A>C	0(3)	0(3)	n.d.			classic	probably damaging	Shabbeer (2006) Hum Genomics 2:297
A285D	c854C>A	0(3)	0(3)	n.d.			classic	probably damaging	Shabbeer (2006) Hum Genomics 2:297
W287X	c860G>A	0(3)	0(3)	n.d.					Davies (1993) Hum Mol Genet 2:1051
M290L	c868A>T	18.5±8.2(3)	22.4±9.6(3)	1.2	0.8 (1)			probably damaging	Ferri (2012) Clin Genet 81, 224
M290I	c870G>A	39.0±1.8(3)	70.5±7.2	1.8			classic	probably damaging	Shabbeer (2006) Hum Genomics 2:297
A291T	c871G>A	13.2±3.4(3)	55.7±0.7(3)	3.7				probably damaging	Zhang (2007) J Nephrol 20:716
L294X	c881T>G	0(4)	0(3)	n.d.					Blaydon (2001) Hum Mutat 18:459
L294S	c881T>C	0(3)	12.4±1.7(3)	n.d.		6.6 (2)		probably damaging	own data
p.F215fs316X	c883delT	0(3)	0(3)	n.d.					Dobrovolny (2008) Am Soc Hum Genet Meet Abs . 150
S297C	c890C>G	0(3)	3.8±0.7(3)	n.d.	121.0 (1)		classic	probably damaging	Germain (2002) Mol Med 8:306
R301G	c901C>G	19.3±4.1(7)	56.5±3.4(3)	2.9			classic	probably damaging	Lai (2001) Hum Genet 109:469
R301Q	c902G>A	8.5±1.8(9)	48.0±2.6(3)	5.6		2.2 (1)	classic/variant	probably damaging	Sakuraba (1990) Am J Hum Genet 47:784
R301P	c902G>C	0(4)	5.0±1.3(3)	n.d.		10.7 (1)	classic	probably damaging	Ashley (2001) J Hum Genet 46:192
L310F	c928C>T	0(4)	4.1±1.1(3)	41.0			classic	probably damaging	Calado (2004) Hum Genet 115:347
L311V	c931C>G	1.9±0.4(3)	40.1±4.9(3)	n.d.	53.6 (1)			probably damaging	own data
D313Y	c937G>T	83.9±21.1(4)	100.3±23.9(3)	1.2	0.5 (27)	0.5 (36)	variant	possibly damaging	Eng (1993) Am J Hum Genet 53:1186
V316I	c946G>A	65.6±22.3(3)	68.3±21.8(3)	n.d.	0.7 (1)		variant	benign	own data
V316G	c947T>G	0(3)	0(3)	n.d.	103.0 (1)	13.0 (1)		probably damaging	own data
p.V316fsX332	c948InsT	0(3)	0(3)	n.d.	75.1 (1)	5.8 (2)			own data
I319T	c956T>C	20.2±3.8(3)	58.3±12.6(3)	2.9		3.0 (2)		probably damaging	Sirrs (2010) Mol Genet Metab 99, 367
N320I	c959A>T	2.0±0.5(3)	31.8±8.9(3)	17.7		2.9 (1)	classic	probably damaging	Schafer (2005) Hum Mutat 25:412
Q321H	c963G>C	3.3±1.9(3)	25.3±7.1(3)	7.7				probably damaging	own data
G325S	c973G>A	25.6±9.6(3)	55.4±14.4(3)	2.2				probably damaging	own data
Q327K	c979C>A	0(4)	0(5)	n.d.			classic	probably damaging	Davies (1993) Hum Mol Genet 2:1051
Q327E	c979C>G	21.5±2.8(3)	80.9±10.0(3)	3.8		1.7 (1)	classic/variant	probably damaging	Schafer (2005) Hum Mutat 25:412
G328A	c983G>C	6.2±1.0(3)	30.0±6.4(3)	4.8			classic	probably damaging	Eng (1993) Am J Hum Genet 53:1186
E341K	c1021G>A	0(3)	0(3)	n.d.	64.4 (2)		classic	probably damaging	Shabbeer (2002) Mol Genet Metab 76:23

E341D	c1023A>C	0(3)	0(3)	n.d.			classic	probably damaging	Shabbeer (2002) Mol Genet Metab 76:23
R342Q	c1025G>A	0(4)	0(3)	n.d.		2.6 (3)	classic	possibly damaging	Ploos van Amstel (1994) Hum Mol Genet 3:503
R342L	c1025G>T	0(3)	0(3)	n.d.	48.3 (2)	3.0 (2)		possibly damaging	own data
L344P	c1031T>C	0(3)	0(3)	n.d.	24.9 (1)		classic	probably damaging	Schafer (2005) Hum Mutat 25:412
S345P	c1033T>C	0(3)	13.3±3.8(3)	n.d.	16.7 (1)		classic	benign	Schafer (2005) Hum Mutat 25:412
W349R	c1045T>A	1.6±1.4(4)	8.9±3.9(4)	5.6				probably damaging	own data
R356W	c1066C>T	16.9±2.3(9)	62.7±3.9(3)	3.7		2.3 (2)	classic	possibly damaging	Bernstein (1989) J Clin Invest 83:1390
R356Q	c1067G>A	89.1±5.0(3)	99.4±4.3(3)	1.1				benign	Hwu (2009) Hum Mutat 30:1397
G360C	c1078G>T	11.9±0.5(3)	26.5±2.7(3)	2.2				probably damaging	Lin (2007) Hum Genet 122:212
G361R	c1081G>C	0(3)	3.7±1.1(3)	n.d.			classic	probably damaging	Davies (1993) Hum Mol Genet 2:1051
R363H	c1088G>A	31.9±2.9(8)	57.9±5.5(3)	1.8			classic/variant	possibly damaging	Cooper (2000) Hum Genet 107:535
A368T	c1102G>A	103.7±33.6(3)	93.3± 5.0(3)	n.d.		0.5 (3)			own data
L372P	c1115T>C	0(3)	2,6±0,7(4)	n.d.				probably damaging	Ebrahim (2012) J Inherit Metab Dis 35, 325
L372R	c1115T>G	0(3)	0(3)	n.d.				probably damaging	Dobrovolny (2008) Am Soc Hum Genet Meet Abs . 150
G373D	c1118G>A	0(4)	0(4)	n.d.				probably damaging	Germain (2001) Hum Mutat 17:353
C378R	c1132T>C	0(3)	0(3)	n.d.				probably damaging	Shin (2001) ASHG Annual Meeting Abstracts. 1799
I384N	c1151T>A	0(3)	0(3)	n.d.	25.1(1)	1.5 (1)	classic	probably damaging	Shabbeer (2002) Mol Genet Metab 76:23
T385A	c1153A>G	45.0±3.7(3)	48.9±7.5(3)	1.1		0.5 (1)		benign	own data
Q386P	c1157A>C	0(3)	0(3)	n.d.			classic	possibly damaging	Shabbeer (2006) Hum Genomics 2:297
P389L	C1166C>T	0(3)	0(3)	n.d.	72.9 (1)			n.a.	own data
V390M	c1168G>A	0(3)	0(4)	n.d.				benign	own data
G395A	c1184G>C	20.1±1.7(6)	23.1±2.0(6)	1.1				probably damaging	Bono (2011) BMC Res Notes 4, 323
F396Y$^{\Phi}$	c1187T>A	87.6±14.2(4)	93.8±15.7(4)	1.1				benign	Novo (1995) Nuceic Acids Research 23. 2636-40
E398A	c1193A>C	48.9±4.7(3)	62.5±7.8(3)	1.3				benign	own data
S405R	c1213A>C	91.0±10.2 (3)	92.7±11.1 (3)			0.5 (1)		probably damaging	own data
p.I407fsX 417	c1221delA	0(4)	0(3)	n.d.		8.2 (2)			own data
T410I	c1229C>T	2.3±0.8(4)	16.1±3.5(4)	n.d.				possibly damaging	own data
L415F	c1243C>T	83.2±5.9(3)	99.5±12.2(3)	1.2		0.5 (1)		benign	own data
L415P	c1244T>C	0(3)	0(3)	n.d.		4.7 (1)		probably damaging	Serebrinsky (2006) Hum Genet 119:361
Q416X	c1246C>T	0(3)	0(3)	n.d.	27.7 (3)	6.7 (1)			own data

Q416P	c1247A>C	0(3)	0(3)	n.d.		benign	own data
E418G	c1253A>G	74.6±15.5(3)	89.1±4.9(3)	1.2	0.5 (1)	benign	own data

Table 10: Mutant enzyme properties. Number of experiments (enzyme activity) and patient numbers (lyso-Gb3) are indicated in brackets. Note: Even though females have much lower values of lyso-Gb3, the 6 mutations that caused no elevated mean lyso-Gb3 value in males likewise caused no pathogenic elevation in females (where data was applicable), indicating that these mutations may not lead to an accumulation. However, the issue of age dependence needs to be addressed separately. A clinical estimation of the novel mutations occurring in the stroke or kidney disease study cohorts was only conducted in personal consultation with the institutional doctor.

[‡] PolyPhen analysis was provided courtesy of Dr. Viatcheslav Saviouk, Centogene GmbH, Rostock, GER

*Mutations have not been described in patients yet

[Φ]F396Y was terminated from HGMD. Not a genomic mutation is responsible for the finding. As underlying mechanism RNA editing was proposed

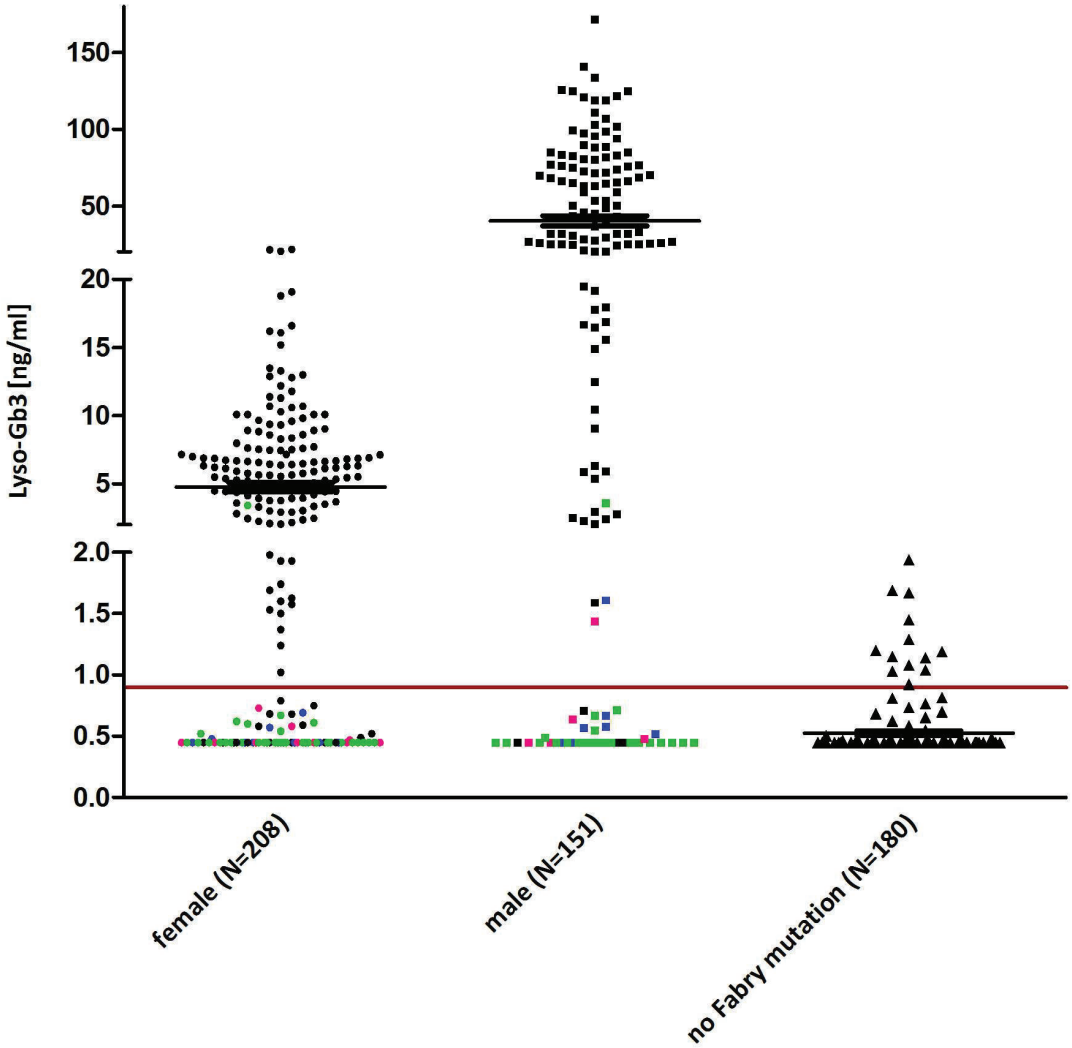


Figure 9: Lyso-Gb3 values for female and male Fabry patients compared to healthy controls. The red line indicates the pathogenic cut-off (0.9 ng/ml plasma). Each data point represents one patient. Indicated in pink are patients with the mutation S126G (8f/5m), in blue A143T (10f/9m) and in green D313Y (34f/25m) to illustrate that most found non-pathogenic mutations belong to either one or the other patient cohort. Other exceptions are: R118C, V316I, E418G (one male patient each) and A20P, D83N, I91T, S102L, R112C, R118C, D175E, G325S, A368T, T385A, W399X, c.1208delT, L415F, (one female patient each) and R252T (4X), N215S (3X). About 180 healthy probands were tested with no Fabry gene variation and had values below 0.9 ng/ml (95 % percentile calculation).

At a first glimpse, high lyso-Gb3 values and critical PolyPhen prediction apparently match low residual enzyme activity (table 10). In the following this was statistically controlled.

3.1.3 Compliance of enzyme measurement with *in vivo* and *in silico* data?

The function of many enzymes is understood very well. In some cases the knowledge about the biochemical and biophysical properties of proteins are used to predict their potential damage upon mutation. The damage state of α-gal was examined computationally before (Saito et al., 2010) and the developed algorithm matched significantly the clinical phenotype. However, this

analysis was applied to known mutations in order to distinguish between classic and atypical mutations. In the present study it was attempted to find a rationale whether a mutation will cause the disease or not. To do so, common disease-related parameters, repeatedly proposed to indicate phenotype severity were collected, opposed and studied.

Table 11: Association of *in vitro* enzyme activity and clinical and computational parameters.

	enzyme activity				
	0 %	>0 - 20 %	≥20 - 60 %	≥60 %	p (for linear trend test)
disease phenotype					
classic	42	8	2	0	<0.001
classic / variant	3	1	3	0	
variant	1	2	6	4	
lyso-Gb3 male (class)					
I	9	0	0	1	<0.001
II	16	3	0	0	
III	4	2	3	1	
IV	0	0	3	3	
lyso-Gb 3 female (class)					
I	5	0	0	0	<0.001
II	13	1	0	0	
III	9	4	4	1	
IV	0	0	4	7	
PolyPhen (class)					
benign	5	2	12	13	<0.001
possibly damaging	8	7	4	1	
probably damaging	68	14	11	2	
DGJ responsiveness					
not responsive (n=90)	68	5	8	8	<0.001
responsive (n=69)	14	24	20	11	

Table 11: Solid statistical association of enzyme activity and other parameters indicative for Fabry disease. Similar sized lyso-Gb3 classes are: I: > 80.0 ng/ml (m), >10.0 ng/ml (f); II: 20.0-80.0 ng/ml (m), 5.0-10.0 ng/ml (f); III: 0.9-20.0 ng/ml (m), 0.9-5.0 ng/ml (f); IV: 0.0-0.9 (m/f).

In a first step it was shown that the measurement of overexpressed α-gal reproduced the enzyme defect adequately. The *in vitro* enzyme activity measurement was associated to the clinical (biomarker divided in female and male) and computational parameters investigated in order to

investigate the coherence among one another. Herein, for simplification, the parameters were grouped in 3-4 “severity categories”. For the categorisation of lyso-gb3-values we used the following cut-points: class I: lyso-Gb3 ≥ 80/10 ng/ml for males/females, class II: lyso-Gb3 20-80/5-10 ng/ml for males/females, class III: lyso-Gb3 0.9-20/0.9-5 ng/ml for males/females, class IV: lyso-Gb3 < 0.9 ng/ml (for males/females).

The matrix (Table 11) illustrates the value of the proposed factors under investigation. The p-values for the performed linear trend test implicated high significance ($p<0.001$).

Finally, to compare the predictive values of female lyso-Gb3 values, male lyso-Gb3 values, *in vitro* enzyme activity and PolyPhen-scores with regard to clinical phenotype on the mutation level we used ordinal regression with only one of the four different prediction measures as the independent variable and with ’clinical phenotype‘ as the outcome. Enzyme activity showed the highest rates of correct classification both for 72 mutations where the clinical estimate was available (data set 1) and for 21 mutations for which all four measures (PolyPhen scores, lyso-Gb3 for females, lyso-Gb3 for males and enzyme activity) were available (data set 2, table 12). The classification rates were 83 % and 86 % for the two subsets of mutations, respectively. Lyso-Gb3 for males and PolyPhen scores showed good prediction quality either: PolyPhen scores led to a slightly higher classification rate of 76 % in the subset compared to 71 % for lyso-Gb3 values for males. With 67 % in the subset of mutations lyso-Gb3 values for females showed the lowest rate of correctly classified mutations.

Table 12: Phenotype prediction quality of distinct parameters

	Lyso-Gb3 for females	**Lyso-Gb3 for males**	***In vitro* enzyme activity**	**Polyphen classes**	**DGJ responsiveness**
data set 1	**N=33**	**N=32**	**N=72**	**N=72**	**n=72**
Nagelkerkes R^2	0.44	0.41	0.53	0.12	0.14
-2 Log Likelihood	12.44	14.43	15.41	16.13	17.37
(df)	(3)	(3)	(3)	(2)	(2)
Mutations correct classified	69.7%	78.1%	83.3%	73.6%	72.2%
data set 2	**N=21**	**N=21**	**N=21**	**N=21**	**n=21**
Nagelkerkes R^2	0.45	0.40	0.82	0.53	0.51
-2 Log Likelihood	12.91	13.44	4.18	7.80	11.38
(df)	(3)	(3)	(3)	(2)	(2)
Mutations correct classified	66.7%	71.4%	85.7%	76.2%	76.2%

Table 12: Comparison of prediction quality for *in vivo*, *in vitro* and *in silico* parameters (co-variates) with regard to phenotype severity (classic, variant, classic/variant) (outcome). Data set 1: All available data in the particular dimension. Data set 2: A subset of 21 mutations that have values in all dimensions (Ulrike Grittner, PhD, Charité University, Berlin).

3.2 Biochemical characteristics of the mutations

3.2.1 Responsiveness to the pharmacological chaperone DGJ

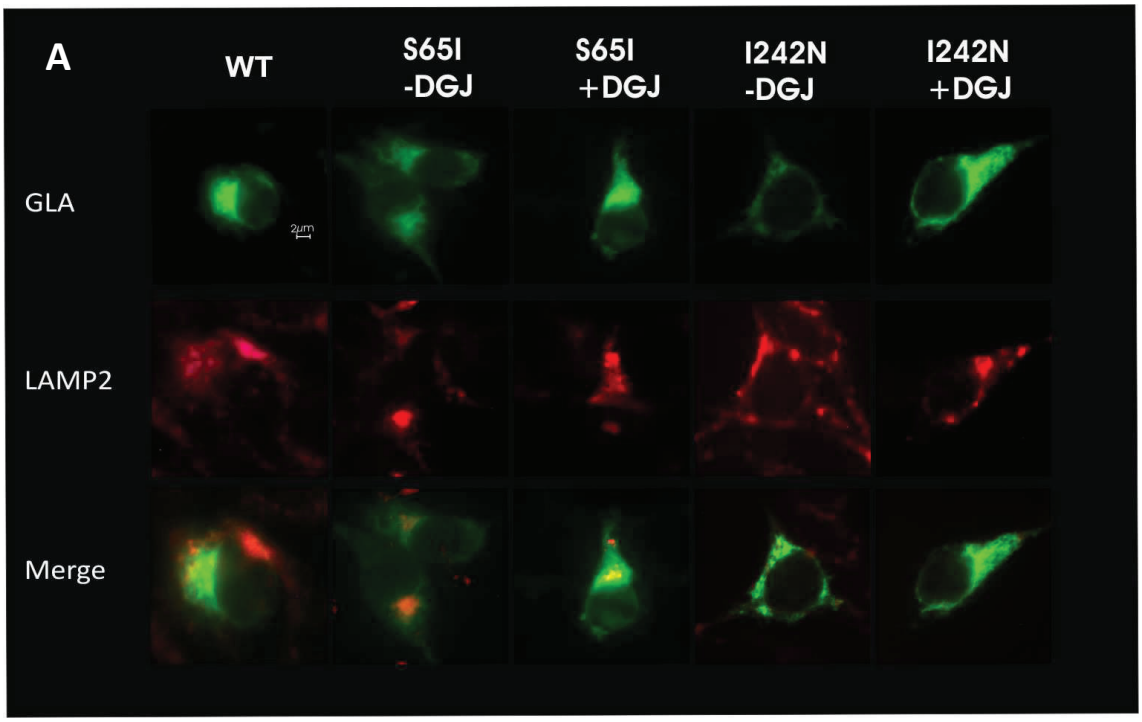
To date, this work is the biggest public source of investigated mutations, especially interesting for the high number of newly described ones. Therefore, we figured it was worthwhile to study the mutations with respect to their behaviour towards the pharmacological chaperone DGJ proposed as an alternative treatment strategy for misfolded and unstable Fabry mutations. As a first screening whether the mutations respond, a similar overexpression system had already proven effective recently (Wu et al., 2011). Measurement of residual plus DGJ-recovered activity of mutant α-gal enzyme revealed 68 (42.8 %) responsive mutations among the missense mutations. It was hypothesised that malfunctioning mutations with a total loss of activity are less responsive to PC treatment than mutations with a significant residual activity. This hypothesis was tested and the responder rate was lower among the severely affected enzymes. Only 14.5 % of class I mutations responded to the PC. Among classes II, III and IV, enzyme activities could be increased in the majority of the mutant enzymes (82.8, 71.4 and 63.4 % respectively). A reason for the drop in category IV could be that a high residual activity is not likely to gain 1.5-fold and a gain of 5-10 % is lost in background noise. Moreover, many of the substituted amino acids in classes III and IV mutations were located on the surface area (table 13) of the enzyme assuming no strong misfolding defect. Therefore, DGJ is not effective on those mutations. Low values for solven-accessible surface (< 1 $Å^2$) indicate that residues are buried in the molecule. The only strong DGJ responder among the mutations depicted in table 13 were I219T and M290I which are buried residues. However, another mutation at position Met290 (M290L, enzyme activity class II) did not respond to DGJ. Only V316I is buried in the molecule and was not responding to DGJ, but retained a high residual activity due likely to the conservative amino acid exchange from valine to isoleucine.

Figure 10 illustrates that DGJ-treated mutants travel along the endocytic route and co-localise with lysosomal marker LAMP2 in immuno stainings. The weaker punctuate signal without the treatment implies less distinct distribution to the target organelle, hence, mis-sorting. The weakness of the signal implies pre-mature degradation of the mutants. The pictures were taken with C-terminal V5-epitope-tagged mutants and in order to verify that this tag did not excessively affected the mutants behaviour, V5 tagged mutants were subjected to enzyme activity measurements and compared to unmodified *GLA* mutations (figure 10B).

Table 13: Mapping of substituted amino acids in mild α-gal mutations.

amino acid substitution	enzyme activity class	localisation	solvent-accessible surface area (Å^2)*	predicted defect**
D83N	class IV	surface	n.a.	opposite charge residue
S102L	class IV	surface	n.a.	exchange with non-polar res.
S126G	class III	n.a.	n.a.	H-bond ablation to Y123/V124
D175N	class IV	surface	n.a.	opposite charge residue
I219T	class III	buried	0.3	creation of new H-bond to N215/Y216
R220Q	class IV	surface	7.6	H-bond ablation to V256/A257 and P259/G260
R252T	class IV	surface, close to ligand binding site	n.a.	exchange with polar residue of opposite charge
M290I	class III	buried	0.8	n.a.
D313Y	class IV	surface	3.5	exchange with polar res.
V316I	class IV	buried	0.1	conservative exchange
T385A	class III	surface	7.2	exchange with non-polar res.
E398A	class IV	surface	16.2	exchange with non-polar res.
L415F	class IV	n.a.	n.a.	altered intramol. distance between β-barrels

Table 13: Location of amino acid residues substituted in mutant enzymes with ≥ 20 % activity (class III-IV). Computational modelling based on crystallographic data of the α-gal protein for 13 selected class IV mutations revealed that most amino acid residues were near-surface. * adapted from Garman et al., 2007; **data courtesy of Arseni Markoff, PhD, Institute of Medical Biochemistry and IZKF, University of Muenster (The PDB template used was 3lxb. Modelling of mutations on this structure and output visualization (not shown) were performed essentially as described in Markoff et al., 2009)



B

	normal [nmol 4-MU/hr/mg protein]		**V5-tagged** [nmol 4-MU/hr/mg protein]	
	- DGJ	**+DGJ**	**- DGJ**	**+DGJ**
WT	8195±2308	8512±428	5097±239	6709±790
S65I	0	835±476	0	463±298
S238N	2497±36	6804±288	959±139	4308±89
I242N	230±18	1877±339	194±8	1730±597
L275F	0	0	0	0
G395A	1897±151	2513±280	814±62	1070±34

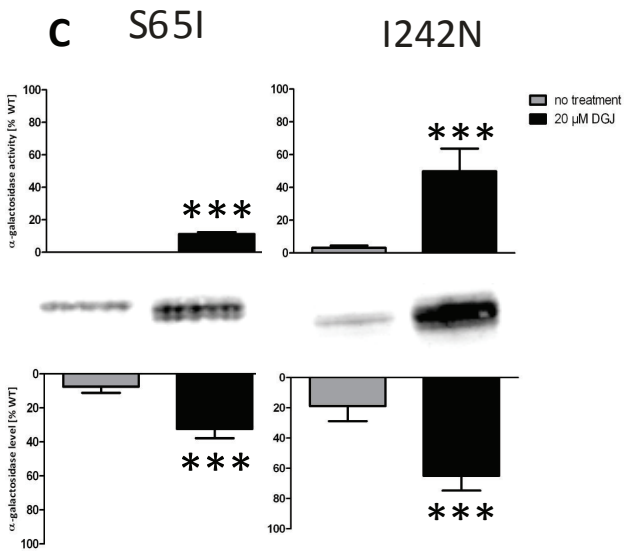


Figure 10: DGJ responsiveness of *GLA* mutations. **A**: Immunofluorescent images of HEK293H cells transfected with α-gal mutant cDNA. The data support the findings of the activity assays. Without DGJ treatment, mutant GLA staining occurred weak, fuzzy and among the whole cell surface instead of co-localised with late endosomal/lysosomal marker protein LAMP2. After DGJ treatment, staining of respective mutants became stronger and more pronounced **B**: Comparison of V5-epitope tagged and native α-gal enzymes. The enzyme activity was substantially decreased when the fusion protein was expressed, but the property of the mutants to respond to DGJ remained intact. **C**: Profile of the mutations S65I and I242N. In accordance with the cellular immunofluorescent images, enzyme activity (upper) and level (lower diagrams) of the mutants rose in dependence on DGJ treatment (black bars). ***p<0.005.

3.2.2 Correlation of enzyme activity and protein level

To collect further arguments for the applied classification, the nature of the defect for the mutations was analysed on the protein level. Mutant enzyme content was compared with the wild type level (examples see figure 10C). Mutations with an activity >6 % of the wild type

correlated with cellular α-gal protein level (Spearman correlation coefficient rs=0.866, p<0.001; figure 11). This evidences that mutants with significant residual activity mainly experience early degradation, because the remaining enzyme is catalytically active. On the contrary, mutations with no or very low activity often showed high amount of enzyme which cannot compensate for the loss of activity.

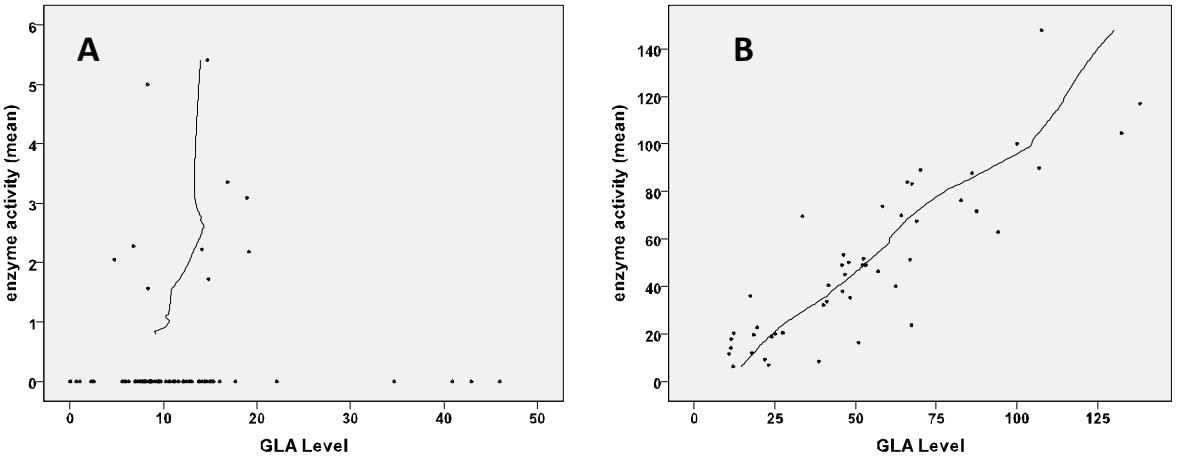


Figure 11: Correlation analysis of mutant enzyme activity and α-gal protein level (semi-quantitative Western blot). **A**: No correlation between protein level and residual activity for mutations possessing less than 6 % residual activity (n=76, Spearman correlation coefficient rs=0.128, p=0.272). **B**: For mutations possessing more than 6 % residual activity (n=48), the *in vitro* enzyme activity and enzyme levels correlated with each other (Spearman correlation coefficient rs=0.866, p<0.001). Diagrams courtesy of Ulrike Grittner

3.2.3 Kinetic properties of the mutant enzymes

The finding that most severe mutations apparently reflect changes in the catalytic core of the enzyme, but can, despite a reduced statistical probability, still be rescued with PC treatment made us think whether there exist transition states between degradation defect and catalytic abolishment. A kinetic assay revealed that most of the tested mutants (that had a significant residual activity -> see exclusion criteria in table 14 legend) had normal K_m and v_{max} values. The mutants E59K and H46P showed increased K_m values and R310P showed impaired v_{max}.

3.3 Pharmacological intervention in assembly and transport of defective α-galactosidase A

It could be demonstrated that many mutations benefited from DGJ administration. Splice site alterations, nucleotide insertions or deletions could not be identified as responders in the present study. Mutations affecting the active site were also excluded as DGJ responders. Still, there is no

evident explanation for a great number of DGJ “non-responders". In the present study, it was aimed to pharmacologically contribute to proper α-gal maturation and trafficking in order to characterise the nature of mutant α-gal defect and possible therapeutical targets.

Table 14: Kinetic properties of mutant α-gal enzyme.

Mutation	K_M (mM)	v_{max} (mmol MU/mg protein/h)
Agalsidase alfa	3.7	1.8
Wild-type	2.9	2.0
H46P	8.6*	2.8
R49G	n.d.	n.d.
E59K	5.0*	1.6
S65I	n.d.	n.d.
A143T	3.6	3.7
A156V	1.8	1.1
D231N	n.d.	n.d.
R301P	2.4	0.3*
R301Q	3.5	1.7
L310F	4.6	1.0
L415P	n.d.	n.d.

Table 14: Kinetic determination of mutant *GLA*. Asterisks indicate a significant change towards the wild type enzyme ($p<0.05$). Agalsidase alfa has been tested to validate consistency of the overexpressed (wild type) and a recombinant, high-purity formulation enzyme. To carry out the experiment, implemented mutants had to have a 2-fold higher enzyme activity than endogenous HEK293H (background noise). R49G, S65I, D231N and L415P failed to comply this criterion and were excluded.

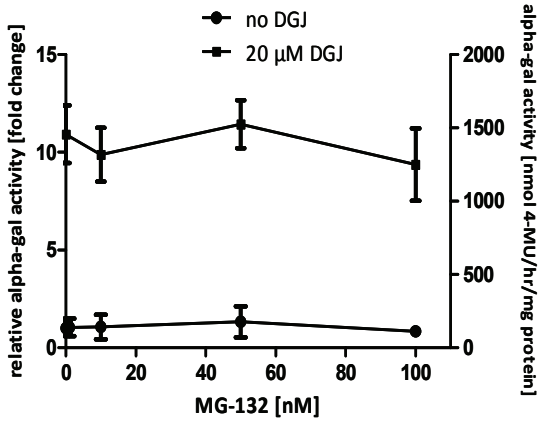
More than 50 % of the missense mutations could not be gainfully treated with DGJ and the definition of a responder in this study might as well be too optimistic since it is a matter of speculation to what activity level a mutation must be brought in order to function without any drawback to the cell, the organ or the patient. In order to obtain a resolvable effect a model mutation (R301Q) was selected for a first compound screening that considerably promised a high capacity to recover lost activity as shown in the DGJ responsiveness experiments. Since many FD related mutations are believed to undergo destabilisation/early degradation of the resulting *GLA* gene product (in this study we propose all mutations above 6 % residual activity (~ 35.1 %), see figure 11). Therefore, besides pharmacological chaperoning, the cancellation of the ERAD process could be benficial for α-galactosidase enzyme recovery.

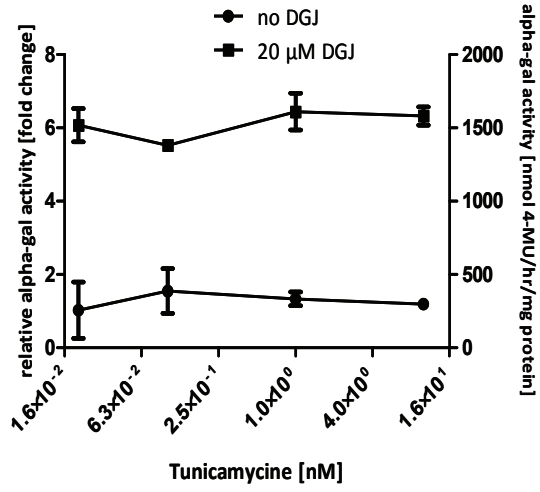
3.3.1 Effect of small molecule modulators of UPR and the ERAD on α-galactosidase mutations

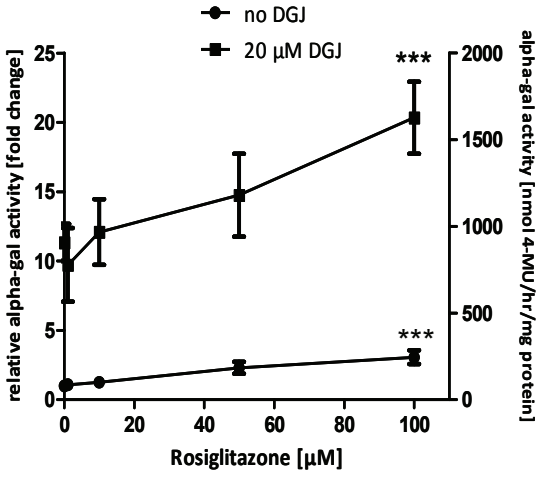
It was demonstrated that small molecules altering the expression profiles of ER-related stress genes, i.e. protein folding assistants (up) or proteasomal degradation inducers (down) can

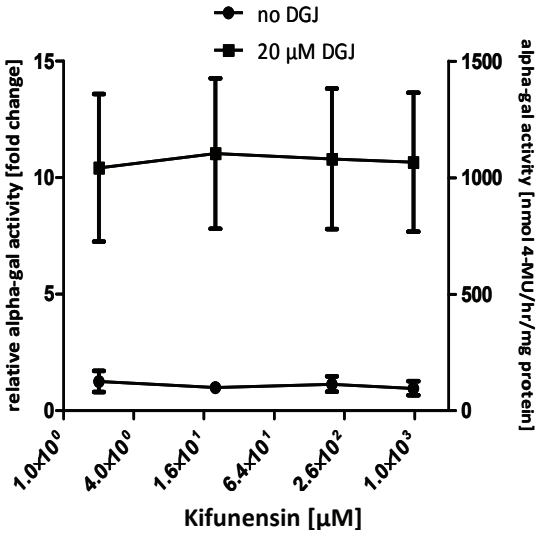
influence the process of mutant folding and further transport (Mu et al., 2008; Wang et al., 2011). In the present study some candidate substances were chosen partially proposed in earlier works and for other related diseases. Concentration series of the respective compounds were applied to HEK293H cells expressing *GLA* mutation R301Q (the model mutation for this screening) with or without DGJ. In this example, the ubiquitination blocker and PPARγ agonist Rosiglitazone (Rosig) proved significant enhancement of the mutants' activity (figure 12). Without the application of DGJ (which alone caused a 10-fold increase of activity), Rosig was able to increase the mutants' activity about 3-fold. Tunicamycine (TM) and Kifunensin (KIF) cause the initiation of ER stress for different reasons, but an effect on α-gal activity could not be detected. Thapsigargin (Tg) treatment leads to a spontaneous and sudden deflation of the ER Ca^{2+} reservoir and a dysregulation of the Ca^{2+} homeostasis due to an inability of the cell to restore it (blockage of influx transporters). The response of other enzymes was not tested, but the α-gal activity significantly dropped after the exposure to this drug. The proteasomal degradation inhibitor (PDi) MG-132 showed no effect either. However, an interesting effect on overall cellular ubiquitination was observed indicating the functionality of MG-132 in the applied dosage of 100 nM.

The Western blot shows protein lysates of pcDNA3.1/GLA[R301Q] transfected HEK293H cells (figure 13). The R301Q expressing cells showed a stronger ubiquitination signal compared to the mock transfected cells (empty vector) indicating ER stress due to the load of misfolded enzyme that had to be processed by the cell. Administration of DGJ remarkably reduced the K48-ubiquitin signal (lane 3). Rosig had a similar effect than DGJ assuming the blockage of the ubiquitin-conjugating machinery. A combinational treatment of both compounds could aggravate the detected effect. Administration of 100 nM or 1 µM of the PDi MG-132 increased the amount of ubiquitinated proteins within the cells indicating the functional inhibition of the 26S proteasome and the subsequent accumulation of no longer degradable peptidic material.









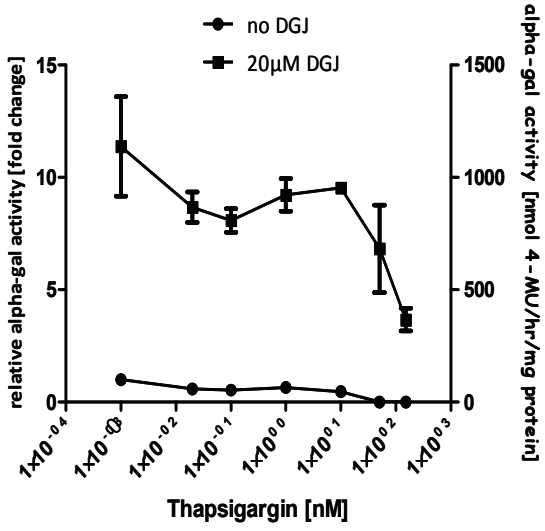


Figure 12: Effect of different UPS disturbing compounds on *GLA*[R301Q]. Graphs show the development of α-gal activity as fold change. DGJ alone usually increased the activity about 10-fold. MG-132, TM and KIF were ineffective to change α-gal activity up to the highest tested concentration (concomitant with the highest dosage tolerated by the cells). Tg treatment led to a substantial loss of activity. The major error bars for KIF are due to few experimental repetitions (N=2). Rosig successively triggered α-gal activity in a concentration-dependent manner. 100 µM of Rosig caused a doubling of measured activity of DGJ-treated and untreated samples. Values are mean ± SD/SEM. *p<0.05, **p<0.01, ***p<0.005.

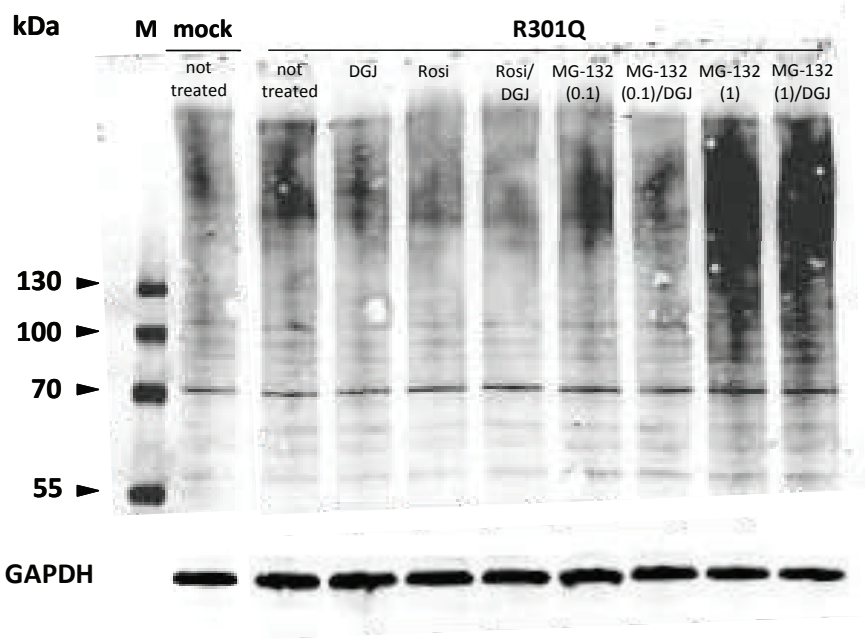


Figure 13: Ubiquitination profile of R301Q mutant expressing HEK293H cells. The overall cellular ubiquitination was altered by overexpressing *GLA*[R301Q]. The ubiquitination signal was reduced due to DGJ treatment (20 µM) indicating relieve of UPR induced ER stress. Also Rosig (100 µM) limited the ubiquitination by an unknown mechanism. MG-132 (0.1 and 1 µM) increased the amount of ubiquitinated proteins. The effect was concentration-dependent and partially revertible by the addition of DGJ. Western blot by Susanne Seemann, MSc.

3.3.2 The use of potential pharmacological chaperone Ambroxol

Currently, only active site-specific chaperones are known in FD addressing the same ligand binding site as DGJ. Consequently, no additional effect can be expected when two drugs are applied with a similar mode of action. The ligand with the higher affinity (under assay condition) would dominate and a combinational administration would presumably show the result of the single treatment The mixed-type GCase inhibitor Ambroxol (ABX) was tested in the cultured cell model of FD on several mutations (figure 14A). The result was negative, but the overexpressed wild type control showed significant response. A dose/respon se curve indicated a rather small therapeutic index and revealed an EC_{50} of 21.2 µM (figure 14B).

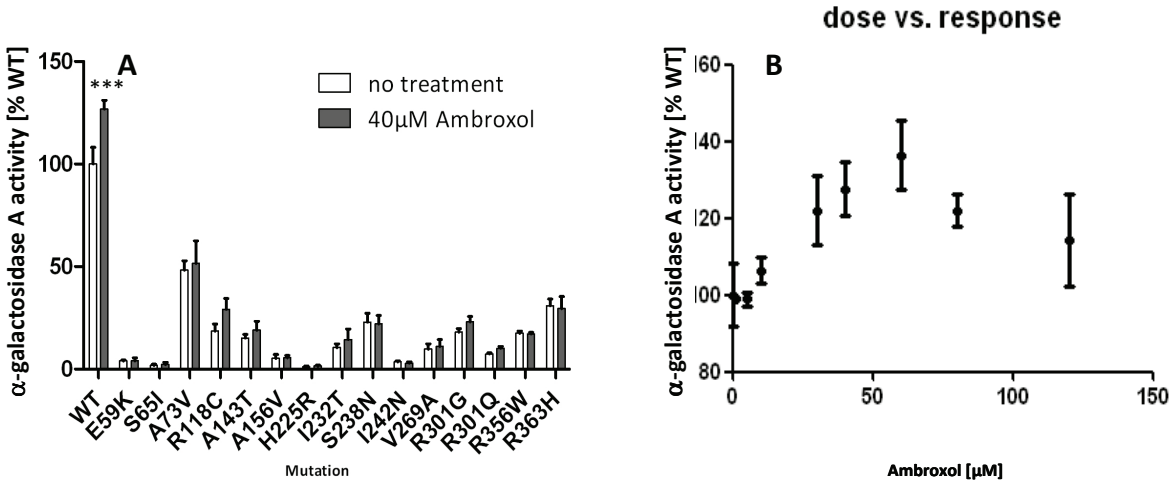


Figure 14: Ambroxol elevates normal α-gal activity. **A**: A subset of 15 mutations was tested for a possible ABX effect. All mutations had significant residual activity and respective enzymes were responsive to DGJ (except for R118C and H225R). It was taken care of this fact to ensure that all mutants had the capacity to change conformation and obtain an enzymatically active state. None of the mutants was enzymatically increased by ABX, but the α-gal wild type enzyme was enhanced approx. 1.3-fold. B: Dose/response curve revealed an optimal concentration of ABX of 60 µM and an EC_{50} of 21.2 µM.

Since it is believed that pharmacological chaperones like DGJ cause the enzyme to undergo a conformational change converting it to a "wild type-like" state, because of a more favourable free energy balance, ABX was additionally administered to mutant expressing DGJ-treated cells. Astonishingly, the DGJ made the mutants "accessible" to the ABX (figure 15). All of the mutations underwent a substantial increase in activity which was reflected on the protein level as evidenced by Western blot analysis (figure 15B). The most pronounced effect was detected for the mutations A156V and R301Q. The wild type enzyme showed no further increase of activity (data not shown). In a next step, the examinations were extended to find other related structures of ABX that possibly lead to a similar outcome. Bromhexine (BHX), of which ABX is a metabolite, with the same medical expectorant function was tested (figure 16). Even though showing a similar effect it appeared to be less effective on the model mutation A156V chosen for its solid ABX effect.

3.3.2.1 Do Ambroxol and Bromhexine derivatives maintain α-galactosidase A activity enhancing ability?

In a collaborative project, Ambroxol and Bromhexine were subjected to chemical derivatisation protocols in order to find other compounds triggering mutant α-gal activity in an analogous way. The compounds were tested on the mutation A156V (figure 17). The substances obtained acronyms SF-[number] according to the chronology they were manufactured. Figure 17 shows the

performances for the compounds in comparison to both lead substances ABX and BHX separated in sub-groups according to origin and the used chemical process for production.

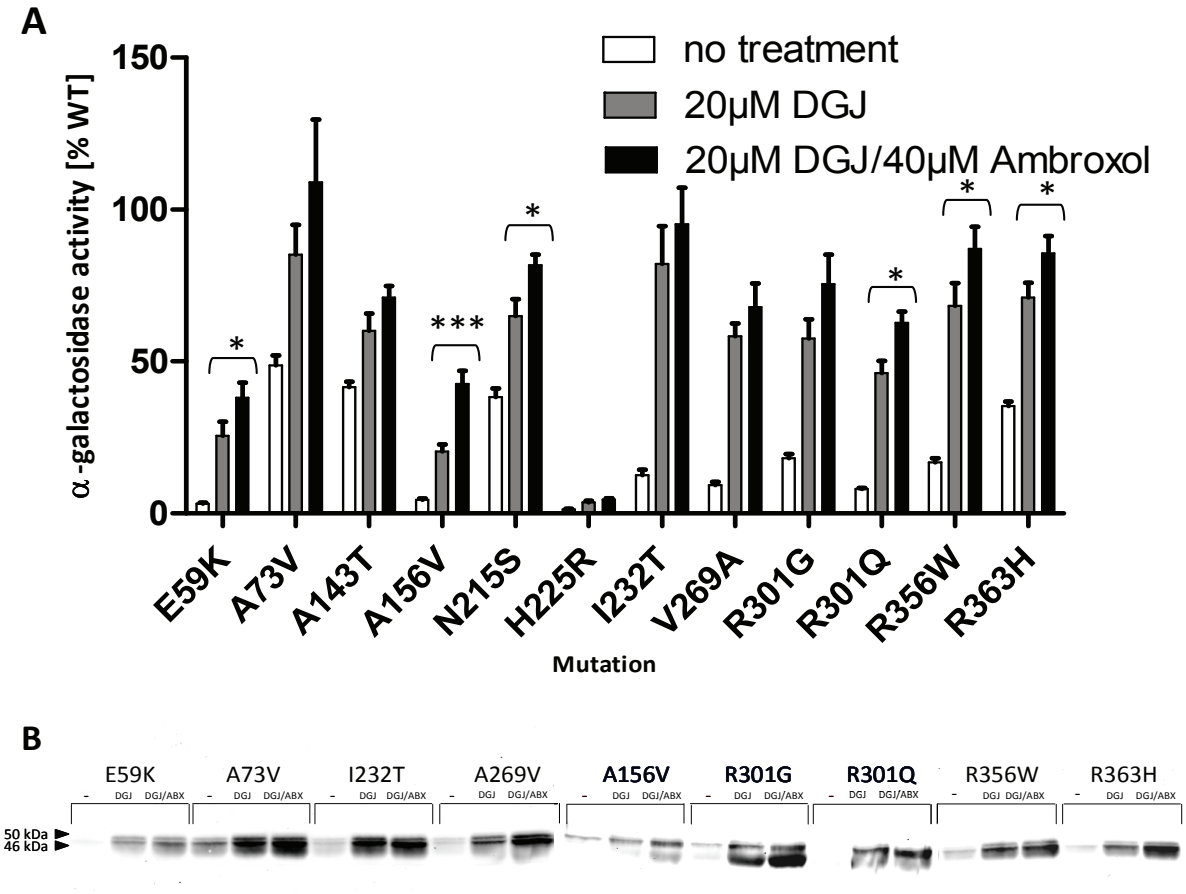


Figure 15: ABX shifts α-gal activity **A**: 12 *GLA* mutations were tested with a combinational treatment of DGJ (20 µM) and ABX (40 µM). Compared to the single treatment, the addition of ABX contributed to the α-gal activity. Mutations E59K, A156V, N215S, R301Q, R356W and R363H showed mathematical significance (paired student´s T-test) after the performed experimental series (N=3-5). **B**: Some mutations were subjected to Western blot analysis which supported the activity examinations. The mature (lysosomal) α-gal form at 46 kDa was amplified, almost no effect could be detected on the ER precursor form of α-gal (50 kDa) which indicated proper trafficking after the stabilisation process. Values are mean ± SEM. *p<0.05, **p<0.01, ***p<0.005.

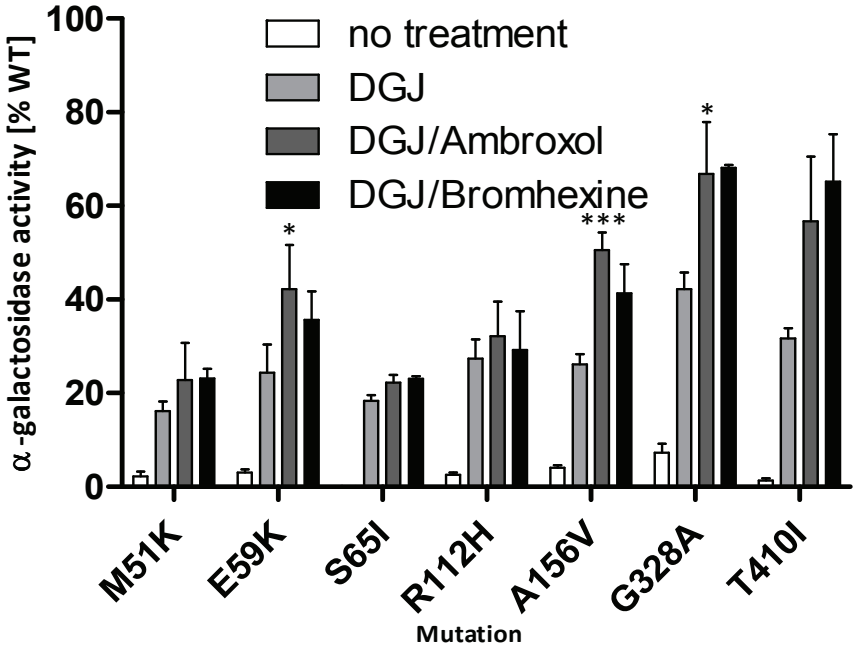


Figure 16: DGJ/ABX vs. DGJ/BHX treatment. A trend to obtain a higher activity after double treatment with 20 µM DGJ/40 µM ABX and 20 µM DGJ/40 µM BHX can be seen. Indicated are the significances for ABX/DGJ versus DGJ single treatment. BHX/DGJ functioned significantly on mutations A156V, G328A and T410I). E59K and A156V showed stronger response to ABX. For mutations G328A and T410I BHX functioned equally well. M51K, S65I and R112H showed only weak responses to both double treatments. Values are mean ± SEM. *p<0.05, **p<0.01, ***<p0.005; N=3-5.

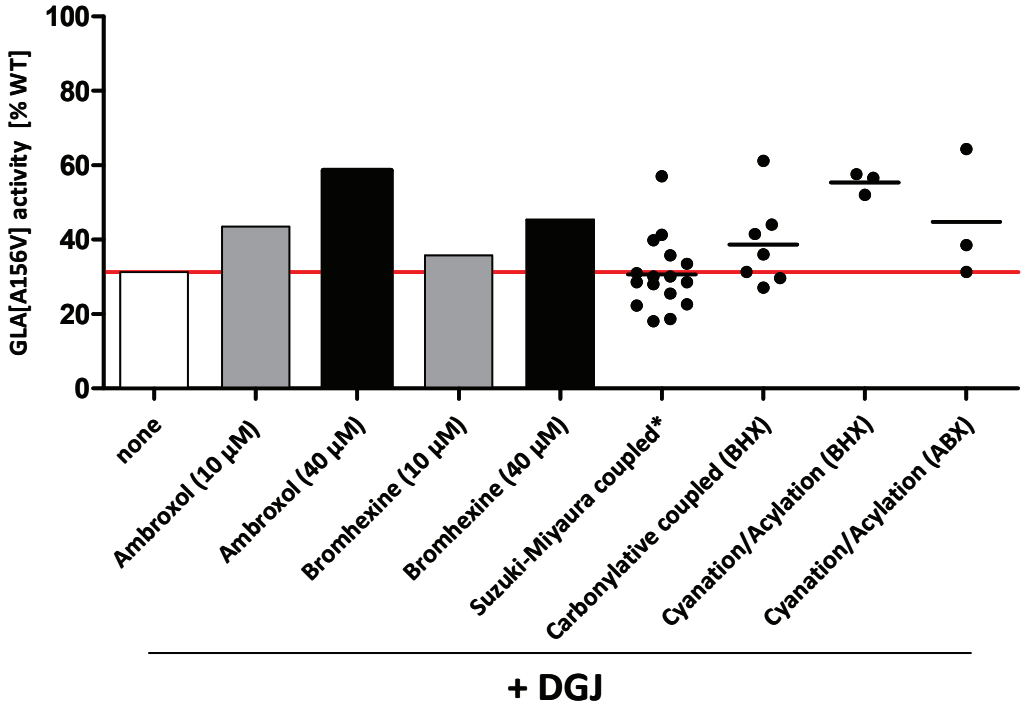


Figure 17: Enhancement of DGJ-elevated GLA[A156V] activity of Ambroxol, Bromhexine and their (28) tested derivatives. Experiments conducted with support of Dajana Großmann, dipl.-biol.

* Substances from this group had to be administered in 10 µM. For a detailed view of the activities of the compounds see Appendix.

Most of the compounds were chemically derived from Bromhexine which was less effective than Ambroxol on the mutant addressed here. Besides, many BHX derivatives, especially the products obtained by S. Miyaura protocol, were harmful to the cells in a concentration of 40 µM. This effect was not examined in more detail. However, some of the substances proved to be more effective than the mother compound. The best effect could be detected among the cyanation and acylation products of ABX and BHX. The results for all tested compounds and their structural formulas can be seen in Appendix table. Whether there exist direct interaction between the compounds and the enzymes remained unclear, but structural similarities among the functional substances are currently under investigation. We aim to find implications for a putative ligand binding site.

3.3.2.2 Ambroxol is functional with active site-specific chaperones

To test whether the effect detected for the DGJ/ABX combinational treatment was alienable to non-iminosugar-based chaperones, galactose was applied to the cell culture media as a substitute for DGJ. The additional effect on the gain of α-gal activity of ABX could be conferred by trend (figure 18) despite limited reruns (N=2). However, specifically A156V did not respond as good to galactose as it did to DGJ.

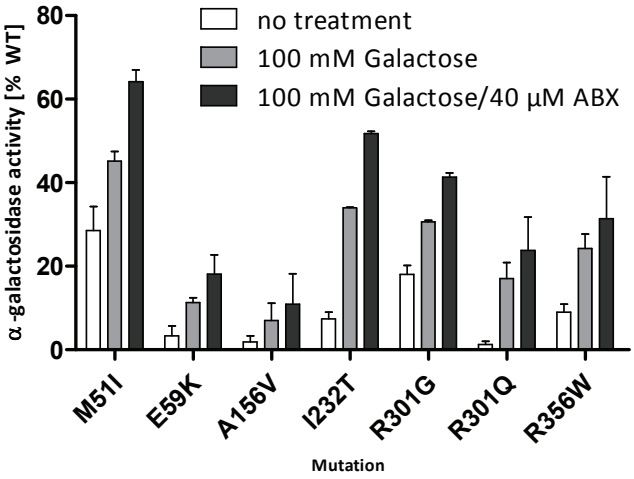


Figure 18: ABX acts with galactose to increase mutant α-gal activity above the value of the single treatment. Values are mean ± SD (N=2).

3.3.2.3 Acid α-glucosidase mutations benefit from a combination with a pharmacological chaperone and Ambroxol

The above findings that ABX and certain derivatives display a beneficial effect on several DGJ-responsive *GLA* mutations surprised especially because of the universality of the effect. The choice of the PC was irrelevant and the diversity of the functioning derivatives with regards to their physico-chemical properties implied a greater application spectrum. A set of iminosugar/chaperone responsive mutations in Pompe disease (PD) was picked (Okumiya et al., 2007, Parenti et al., 2007) to test whether the ABX effect was transferable to another LSD based

on a glycosylase defect in the same HEK293H cell based assay. The mutations were produced according to the same methodology as the *GLA* mutations (see materials and methods section and Appendix for the respective primers). Initially, the iminosugar N-Butyl-deoxynojirimycine (NB-DNJ) was chosen as the inhibitor/PC. The wild type α-glucosidase activity dropped to 65-70 % upon administration of NB-DNJ. This finding was unexpected but allegeable taken into consideration the inhibitory nature of this compound *in vitro*. Likewise, no optimisation experiment for optimal dosage of NB-DNJ was conducted which could explain that the likely optimal dosage of NB-DNJ lies beneath the administered 20 µM which was just adopted from the literature. However, ABX did not change the behaviour of the wild type significantly. In accordance with the common level of knowledge, R375L was not an NB-DNJ responder and did not experience any benefit from ABX or NB-DNJ/ABX as well. Y575S did not respond which stands in contrast to the findings of Flanagan et al., 2009. Among the found responders, L552P indeed displayed an additional effect upon double treatment and experienced a 2.2-fold activity increase (NB-DNJ: 6.9 %, NB-DNJ/ABX: 15.3 %).

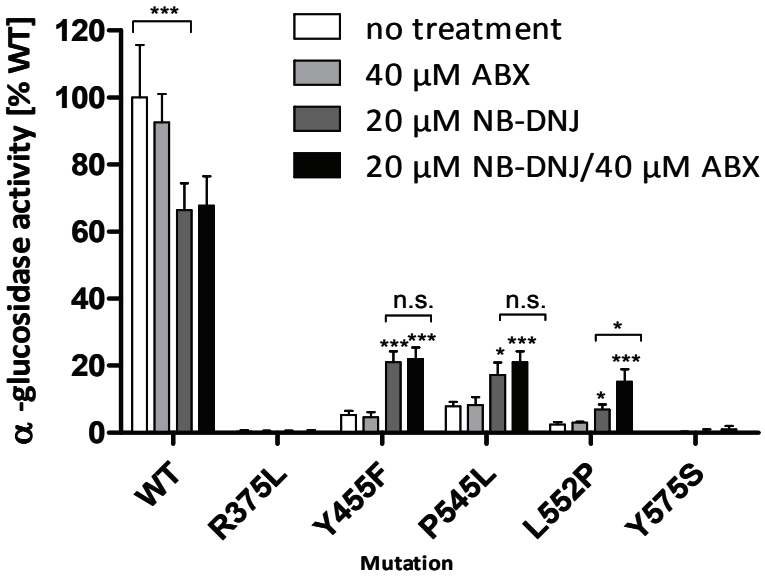


Figure 19: NB-DNJ enhances α-glucosidase mutants but concurs only on L552P with ABX. NB-DNJ even displays an activity diminishing effect on the wild type α-glucosidase in this cell based assay. Activity of the mutants R375L and Y575S was not significantly elevated by NB-DNJ. Y455F, P545L and L552P were enhanced. Only L552P showed additional gain upon NB-DNJ/ABX combinational treatment compared to the single treatment. Values are mean ± SEM. *p<0.05, **p<0.01, ***p<0.005, n.s.: not significant. Experiment conducted by Susann Pohlers, MSc; N=3-5.

With the knowledge of this weak, however stunning, additional effect of the NB-DNJ/ABX double treatment, the PC deoxynojirimycine (DNJ) was also applied in conjunction with ABX. The stronger effect of the DNJ to the subset of NB-DNJ responders from figure 19 (along with Y575S) was

obvious. The strong effect of the double treatment was even more promising (figure 20). The *GAA* mutations showed a substantial increase of activity after DNJ treatment. By the addition of ABX, the activities rose for Y455F, P545L and L552P 1.7-, 2.3- and 2.5-fold, respectively.

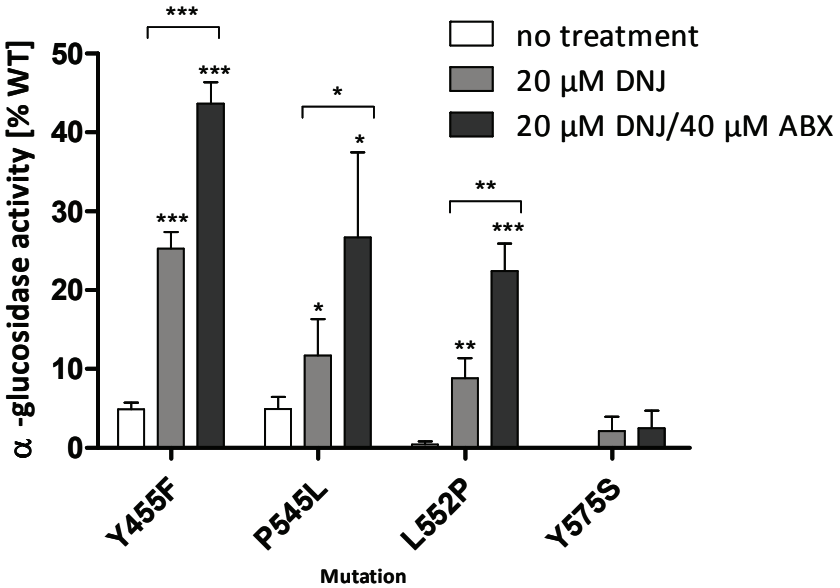


Figure 20: DNJ and ABX elevate mutant α-glucosidase activity above the level of the single PC treatment. DNJ is able to increase mutant *GAA* activity effectively. Also Y575S showed a weak, however not significant, effect after administration of 20 µM DNJ. Except for this mutation, the remainder of the mutants showed roughly a doubling of enzyme activity with the addition of ABX. Values are mean ± SEM. *p<0.05, **p<0.01, ***p<0.005; N=3-5.

3.3.2.4 Ambroxol, not Bromhexine, increases mutant glucocerebrosidase activity

Because many ABX/BHX derivatives were synthesized and found to display a similar effect on mutant α-gal enzyme in a cell-based assay the "*vice versa*" approach was applied to those derivatives in order to identify compounds that are still capable of stabilising mutant glucocerebrosidase (GCase) and enhancing the activity such as the mother compound ABX. For this purpose, a Gaucher disease patient-derived fibroblast line (GM00852) harbouring one allele of the ABX-responsive mutation N370S related to non-neuronopathic GD type 1. The choice of the substance was done according to the results from the FD screening, e.g. compound SF-54B performed equally well as ABX on the mutant *GLA*[A156V] increasing its activity to 57.6 % compared to 31.2 % (DGJ alone) and 58.5 % (DGJ/ABX), respectively. It turned out that among the tested 8 ABX/BHX derivatives (along with ABX and BHX, figure 21) only SF-153B retained the capacity to increase endogenous mutant GCase with a comparable efficiency. Even BHX, found to inhibit wild type GCase *in vitro*, did not show a positive result. MG-132 was used in 100 nM for three days of treatment. When administered at a dosage of 1 µM the positive effect was lost (data not shown). The iminosugar NB-DNJ, shown to increase mutant GCase activity did not

increase the activity in this setting. Maegawa and colleagues (2009) observed a ~3-fold increase of activity in a cell line homozygous for the examined mutation N370S. In the present experiment only one allele harboured the responsive N370S mutation, the other allele was disrupted by a nucleotide insertion (common GD mutation 84GG) explaining the lower increase of activity by 2.1-fold.

Subsequently, it was tested whether the substances still exhibit the inhibitory effect on wild type GCase. The medical formulation of GCase (Cerezyme®, Cambridge, USA) was used to illustrate the *in vitro* inhibitory effect of ABX and the derivatives thereof. As expected, ABX inhibited the enzyme in a concentration-dependent manner (figure 22). The figure exemplifies the assay outcome for some chosen compounds. SF-170C and SF-110D retained the inhibitory function of the lead substance whereas SF-153B, the only substance that performed well and led to a 1.8-fold increase in GBA[N370S] in the cell-based assay showed a strong reduction of inhibitory function.

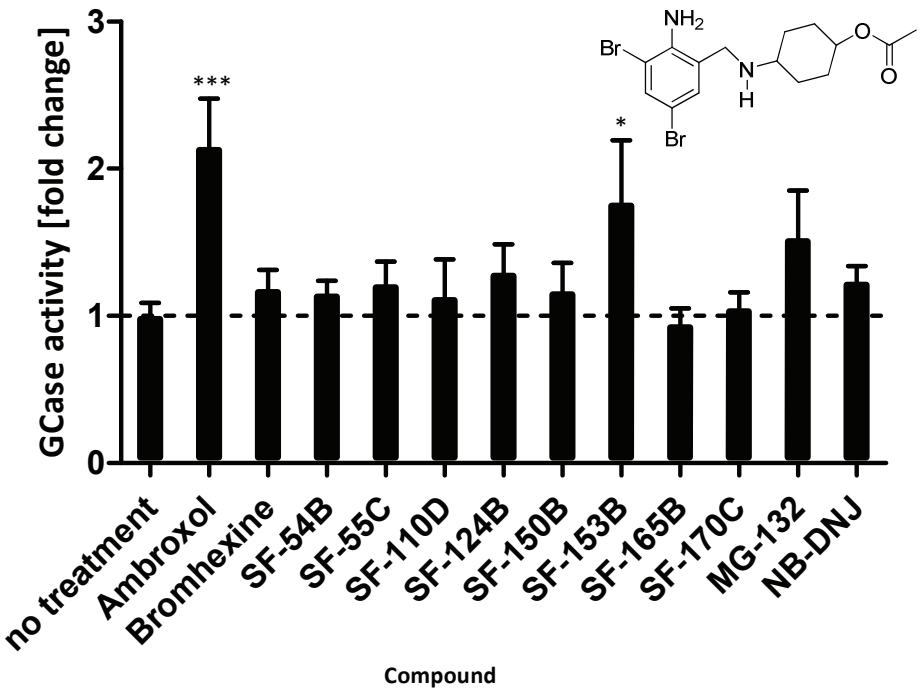


Figure 21: ABX and derivatives were tested on *GBA*[N370S] in a GD fibroblast cell line (GM00852, Coriell cell repository, Camden, USA). Mutant activity was elevated significantly by ABX and SF-153B (Structure of the acylation product of ABX (SF-153B) see inlay upper right). All substances were applied at a concentration of 40 µM. MG-132 (100 nM) was close to mathematical significance. The remainder of the substances were considered ineffective. Values are mean ± SEM. *p<0.05, **p<0.01, ***p<0.005; N=4.

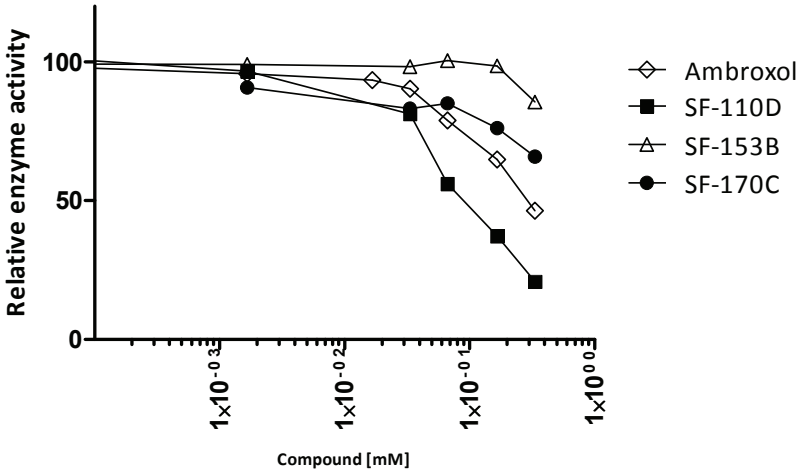


Figure 22: Inhibition of wild type GCase (Cerezyme®, Cambridge, USA). The inhibition assay was conducted at pH 6.7. ABX reduced the enzyme activity to 46 % at a concentraion of 0.33 mM. At this concentration, SF-110D was more potent diplaying only 21 % residual activity. SF-153B, as a functional chaperone lost its ability to inhibit the GCase in vitro showing only a mild reduction of activity to 86 % at the highest administered dosage. Values are obtained from two independent experiments.

3.3.2.5 *In vivo* test of Ambroxol in Fabry patient B-lymphocytes

The effectivity of ABX was also tested in two FD patient-derived cell lines harbouring the mutations A143T and N215S (for a protocol of Epstein Barr Virus- (EBV-) based cell immortalisation see method in the Appendix section). The *GLA*[A143T] patient cells did only modestly respond to DGJ and showed no additional benefit from the DGJ/ABX double treatment (figure 23). The best effect was unexpectedly obtained with the application of 40 µM ABX alone. The cell line generated from the *GLA*[N215S] carrying patient responded to DGJ, but did not show a response to ABX alone or in combination.

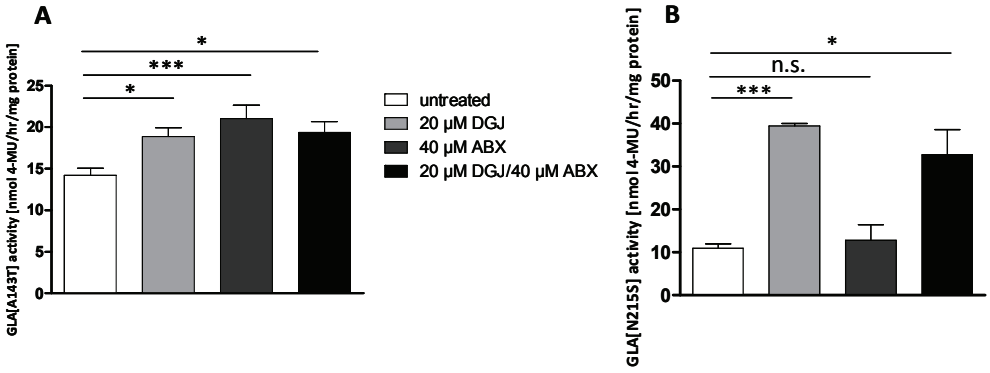


Figure 23: Patient-derived B-lymphocytes responded to DGJ, but showed no benefit to the DGJ/ABX administration. Patient cells were treated for 5 days with the respective small molecule. **A**: *GLA*[A143T] cells showed a modest increase in activity with 20 µM DGJ and no further increase after additional administration of ABX. On the contrary, ABX alone showed the strongest positive result in these cells. **B**: *GLA*[N215S] cells responded strongly and with a high statistical significance to 20 µM DGJ. ABX alone and

the combination of DGJ and ABX did not lead to higher enzyme activity. Values are mean ± SEM. *p<0.05, **p<0.01, ***p<0.005; N=5.

4 Discussion

4.1 Establishment of an *in vitro* α-galactosidase A overexpression system in HEK293H cells

An *in vitro* overexpression assay was established in which mutated *GLA* enzymes could be characterised biochemically. The system was utilised to measure the residual activity of the mutant enzymes, to determine the nature of the defect of the enzyme (catalytic or stability) and the responsiveness towards the pharmacological chaperone DGJ. The system was validated on the basis of the following requirements:

- high standardisation

- patient-independent system (objectivity)

- easy handling

- good reproducibility

- good signal-to-noise ratio

- fast processing

Comparable systems were described before (Okumiya et al., 1995; Ishii et al., 2007; Wu et al. 2011). In our laboratory, we reached a high degree of standardisation of the protocol. Usually, an average increase of about 50-fold for intracellular α-gal activity was found when cells were transfected with a plasmid harbouring the wild type cDNA sequence. In a systematic examination endogenous α-gal ("noise") and the overexpressed wild type gene had activity values of 97.5±11.2 and 4844.4±399.3 4-MU nmol/hr/mg protein, respectively (N=30 independent experiments). This difference determined the ability to distinguish between reduced mutant enzyme activity compared to the wild type. The found basic data were superior to other tested systems (i.e. Cos7 cells). The most stable and reliable results were obtained 66-72 hrs post transfection indicating that the intracellular synthesis and processing of the enzyme reached a plateau-phase (see experimental scheme on page 29). This time point was retained throughout the whole study unless indicated differently.

The examined subset of 171 mutations showed the whole spectrum from none to normal activity. In individual cases mutant enzyme activity exceeded wild type activity (e.g. N139S). However, wild type activity already exhausted the detection range of the system and mutations with high activities showed high variance after few experiments (commonly N=3-5). For some of the mutations negative results were obtained after subtraction of endogenous α-gal activity. One

possible explanation is that the endogenously expressed α-gal molecules form homodimers with the mutant monomer forms present in high numbers therefore affecting their ability to catalyse the hydrolytic reaction whereas the homodimers being formed in the empty vector transfected cells exclusively consist of wild type subunits. All of the negative values were normalised to "0". The normalisation is explained under **2.2.4**.

4.2 Classification of α-galactosidase A mutations

4.2.1 Characterisation and classification by *in vitro* enzyme activity

For many lysosomal hydrolases it is controversially discussed how much residual activity is needed to avoid symptom onset in the patients. The considerations comprise experience from clinical studies as well as evidence from cell biological and biochemical approaches. It is commonly agreed that the catabolic processes lysosomal glycosylases like α-gal are involved in can ensure proper functionality due to abundant α-gal activity. How much "excess" activity is provided by α-gal, hence, how much residual activity a mutated enzyme needs is not known and might be differing between individuals. Herein, life conditions such as diet and genetic background may play a role. Leinekugel and colleagues (1992) proposed that a residual activity of 10-15 % retains cellular substrate turnover. This could be regarded as a definite quantitative link between enzyme activity and sustained cellular function for lysosomal β-hexosaminidase A and arylsulfatase A enzymes. However, researchers defined different cut-points ranging from 10 % as the assumingly required activity a hemizygote patient should display to reduce FD symptoms (Fan and Ishii, 2007) to 15 % indicated as the minimum activity level to prevent classic symptoms (Andreotti et al., 2011). In another application 50 % of activity was considered of therapeutic merit (Shin et al., 2007). Furthermore, mutations with a residual activity of 20-40 % (in a transient expression system) were related to later-onset FD (Spada et al., 2006). In the present study, reasonable boundaries were selected to classify the mutations for their behaviour in the established activity determination system. Four classes were defined for enzymes with:

class I: no residual activity

class II: >0-20 %

class III: ≥20-60 %

class IV: ≥60 %

preliminary to statistical evaluation. Class I considers mutations believed to display the classic disease phenotype. Indeed, all of the mutations already clinically described within this class were

classified "classic" according to former studies. Only L129P and L275F were classified classic/variant due to own patient-derived data. However, both mutations were found in female heterozygotes which explains the milder disease course. This finding argues that clinical phenotype assessment should be limited to hemizygote males in future work. Class II uses lower and upper cut-points designed to harbour classic and variant mutations equally. The exact upper cut-point was utilised because the formerly classified late onset/oligosymptomatic mutations R118C and A143T had activities around 20 %. Apparently, all of the mutations within this class showed gender-independently increased biomarker levels indicating that the patients were affected. Moreover, none of the described mutations assigned to class II was related to an equivocal phenotype. In order to discriminate between mutations with a rather high residual activity and those showing only a very moderate decrease we defined another cut-point at 60 % residual activity. Indeed, many mutations were found to be novel within class IV which evidences that these mutations could have been overlooked in symptom-free or very mildly affected patients so far. Among the described mutations, only N139S and D313Y had a very high activity and were consequently assigned to this group. The mutation D313Y was considered as a single nucleotide polymorphism (SNP) after the finding that a second mutation in cis of the same gene (G411D) accounted for the strong activity diminishment *in vitro* (Froissart et al., 2003; Yasuda et al., 2003). However, there is evidence that D313Y is related to FD symptoms (Brouns et al., 2010; Wittmann et al., 2012; Lenders et al., 2013). The majority of class III mutations were found in FD patients yet (16 of 28) (see literature references in table 10). Moreover, only positions Trp24 was not formerly described to be involved in a disease causing amino acid substitution.

4.2.2 Validation of biomarker lyso-Gb3

In the Albrecht-Kossel-Institute's diagnostic department FD biomarker plasma lyso-Gb3 is routinely tested following genetic diagnosis (which is still the preceding imperative procedure). Being aware of the shortcomings of patient-dependent data, the performance of the FD biomarker lyso-Gb3 was tested with respect to sensitivity, specificity and accuracy (figure 9). A significant value was found for lyso-Gb3 derived from females and males to detect patients with α-gal mutations. For each mutation studied lyso-Gb3 mean values were always higher in male than in female patients, but the values obtained in females also reflected disease pathology. It can be pointed out that every mutation causing a pathological lyso-Gb3 mean value in males also demonstrates with a pathologically elevated mean value in females indicating that the outbreak of the disease is gender-independently very probable and invariable. However, in the case of mutation N215S that has a higher frequency in the population and is related to a milder FD course, showed pathologically (but moderately) elevated lyso-Gb3 values in all 7 male individuals

tested (mean = 4.2 ng/ml) whereas among the 6 female patients tested only 3 displayed an increased lyso-Gb3 value (mean = 1.1 ng/ml). The 3 individuals showed values of 0.79 ng/ml, 0.68 ng/ml and one was below limit of quantification (<LLOQ), respectively. Other mutations such as S126G, A143T and D313Y did not lead to elevation of lyso-Gb3 (i.e. mean values are non-pathological, see table 10). For the mutation D313Y this is in accordance with results recently reported (Niemann et al., 2013). However, the conclusion is not necessarily that a mutation with a non-pathological lyso-Gb3 mean value cannot cause disease since (1) single individuals showed elevation and (2) D313Y, S126G and A143T had highly different residual enzyme activities of about 85 %, 50 %, and 20 %, respectively, and it is therefore unlikely that there is no harmful effect to the body relying merely on unobtrusive biomarker. Essentially, lyso-Gb3 is a marker of classic rather than variant forms of FD. However, we conclude that it reflects disease severity after all.

4.2.3 *In vitro* enzyme activity is a disease phenotype predictor

Statistical analysis revealed that the predictive models proposed earlier (Kuipers et al., 2010; Saito et al., 2010; Andreotti et al., 2011) , based on *in vivo* and *in silico* data, were associated with *in vitro* enzyme activity measurement data indicating that all parameters were potentially related to disease severity. The single parameters were individually tested for their predictive value. The PolyPhen algorithm successfully predicted 73.6 % of the 72 mutations where phenotype data was available (76.2 % of the subset of 21 mutations) a value below the reported by authors stating a sensitivity and specificity of PolyPhen about 80 % (Ramensky et al., 2002). However, this might be due to the fact that we were not trying to discriminate between disease causing and not disease causing (SNP) mutations, but between severe and mild disease forms which may deteriorate prediction accuracy.

Biomarkers should detect the disease state with the highest possible accuracy. A perfect diagnostic test has an accuracy of 1.0 determined from the dimensions specificity and sensitivity (Simundic, 2008). The values obtained for female and male lyso-Gb3 were 0.85 and 0.86, respectively. Therefore, biomarker data as assessed in the routine laboratory of the Albrecht-Kossel-Institute is of "*very good*" predictive accuracy discriminating individuals in the disease condition from healthy individuals (Simundic, 2008). The discrimination between the three phenotypic emergences classic, variant and classic/variant was efficient for 69.7 % (for 33 mutations where data was available) and 78.1 % (for 32 mutations where data was available) of the mutations, respectively.

The best quality was obtained with the measurement of α-gal activity correctly identifying 83.3 % of the 72 tested mutations. This indicates that compared to patient-derived data *in vitro* data

generation objectifies the biological consequence of *GLA* mutations and creates a quantifiable model overcoming the problem of individual patient constitution leading to non-pathogenic biomarker data for otherwise pathogenic mutations (as observed for example for the mutation N215S). As for therapeutic decisions, it is admittedly difficult to define a critical value for residual enzyme activity below which FD will break out. However, from the obtained data it can be proposed that 20 % of residual activity can be regarded as a very critical measure, because all mutations within this range (class I and class II) can be classified as "certainly disease-causing" to our current knowledge. Above this value it might be a matter of individual patient constitution whether the patients develop symptoms. Mutations with a residual activity within a range of 20-60 % (class III) may be within a twilight zone harbouring mutations that lead to disease (A143T, N215S) or may remain symptom-free (perhaps S126G). The mutations with higher than 60 % activity with the proposed single nucleotide polymorphism D313Y being one of them need to be studied epidemiologically, hence by frequence in the population, because it is thoroughly possible that no phenotype is provoked in the majority of cases.

4.3 Biochemical analysis of mutant α-galactosidase A

It is important to state at this point that we followed the assumption that all mutations potentially lead to a negative outcome, hence Fabry disease, for the patients even though for some of the mutations a link between the mutation and the observed phenotype (see table 15) was not proven. One way to prove this assumption could be an epidemiological study that would reveal the incidence of the mutations in healthy individuals. This would not, however, eliminate the possibility that a proband/patient healthy at the time point of examination could develop FD symptoms later on in life.

Table 15: List of mutations in the GLA gene leading to obscure clinical consequences

Mutation (number of patients)	observed with phenotype*
D83N(1), S102L(1), N139S(5), R252T(4), V316I(1), L415F(1), E418G(1)	cerebrovascular
A37T(1), H225D(1) E398A(1)	renal
R220Q(1)	cardiac

Table 15: List of mutations and the assigned clinical symptom that led to genetic analysis. Mutations depicted here had high residual activities and were not reported as disease-causing before (exception: N139S, Havndrup, 2010). D313Y was found likewise in mono-symptomatic patients with renal, cardiac and cerebrovascular involvement. * no detailed clinical description, see also table 10.

With this reasoned, we conducted experiments to in-depth characterise mutations that "fail" to show pathogenecity in any of the proposed *in vitro*, *in vivo* and *in silico* parameters in order to find evidence for a possible enzyme damage. As shown before, mutation E59K showed significant activity (Ishii et al., 2007), but is catalytically affected showing an impaired K_m value (Ishii et al., 2007; Andreotti et al., 2011). Mutation S65T has no obvious structural damage according to crystallographic data (Garman et al., 2007), but was shown to destabilise the enzyme (Ishii et al., 2000).

4.3.1 Early degradation as a main cause for the pathogenecity of α-galactosidase A mutations

Western blot and kinetic examinations of the mutant proteins suggested that a high number of enzymes were adversely affected by a loss of enzyme level either due to early degradation by ERAD or pH dependent disaggregation in the neutral pH of the ER. This holds especially true for mutant enzyme forms with a significant rest activity (<6 %). In the case of E59K which actually had a lower than 6 % activity and H46P which would potentially belong to the assumed degradation prone mutations an altered substrate turnover could be evidenced (table 14). This illustrates that an early degradation defect can be accompanied by a catalytic defect. A minor degradation defect was shown for mutation D231N (46 % of wild type enzyme level), an active site residue mutant that had no residual activity. This mutant form was stabilised with DGJ (66.7 % of wild type), but did not recover activity. However, for mutant form H46P characterised with a mild decrease in enzyme activity (class III), a benign *in silico* forecast (and strong responsiveness to DGJ, see next paragraph) this analysis could provide significant evidence for the occurrence of patients with this mutation, because an increased K_M value could lead to diminished intra-lysosomal substrate turnover despite normal performance in the *in vitro* assay where excess substrate was added. It is worthwhile to examine more of those mildly damaged mutations in this assay.

4.3.2 Determination of DGJ responsiveness contributes to future therapeutic treatment strategies and is a tool for enzyme damage depiction

The pharmacological chaperone DGJ is a compound that increases mutant α-gal activity exploiting the capacity of the patients α-gal variant to catalyse the natural reaction in the lysosomes. To do so, the galactose analogue DGJ binds to the catalytic centre of the enzyme and stabilises its folding state (Ishii et al., 2007). In comparison to the conventional ERT this strategy has been argued to benefit to the patients condition by several means:

- A patient receiving ERT needs to acquire nursing service on a regular basis for intravenous infusions of the drug (Replagal: administered at a dose of 0.2 mg/kg body weight over 40 minutes; Fabrazyme: administered at a dose of 1.0 mg/kg body weight over > 90 minutes).

This particular burden should not be underestimated. An orally available drug would overcome this cost-intensive care and could reduce the costs of the therapy, make it affordable in countries with less developed health care systems.

- Besides this economical aspect, the PCT has the specific advantage over the ERT to use the body's own resources to ameliorate disease symptoms. In common sense, the lack of enzyme activity was called to account for FD onset and progression. To what extent the aggregation of incorrectly folded protein in the ER (Ishii et al., 1996) contributes to the fatal emergence of certain symptoms remains unknown.
- Additionally, ERT was shown to cause immune reactions diminishing enzyme activity (Ohashi et al., 2008), the enzyme has a very short half-life and only limited efficacy in patients with renal failure (Choi et al., 2010) which can abate the beneficial outcome.
- However, one possible drawback of a small molecule inhibitor is the danger of adverse effects in consequence of overdosing (Steet et al., 2006).

In this study, a responder was defined as a mutation that was able to gain a 1.5 fold increase in enzyme activity or at least 5 % in comparison to the untreated state. Those parameters obviously fit the course by others, because DGJ responsiveness was confirmed in most cases when ascertained earlier (Shin et al., 2007/2008; Wu et al., 2011). Altogether, among the tested missense mutations in this study, 42.8 % were responding to the DGJ treatment. This stands in good agreement with literature data obtained from another subset of 81 mutant enzyme forms (Wu et al., 2011). The responsiveness of 42.8 % of the tested missense mutations indicated that those mutations display a misfolding phenotype rather than catalytic damage which is in accordance with the finding that mutations with a residual activity >6 % (60/171 ~ 35.1 %) showed correlation with intracellular α-gal level. As expected, none of the nonsense or frameshift causing deletion/insertion mutations could partially recover enzyme activity. DGJ responsiveness was suggested as a potential measure for the deleteriousness of a mutation before since mutations with a residual enzyme activity which are related to the variant Fabry disease form are believed to be more likely responding to PCT (Andreotti et al., 2010).

We utilised DGJ as a potential measure for the deleteriousness of a mutation. Evidently, the number of DGJ responders was lower in class I (no residual activity) compared to the other classes. Even though the increase of DGJ responders was not linear across the classes, a significant association was found to the residual enzyme activity (table 11). Still, the high predictive quality of this measure surprised. About 75 % of the mutations were classified correctly into classic and variant phenotype classes which are about the same as PolyPhen and biomarker data (compare

table 12). The rather moderate rate of DGJ responding mutations in class IV (enzymes with > 60% activity) can be explained by the high number of surface residues affected within this class. Amino acid location (on the surface or buried) is critical for the prediction of pharmacological chaperone responsiveness since superficial alterations of the α-gal might not cause folding defects addressable by PC. A closer look at positions Arg118 (buried residue), Ser126 (stabilising loop) and Asp264 (close active site) showed that mutation at those sites encoded enzymes that displayed an overall minor or no increase of activity through DGJ treatment indicating that the moieties are generally located in positions where treatment with the PC is not favourable for α-gal activity elevation and the type of amino acid exchange is of less importance apparently (exception: D264N, see table 10).

Wu et al. (2011) found a general consistency of results obtained from a HEK293-cell based overexpression system and the observed DGJ responsiveness of the mutations in patient-derived cells. Therefore, it was suggested that the introduced system provides results conferrable to the *in vivo* situation of the respective mutations and is suitable for primary screening of new compounds able to modify mutant α-gal activity and give hints for novel mechanisms to modulate mutant α-gal activity. The aim of this study was to find compounds that can address DGJ non-responding mutant α-gal forms or that can increase the efficacy of the DGJ in order to make small molecules applicable for a higher number of FD mutations (see **4.1.3**).

4.3.3 *In vitro* identification of mutant α-galactosidase A activity augmenting substances

4.3.3.1 Ambroxol as a lead structure

Compared to other screening systems which only allow for detection of substances directly interacting with the enzyme of interest ultimately leading to modulation of its activity (Zheng et al., 2007), the present system enables to consider the manipulation of mechanisms and pathways profitable in the complex cellular environment. Still, in a first approach a putative pharmacological chaperone was examined found to inhibit wild type glucocerebrosidase *in vitro* while enhancing mutant forms of the enzyme in a cellular assay: Ambroxol. In this case of ABX, a possible functionality towards normal and mutant α-gal was presumed PC-like due to a desired cross-reactivity event. Indeed, ABX raised mutant α-gal activity of several DGJ responsive enzyme forms significantly above the level of the DGJ single treatment (figs. 13/14). The dose/response curve showed a typical course depicting low-dose stimulatory and high-dose inhibitory response (inverted U-shape, fig. 12B). But it was assessed that the decrease of activity was rather due to a harmful effect to the cells (in concentrations ≥ 100 µM) than a specific inhibition. Preliminary data indicate that the ABX function is apparently not based on α-gal inhibition since it was

demonstrated that it displayed neither inhibiting nor stimulating effect on the enzyme in a cell-free assay (data not shown). Thus, in a conventional *in vitro* screening system for inhibitors/stimulators, ABX would have been missed as a potential enzyme enhancer in a cellular system. Whether ABX exerts its effect on α-gal activity *via* direct ligand/receptor binding or whether it employs other cellular components is currently under investigation.

A combination of 20 µM DGJ and 40 µM ABX was found most efficacious since higher ABX concentrations led to a higher mean variation; higher DGJ concentrations were not tested. Bromhexine was tested in the same experimental set-up, because a similar effect was postulated. The results for BHX outlined in figure 16 showed a weaker effect on mutant enzyme forms that showed the most significant ABX results (E59K, A156V). The mutation T410I responded stronger to BHX. However, the differences between DGJ/ABX and DGJ/BHX were not significant. Mutant enzymes S65I and R112H showed only a mild tendency to benefit from the combinational treatment. Therefore, we concluded that (1) mutations seem to have different preferences for either ABX or BHX and (2) the mechanism of action is apparently dependent on the nature of the mutation.

4.3.3.1.1 Ambroxol and Bromhexine derivatives enhance α-galactosidase A activity

As indicated before, ABX was tested due to the findings that it was an enzyme enhancing agent for glucocerebrosidase, the mutant enzyme in Gaucher disease. Structural data suggested that it acts as a pharmacological chaperone (Maegawa et al., 2009). Furthermore, Ambroxol had been described as an inhibitor of nitric oxide induced human platelet soluble guanylate cyclase and rat lung soluble guanylate cyclase activity (Severina et al., 2000) and blocks neuronal voltage-gated sodium channels (Weiser and Wilson, 2002). Moreover, Ambroxol shares a typical inhibitory structure motif with imipramine and chloroquine enabling the inhibition of lysosomal phospholipase A *in vitro* (Heath and Jacobson, 1985). However, the structural feature necessary for the pharmacological chaperone function on glucocerebrosidase is not known. Therefore, a derivatisation program was started as a collaborative project in order to alter specific sites of the ABX/BHX lead structures (figure 24) molecule and subsequent testing for α-gal enhancing function. Several of the tested compounds retained activity towards overexpressed GLA mutation A156V (figure 17). Evidently, cyanation products SF-54B and SF-55C and acylation products SF-150B and SF-153B performed just as well as the lead compounds. Notably, SF-54B, 55C and 150B are BHX derivatives that even enhanced the activity of the lead structure.

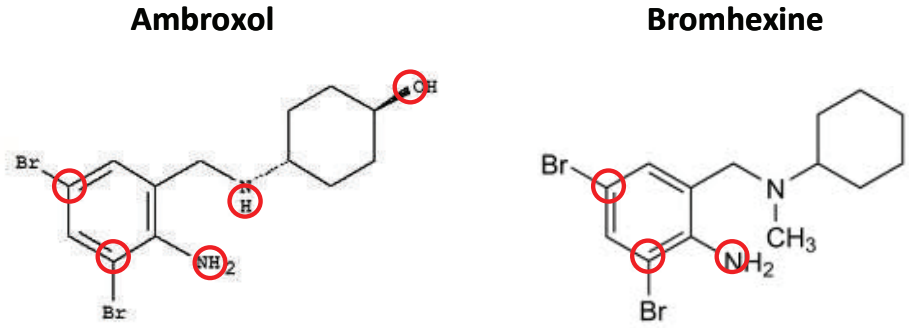


Figure 24: Derivatisation sites of Ambroxol and Bromhexine. The compounds tested in the *in vitro* α-gal enzyme activity assay are indicated with red circles. Ambroxol had two additional coupling sites, the hydroxy-group at the cylohexanol ring and the functionally active (benzyl-) amine group.

No compound could be identified increasing the effect exerted by ABX on mutant α-gal form A156V. Common structure elements of the functional compounds are currently investigated. Next, the question was addressed whether one of the compounds was a more effective PC on mutant glucocerebrosidase. A system based on cultured patient-derived fibroblasts was utilised as described before (Bendikov-Bar et al., 2013). It was shown that mutant forms of glucocerebrosidase could be triggered. The strength of the ABX effect was dependent on the mutation and cell line. Interestingly, cell lines harbouring the Gaucher disease causing gene variant N370S homozygously displayed variable data ranging from 1.4 (cell line originating from NYU Medical Center, NY, USA, Bendikov-Bar et al., 2013) to 3.0 (cell lines provided by the Hospital for Sick Children Tissue Culture Facility, MA, USA, Maegawa et al., 2009) fold increase. Despite different assay conditions (treatment duration, enzyme activity measurement, ABX concentration) it is likely that different individuals (patients/patient cell lines) display varying beneficial effects upon treatment. To our knowledge, no other compound analogue than ABX itself has been applied to Gaucher disease cells so far. Even though it is known that BHX is a potent glucocerebrosidase inhibitor *in vitro* there is no present publication that reports its functionality as a pharmacological chaperone. The compounds BHX and ABX were administered to cell line GM00852 (Coriell cell repository, NJ, USA) harbouring the genotype N370S/c.84_85insG). Moreover, selected derivatives and the inhibitor NB-DNJ and the proteasomal degradation inhibitor MG-132 were analysed as well. To begin with, BHX did not significantly change glucocerebrosidase activity within the cells in contrast to ABX which leads to a 2.1-fold increase. The finding that NB-DNJ did not lead to a substantially increased N370S activity surprises: In a Cos7 cell based overexpression assay it was shown to increase *GBA*[N370S] mutant activity by a factor of 2.1 (Alfonso et al., 2005). The peptidic proteasomal inhibitor n-benzoyloxycarbonyl (Z)-Leu-Leu-leucinal (MG-132) could slightly increase cellular glucocerebrosidase activity in a concentration evidently lower than necessary to inhibit proteasomal activity in the tested cell line

(100 nM). A higher concentration (1 µM) did not recover mutant activity. It was shown that 0.4 µM (Wang et al., 2011) and 15 µM (Ron and Horowitz, 2005) concentrations of MG-132 were beneficial in cultured skin fibroblast Gaucher disease models. Again, strong inter-individual variability of the cells, different culture and assay conditions can be accountable for the detected differences, but it should be taken into consideration that rather induced gene regulation (Kim et al., 2011; Wang et al., 2011) than proteasomal inhibition could be the responsible mechanism for the observed pharmaceutical effect of the drug.

The main focus of the study was on the ABX/BHX derivatives. Among the tested ones only Ambroxol analogue SF-153B was able to stimulate the enzyme activity. The compounds tested herein were selected by their ability to stimulate mutant α-gal activity lacking any further rationale on a structural basis. The results suggest that the physico-chemical properties that specify ABX and its derivatives as enzyme enhancing molecules for α-gal are considerably different from that ones identifying ABX as a PC in Gaucher disease. Therefore, selection criteria have to be re-evaluated. It is likely that derivatives sent off from ABX as the lead structure are the better choice for a PC screening in Gaucher disease. The derivatives were subjected to an *in vitro* inhibition assay on recombinant glucocerebrosidase (imiglucerase) in order to examine whether the derivatisation process was accompanied by loss of inhibitory function as well. Most of the tested substances (excerpted results are shown in figure 22) retained inhibitory function without undergoing significant changes in efficiency. Substance SF-110D showed an increased ability to block enzyme function in this experiment, but it did not increase *GBA*[N370S] activity intracellularly. By contrast, SF-153B inhibited the recombinant enzyme only mildly and starting at the highest concentration applied (0.33 µM). A possible explanation for the intracellular functionality of SF-153B as a pharmacological chaperone is that the compound is supposed to act as a pro-drug of ABX (personal communication with Prof. M. Beller, PhD, Leibniz Institute for Catalysis, Rostock, Ger) which becomes activated only within the cells. The discovery of a pharmacological chaperone that does not act as an enzyme inhibitor *in vitro* may improve drug safety of a small molecule in clinical practice (Steet et al., 2007).

It was not possible to reproduce the elevation of enzyme activity in patient-derived B-lymphocyte cells. It is thus important to reveal the mechanism of the observed enzyme enhancing effect of the DGJ/ABX combination. It could be that the chosen mutations A143T and N215S were not the optimal model. Regrettably, cells harbouring model mutations A156V and R301Q were not available. However, the author of these lines may be allowed to speculate that the failure to reproduce the demonstrated effect in the HEK293H cell system is due to too low expression represented by an average enzyme activity of $\sim$ 30 nmol/hr/mg protein in human B-lymphocytes

diminished to about 14 and 10 nmol/hr/mg protein by the mutations A143T and N215S, respectively. The HEK293H cells had an endogenous activity of 97.5 nmol/hr/mg protein. Administration of 60 μM ABX increased the activity to 134.6 nmol/hr/mg protein. Therefore, the endogenously expressed wild type enzyme of the HEK293H cells which showed a ~3-fold higher basal level benefited from ABX. Of course, another possible explanation involves cell line specific differences rendering the cells ABX responsive or mediating insensibility for any reason.

4.3.3.1.2 Consideration of Ambroxol as a universal chemical chaperone

It was also demonstrated that ABX enhances mutant acid glucosidase alpha activity (figures 19, 20). In an analogous screening system, mutant GAA forms were overexpressed in HEK293H cells and treated with a pharmacological chaperone (NB-DNJ or DNJ) and a combination of the PC and ABX. PC-responsive mutations tended to be further stimulated by additional ABX administration. Of note, none of the tested derivatives could be shown to be effective on the acid alpha-glucosidase mutant forms (data not shown). Please note, that Bromhexine did not show a beneficial effect on the enzyme and that all tested compounds (SF-54B, SF-55C, SF-67A, SF-80A) were derivatives thereof. As for the glucocerebrosidase this indicates a strict demand for ABX and maybe derivatives thereof (e.g. SF-153B must be tested).

The data show that ABX has a wide area of action which makes it a universal chemical chaperone comparable to dimethyl sulfoxide (DMSO), trimethylamine n-oxide (TMAO), glycerol, and other small molecules with low specificity. Furthermore, the functionality of ABX on different acid hydrolases of the lysosomal enzymatic complex emphasises the generality of the pathway and resulting therapeutic strategies (Mu et al., 2008). An advantage in considering ABX as a putative drug to treat LSDs like Gaucher (where surely the best data and rationale for this consideration exist), Fabry and Pompe disease is its clinical approval and good tolerance even in high dosages.

However, it appears that ABX demands for a functional pharmacological chaperone since treatment prosperity of ABX is hampered when administered with a less effective chaperone such as NB-DNJ on PD mutations. This contingency is one limiting point. It could be interesting to test ABX in combination with a general chaperone like 4-Phenylbutyrate that was able to overcome the trafficking defect of α-gal mutant enzymes, but did not lead to the generation of functional enzyme at the destination organelle. The contiguous effect of DMSO was shown by using it as a solvent for ABX. This combination showed a slightly lower effect of the DGJ/ABX combination when compared to ultrapure water-dissolved ABX. DMSO had to be used as the solvent for BHX and the derivated compounds which showed poor solubility in water. Therefore, enzyme activity

"at max" may be lower than it could be if the form of administration to cultured cells can be improved.

Intrinsic aggregation propensity of the mutant α-gal forms was recently proposed as a possible threat of the mutant enzyme forms to cause FD pathogenicity (Siekierska et al., 2012). Siekierska and colleagues concluded: “Combining pharmacological chaperones treatment with suppression of aggregation might be beneficial for future therapeutic strategy against Fabry disease”. It is possible though speculative that ABX functions as an aggregation suppressor since there is no evidence for direct interaction to the enzyme *in vitro*. It is also unlikely that ABX expresses its function *via* gene regulatory mechanisms since none of the proposed interacting pathways of lysosomal enzyme maturation was yet reported to be modulated by ABX. Future work will show whether ABX functionality is associated with enzyme forms undergoing strong aggregation.

4.3.3.2 Rosiglitazone elevates mutant *GLA*[R301Q] activity

In an approach to test substances that manipulate the cellular ER stress response and the UPS, several compounds were selected to be tested in the presented screening system (figure 12).

Kifunensin (KIF) did not display any effect on α-gal activity in the presented study. KIF (1-Deoxymannosid) is a mannose homologue functioning as mannosidase inhibitor which in turn causes ER stress. KIF was shown to increase the cellular level of mutant a-gal (Ishii et al., 2007). A similar effect was observed with the irreversible proteasomal degradation inhibitor Lactacystin in the same study. Since α-gal is a likely target enzyme of mannosidases this enzyme´s activity may be necessary for proper α-gal trafficking. The same holds true for ER stress mediator Tunicamycin (TM), the blockage of N-glycosylation might antagonise proper maturation and transport of α-gal and therefore adversing possible beneficial expression of the UPR genes. Thapsigargin (Tg) may jeopardise contributional ER gene expression by creating a Ca^{2+} ion depleted environment in the ER which does not allow for proper protein folding. Possibly, a shorter treatment time followed by immediate withdrawal of these compounds may be more helpful than a continuous administration. In a related study, the influence of the substances TM and Tg did also no benefit to mutant forms of glucocerebrosidase (Wang et al., 2011). The reversible proteasomal degradation inhibitor MG-132 was tested, because another inhibitor (Lactacystin) showed promising results: The enzyme amount was increased by treatment with Lactacystin on mutant forms E66Q, F113L, N215S, M296I and R301Q, leading to a decrease of premature degradation (Ishii et al., 2007). Moreover, MG-132 provoked a cellular increase of catalytically active glucocerebrosidase mutations. There was no detectable effect on the mutant α-gal form under examination on the activity level whereas a mild effect could be demonstrated on the protein

level (data not shown). Since Lactacystin did not show any effect on a broad spectrum of GLA mutations (E59K, M72V, I91T, A97V, R112H, L166V, Q279E, M296V, R356W, G373D and G373S) it is likely that MG-132 also selectively affects mutant enzymes in FD and the mutation R301Q was not the right model.

Rosiglitazone (Rosig) is a ubiquitination inhibitor. Without a proper ubiquitinating system the cellular UPS cannot function properly. In the presented system FD related mutant enzyme form R301Q responded to Rosig and a combination of DGJ and Rosig with enhanced activity (figure 12). Rosig was the only UPS-disrupting compound that showed a beneficial effect on mutant α-gal activity. Other compounds with a distinct mechanistic force but leading to UPS inhibition (e.g. Bortezomib) were tested without an effect (data not shown). The α-gal enzyme is synthesised as an immature protein of 50 kDa. This "ER-form" gets N-terminally processed followed by conjugation of high-mannose or complex oligosaccharides at its *Asn-139*, *Asn-192* and *Asn-215* residues (Matsuura et al., 1998, Ioannou et al., 1998). The mature lysosomal enzyme appears as a 46 kDa band on the Western blot. Some mutations such as the R301Q lead to a maturation defect of the corresponding enzyme and, hence, merely immature form can be detected. Besides the increase of activity Rosiglitazone predominantly elevated the cellular amount of the immature form of GLA[R301Q] (data not shown). In combination with DGJ, Rosiglitazone significantly increased the mature form above the R301Q mutant form in comparison to the DGJ single treatment. We assume a mechanism by which a great portion of mutant α-gal enzyme destined for proteasomal degradation within the ER at first, translocates to the cytosol where ubiquitination can no longer take place. A retrograde transport process back to the ER lumen may make the protein amenable for further transport. The supply of unfolded α-gal enzyme is consequently increased in the ER and the DGJ supports the course of correct folding and trafficking. Alternatively, one could think of a disturbed translocation (e.g. exhausted capacity) of unfolded/immature α-gal enzyme from the ER to the cytosol due to the failure to ubiquitinate and degrade the bulk of protein. Possibly, both mechanisms run simultaneously. In any case, a higher amount of α-gal is accessible to the PC and make the DGJ function more efficiently. Subsequently, it becomes a more probable statistical event α-gal can bypass ER retention and degradation. A rapid kinetic of DGJ-recovered R301Q activity in Rosig pre-treated cell cultures (data not shown) supports this hypothesis.

However, it is plausible that Rosiglitazone functions *via* mechanisms other than ubiquitination circumvention. First, Rosiglitazone displays agonist action on Peroxisomal proliferator-activated receptor gamma (PPARγ) which regulates fatty acid and carbohydrate metabolism related genes and mediates glucose uptake of cells which may have an influence on *GLA* expression, maturation

and trafficking of the enzyme. Moreover, the ubiquitination inhibition causes the expression of ER stress genes (Weber et al., 2004) which is a supposed mechanism for MG-132 besides the proteasomal inhibition in Gaucher disease (Kim et al., 2011; Wang et al., 2011). The influence of glucose should be tested on the ability of Rosig to display its function on α-gal, since a significantly lower activity of α-gal is observed in normal glucose medium. The use of glucose depleted medium on *GLA* mutations expressing cells treated with Rosig could clarify this point. Second, an *in silico* search for putative ubiquitination sites (http://www.ubpred.org/) in α-gal gave no positive results indicating that degradation of α-gal may take place by a different mechanism than ubiquitin-mediated ERAD. For comparison, human glucocerebrosidase, known to be ubiquinated (Ron and Horowitz, 2005) was probed as well. Examination resulted in the identification of three potential ubiquitination sites (Lys13, Lys237, Lys480).

A potential application for Rosig could be DGJ non-responding mutations that have a significant residual activity such as R118C, M267T, T385A and others broadening the spectrum of small molecule addressable mutations. In order to separate the two likely mechanistic functions of Rosig occasionally leading to increased α-gal activity PPARγ agonists (e.g. Bezafibrate) and ubiquitination inhibitors (e.g. PYR-41, SMER3) will be tested.

Summary

Mutations within the α-galactosidase A gene (*GLA*) lead to Fabry disease. Despite the X-chromosomal recessive mode of inheritance, female carriers can develop symptoms. However, diagnosis (especially) of females is complex due to the inactivation of parts of the X-chromosome observed in female patients. In the case of novel found mutations specimen from female donors are unfeasible for biochemical examination. Therefore, this study was designed to facilitate diagnostic decisions and disease prognosis by characterising multiple mutant forms of the enzyme. The utilised overexpression system in HEK293H cells has the capacity to provide information about the level of damage of the mutations. On the basis of a comparative model, the phenotype prediction values of *in vivo* biomarker and a computational prediction algorithm (PolyPhen) revealed that enzyme damage as obtained by the *in vitro* activity measurement was most accurate in assigning the mutations to the correct disease phenotype group. Moreover, evidence could be found that a detailed biochemical characterisation (e.g. kinetic properties) of the mutations might reveal unforeseen damage that likely contributes to clinical consequences.

The routine evaluation of the responsiveness to pharmacological chaperone DGJ showed that about half of the missense mutations can be assumed defective *via* misfolding and instability rather than catalytic damage. This indicates that there is a great potential of patients to be treated with an alternative small molecule therapy.

The finding that inhibitors of the proteasomal degradation process (e.g. MG-132) and compounds provoking the ER stress response did not lead to a marked change in mutant (R301Q) α-gal activity illustrates the differences in the processes underlying α-gal and glucocerebrosidase maturation even though both enzymes need to be transported to the lysosome. It is worthwhile to consider those processes in more detail in order to find therapeutic targets addressable by small molecules. By what means the PPARγ agonist Rosiglitazone, a drug used for the treatment of type II diabetes, helps to increase mutant α-gal activity needs to be determined. However, in this study it was used, because it was believed to interfere with the ubiquitin proteasome system. The potential pharmacological chaperone Ambroxol displayed the ability to increase the activity of several mutant α-gal forms in combination with DGJ above the level of the single DGJ treatment which suggests that even non-iminosugar compounds can act as molecular enhancers of mutant α-gal forms in Fabry disease.

6 Literature

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Online sources

http://www.focusonfabry.co.uk/healthcare_symptoms.aspx?mode=print

http://www.hgmd.org/

http://fabry-database.org/mutants/

7 Appendix

7.1 List of primers

No.	Name	Sequence	purpose
			GLA Mutagenesis
1	E7X frw	gctgaggaacccataactacatctggg	
2	E7X rev	cccagatgtagttatgggttcctcagc	
3	A15E frw	ctgcgcgcttgagcttcgcttcc	
4	A15E rev	ggaagcgaagctcaagcgcgcag	
5	W24C frw	ccctcgtttcctgtgacatccctgggg	
6	W24C rev	ccccagggatgtcacaggaaacgaggg	
7	G35R frw	agcactggacaatagattggcaaggac	
8	G35R rev	gtccttgccaatctattgtccagtgct	
9	L36S frw	actggacaatggatcggcaaggacgccta	
10	L36S rev	taggcgtccttgccgatccattgtccagt	
11	A37T frw	ggacaatggattgacaaggacgcctac	
12	A37T rev	gtaggcgtccttgtcaatccattgtcc	
13	M42V frw	aaggacgcctaccgtgggctggctgca	
14	M42V rev	tgcagccagcccacggtaggcgtcctt	
15	G43S frw	gacgcctaccatgagctggctgcactg	
16	G43S rev	cagtgcagccagctcatggtaggcgtc	
17	H46P frw	ccatgggctggctgcctgggagcgcttcat	
18	H46P rev	atgaagcgctcccaggcagccagcccatgg	
19	R49G frw	gctgcactgggaggcttcatgtgcaa	
20	R49G rev	ttgcacatgaagcctcccagtgcagc	
21	R49C frw	gttgcacatgaagcactcccagtgcagcc	
22	R49C rev	ggctgcactgggagtgcttcatgtgcaac	
23	M51K frw	Ctgggagcgcttcaagtgcaaccttgact	
24	M51K rev	agtcaaggttgcacttgaagcgctcccag	
25	M51I frw	tgggagcgcttcatatgcaaccttgactg	
26	M51I rev	cagtcaaggttgcatatgaagcgctccca	
27	C52W frw	gagcgcttcatgtggaaccttgactgcca	
28	C52W rev	tggcagtcaaggttccacatgaagcgctc	
29	E59K frw	ttgactgccaggaaaagccagattcctgc	
30	E59K rev	gcaggaatctggctttttcctggcagtcaa	
31	C63Y frw	gagccagattcctacatcagtgagaag	
32	C63Y rev	cttctcactgatgtaggaatctggctc	
33	S65I frw	cagattcctgcatcattgagaagctcttcat	
34	S65I rev	atgaagagcttctcaatgatgcaggaatctg	
35	E66K frw	attcctgcatcagtaagaagctcttcatg	
36	E66K rev	catgaagagcttcttactgatgcaggaat	
37	L68F frw	gcatcagtgagaagttcttcatggagatg	
38	L68F rev	catctccatgaagaacttctcactgatgc	
39	A73V frw	cttcatggagatggtagagctcatggtct	
40	A73V rev	agaccatgagctctaccatctccatgaag	
41	K82X frw	tctcagaaggctggtaggatgcaggttat	
42	K82X rev	ataacctgcatcctaccagccttctgaga	
43	D83N frw	tcagaaggctggaagaatgcaggttatgag	

44	D83N rev	actcataacctgcattcttccagccttctg	
45	Y86D frw	ggaaggatgcaggtgatgagtacctctgc	
46	Y86D rev	gcagaggtactcatcacctgcatccttcc	
47	L89H frw	caggttatgagtaccactgcattgatgact	
48	L89H rev	cagtcatcaatgcagtggtactcataacctg	
49	276InsTGAT frw	tacctctgcattgattgatgactgttggatggct	
50	276InsTGAT rev	agccatccaacagtcatcaatcaatgcagaggta	
51	D93Y frw	tacctctgcattgattactgttggatggctc	
52	D93Y rev	gagccatccaacagtaatcaatgcagaggta	
53	D93E frw	cctctgcattgatgagtgttggatggctc	
54	D93E rev	gagccatccaacactcatcaatgcagagg	
55	W95L frw	cattgatgactgtttgatggctccccaaa	
56	W95L rev	tttggggagccatcaaacagtcatcaatg	
57	W95C frw	gcattgatgactgttgtatggctccccaaagag	
58	W95C rev	ctctttggggagccatacaacagtcatcaatgc	
59	R100T frw	gatggctccccaaacagattcagaaggca	
60	R100T rev	tgccttctgaatctgtttggggagccatc	
63	S102L frw	ctccccaaagagatttagaaggcagacttca	
64	S102L rev	tgaagtctgccttctaaatctctttggggag	
65	E103X frw	cccaaagagattcataaggcagacttcag	
66	E103X rev	ctgaagtctgccttatgaatctctttggg	
67	R112C frw	caggcagaccctcagtgctttcctcatggga	
68	R112C rev	tcccatgaggaaagcactgagggtctgcctg	
69	R112H frw	gcagaccctcagcactttcctcatggg	
70	R112H rev	cccatgaggaaagtgctgagggtctgc	
71	R118S frw	gctttcctcatgggattagccagctagctaattat	
72	R118S rev	ataattagctagctggctaatcccatgaggaaagc	
73	R118G frw	gctttcctcatgggattggccagctagctaattat	
74	R118G rev	ataattagctagctggccaatcccatgaggaaagc	
75	R118C frw	ctttcctcatgggatttgccagctagctaatta	
76	R118C rev	taattagctagctggcaaatcccatgaggaaag	
77	R118H frw	ctttcctcatgggattcaccagctagctaattatg	
78	R118H rev	cataattagctagctggtgaatcccatgaggaaag	
79	R118P frw	ctttcctcatgggattcccagctagctaattatg	
80	R118P rev	cataattagctagctgggaatcccatgaggaaag	
81	R118L frw	ctttcctcatgggattctccagctagctaattatg	
82	R118L rev	cataattagctagctggagaatcccatgaggaaag	
83	L120V frw	catgggattcgccaggtagctaattatgttc	
84	L120V rev	gaacataattagctacctggcgaatcccatg	
85	H125P frw	ctagctaattatgttccccagcaaaggactgaag	
86	H125P rev	cttcagtcctttgctgggaacataattagctag	
87	S126R frw	agctaattatgttcaccgcaaaggactgaagct	
88	S126R rev	agcttcagtcctttgcggtgaacataattagct	
89	S126G frw	agctaattatgttcacggcaaaggactgaagct	
90	S126G rev	agcttcagtcctttgccgtgaacataattagct	
91	S126C frw	ctagctaattatgttcactgcaaaggactgaagctag	
92	S126C rev	ctagcttcagtcctttgcagtgaacataattagctag	
93	S126N frw	tagctaattatgttcacaacaaaggactgaagctagg	
94	S126N rev	cctagcttcagtcctttgttgtgaacataattagcta	

95	S126T frw	tagctaattatgttcacaccaaaggactgaagctagg	
96	S126T rev	cctagcttcagtcctttggtgtgaacataattagcta	
97	S126I frw	tagctaattatgttcacatcaaaggactgaagctagg	
98	S126I rev	cctagcttcagtcctttgatgtgaacataattagcta	
99	L129P frw	tcacagcaaaggaccgaagctagggattt	
100	L129P rev	aaatccctagcttcggtcctttgctgtga	
101	L131P frw	gcaaaggactgaagccagggatttatgcaga	
102	L131P rev	tctgcataaatccctggcttcagtcctttgc	
103	G132R frw	caaaggactgaagctaaggatttatgcagatgt	
104	G132R rev	acatctgcataaatcctagcttcagtcctttg	
105	G132E frw	aggactgaagctagagatttatgcagatg	
106	G132E rev	catctgcataaatctctagcttcagtcct	
107	A135V frw	gctagggatttatgtagatgttggaaata	
108	A135V rev	tatttccaacatctacataaatccctagc	
109	D136E frw	agggatttatgcagaagttggaaataaaacc	
110	D136E rev	ggttttatttccaacttctgcataaatccct	
111	G138R frw	tttatgcagatgttagaaataaaacctgc	
112	G138R rev	gcaggttttatttctaacatctgcataaa	
113	N139S frw	tgcagatgttggaagtaaaacctgcgcag	
114	N139S rev	ctgcgcaggttttacttccaacatctgca	
115	T141I frw	gatgttggaaataaaatctgcgcaggcttccct	
116	T141I rev	agggaagcctgcgcagattttatttccaacatc	
117	C142R frw	ttggaaataaaaccgcgcaggcttccctgg	
118	C142R rev	ccagggaagcctgcgcgggttttatttccaa	
119	A143T frw	ggaaataaaacctgcacaggcttccctggga	
120	A143T rev	tcccagggaagcctgtgcaggttttatttcc	
121	A143P frw	ggaaataaaacctgccccaggcttccctggga	
122	A143P rev	tcccagggaagcctggcaggttttatttcc	
123	G147R frw	cgcaggcttccctaggagttttggatact	
124	G147R rev	agtatccaaaactcctagggaagcctgcg	
125	A156D frw	ctacgacattgatgaccagacctttgctg	
126	A156D rev	cagcaaaggtctggtcatcaatgtcgtag	
127	A156V frw	ctacgacattgatgtccagacctttgctg	
128	A156V rev	cagcaaaggtctggacatcaatgtcgtag	
129	W162G frw	gacctttgctgacgggggagtagatct	
130	W162G rev	agatctactccccgtcagcaaaggtc	
131	D165H frw	gctgactggggagtacatctgctaaaatttg	
132	D165H rev	caaattttagcagatgtactccccagtcagc	
133	D165V frw	tgactggggagtagttctgctaaaatttg	
134	D165V rev	caaattttagcagaactactccccagtca	
135	D165Y frw	gctgactggggagtatatctgctaaaattt	
136	D165Y rev	caaattttagcagatatactccccagtcagc	
137	D170N frw	gatctgctaaaatttaatggttgttactgtg	
138	D170N rev	cacagtaacaaccattaaattttagcagatc	
139	C172G frw	gctaaaatttgatggtggttactgtgacagttt	
140	C172G rev	aaactgtcacagtaaccaccatcaaattttagc	
141	C172Y frw	ctaaaatttgatggttattactgtgacagtttg	
142	C172Y rev	caaactgtcacagtaataaccatcaaattttag	
143	c521InsT frw	aatttgatggttgttacttgtgacagtttggaaaatt	

144	c521InsT rev	aattttccaaactgtcacaagtaacaaccatcaaatt	
145	D175N frw	ttgatggttgttactgtaacagtttggaaaatttg	
146	D175N rev	caaattttccaaactgttacagtaacaaccatcaa	
147	D175E frw	tggttgttactgtgaggagtttggaaaatttg	
148	D175E rev	caaattttccaaactctcacagtaacaacca	
149	c525-526delCA	tgatggttgttactgtgaˆgtttggaaaatttggcag	
150	c525-526delCA	ctgccaaattttccaaacˆtcacagtaacaaccatca	
151	G183V frw	gaaaatttggcagatgttataagcacatgtcct	
152	G183V rev	aggacatgtgcttataaacatctgccaaattttc	
153	M187T frw	tggttataagcacacgtccttggccctga	
154	M187T rev	tcagggccaaggacgtgtgcttataacca	
155	L191P frw	atgtccttggcccgaataggactggc	
156	L191P rev	gccagtcctattcggggccaaggacat	
157	W204C frw	ctcctgtgagtgcctctttatatg	
158	W204C rev	catataaagagggcactcacaggag	
159	K213M frw	gtggccctttcaaatgcccaattatacag	
160	K213M rev	ctgtataattgggcatttgaaagggccac	
161	N215S frw	cctttcaaaagcccagttatacagaaatccg	
162	N215S rev	cggatttctgtataactgggcttttgaaagg	
163	I219T frw	ccaattatacagaaacccgacagtactgcaa	
164	I219T rev	ttgcagtactgtcgggtttctgtataattgg	
165	R220Q frw	aattatacagaaatccaacagtactgcaatcac	
166	R220Q rev	gtgattgcagtactgttggatttctgtataatt	
167	N224S frw	ccgacagtactgcagtcactggcgaaatt	
168	N224S rev	aatttcgccagtgactgcagtactgtcgg	
169	H225D frw	gacagtactgcaatgactggcgaaatttt	
170	H225D rev	aaaatttcgccagtcattgcagtactgtc	
171	H225R frw	acagtactgcaatcgctggcgaaattttg	
172	H225R rev	caaaatttcgccagcgattgcagtactgt	
173	R227Q frw	ctgcaatcactggcaaaattttgctgaca	
174	R227Q rev	tgtcagcaaaatttgccagtgattgcag	
175	D231N frw	ggcgaaattttgctaacattgatgattcc	
176	D231N rev	ggaatcatcaatgttagcaaaatttcgcc	
177	I232T frw	gaaattttgctgacactgatgattcctggaa	
178	I232T rev	ttccaggaatcatcagtgtcagcaaaatttc	
179	W236C frw	cattgatgattcctgcaaaagtataaagagt	
180	W236C rev	actctttatacttttgcaggaatcatcaatg	
181	S238N frw	gatgattcctggaaaaatataaagagtatcttg	
182	S238N rev	caagatactctttatattttccaggaatcatc	
183	I242N frw	aaagtataaagagtaacttggactggacatc	
184	I242N rev	gatgtccagtccaagttactctttatacttt	
185	R252T frw	tcttttaaccaggagacaattgttgatgttgct	
186	R252T rev	agcaacatcaacaattgtctcctggttaaaaga	
187	I253S frw	taaccaggagagaagtgttgatgttgctg	
188	I253S rev	cagcaacatcaacacttctctcctggtta	
189	G261V frw	gctggaccaggggttttggaatgaccca	
190	G261V rev	tgggtcattccaaacccctggtccagc	
191	W262C frw	gaccaggggttgcaatgacccagata	
192	W262C rev	tatctgggtcattgcaacccccctggtc	

193	D264N frw	caggggttggaataacccagatatgtta	
194	D264N rev	taacatatctgggttattccaacccccctg	
195	D264H frw	caggggttggaatcacccagatatgtta	
196	D264H rev	taacatatctgggtgattccaacccccctg	
197	D264Y frw	caggggttggaattacccagatatgtta	
198	D264Y rev	taacatatctgggtaattccaacccccctg	
199	D264A frw	aggggttggaatgccccagatatgttag	
200	D264A rev	ctaacatatctggggcattccaacccccct	
201	D264G frw	aggggttggaatggcccagatatgttag	
202	D264G rev	ctaacatatctgggccattccaacccccct	
203	D264V frw	aggggttggaatgtcccagatatgttag	
204	D264V rev	ctaacatatctgggacattccaacccccct	
205	D264E frw	aggggttggaatgaaccagatatgttagtg	
206	D264E rev	cactaacatatctggttcattccaacccccct	
207	M267T frw	gaatgacccagatacgttagtgattggca	
208	M267T rev	tgccaatcactaacgtatctgggtcattc	
209	L268S frw	gacccagatatgtcagtgattggcaac	
210	L268S rev	gttgccaatcactgacatatctgggtc	
211	V269M frw	gacccagatatgttaatgattggcaactttg	
212	V269M rev	caaagttgccaatcattaacatatctgggtc	
213	V269A frw	acccagatatgttagcgattggcaactttgg	
214	V269A rev	ccaaagttgccaatcgctaacatatctgggt	
215	c807delG frw	Cccagatatgttagt^attggcaactttggc	
216	c807delG rev	Gccaaagttgccaat^actaacatatctggg	
217	I270M frw	agatatgttagtgatgggcaactttggcc	
218	I270M rev	ggccaaagttgcccatcactaacatatct	
219	N272S frw	gttagtgattggcagctttggcctcagct	
220	N272S rev	agctgaggccaaagctgccaatcactaac	
221	F273L frw	gtgattggcaacttaggcctcagctggaa	
222	F273L rev	ttccagctgaggcctaagttgccaatcac	
223	L275F frw	attggcaactttggcttcagctggaatcagc	
224	L275F rev	gctgattccagctgaagccaaagttgccaat	
225	L275H frw	tggcaactttggccacagctggaatcagc	
226	L275H rev	gctgattccagctgtggccaaagttgcca	
227	T282I frw	gaatcagcaagtaattcagatggccctct	
228	T282I rev	agagggccatctgaattacttgctgattc	
229	Q283P frw	tcagcaagtaactccgatggccctctggg	
230	Q283P rev	cccagagggccatcggagttacttgctga	
231	A285D frw	agtaactcagatggacctctgggctatca	
232	A285D rev	tgatagcccagaggtccatctgagttact	
233	W287X frw	cagatggccctctgagctatcatggctgc	
234	W287X rev	gcagccatgatagctcagagggccatctg	
235	M290L frw	ctctgggctatcttggctgctcctt	
236	M290L rev	aaggagcagccaagatagcccagag	
237	M290I frw	cctctgggctatcatagctgctcctttattc	
238	M290I rev	gaataaaggagcagctatgatagcccagagg	
239	A291T frw	ctgggctatcatgactgctcctttattca	
240	A291T rev	tgaataaaggagcagtcatgatagcccag	
241	L294X frw	catggctgctccttgattcatgtctaatg	

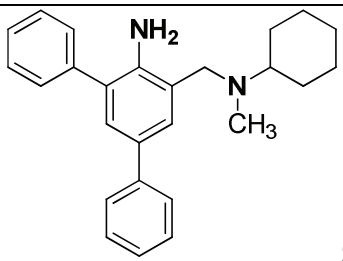
242	L294X rev	cattagacatgaatcaaggagcagccatg	
243	L294S frw	catggctgctccttcattcatgtctaatg	
244	L294S rev	cattagacatgaatgaaggagcagccatg	
245	c883delT frw	tggctgctcccttta^tcatgtctaatgacct	
246	c883delT rev	aggtcattagacatga^taaaggagcagcca	
247	S297C frw	tcctttattcatgtgtaatgacctccgac	
248	S297C rev	gtcggaggtcattacacatgaataaagga	
249	R301G frw	ctaatgacctcggacacatcagccc	
250	R301G rev	gggctgatgtgtccgaggtcattag	
251	R301Q frw	ctaatgacctccaacacatcagccc	
252	R301Q rev	gggctgatgtgttggaggtcattag	
253	R301P frw	ctaatgacctcccacacatcagccc	
254	R301P rev	gggctgatgtgtgggaggtcattag	
255	L310F frw	ctcaagccaaagcttccttcaggataag	
256	L310F rev	cttatcctgaaggaaagctttggcttgag	
257	L311V frw	aagccaaagctctcgttcaggataaggac	
258	L311V rev	gtccttatcctgaacgagagctttggctt	
259	D313Y frw	caaagctctccttcagtataaggacgtaattgc	
260	D313Y rev	gcaattacgtccttatactgaaggagagctttg	
261	V316I frw	cttcaggataaggacataattgccatcaatc	
262	V316I rev	Gattgatggcaattatgtccttatcctgaag	
263	V316G frw	tcaggataaggacggaattgccatcaatc	
264	V316G rev	gattgatggcaattccgtccttatcctga	
265	c948InsT frw	caggataaggacgttaattgccatcaatc	
266	c948InsT rev	gattgatggcaattaacgtccttatcctg	
267	I319T frw	ggacgtaattgccaccaatcaggacccct	
268	I319T rev	aggggtcctgattggtggcaattacgtcc	
269	N320I frw	gtaattgccatcattcaggacccctg	
270	N320I rev	caaggggtcctgaatgatggcaattac	
271	Q321H frw	ttgccatcaatcacgacccctgggca	
272	Q321H rev	tgcccaaggggtcgtgattgatggcaa	
273	G325S frw	atcaggacccctgagcaagcaagggtac	
274	G325S rev	gtaccttgcttgctcaaggggtcctgat	
275	Q327K frw	ccccttgggcaagaaagggtaccagct	
276	Q327K rev	agctggtacccttttcttgcccaagggg	
277	Q327E frw	ccttgggcaaggaagggtaccag	
278	Q327E rev	ctggtacccttccttgcccaagg	
279	G328A frw	cttgggcaagcaagcgtaccagcttagac	
280	G328A rev	gtctaagctggtacgcttgcttgcccaag	
281	E341K frw	ctttgaagtgtggaaacgacctctctc	
282	E341K rev	gagagaggtcgtttccacacttcaaag	
283	E341D frw	ctttgaagtgtgggaccgacctctctcaggc	
284	E341D rev	gcctgagagaggtcggtcccacacttcaaag	
285	R342Q frw	tgaagtgtgggaacaacctctctcaggct	
286	R342Q rev	agcctgagagaggttgttcccacacttca	
287	R342L frw	tgaagtgtgggaactacctctctcaggct	
288	R342L rev	agcctgagagaggtagttcccacacttca	
289	L344P frw	ggaacgacctcctcaggcttag	
290	L344P rev	ctaagcctgaggaggtcgttcc	

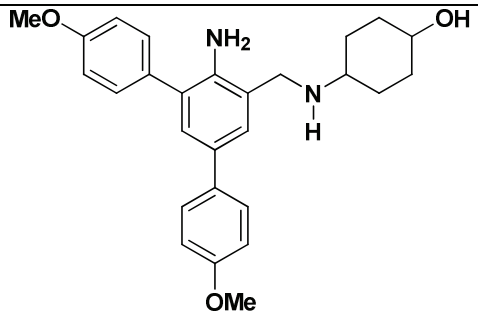
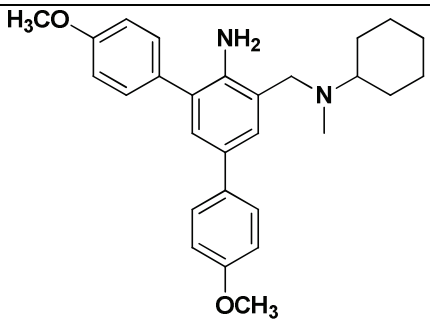
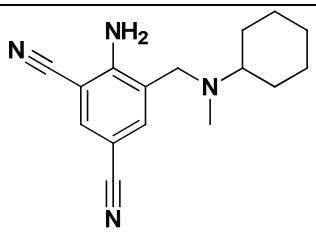
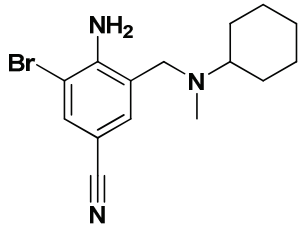
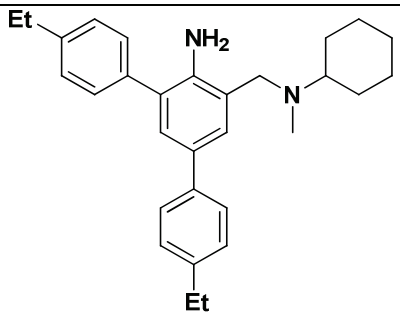
291	S345P frw	gaacgacctctcccaggcttagcct	
292	S345P rev	aggctaagcctgggagaggtcgttc	
293	W349R frw	tctcaggcttagccagggctgtagctatg	
294	W349R rev	catagctacagccctggctaagcctgaga	
295	R356W frw	gtagctatgataaactggcaggagattgg	
296	R356W rev	ccaatctcctgccagtttatcatagctac	
297	R356Q frw	gtagctatgataaaccagcaggagattggtg	
298	R356Q rev	caccaatctcctgctggtttatcatagctac	
299	G360C frw	aaccggcaggagatttgtggacctcgctctt	
300	G360C rev	aagagcgaggtccacaaatctcctgccggtt	
301	G361R frw	ggcaggagattggtcgacctcgctcttat	
302	G361R rev	ataagagcgaggtcgaccaatctcctgcc	
303	R363H frw	gattggtggacctcactcttataccatcg	
304	R363H rev	cgatggtataagagtgaggtccaccaatc	
305	A368T frw	gctcttataccatcacagttgcttccctg	
306	A368T rev	cagggaagcaactgtgatggtataagagc	
307	L372P frw	tcgcagttgcttcccgggtaaaggagtggc	
308	L372P rev	gccactcctttacccggggaagcaactgcga	
309	L372R frw	cgcagttgcttcccggggtaaaggagtgg	
310	L372R rev	ccactcctttacccgggaagcaactgcg	
311	G373D frw	agttgcttccctggataaaggagtggcct	
312	G373D rev	aggccactcctttatccagggaagcaact	
313	C378R frw	taaaggagtggcccgtaatcctgcctg	
314	C378R rev	caggcaggattacgggccactccttta	
315	I384N frw	tcctgcctgcttcaacacacagctcctcc	
316	I384N rev	ggaggagctgtgtgttgaagcaggcagga	
317	T385A frw	tgcctgcttcatcgcacagctcctccc	
318	T385A rev	gggaggagctgtgcgatgaagcaggc	
319	Q386P frw	ctgcttcatcacaccgctcctccctgtga	
320	Q386P rev	tcacagggaggagcggtgtgatgaagcag	
321	L389P frw	cacacagctcctccttgtgaaaaggaagc	
322	L389P rev	gcttccttttcacaggaggagctgtgtg	
323	V390M frw	cacagctcctcccatgaaaaggaagcta	
324	V390M rev	tagcttccttttcatagggaggagctgtg	
325	G395A frw	gtgaaaaggaagctagcgttctatgaatggact	
326	G395A rev	agtccattcatagaacgctagcttccttttcac	
327	F396Y frw	tgaaaaggaagctagggtactatgaatggacttcaag	
328	F396Y rev	cttgaagtccattcatagtaccctagcttccttttca	
329	E398A frw	agctagggttctatgcatggacttcaaggtt	
330	E398A rev	aaccttgaagtccatgcatagaaccctagct	
331	S405R frw	tcaaggttaagctgtcacataaatc	
332	S405R rev	gatttatgtgacagcttaaccttga	
333	c1221delA	gttaagaagtcacataa^tcccacaggcactgttt	
334	c1221delA	aaacagtgcctgtggga^ttatgtgacttcttaac	
335	T410I frw	gtcacataaatcccataggcactgttttgct	
336	T410I rev	agcaaaacagtgcctatgggatttatgtgac	
337	L415F frw	cacaggcactgttttgtttcagctagaaaatac	
338	L415F rev	gtattttctagctgaaacaaaacagtgcctgtg	
339	L415P frw	caggcactgttttgcctcagctagaaaatac	

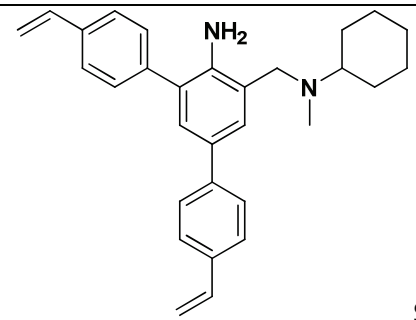
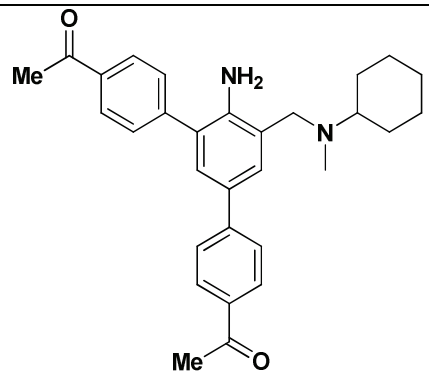
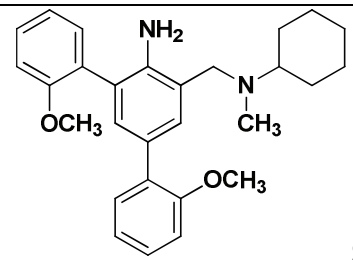
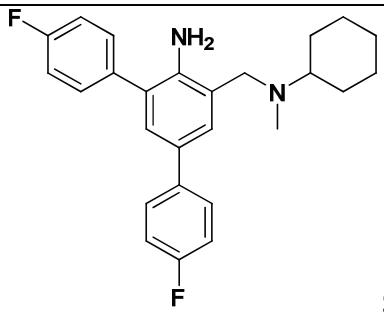
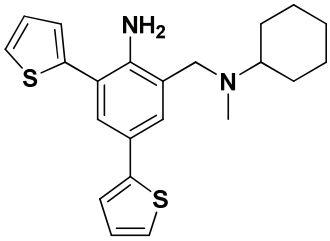
340	L415P rev	gtattttctagctgaggcaaaacagtgcctg	
341	Q416X frw	aggcactgttttgctttagctagaaaatacaat	
342	Q416X rev	attgtattttctagctaaagcaaaacagtgcct	
343	Q416P frw	cactgttttgcttccgctagaaaatacaat	
344	Q416P rev	attgtattttctagcggaagcaaaacagtg	
345	E418G frw	gttttgcttcagctaggaaatacaatgcagatg	
346	E418G rev	catctgcattgtatttcctagctgaagcaaaac	
			GAA Mutagenesis
347	R375L frw	cttccacctgtgcctctggggctactcct	
348	R375L rev	aggagtagccccagaggcacaggtggaag	
349	Y455F frw	ctgccgggagcttcaggccctacga	
350	Y455F rev	tcgtagggcctgaagctcccggcag	
351	P545L frw	caccctacgtgcttggggtggttgg	
352	P545L rev	ccaaccaccccaagcacgtagggtg	
353	L552P frw	ttgggggggacccccaggcggccac	
354	L552P rev	gtggccgcctgggggtccccccaa	
355	Y575S frw	ctgcacaacctctccggcctgaccgaa	
356	Y575S rev	ttcggtcaggccggagaggttgtgcag	
			Cloning
357	GAA frw	taggagctgtccaggccatc	GAA
358	GAA rev	gagagactaacacactccgc	GAA
359	GLA frw	aggtcggatccgacaatgcagctgaggaacc	GLA
360	GLA rev (*Bst* BI)	ggtgttcgaattaaagtaagtcttttaatgacatctgca	GLA
361	GLA rev V5-tag	ggtcgaatgaaagtaagtcttttaatgacatctgca	GLA + epitope tag
			Sequencing primers*
362	T7 frw	taatacgactcactataggg	all used vectors
363	BGH rev	tagaaggcacagtcgaggc	all used vectors

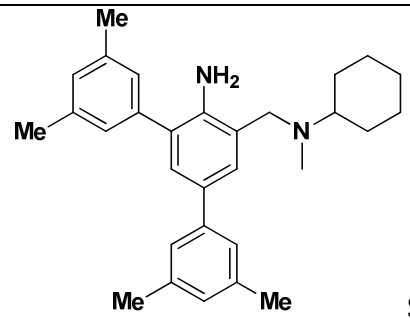
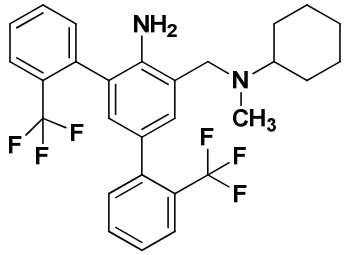
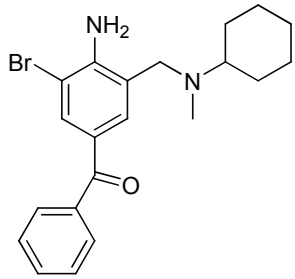
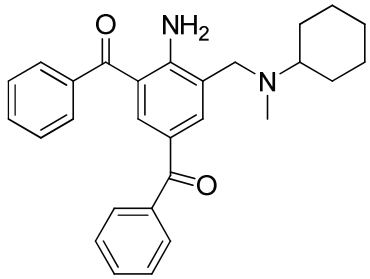
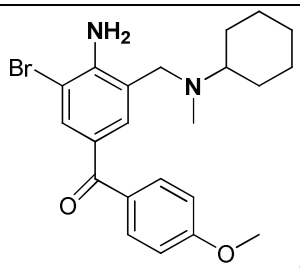
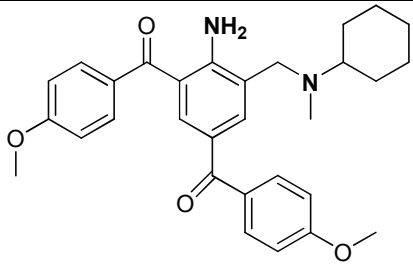
* For inserts > 1 kb, internal primers were generated to cover the whole sequence

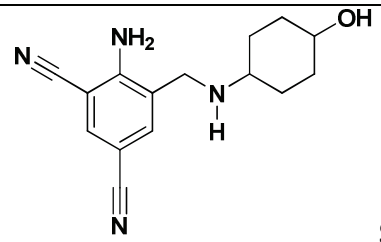
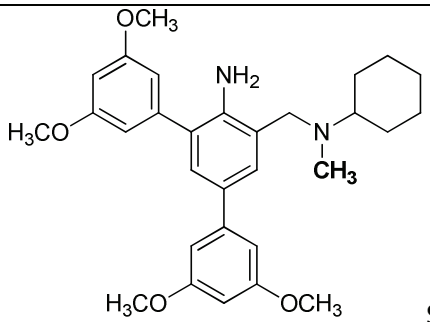
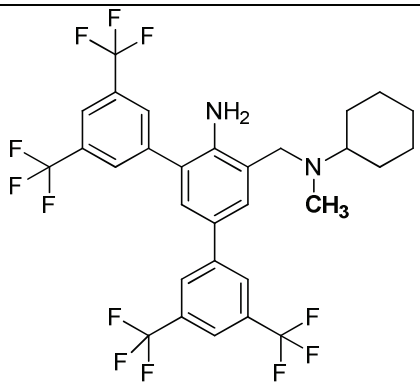
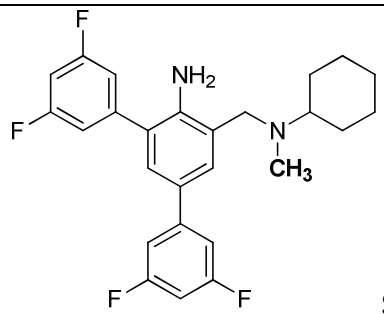
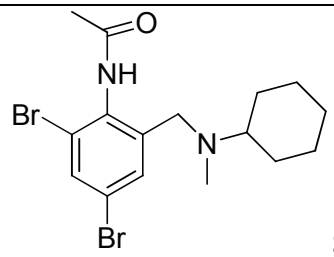
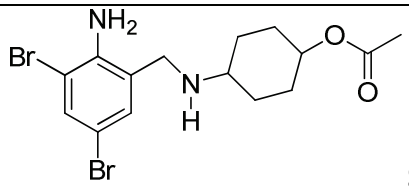
7.2 Table of ABX/BHX derivatives and the effect on mutant form A156V of α-galactosidase A

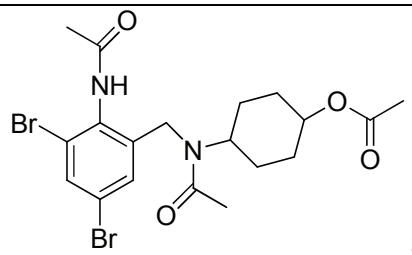
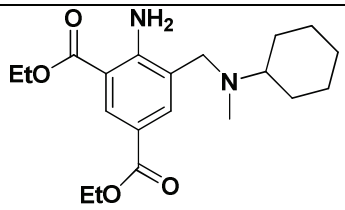
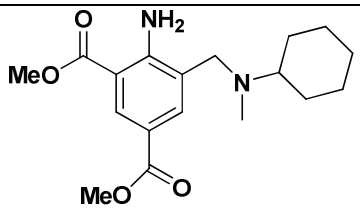
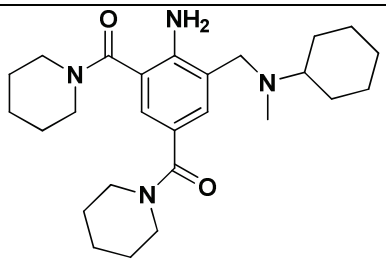
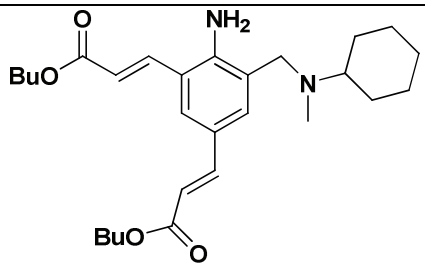
Compound (in order of running number)	**Activity** (1. 10. 40 µM. values are mean ± SEM)	**Derived from**
 **SF-18A**	**33.7 ± 4.8** **31.0 ± 2.2** **n.a.**	**Bromhexine**

 SF-21A	**29.4 ± 0.4** **24.2 ± 3.8** **n.a.**	**Ambroxol**
 SF-22B	**26.5 ± 7.8** **25.5 ± 3.3** **3.7 ± 3.7**	**Bromhexine**
 SF-54B	**31.8 ± 6.0** **51.9 ±9.3** **57.6*** ±5.1**	**Bromhexine**
 SF-55C	**40.3 ± 8.5** **47.2** ± 5.2** **52.0*** ± 1.9**	**Bromhexine**
 SF-67A	**45.3* ± 6.3** **39.9* ± 1.7** **17.7 ±3.0**	**Bromhexine**

 **SF-68A**	**3.7 ± 4.0** **28.6 ± 4.3** **17.5 ± 2.7**	**Bromhexine**
 **SF-75B**	**36.4 ± 4.8** **28.6 ± 4.3** **23.3 ± 3.9**	**Bromhexine**
 **SF-76A**	**20.6 ± 2.5** **30.1 ± 5.3** **11.5 ± 3.6**	**Bromhexine**
 **SF-77**	**28.4 ± 5.0** **35.8 ± 4.9** **21.7 ± 3.3**	**Bromhexine**
 **SF79B**	**40.5 ± 5.3** **41.3*** ± 2.3** **34.8 ± 7.2**	**Bromhexine**

 **SF-80**	**49.7 ± 1.2** **57.0** ± 4.3** **30.6 ± 4.2**	**Bromhexine**
 **SF-89**	**30.7 ± 6.3** **18.1 ± 3.7** **n.a.**	**Bromhexine**
 **SF-104A**	**21.5 ± 3.8** **25.8 ± 5.3** **27.1 ± 4.7**	**Bromhexine**
 **SF-104B**	**16.3 ± 2.5** **27.8 ± 3.6** **36.0 ± 2.1**	**Bromhexine**
 **SF-110C**	**25.9 ± 4.7** **32.1 ± 2.9** **31.4 ± 6.3**	**Bromhexine**
 **SF-110D**	**29.9 ± 4.9** **39.9** ± 3.7** **44.0*** ± 3.4**	**Bromhexine**

 **SF-124B**	**27.7 ± 4.1** **31.7 ± 4.6** **31.4 ± 6.5**	**Ambroxol**
 **SF-146C**	**23.2 ± 7.1** **22.3 ± 6.2** **23.6 ± 7.1**	**Bromhexine**
 **SF-147A**	**21.3 ± 6.1** **22.6 ± 7.2** **19.5 ± 6.1**	**Bromhexine**
 **SF-148A**	**20.3 ± 6.4** **18.7 ± 5.6** **15.6 ± 6.6**	**Bromhexine**
 **SF-150B**	**31.0 ± 6.0** **39.8 ± 6.0** **56.6*** ± 6.7**	**Bromhexine**
 **SF-153B**	**32.0 ± 5.0** **42.2 ± 7.5** **64.4* ± 11.2**	**Ambroxol**

 **SF-154C**	**37.5 ± 6.0** **29.0 ± 3.8** **31.4 ± 5.3**	**Ambroxol**
 **SF-164C**	**49.8 ± 6.7** **48.3 ± 4.0** **41.5 ± 1.4**	**Bromhexine**
 **SF-165B**	**n.a.** **41.0 ± 8.9** **38.3 ± 3.5**	**Bromhexine**
 **SF-170C**	**n.a.** **37.5 ± 4.2** **60.6* ± 8.6**	**Bromhexine**
 **SF-183A**	**n.a.** **39.2 ± 8.2** **29.7 ± 4.3**	**Bromhexine**

All 28 compounds were administered in combination with 20 µM DGJ. Mutant α-gal form A156V had a reduced enzyme activity of 4.5 % wild type. DGJ alone increased the activity to 31.41 %. A significant increase of the single administration of DGJ is indicated: *p<0.05; **p<0.01; ***p<0.005

7.3 Immortalisation of peripheral B-lymphocytes from blood via Epstein-Barr Virus transformation

(1) *Preparation of EBV*

Callitrichidae cells containing EBV strain B95-8 were recovered from liquid nitrogen and brought to cultivation in 5 ml RPMI supplemented with 20 % FBS I T25 cell culture flasks over night (37°C, 5 % CO_2). At day 2, cells were transferred to a T75 flask and additional 5 ml culture medium were added. After cultivation for an additional 5-7 days the culture was processed as follows:

- Centrifugation at 1,200 rpm for 6 min (RT) in a benchtop centrifuge
- The supernantant containing the viral particles was filtered twice (0.45 µm pore size) and stored at 4°C. The filtrate is good for 4 weeks
- The cell pellet was resuspended in 1 ml of freezing medium (RPMI culture medium supplemented with 10 % DMSO and 5 % glycerol)

Plastic disposables were cleaned in agreement with the local authorities on the basis of the Gentechniksicherheitsverordnung (GenTSV).

(2) *Isolation of lymphocytes from patient blood*

Venous heparan-preserved blood was diluted 1:1 in RPMI-medium (w/o supplemtents). 15 ml tubes were prepared with 5 ml Lymphoflot (Biotest Diagnostics, Frankfurt am Main, Germany). Lymphoflot was carefully layered with a cover of the diluted blood (avoid mixing, hold tube angular!). The tube was spun at 1,200 rpm for 21 min (RT, use minor brake power). The middle layer (indicated as a white ring from lymphocytes) was recovered with a 1 ml Eppendorf pipet and transferred to a fresh centrifuge tube. RPMI medium (w/o supplemtents) was added up to 15 ml followed by a centrifugation step (6 min, 1,200 rpm, RT). The supernatant was discarded and this wahs step was repeated twice. Finally, the cell pellet was resuspended in 1 ml culture medium (RPMI, 20 % FBS, 1 % P/S) additionally supplemented with 100 µg/ml cyclosporin A.

(3) *EBV transformation*

The lymphocyte culture obtained in step 2 was pipetted in slant tubes and mixed with 1 ml (1:1) EBV particle containing solution from step 1. After 2 days of incubation (37°C, 5 % CO_2), 1 ml of the culture medium was carefully removed without and 1 ml of fresh culture medium containing cyclosporin A was added. Another 5 days later (day 7 from the start) this procedure was repeated. After one more week (day 14), the tubes were spun (6 min, 1,200 rpm, RT) and the supernatant was discarded. Fresh RPMI (+ 20 % FBS, 1 % P/S) without cyclosporin was added and the cells were transferred to a T25 flask and incubated about one more week. After growth of a sufficient

number of clones (the medium appears slightly yellow), cells were splitted. After expansion, the cells can be deep frozen and applied to the experimental procedures.

7.4 Acknowledgements

It would not have been possible to write this doctoral thesis without the help and support of my wife Inga. I am grateful for her patience and advice. I am grateful to my parents as well, who kept other stuff off my back whenever possible.

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Excellent technical support was bestowed by Tina Czajka, Mandy Loebert and Norman Krüger.
I apologise for any inadequacy that may (still) be contained in this work. It is entirely my own and I want to thank the reviewers for not paying too much attention.

7.5 List of publications

Hovakimyan M, Stachs O, Reichard M, Mascher H, **Lukas J**, Frech MJ, Guthoff R, Witt M, Rolfs A, Wree A (2011) “Morphological alterations of the cornea in the mouse model of niemann-pick disease type c1”, Cornea 30(7):796-803

Yan X, **Lukas J**, Witt M, Wree A, Hübner R, Frech M, Köhling R, Rolfs A, Luo J (2011) “Decreased expression of myelin gene regulatory factor in Niemann-Pick type C 1 mouse”, Metab Brain Dis 26(4):299-306

Hovakimyan M, Petersen J, Maass F, Reichard M, Witt M, **Lukas J**, Stachs O, Guthoff R, Rolfs A, Wree A (2011) “Corneal alterations during combined therapy with cyclodextrin/allopregnanolone and miglustat in a knock-out mouse model of NPC1 disease”, PLos One 6(12):e28418

Avchalumov Y, Kirschstein T, **Lukas J**, Luo J, Wree A, Rolfs A, Köhling R (2012) “Increased excitability and compromised long-term potentiation in the neocortex of NPC1(-/-) mice”, Brain Res. 20;1444:20-6

Wittmann J, Karg E, Turi S, Legnini E, Wittmann G, Giese A-K, **Lukas J**, Goelnitz U, Klingenhaeger M Bodamer O, Muehl A, Rolfs A (2012) "Newborn Screening for Lysosomal Storage Disorders in Hungary", JIMD Rep 6:117-25

Lukas J, Torras J, Navarro I, Giese A-K, Boettcher T, Mascher H, Lackner KJ, Fauler G, Paschke E, Cruzado JM, Dudesek A, Wittstock M, Meyer W, Rolfs A (2012) "Broad spectrum of Fabry disease manifestation in an extended Spanish family with a new deletion in the *GLA* gene", Clin Kidney J 5(5):395-400

7.6 Submitted papers

Sharif M, Pews-Davtyan A, **Lukas J**, Schrank J, Langer P, Rolfs A, Beller M. Palladium-Catalysed Carbonylative Transformations of Bromhexine to New Bioactive Compounds as Glucocerebrosidase Inhibitors. Organic and Biomolecular Chemistry**,** 2013

Lukas J, Giese AK, Markoff A, Grittner U, Kolodny E, Mascher H, Lackner KJ, Meyer W, Wree P, Saviouk V, Rolfs A. Functional characterisation of α-galactosidase mutations as a basis for a new classification system in Fabry disease. PLoS Genetics, 2013

Hovakimyan M, Meyer A, **Lukas J**, Luo, J, Hummel T, Rolfs A, Wree A, Witt M. Olfactory deficits in Niemann-Pick Type C1 (NPC1) disease. Brain Structure & Function, 2013

7.7 Posters and oral presentations at Scientific Meetings

Lukas J, Beller M, Uhrmacher A and Rolfs A "An approach to improve the efficacy of Pharmacological Chaperone Therapy in Anderson Fabry Disease", 5th International Stem Cell School in Regenerative Medicine, Berlin, Germany, 2008

Lukas J "Pharmacological Chaperone Therapy in Fabry Disease" SIFAP - 4th Investigator Meeting Neuruppin, Germany, 2009

Lukas J, Frahm J, Goelnitz U, Wree A, Köhling R, Rolfs A "Evaluation of disease relevant mutations in Anderson Fabry Disease - how to proof the medical consequences?" The European Human Genetics Conference, Vienna, Austria, 2009

Lukas J, Goelnitz U, Wree A, Köhling R, Rolfs A "Evaluation of mutant a-galactosidase A activity - a rapid screening method to determine disease relevant defects", The American Society of Human Genetics 59th Annual Meeting, Honolulu, USA

Lukas J "Investigating the severity of α-galactosidase mutations in Fabry Disease (FD) – a quick screening method to test for mutant enzyme activity" Lysosomal Storage Disorders and the Nervous System: 2nd Internatational Postgraduate Course

Lukas J, Goelnitz U, Wree A, Köhling R, Rolfs A "Quick evaluation and classification of alpha Galactosidase A mutants in Anderson Fabry Disease - Prediction of disease relevant mutations", The European Human Genetics Conference, Gothenburg, Sweden, 2010

Lukas J "Functional consequences of mutations in Fabry Disease", Lysosomal Storage Disorders: From science into clinic: 3rd Internatational Postgraduate Course

Lukas J, Ernst M, Fuellen G, Yen X, Luo J, Koczan D, Rolfs A "Differential expression of Cell Migration, Differentiation/Proliferation and Apoptosis related genes in NPC1 mouse brain", Michael, Marcia and Christa Parseghian Scientific Conference on Niemann-Pick Type C Research, South Bend, USA, 2011

Lukas J, Beller M, Schmoele A, Frech M, Rolfs A "How to increase mutant enzyme activity in Anderson Fabry Disease (AFD)", Metabolic aspects of chronic brain diseases. FENS-IBRO Partially Supported School, Reisensburg-Günzburg, Germany, 2011