In Vitro and *In Vivo* Amenability to Migalastat in Fabry Disease

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Migalastat (1-deoxygalactonojirimycin) is approved for the treatment of Fabry disease (FD) in patients with an amenable mutation. Currently, there are at least 367 amenable and 711 non-amenable mutations known, based on an *in vitro* good laboratory practice (GLP) assay. Recent studies demonstrated that *in vitro* amenability of mutations did not necessarily correspond to *in vivo* amenability of migalastat-treated patients. This discrepancy might be due to (methodological) limitations of the current GLP-HEK assay. Currently, there are several published comparable cell-based amenability assays, with partially different outcomes for the same tested mutation, leading to concerns in FD-treating physicians. The aim of this review is to elucidate the idea of amenability assays from their beginning, starting with patient-specific primary cells to high-throughput assays based on overexpression. Consequently, we compare methods of current assays, highlighting their similarities, as well as their pros and cons. Finally, we provide a literature-based list of α-galactosidase A mutations, tested by different assays to provide a comprehensive overview of amenable mutations as a good basis for the decision-making by treating physicians. Since *in vitro* amenability does not always correspond with *in vivo* amenability, the treating clinician has the responsibility to monitor clinical and laboratory features to verify clinical response.

Fabry disease (FD; Online Mendelian Inheritance in Man [OMIM] #301500) is an X chromosome-linked inborn error due to various mutations within the α-galactosidase A (GLA/AGAL) gene, resulting in deficient enzymatic AGAL activity. Fabry-specific manifestations are a consequence of systemic accumulation of glycolipids (mainly globotriaosylceramide [Gb_3]) in various tissues and cell types.[1] The progressive Gb_3 accumulation is accompanied by a high risk of early onset of stroke, life-threatening arrhythmia, myocardial infarction, or cardiac and renal failure. Since 2001, enzyme replacement therapy (ERT) with agalsidase-alfa (Replagal, Shire/Takeda) and agalsidase-beta (Fabrazyme, Sanofi Genzyme) is available for treatment, resulting in intracellular Gb_3 reduction, leading to a clinical stabilization or at least a slowed disease progression in males and females.[2–9] A second treatment option based on a pharmaceutical chaperone (migalastat, 1-deoxygalactonojirimycin [DGJ]; Galafold, Amicus Therapeutics) has been approved in Europe since May 2016, in Canada since September 2017, in Japan since March 2018, and in the United States since August 2018 for long-term treatment of FD in adults (≥ 18 years of age in United States and Canada, ≥ 16 years in other countries) for patients with an amenable mutation and an estimated glomerular filtration rate (eGFR) ≥ 30 mL/min per 1.73 m^2. Amenability, which means the response in terms of increasing enzymatic activities of an AGAL mutation to migalastat, is currently being tested in a cell culture-based good laboratory practice (GLP) assay.[10] Several amenability assays have been published during the recent years, partially with different outcomes for the same mutation. This review aims to provide background information, including the history of migalastat, and focus on the current referred amenability assays to explain their function, similarities, and differences and their pros and cons, which might explain observed inter-assay results.

Identification of Migalastat as a Pharmaceutical Chaperon to Treat Patients with FD

Most missense *GLA* mutations result in an unstable and misfolded protein, leading to reduced AGAL activities within affected cells. Misfolded proteins will not pass the protein quality control mechanism within the endoplasmic reticulum (ER), resulting in a premature degradation before reaching the lysosomes.[11,12] To restore folding and stability of the protein, pharmacological chaperones can be used, which bind reversibly to the active center of the protein.[13] The first *in vitro* studies on FD were performed by adding galactose as a chaperone to fibroblasts carrying the mutation p.Q279E, resulting in an increase of AGAL activity by 15%.[14] Infusion of galactose (1 g/ kg body weight) in a patient with p.G328R and a cardiac phenotype was effective but not practically implementable due to the high frequency and durations of infusions (every other day, 4 h).[15] The small molecule DGJ is an iminosugar, which was initially identified as a competitive inhibitor of the AGAL enzyme. However, at sub-inhibitory concentrations (extracellular, 20–100 μM), a binding to the enzyme's catalytic center (wild-type and amenable mutations) can facilitate proper protein folding in the ER and an accelerated maturation and trafficking to the lysosome.[16,17] This resulted in an increase of enzymatic AGAL activity in healthy control cells and, importantly, in Fabry patient-derived lymphoblasts carrying the first described

responsive mutations p.R301Q and p.Q279E.[16,17] Daily oral intake resulted in increased AGAL activities in the heart, kidneys, liver, and spleen in heterozygous as well as homozygous transgenic mice with a mutant p.R301Q background.[18,19] Stimulation of these transgenic mice with daily orally-applied DGJ during 2 weeks finally led to a decreased Gb_3 storage in kidneys.[19] Prolonged treatment durations and dose optimization resulted in a Gb_3 clearance of additional FD-relevant tissues such as the brain and the heart, especially in aged transgenic mice, indicating a superior effect of every other day treatment over a daily intake.[20] In contrast to ERT, increased AGAL activities and decreased Gb_3 concentrations within the brain further demonstrated the ability of DGJ to cross the blood-brain barrier.[20] Of note, the co-administration of ERT and migalastat resulted in an increased AGAL tissue uptake and improved Gb_3 reduction in Fabry mice[21] as well as higher systemic exposures and tissue levels of AGAL in Fabry patients.[22] Unfortunately, this potentially very effective combination strategy was not followed up.

Impact of Migalastat on Clinical Outcomes

The safety and efficacy of oral migalastat at 123 mg every other day in patients with genetically confirmed FD were assessed in two randomized, multi-center, placebo-controlled (FACETS)[23] or active comparator-controlled (ATTRACT)[24] phase III trials. Migalastat was efficacious in ERT-naive and ERT-experienced patients with amenable AGAL mutations, resulting in reduced cardiac mass, stable renal function, and increased and sustained endogenous AGAL activity levels, with reductions in Gb_3 accumulation in renal tissue. Plasma globotriaosylsphingosine (lyso-Gb_3) levels in previously untreated patients decreased[23] or remained constant in ERT-pretreated patients[24] under migalastat. Real-world data from a single center as well as a multi-center study confirmed the safety, a reduction of cardiac mass, and biochemical outcomes after 12 months of migalastat treatment.[25,26] In contrast, a stabilization of renal function was not observed in either of the two studies. However, due to the currently limited number and duration of studies,[27] short-term observations and heterogeneous and small patient cohorts might have influenced partially different outcomes, and results should be interpreted carefully. Future studies including well-characterized patient cohorts (separated by sex, phenotype, and other factors) and longer observational periods are now warranted to further clarify the impact of migalastat in patients with amenable mutations.
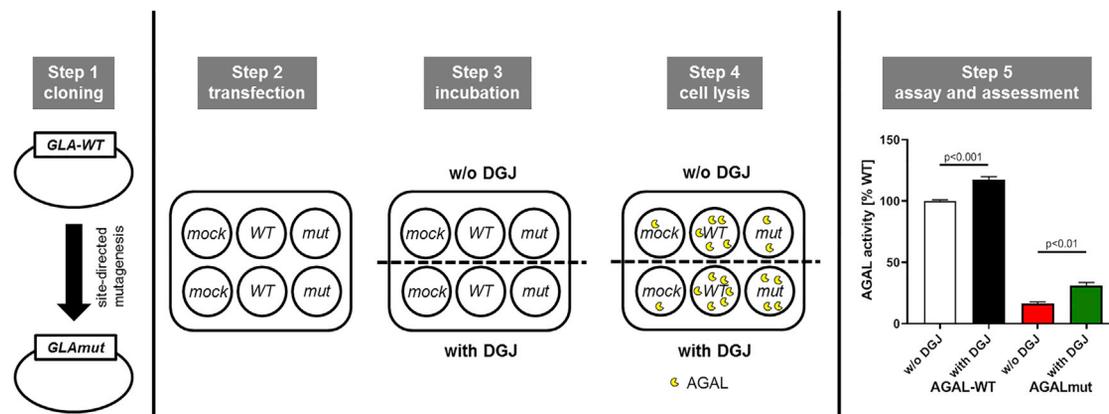
First Amenability Assays

A response (currently termed amenability) of AGAL mutations to sub-inhibitory concentrations of DGJ had (and still has) to be checked individually for every (missense) mutation. First characterizations were performed using patient-specific cell lines identifying p.R301Q, p.Q279E,[16] as well as p.T194I and p.V390fsX8[12] as amenable. Later on, Benjamin et al.[28] additionally analyzed the amenability of 75 different AGAL mutations in patient-derived lymphoblasts. However, the large variety of individual mutations required a more feasible method with a less time- and money-consuming approach to measure the amenability of AGAL variations to DGJ. In this respect, Shimotori et al.[29] used COS7 cells, individually

transfected with AGAL mutants identified in their patient cohort and incubated with 10 μM DGJ during 3 days, to identify 11 missense mutations as amenable. Since a response of overexpressed AGAL mutants in cell culture to DGJ did not necessarily reflect a comparable situation in FD-specific cells, another cell-based assay in human embryonic kidney (HEK)293 cells was designed, and outcomes were compared to appropriate male Fabry patient-derived lymphoblasts also incubated with DGJ.[30] Overexpression and subsequent incubation of 81 mutants with different DGJ concentrations for 4–5 days identified 49 mutations with a significant response.[30] The combination of HEK293 cells and overexpression constructs is the basis for the currently used amenability assays. Two open-label uncontrolled phase II studies of 12 and 24 weeks in nine males with FD documented that a treatment with 150 mg of migalastat every other day led to increased AGAL activities and decreased Gb_3 concentrations in six of nine patients.[31] As inclusion criteria, a missense *GLA* mutation, residual AGAL activity of at least 3% of normal, and an increase in AGAL activity by at least 20% in the presence of 20 μM migalastat in patients' cultured lymphocytes were required.[31] Of note, the three patients who did not show a consistent response *in vivo* after migalastat intake had AGAL mutations that did not respond to migalastat in transfected HEK293T cells.[31] However, the positive biochemical outcomes were subsequently confirmed in females[32] and further supported by reduced plasma lyso-Gb3 levels in patients with amenable mutations from a phase II study.[33]

Current Amenability Assays

Evolving numbers of newly identified *GLA* mutations over time required high-throughput (HTP) assays, which are able to measure a response of an AGAL mutation without the need of patient-specific material. In addition, inconsistencies between *in vitro* and *in vivo* amenability required further improved assays. In general, all currently used assays are based on HEK293 cells, which are transfected with individual *GLA* mutations and subsequently incubated with DGJ to measure an increase of enzymatic activity (Figure 1). In the following discussion, the most recent HTP assays are presented and discussed concerning their pros and cons (Table 1).

One of the first assays was established by Lukas et al.[34] using HEK293H cells transfected with *GLA* mutations, which were subsequently incubated with 20 μM DGJ for 60 h (Table 1). The amount of 20 μM chaperone did not reflect the average maximum concentration in the plasma following a single dose (150 mg) of migalastat, which has been calculated as 10 μM.[38] Although not directly stated, the authors used 20 μM DGJ probably according to the original studies by Fan et al.[16] Amenability criteria were defined as a 1.5-fold increase of AGAL activity over baseline or >5% activity compared to the untreated control.[34] In their recent meta study, the authors adopted the current GLP-HEK assay criteria (absolute increase by ≥3% of wild-type AGAL activity and a ≥1.2-fold increase of baseline enzymatic activity) and identified 89 out of 178 mutations as amenable.[34,35,39] Although used as a basis for future assays, the setup has some limitations that need to be considered. Since migalastat is an inhibitor, a wash-out step after incubation is very important.[12,16]

Figure 1. Schematic Overview of the Basic Principles of HEK293 Amenability Assays

HEK assays can be separated into five steps. Step 1: cloning of *GLA* mutations using site-directed mutagenesis. Step 2: transfection of seeded HEK293 cells with a mock control (negative control; necessary for AGAL background substraction), wild-type AGAL (positive control; reference activity is set to 100%), and the *GLA* mutation of interest. Step 3: incubation of cells with or without DGJ. Step 4: cell lysis to release overexpressed AGAL. Step 5: AGAL activities are measured and compared to each other. Depending on the assay protocols, various sub-steps can be included in between, such as wash-outs, determination of transfection efficiencies, among others. AGAL/GLA, α-galactosidase A; DGJ, 1-deoxygalactonojirimycin; WT, wild-type.

Wash-out to remove traces of DGJ was not reported,[34] nor was a specific inhibition of α-galactosidase B (NAGA). NAGA was demonstrated to degrade the artificial substrate 4-methylumbelliferyl-α-D-galactopyranoside,[40] which was used in the fluorimetric-based enzyme activity assay. An insufficient competitive inhibition of NAGA may lead to slightly increased AGAL activities,[41] increasing the risk for false-positive results, especially for AGAL mutations with low residual activities. A further critical issue is the lack of proper transfection efficacy controls. Only a semiquantitative western blot to detect protein level changes was applied,[34] which is not a proper tool to assess transfection efficiencies in general.

Due to the lack of comprehensive transfection controls in their initial work, a GLP-HEK assay was established by Benjamin et al.[10] based on the same assay but featuring some improvements (Table 1). This GLP-HEK assay is the basis for the accreditation, and only patients with amenable mutations according to this assay are approved to be treated with migalastat. Most importantly, the transfection efficacy for every transfection was now generally controlled by qPCR, and cells were stimulated with 10 μM chaperone. However, the stimulation duration was now prolonged to 5 days combined with a wash-out of 2 h. Of note, considering the approved medication (every other day) for migalastat, the prolonged stimulation (5 days) seems to be a long period and does not reflect the *in vivo* situation correctly. However, due to the small-scale setup in 96-well plates, these changes were probably required to detect sufficient activity changes. In contrast to amenability criteria by Lukas et al.,[34,35] mutations were, and still are, defined as amenable when AGAL activity is ≥1.2-fold over baseline with an additional absolute increase of ≥3% wild-type AGAL activity in the presence of 10 μM migalastat.[10] Based on this assay and definition, 1,384 amenable and 754 non-amenable theoretical mutations have been identified so far, for which migalastat treatment is approved (https://www.galafoldamenabilitytable.com/; last updated in May 2020).

Recently, a comparable amenability assay was implemented by Oommen et al.[36] to detect potential inter-assay variability among AGAL mutations (Table 1). In accordance with the GLP-HEK assay, the authors used the same experimental setup, including stimulation with 10 μM DGJ for 5 days.[36] Using the same amenability criteria, the authors identified 6 out of 59 tested mutations that did not match the classification of amenability reported using the GLP-HEK assay.[10] In contrast to the GLP-HEK assay, four mutations were identified as not amenable and two mutations were identified as amenable.[36] A comparison to the GLP-HEK assay-derived data revealed poor assay reproducibility. In contrast to qPCR, secreted embryonic alkaline phosphatase (SEAP) was used to measure and correct for transfection efficacy.[36] However, a possible impact of the overexpressed mutant AGAL on trafficking and secretion of SEAP and vice versa was not completely excluded until now and is discussed as a limitation of the assay.[42] In addition, a wash-out of chaperone was not explicitly mentioned by the authors.

Although all three assays share the advantage of an HTP tool, without the need of patient-specific material, they share a major disadvantage. They are based on an artificial overexpression model, not reflecting the *in vivo* situation in a patient or of patient-derived cells. Furthermore, the assays are performed in wild-type HEK cells with endogenous AGAL activity, requiring the subtraction of background activity from transfected cells. In addition, the presence of a wild-type AGAL might also lead to a heterodimerization of the wild-type and the mutated AGAL, as observed for other proteins previously.[43] This might decrease assay sensitivity and lead to false-positive results, especially for mutations with very low AGAL activities, as recently demonstrated for some mutations.[26,37] The latter is addressed by a recent assay based on CRISPR-Cas9-mediated *GLA*-knockout HEK293T cells (Table 1).[37] Although this assay also depends on overexpression, an effect of endogenous AGAL can be excluded, increasing the sensitivity especially for mutations with low AGAL activities. Furthermore, cells

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Table 1. Comparison of the Currently Used Cited In-House and Good Laboratory Practice Amenability Assays

Assay	Lukas et al.[34,35]	Benjamin et al.[10] (GLP-HEK Assay)	Oommen et al.[36]	Lenders et al.[37] (HEK293T GLA Knockout Assay)	Lenders et al.[37] (Patient-Derived Urinary Cells)
Cells	HEK293H	HEK293	HEK293H (GripTite 293 MSR)	HEK293T	fibroblast-like primary immortalized cell line
Duration (days)	2.5	5	5	2	2
DGJ (μM)	20	10	10	10 (20)	10 + 20
Expression plasmid	pcDNA3.1/V5-His6	pcDNA6	pcDNA6/V5-His	pcDNA3.1	not required
Transfection efficiency	quantitative western blots	qPCR	commercial SEAP	commercial luciferase	not required
Amenability criteria	1.5-fold over baseline or >5% compared to untreated value	$\geq$1.2-fold over baseline + absolute increase of $\geq$3% wild-type activity			
Pros	high-throughput screening				no overexpression model
	no patient samples required				patient-specific mutation in appropriate genetic background
	identification of amenability in mutations with high residual activity			identification of amenability in mutations with high and low residual activity	
		DGJ wash-out (2 h)		DGJ wash-out	
				no endogenous AGAL activity	assessment of potential Gb$_3$ depletion
				no heterodimerization between wild-type and mutant AGAL	
Cons	overexpression model				requires patients' urine
	high background due to endogenous AGAL activity				immortalization process
	heterodimerization between wild-type and mutant AGAL				time consuming
	no NAGA inhibition mentioned		overexpression of mutant AGAL might affect SEAP trafficking and secretion	overexpression of mutant AGAL might affect luciferase trafficking and secretion	expensive
	no DGJ wash-out mentioned		no DGJ wash-out mentioned		

AGAL, α-galactosidase A; DGJ, 1-deoxygalactonojirimycin; Gb$_3$, globotriaosylceramide; HEK, human embryonic kidney; NAGA, α-galactosidase B; SEAP, secreted embryonic alkaline phosphatase.

were stimulated for 2 days, making this setup more comparable to the *in vivo* situation.[23,24] A luciferase assay system to examine transfection efficacy was used and, as criticized for the SEAP, it is not clear whether overexpressed mutant AGAL might impair the reporter protein and vice versa. However, based on this next-generation assay, mutations of patients who did not biochemically respond to migalastat were identified as not amenable.[26,37] To further substantiate these data, a more specific assay based on patient-derived urinary cells[37] was designed (Table 1). Comparable to the assays of the pre-HTP area, this assay uses patient-derived (urinary fibroblast-like cells) incubated either with 10 or 20 μM chaperone for 2 days. Using the same amenability criteria as for the GLP-HEK assay, it could be demonstrated that AGAL activity of an amenable mutation increased and intracellular Gb$_3$ inclusions decreased after DGJ stimulation, whereas a mutation previously termed amenable did not respond to DGJ, and appropriate cells accumulated Gb$_3$.[37] Due to the time-consuming extraction and cultivation of primary cells and subsequent immortalization, this assay does not provide the advantages of HTP assays. Similar to the pre-HTP assays, this assay can be used to investigate the real AGAL activity of a specific mutation and provides an impression on the potential

biochemical outcomes in terms of intracellular Gb$_3$ inclusions as well. Hence, this assay seems to be a patient- and mutation-specific tool for mutations with low AGAL activities.

Comparisons between the amenability assays in wild-type HEK cells demonstrated significant inter-assay discrepancies. Table 2 provides a comprehensive overview of amenable AGAL mutations (according to the GLP-HEK assay) in comparison to the other three in-house assays. Interestingly, when comparing outcomes for amenable mutations (according to the GLP-HEK assay) with data from the in-house assays by Lukas et al.[39] and Oommen et al.,[36] the in-house assay by Lukas et al. fits better to the GLP-HEK assay (Figure 2). Amenability data by Lenders et al.[26] correlated best with the GLP-HEK assay, but results should be carefully interpreted due to the limited number of tested mutations. In conclusion, all observed discrepancies between the assays are probably due to several reasons, including different plasmids used for overexpression, distinct concentrations of DGJ, differences in incubation times, missing wash-outs and absent or different transfection controls, and the potential heterodimerization of endogenous wild-type AGAL (functional) and overexpressed

Table 2. Comparison of Amenability Outcomes Between a GLP-HEK Assay and In-House Assays

Assay	Benjamin et al.[10]		Lukas et al.[39]		Oommen et al.[36]		Lenders et al.[26]		Benjamin et al.[10]	Lukas et al.[39]	Oommen et al.[36]	Lenders et al.[26]
Amino Acid	w/o DGJ	Absolute Increase % WT, (n-Fold)	w/o DGJ	Absolute Increase %WT, (n-Fold)	w/o DGJ	Absolute Increase %WT, (n-Fold)	w/o DGJ	Absolute Increase %WT, (n-Fold)	Amenable (Yes/No)			
p.L3P	71.9	20.3 (1.3)	117.7	11.3 (1.1)	69.4	37.1 (1.5)			yes	yes	yes	
p.A13T	51.7	10.4 (1.2)			61.9	19.6 (1.3)			yes		yes	
p.A15G	19.0	9.0 (1.5)			15.5	15.5 (2.0)			yes		yes	
p.A15T	39.1	18.4 (1.5)			12.4	85.2 (7.8)			yes		yes	
p.A20D	4.3	5.7 (2.4)			5.8	10.6 (2.8)			yes		yes	
p.A20P	11.5	4.4 (1.4)			13.6	22.6 (2.7)			yes		yes	
p.W24R	52.6	10.9 (1.2)			50.5	20.6 81.4)			yes		yes	
p.D33G	29.3	41.3 (2.4)	37.4	24.6 (1.7)					yes	yes		
p.N34S	0.6	16.1 (29.2)			2.0	14.1 (8.2)	0.9	1.5 (2.7)	yes		yes	no
p.L36W	0.7	15.9 (23.7)	2.3	20 (9.7)					yes	yes		
p.A37T	48.9	47.5 (2.0)	69.6	63.3 (1.9)					yes	yes		
p.M42L	38.8	21.3 (1.5)			16.5	26.5 (2.6)			yes		yes	
p.M42V	0.5	3.8 (8.6)	0	7.2 (n/c)					yes	yes		
p.M42T	2.5	17.8 (8.1)	2.9	18.5 (7.4)					yes	yes		
p.H46P	31	75.9 (3.4)	40.1	58.7 (2.5)					yes	yes		
p.M51K	6.3	15.8 (3.5)	0	8.7 (n/c)					yes	yes		
p.M51I	22.3	24.8 82.1)	37.4	24.6 (1.7)					yes	yes		
p.C56Y	N/A	7.3 (n/c)			0.0	6.6 (n/c)			yes		yes	
p.Q57L	71.6	21.7 (1.3)			52.9	28.4 (1.5)			yes		yes	
p.E59K	8.6	8.9 (2.0)	2.2	16.3 (8.4)					yes	yes		
p.P60L	21.7	39.3 (2.8)	15.6	17.5 (2.1)					yes	yes		
p.E66G	34.2	11.9 (1.4)			28.9	37.0 (2.3)			yes		yes	
p.E66K	4.8	8.1 (2.7)	6.8	11.5 (2.7)	6.9	8.8 (2.3)			yes	yes	yes	
p.M72I	54.7	22.8 (1.4)			106.5	101.9 (2.0)			yes		yes	
p.A73V	53.6	33.3 (1.6)	44	20.7 (1.5)					yes	yes		
p.D83N	69.2	23.8 (1.3)	62.9	8.7 (1.1)	187.5	54.3 (1.3)			yes	yes	yes	
p.G85M	7.9	4.0 (1.5)			6.5	15.1 (3.3)			yes		yes	
p.G85S	12.4	6.7 (1.5)			7.6	12.2 (2.6)			yes		yes	
p.I91T	0.9	11.7 (14.0)	0.7	6.3 (10.0)					yes	yes		
p.S102L	19.9	42.9 (3.2)	71.6	7.3 (1.1)					yes	yes		
p.A108T	57.1	23.7 (1.4)			73.3	11.2 (1.15)			yes		no	
p.R112H	2.6	14.8 (6.7)	1.6	17.8 (12.1)					yes	yes		
p.R118C	24.0	5.5 (1.3)			5.3	5.8 (2.1)	45.4	20.1 (1.4)	yes		yes	yes
p.A121T	18.9	49.0 (3.6)	50	5.5 (1.1)					yes	yes		
p.S126G	83.7	30.2 (1.4)	51.3	16.1 (1.3)	143.7	26.9 (1.19)			yes	yes	no	
p.G128E	45.2	13.4 (1.3)			26.4	17.4 (1.7)			yes		yes	
p.A135V	0	3.7 (n/c)	0	6.9 (n/c)			0.1	0.1 (2.0)	yes	yes		no
p.D136E	1.4	11.5 (9.2)	0	31.3 (n/c)					yes	yes		
p.N139S	65.5	13.6 (1.2)	64.6	9.7 (1.2)	70.1	47.6 (1.7)	103.4	24.0 (1.2)	yes	yes	yes	yes
p.A143T	21.4	22.4 (2.0)	31.3	18.1 (1.6)					yes	yes		
p.G144D	50.2	26.3 (1.5)			28.8	16.1 (1.6)			yes		yes	
p.P146S	41.9	22.2 (1.5)			17.9	22.7 (2.3)			yes		yes	

(Continued on next page)

Table 2. Continued

Assay	Benjamin et al.[10]		Lukas et al.[39]		Oommen et al.[36]		Lenders et al.[26]		Benjamin et al.[10]	Lukas et al.[39]	Oommen et al.[36]	Lenders et al.[26]
p.A156V	1.2	11.6 (10.7)	4.3	12.5 (3.9)					yes	yes		
p.W162G	0.8	5.1 (7.4)	0	5.2 (n/c)			1.4	1.5 (2.1)	yes	yes		no
p.D165H	1.3	7.0 (6.4)	3.4	8.5 (3.5)					yes	yes		
p.D175E	44.3	9.1 (1.2)			57.9	9.6 (1.17)			yes		no	
p.G183A	22.4	34.0 (2.5)	10	36.6 (4.6)					yes	yes		
p.M187V	1.3	13.6 (11.5)	22.8	44.2 (2.9)	12.2	26.5 (3.2)			yes	yes	yes	
p.M187I	5.1	25.6 (6.0)	3.1	28.1 (10.1)					yes	yes		
p.I198T	64.7	30.8 (1.5)	38.7	11.7 (1.3)	58.3	58.5 (2.0)			yes	yes	yes	
p.E203V	43.0	21.5 (1.5)			144.6	72.1 (1.5)			yes		yes	
p.P205T	14.4	34.4 (3.4)	10.2	60.2 (6.9)					yes	yes		
p.P210S	75.2	38.2 (1.5)			41.9	49.5 (2.2)			yes		yes	
p.K213M	43.2	12.7 (1.3)			31.5	12.9 (1.4)	61.6	21.6 (1.4)	yes		yes	yes
p.P214S	22.4	60.1 (3.7)	18.1	43.5 (3.4)					yes	yes		
p.P214L	33	58.6 (2.8)	19.4	44.7 (3.3)					yes	yes		
p.N215D	43.8	14.5 (1.3)			41.1	22.4 (1.5)			yes		yes	
p.N215S	15.6	20.0 (2.3)	36.7	24.9 (1.7)	27.1	28.1 (2.0)	26.8	57.5 (3.1)	yes	yes	yes	yes
p.Y216C	2	18.7 (10.4)	2.3	23.6 (11.2)					yes	yes		
p.I219T	55.8	37.8 (1.7)	53.3	32 (1.6)					yes	yes		
p.R220Q	45.2	16.5 (1.4)			115.5	38.7 (1.3)			yes		yes	
p.N224S	10.3	19.4 (2.9)	31.1	51.1 (2.6)					yes	yes		
p.H225D	43.8	66.8 (2.5)	32.2	28.3 (1.9)					yes	yes		
p.N228S	124.5	44.7 (1.4)	59.5	11.1 (1.2)	89.6	54.9 (1.6)			yes	yes	yes	
p.I232T	15	70.0 (5.7)	11.5	50.1 (5.4)					yes	yes		
p.S238N	37.1	59.3 (2.6)	36	58.3 (2.6)					yes	yes		
p.I239T	37.7	55.1 (2.5)	26.6	58.3 (3.2)					yes	yes		
p.K240N	N/A	> 3% (≥1.2)					33.4	24.4 (1.7)	yes			yes
p.I242N	7.6	59.8 (59.8)	3.1	46.7 (16.1)					yes	yes		
p.L243F	7.9	34.4 (5.4)	11.4	59.4 (6.2)					yes	yes		
p.W245G	44.1	18.9 (1.4)			51.3	229.9 (5.5)			yes		yes	
p.N249K	17.9	17.3 (2.0)	23.7	30.9 (2.3)					yes	yes		
p.Q250P	24.8	33.9 (2.4)	18.5	42.5 (3.3)					yes	yes		
p.I253T	38.9	41.3 (2.1)	73	42.8 (1.6)					yes	yes		
p.I253S	3.3	27.9 (9.5)	4.4	49 (12.1)					yes	yes		
p.P259R	23.3	37 (2.6)	20.5	19.5 (2.0)					yes	yes		
p.N263S	15.8	64.7 (5.1)	6.7	57.8 (9.7)					yes	yes		
p.D264Y	0.5	5.7 (12.9)	0	5.4 (n/c)	2.8	8.6 (4.0)			yes	yes	yes	
p.V269M	4.4	21.5 (5.9)	0	17.3 (n/c)					yes	yes		
p.V269A	0	7.8 (n/c)	9	36 (5.0)					yes	yes		
p.G271D	1.5	30.7 (21.5)	3.1	34.8 (12.3)					yes	yes		
p.S276N	2.3	7.0 (3.9)			4.6	40.4 (9.9)			yes		yes	
p.T282I	5.2	18.5 (4.6)	5	42.7 (9.5)			12.6	9.2 (1.7)	yes	yes		yes
p.M284V	25.2	37.9 (2.5)	16.2	27.2 (2.7)					yes	yes		
p.M290L	58.6	52.8 (1.9)					69.4	53.1 (1.8)	yes			yes

(Continued on next page)

Table 2. Continued

| Assay	Benjamin et al.[10]		Lukas et al.[39]		Oommen et al.[36]		Lenders et al.[26]		Benjamin et al.[10]	Lukas et al.[39]	Oommen et al.[36]	Lenders et al.[26]
p.A291T	16.5	24.0 (2.5)	13.2	42.5 (4.2)					yes	yes		
p.L294S	0	4.9 (n/c)	0	12.4 (n/c)			0.2	0.3 (2.5)	yes	yes		no
p.R301G	19.1	45.6 (3.4)	19.3	37.2 (2.9)					yes	yes		
p.R301Q	5.5	39.0 (8.1)	8.5	39.5 (5.6)					yes	yes		
p.R301P	0	4.2 (n/c)	0	5 (n/c)					yes	yes		
p.S304N	94.1	27.7 (1.3)			77.8	12.5 (1.16)			yes		no	
p.S304T	76.4	40.5 (1.5)			56.7	23.4 (1.4)			yes		yes	
p.L310F	0.8	10.8 (14.5)	1.6	26.1 (17.3)	1.8	24.5 (14.6)			yes	yes	yes	
p.L311V	2	16.0 (9.0)	1.9	38.2 (21.1)					yes	yes		
p.D313G	25.5	14.9 (1.6)			25.1	25.4 (2.0)			yes		yes	
p.D313Y	59	21.9 (1.4)	83.9	16.4 (1.2)	54.2	28.9 (1.5)			yes	yes	yes	
p.V316I	92.1	34.0 (1.4)			99.8	33.4 (1.3)			yes		yes	
p.I317T	6.5	17.0 (3.4)			2.7	15.2 (6.7)			yes		yes	
p.I319T	10.3	17.7 (2.7)	20.2	38.1 (2.9)					yes	yes		
p.N320I	1.4	15.7 (12.2)	2	29.8 (15.9)			6.0	14.3 (3.4)	yes	yes		yes
p.Q321H	1.9	17.9 (10.4)	3.3	22.0 (7.7)					yes	yes		
p.D322N	36.1	10.3 (1.3)			17.8	13.3 (1.8)			yes		yes	
p.G325S	24.7	37.8 (2.5)	25.6	29.8 (2.2)					yes	yes		
p.Q327E	22.9	27.5 (2.2)	21.5	59.4 (3.8)					yes	yes		
p.G328A	6.9	21.8 (4.2)	6.2	23.8 (4.8)					yes	yes		
p.G334E	86.7	18.8 81.2)			57.5	23.4 (1.4)			yes		yes	
p.P343L	36.6	13.0 (1.4)			39.6	22.6 (1.6)			yes		yes	
p.S345P	0	3.7 (n/c)	0	9.7 (n/c)					yes	yes		
p.R356W	11	38.1 (4.5)	16.9	45.8 (3.7)					yes	yes		
p.R356Q	36.1	39.0 (2.1)	33.3	33.0 (2.0)					yes	yes		
p.G360C	8.7	8.1 (1.9)	16.2	16.7 (2.0)	5.3	9.9 (2.9)			yes	yes	yes	
p.R363H	20	30.5 (2.5)	31.9	26.0 (1.8)					yes	yes		
p.A368T	54.6	18.0 (1.3)			79.6	21.3 (1.3)			yes		yes	
p.T385A	57.3	16.1 (1.3)			28.6	17.3 (1.6)	68.1	9.6 (1.1)	yes		yes	no
p.G395A	24.4	6.3 (1.3)			13.4	5.2 (1.4)			yes		yes	
p.L403S	14.0	4.9 (1.4)			13.2	13.0 (2.0)			yes		yes	
p.T410I	0.4	11.8 (30.5)	2.3	13.8 (7.0)					yes	yes		
p.E418G	67.5	21.9 (1.3)	74.6	14.5 (1.2)	140.7	51.8 (1.4)			yes	yes	yes	
p.M421V	83.0	25.6 81.3)			58.3	26.0 (1.5)			yes		yes	

Only amenable mutations according to the GLP-HEK assay were included. According to the GLP-HEK assay, an amenable mutation responds with an absolute increase by ≥3% of wild-type AGAL activity and a ≥1.2-fold increase of baseline enzymatic activity in the presence of 10 μM migalastat. w/o, without; WT, wild-type; NA, not available; n/c, not calculated.

mutant AGAL, which might result in slightly higher measured AGAL activities. This is especially important for AGAL mutations with very low enzymatic activities, in which cases the mutant activity will be measured incorrectly high.

In Vivo Amenability

Despite their advantages and disadvantages, current amenability assays can be used to identify potentially amenable mutations. However, they do not represent *in vivo* conditions in patients, since measurement of AGAL activity is based on an artificial substrate, and stimulation is performed in a cell culture background. The *GLA*-knockout HEK293T assay seems to be a more patient-specific approach, especially for mutations with very low enzymatic activities, although no direct conclusion can be drawn about the extent of Gb₃ depletion in individual patients. Furthermore, future studies are warranted to generate data for additional mutations. Unfortunately, the cutoff value of required
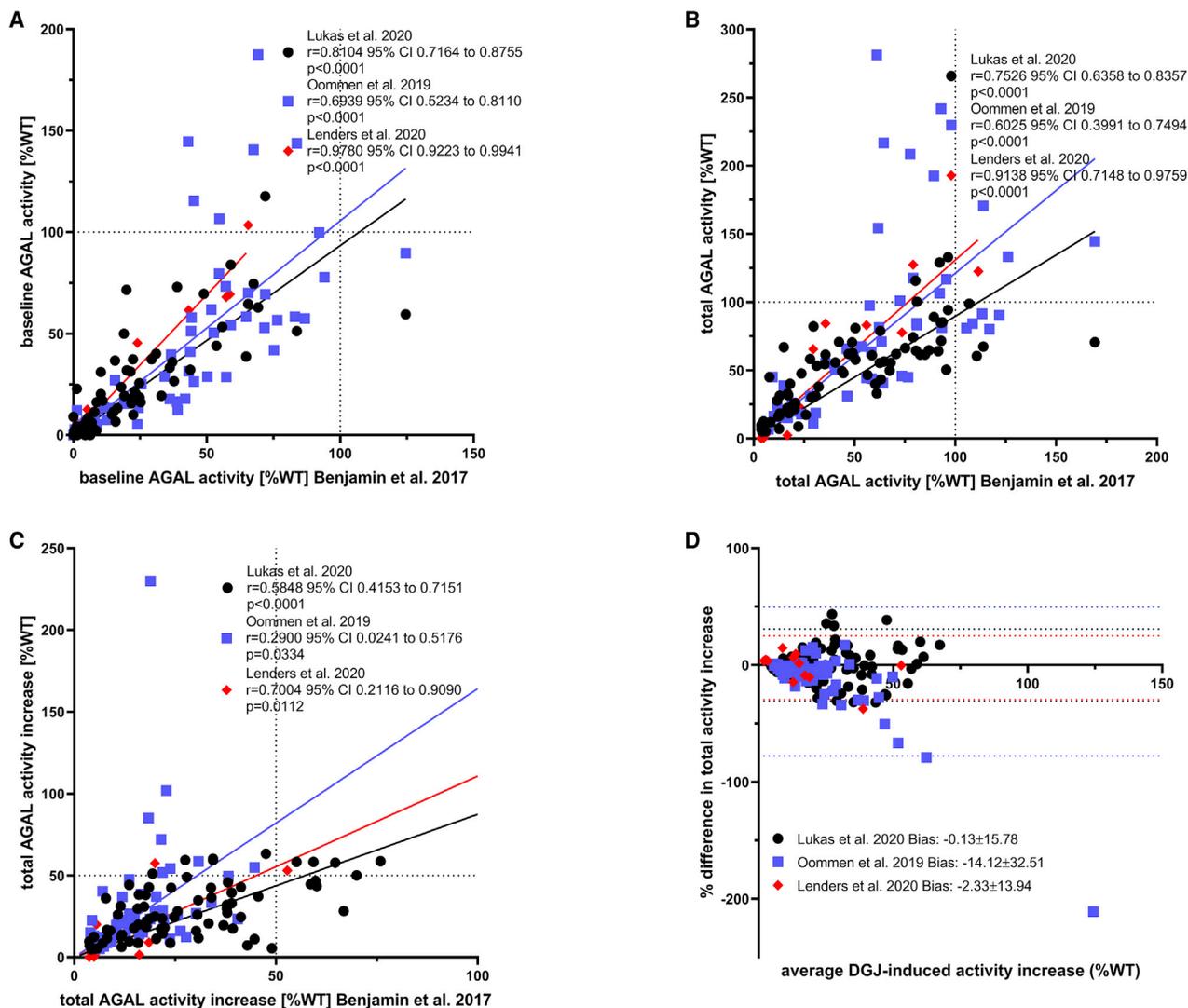
**Figure 2. Comparison of the GLP-HEK Assay with In-House Assays**

(A–C) Pearson correlation of α-galactosidase A activities presented from (A) baseline activities without DGJ, (B) total activities after incubation with DGJ, and (C) total activity increase over baseline in % of wild-type (WT) activity after incubation with DGJ. (D) Bland-Altman analysis of the in-house assays compared to the GLP-HEK assay for total activity increase over baseline. The dotted lines represent the 95% confidence intervals. Only amenable mutations according to the GLP-HEK assay were included and compared to data available from in-house assays (Lukas et al.,[39] n = 77 mutations; Oommen et al.,[36] n = 54 mutations; Lenders et al.,[26] n = 12 mutations). Data were extracted from Benjamin et al.,[10] Lukas et al.,[39] Oommen et al.,[36] and Lenders et al.[26] GraphPad Prism v8.0 software (GraphPad, La Jolla, CA, USA) was used for statistical analysis and visualization.

intracellular and lysosomal AGAL activities after migalastat stimulation to achieve a beneficial therapeutic response in patients remains elusive. Currently, low baseline activities, absent or low biochemical responsiveness, and inter-assay discrepancy are alarm signals for the potential misclassification of a genetic variant/mutation.[39] Therefore, amenability assays can only give an indication of a potential treatability of the patient with a possible therapy efficiency.

According to current knowledge, "amenable" mutations can be divided into four groups as follows: (1) non-pathogenic amenable genetic variants where patients should not be treated, (2) good responding mutations (e.g., p.N215S) where patients benefit very well from migalastat therapy, (3) mutations (e.g., p.N34S, p.W162G, p.L294S) that do not respond or respond poorly, and (4) theoretically a group of mutations is conceivable, in which migalastat acts as an inhibitor with a very strong affinity to the active center, leading to a potential increase of (lyso-)Gb₃, with the clinical consequence of therapy discontinuation. As a consequence, we propose the following workup for the treatment of patients with migalastat (Figure 3).

After diagnostic confirmation of a patient in need of treatment[44,45] with a FD-causing mutation, the physician should check the

in vitro amenability   *in vivo* / clinical amenability
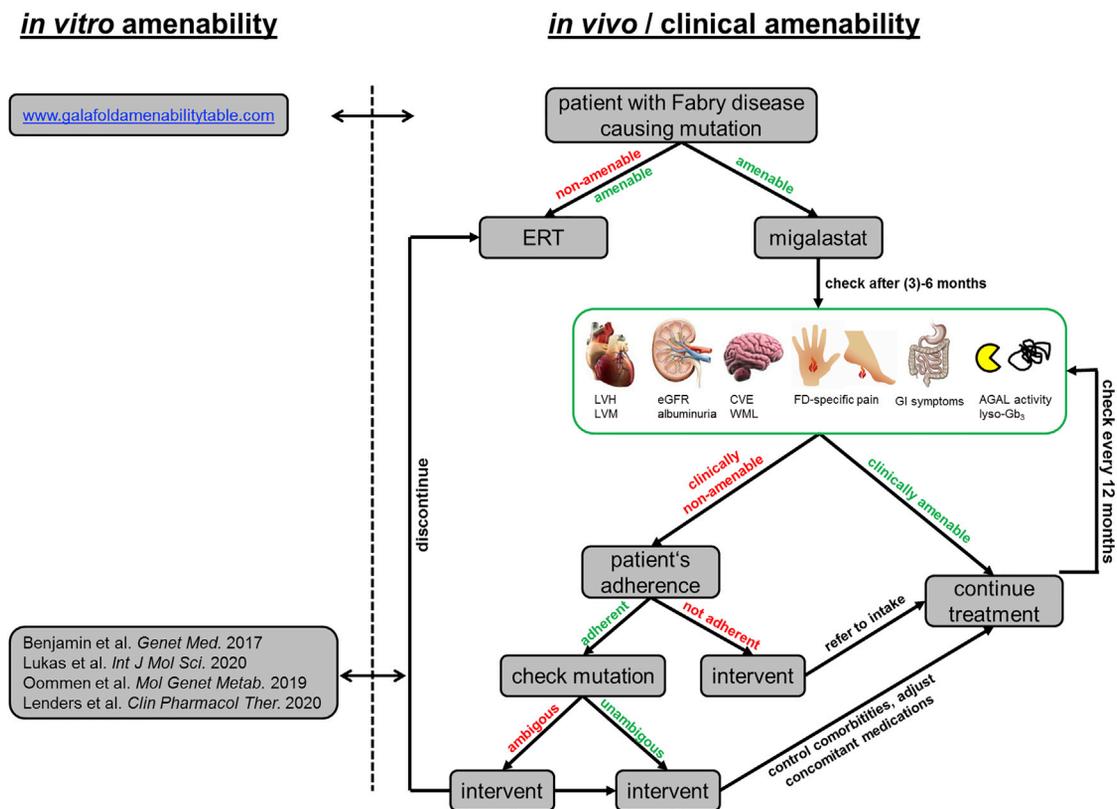
Figure 3. Work-Up to Assess Clinical Amenability in Patients Treated with Migalastat

Biochemical response after migalastat initiation should already be measurable after 3 months of treatment by increasing AGAL activities and decreasing plasma lyso-Gb$_3$ levels. Regular follow-up examinations should include echocardiography of the heart to assess left ventricular hypertrophy (LVH) and left ventricular masses (LVMs), determination of eGFR and proteinuria/albuminuria measures for kidney function, brain MRI to assess cerebrovascular events (CVEs) and white matter lesions (WMLs), a pain questionnaire for FD-specific pain and gastrointestinal symptoms, and α-galactosidase A (AGAL) activity and globotriaosylsphingosine (lyso-Gb$_3$) measurements in blood.

amenability of the *GLA* mutation via the appropriate website (https://www.galafoldamenabilitytable.com/hcp; last accessed on June 10, 2020). If amenable, a decision needs to be made either for ERT or migalastat treatment. If migalastat is chosen, at least after 6 and 12 months a follow-up examination should be performed to control whether current therapeutic goals[46] of a Fabry-specific therapy are fulfilled, which, in the context of multimodal care, should include (1) prevention/delay of the progression of organ manifestations (especially in the kidney, heart, and central nervous system), (2) reduction of complaints (especially neuropathic pain reduction), and (3) improvement of quality of life.[45,46]

Furthermore, the biochemical response to migalastat should be accessed by measuring AGAL activity and plasma lyso-Gb$_3$ in the blood, since levels already change after 3 months.[25,37] Increasing AGAL activities are associated with a decrease of myocardial mass[25] so that AGAL activity (and lyso-Gb$_3$ values) might serve as potential markers for therapy efficiency. If most of these aims are achieved, the patient is clinically amenable (*in vivo* amenability) and treatment should be continued. If a patient is clinically not amenable, the patient's adherence must be checked. In case of nonadherence, the physician should encourage the

patient to take the oral chaperone consistently. If adherence is ensured, a physician (Fabry expert) of an interdisciplinary Fabry center should verify the *in vitro* amenability of the *GLA* mutation using secondary literature and comparing *in vitro* data (if possible). If the *in vitro* amenability is ambiguous, the physician should consider switching from migalastat to ERT. In addition, control for comorbidities and/or adjustment for concomitant medications such as renin-angiotensin-aldosterone system (RAAS) blockers and others are warranted.

In conclusion, migalastat seems to be an attractive alternative to ERT for many FD patients with amenable mutations.[23–26] Amenability assays can only give an indication of a potential treatability of the patient with possible therapy efficiency. The physician has the responsibility to verify the clinical responsiveness of the patient through regular clinical follow-ups in an interdisciplinary Fabry center by an examination of the three main organ systems (kidney, heart, and central/peripheral nervous system) as well as AGAL activity and (lyso-)Gb$_3$ levels.

CONFLICTS OF INTEREST

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