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Investigating Fabry disease - some lessons learned

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Abstract

Despite recent advances, there is still much to be learned about the pathogenesis of Fabry disease. The categorization of *GLA* gene missense mutations has been complicated by the fact that some missense variants may fall into more than one category. For instance, the A143T variant may cause late-onset Fabry disease in some subjects and not result in Fabry disease in others (pseudo-deficient). Efforts to mitigate the pathobiology of α-galactosidase A deficiency should differentiate between damaging (maladaptive) consequences and compensatory (adaptive) changes. Current therapy leaves a significant unmet need, especially concerning cardiovascular complications and cardiological clinical outcomes. Non-Fabry-specific therapy is necessary and quite beneficial and must be utilized. Its contribution should be considered when trying to assess the net effect of Fabry-specific therapy. Enzyme replacement therapy (ERT) can be administered to patients independently of their *GLA* genotype, as it slows the decline of kidney function in most patients if initiated sufficiently early in the disease course. Migalastat has better tissue penetration than ERT, but its usefulness is restricted to patients with amenable missense *GLA* variants. However, it is important to realize that in a substantial proportion of common amenable mutations, migalastat increases α-galactosidase A activity level beyond the disease threshold and thus eliminates the metabolic disturbance that is at the center of Fabry disease. Substrate reduction therapy and gene therapy approaches are being developed, but these therapeutic modalities have their own limitations and difficulties.

Keywords: Pathogenesis, lysosomal disease, ERT, pharmacological chaperone, gene therapy, *GLA* variant

INTRODUCTION

The goal of this update is to highlight and comment on recent advances in the pathogenesis and treatment of Fabry disease by either describing novel observations or emphasizing and re-interpreting previously published data.

The most important predictor and modifier of the age of clinical disease onset and overall disease severity is residual α-galactosidase A activity[1]. Patients with the classic form have no residual enzyme activity as measured in peripheral blood white cells, while those with so-called late-onset (sometimes referred to as atypical) Fabry disease have real measurable residual enzyme activity[2]. Although disease severity tends to decrease with increased residual α-galactosidase A activity, there is no strict linear correlation between residual enzymatic activity and severity of clinical features, probably because of the presence of various modifiers. What is often not appreciated is that there is a "disease threshold" of about 35% of mean normal[2]. In other words, α-galactosidase A activity below normal but above 35% of mean normal is not thought to be associated with increased risk of clinical Fabry disease-related complications. This has important implications when evaluating a *GLA* variant of unknown significance (VUS) and deciding if a person with a particular *GLA* variant should be considered for therapy such as ERT or pharmacological chaperone[3,4]. Patients with late-onset Fabry disease may have any of the same organ/system complications (heart, kidney, cerebrovascular, peripheral nerve) as patients with the classic form but typically develop those complications at a later age, with the possible exception of late-onset female heterozygotes who rarely if ever develop renal insufficiency[1].

The main reason for delay in diagnosis is the non-specific nature of the complications of Fabry disease[5]. The characteristics of a cerebrovascular stroke, the nephropathy, the small-fiber neuropathy, and most cardiac manifestations in patients with Fabry disease are like those that occur in patients who have other disorders - for example, diabetes mellitus - and are in many ways a phenocopy of Fabry disease[5].

PHENOTYPIC CATEGORIZATION OF *GLA* VARIANTS

As mentioned above, *GLA* gene variants associated with low residual α-galactosidase A activity may be pathogenic if enzyme activity is below the disease threshold and are presumed non-pathogenic if it is higher. However, some such variants may have low enzyme activity in some individuals and high (not disease-causing) in others. The best example is the common A143T variant. It is considered a benign variant in some publications but has been shown to be pathogenic in others[6-9]. The T419A variant was shown to be associated with 32% of mean normal activity in one male and 0% activity in two of his grandsons. Therefore, both late-onset and classic phenotype co-occurred within the same family[10]. The D313Y variant may be another such example[11]. Therefore, it is important to measure α-galactosidase A activity in blood leukocytes of males in the family before concluding whether a *GLA* gene variant is disease-causing or not. The pathogenicity of the mutation may be uniform in a particular family, but determination in female heterozygotes is difficult since measurements of enzyme activity in peripheral blood white cells are not useful in females, and levels of plasma lyso-Gb3 are often not elevated in such patients[7,10,12]. Since the A143T variant is often detected by newborn screening, follow-up enzyme activity testing in blood leukocytes of newborn males (and expressed as percent of mean normal of the same laboratory) would help decide if the child (and therefore possibly his relatives) may be at risk for Fabry disease-related manifestations.

Unfortunately, this is often not done for subjects born with this variant[13-15]. The cause of the large individual variability in residual enzyme activity of some *GLA* variants has not yet been established. It is likely that the ability of the cell to promote adequate protein folding and chaperoning of mutated

α-galactosidase A molecules varies from one person to another, which leads to a rather wide spectrum of enzyme activity of certain *GLA* variants[16].

UNDERSTANDING SECONDARY BIOLOGICAL ABNORMALITIES

Despite a fairly large number of efforts in the past 30 years to understand the pathogenic mechanism of Fabry disease, we still have a poor grasp of how an increase of certain glycosphingolipids leads to multi-organ dysfunction. One approach has been to look for disruptions in the biology of the cell or organ involved in the disease in comparison with healthy controls[17-19]. The tendency, in many cases, is to consider any deviation from the norm of protein or gene expression as a biological abnormality caused by the disease that needs to be corrected or negated in some way. That may be true if the biological dysfunction is maladaptive. However, "correcting" an adaptive (or even a neutral) cellular response may aggravate the disease process. For example, depending on the biological context, autophagy can be either adaptive or maladaptive[20]. Another example of the importance of determining the significance of a biochemical aberration involves the anionic lipid lysobisphosphatidic acid (LBPA), also called bis[(monoacylglycerol)phosphate]. It was found to be increased in both rare and common neurodegenerative disorders, including in lysosomal diseases[21]. This lipid was initially thought to be part of the harmful lysosomal dysfunction seen in these disorders. Indeed, decreasing LBPA was thought to be beneficial based on studies in a macrophage cell line in Gaucher disease[22]. However, it was later found that its accumulation in certain diseases is an ameliorative response to lysosomal dysfunction, including increased autophagy, suggesting that augmenting its synthesis may have a major therapeutic benefit in a wide spectrum of human diseases[23-25]. Interestingly, it was recently discovered that LBPA deficiency is the direct cause of *CLN5* neuronal ceroid lipofuscinosis due to a deficiency of its synthetic enzyme[23].

The need to distinguish between adaptive and maladaptive responses is equally important in Fabry disease research. Researchers have recently described an overexpression of α-synuclein in glomeruli of kidney biopsies and in a podocyte cell line from patients with Fabry disease[17]. The podocyte structure and function did not completely normalize with ERT. This finding led the authors to develop a knockdown model of podocyte damage in Fabry disease using a podocyte cell line[17]. Increased numbers of lysosomes, their pH, and increased oxidative stress via reactive oxygen species were defined as the Fabry phenotype of this podocyte line. Adding exogenous α-galactosidase A, using substrate reduction or a pharmacological chaperone, reversed the abnormal expression of most proteins in this cell line but not the over-expression of α-synuclein. On the other hand, knocking down α-synuclein in the Fabry podocyte cell line was associated with a reduction in lysosomal area, lysosomal pH, and reactive oxygen species accumulation, while its over-expression enhanced this cellular phenotype. However, the phenotypic abnormalities defined in the cell line *in vitro* do not model the filtering function of podocytes in renal glomeruli in situ. In addition, it is impossible to determine whether the over-expression of α-synuclein, as well as the combination of increased lysosomes, lysosomal pH, and reactive oxygen species, are adaptive or maladaptive cellular responses in the podocyte line, especially in podocytes in the intact glomerulus. Reactive oxygen species signaling plays an important role in both cellular physiology and pathophysiology[26], as its function in both the innate and even in the adaptive immune response illustrates[27]. Additional doubt about the putative pathogenic role of renal α-synuclein is raised by its apparent protective function in renal proximal tubules in the pathogenesis of kidney fibrosis[28] and by the absence of kidney disease in patients with Parkinson's disease due to triplication of *SNCA* locus and the consequent two-fold α-synuclein overexpression[29,30]. Interestingly, similar findings including α-synuclein overexpression were found in the brain of the Fabry knockout mouse model, but with no apparent neurological dysfunction or neurodegeneration[31-33].

THERAPY FOR FABRY DISEASE - PRESENT AND FUTURE

Therapy for Fabry disease is best divided into non-Fabry-specific therapy (medical or surgical) and Fabry-specific therapy[34]. The organ complications of Fabry disease obey the same physiologic rules as those that govern similar illnesses in the general population. Therefore, standard medical therapy used to prevent stroke, kidney failure, and cardiomyopathy caused by common diseases is likely to be useful in Fabry disease[35]. Non-Fabry-specific therapy includes antiproteinuric medications and renal transplantation for kidney involvement[36]; various forms of anti-arrhythmic therapies medications, implants, and surgical approaches (e.g., myectomy and heart transplantation) for cardiac disease[37]; anti-platelet agents to prevent ischemic strokes and various antalgic medications for the symptoms of small-fiber neuropathy[38,39]; and even simple interventions such as cholestyramine for gastrointestinal symptoms of Fabry disease (personal observation). Non-Fabry-specific therapy is often very effective and less costly than Fabry-specific therapy. Its effect on organ function, however, often confounds the assessment of the net benefits of Fabry-specific therapy. The effort to optimize and then keep constant antiproteinuric therapy in clinical trials that use kidney function as a major outcome is an example of attempts to minimize the confounding effect of standard non-specific therapy.

Currently, Fabry-specific therapy consists of either ERT or pharmacological chaperone. ERT's main goal is to prevent organ dysfunction. The current consensus is that ERT in Fabry disease slows the decline of the glomerular filtration rate in patients with low proteinuria and decreases neuropathic pain and gastrointestinal symptoms in some patients[34]. However, it has no proven effect on cardiac outcome or on the risk of cerebrovascular stroke[40,41], although some papers suggest a small effect[42,43]. The reasons for the limited effect of ERT likely include the common initiation of therapy too late in life, the limited access of the infused enzyme to critical cells in affected organs such as cardiomyocytes, too low a dose or insufficient frequency of administration, as well as the frequent presence of neutralizing anti-enzyme antibodies, particularly in males with classic Fabry disease[44-47]. In the US, there are two ERT preparations for Fabry disease - agalsidase beta and pegunigalsidase alfa-iwxj. Outside of the US, there is also agalsidase alfa. The FDA and EMA have recently approved pegunigalsidase alfa-iwxj as ERT for Fabry disease[48,49]. This product has a mean circulation half-life of the active enzyme of 80 hours compared to about one hour of agalsidase beta[50]. Its approval was mainly based on the reduction of lysosomal inclusions in kidney capillary endothelial cells. It was hypothesized that the long circulating half-life would be advantageous and lead to a greater therapeutic effect, particularly in slowing the decline of renal function by improving the eGFR slope[47,51]. Therefore, a trial involving this new ERT called the BALANCE study (NCT02795676) was conducted and showed pegunigalsidase alfa-iwxj to be non-inferior to agalsidase beta in the estimated glomerular filtration rate (eGFR) slope over two years[51]. Patients in this study were selected to have an eGFR slope at the entry that was more negative than -2 mL/min/1.73 m^2/year, and indeed, at baseline, both pegunigalsidase alfa-iwxj and agalsidase beta groups had a mean eGFR slope of about -8 mL/min/1.73 m^2/year[51]. However, throughout the study, both groups had a much better eGFR slope, with a mean slope of -3.6 and 3.2, respectively, over the two years. While this is a slower eGFR decline than measured at study entry, it is still faster than normal, or about -1 mL/min/1.73 m^2/year[52]. The reason for the apparent 'slowing' of the renal decline in the agalsidase beta group is not easily explained. It may be that the eGFR slope for inclusion was not accurate, and in fact, these patients were slower progressors. Alternatively, it represents a type of Hawthorne effect[53]. A more careful evaluation, possibly a case-control study, comparing not only between patient groups but also tracking the effect of a switch from agalsidase beta to pegunigalsidase alfa-iwxj on the eGFR slope of the same subject may help shed further light on the possible differential effect in eGFR slope between intermittent (short circulating half-life) and continuous (long circulating half-life) supply of α-galactosidase A to the kidney. For increased patient convenience, this product can also be administered every four weeks at a dose of 2 mg/kg. An open-label study shows that this

regimen is promising[54]. Unfortunately, the regulatory agencies have not yet approved the every-four-weeks regimen.

The pharmacological chaperone migalastat does not share some of the drawbacks of ERT that are mentioned above. It is a small molecule, the size of glucose, that is widely distributed in the body - including into the heart and kidney - in comparison to the limited penetration of ERT into the heart (cardiomyocytes) and kidney podocytes[44,55]. The administration of migalastat every other day is calculated to lead to an overall stable increase of endogenous α-galactosidase A levels in contrast to ERT administration every 14 days. ERT administration (agalsidase beta) is associated with low or no increase in α-galactosidase A activity in the 5-7 days that precede the following enzyme infusion[56,57]. In addition, migalastat has not been reported to induce an immune response to itself or to the chaperoned endogenous α-galactosidase A.

On the other hand, migalastat is effective only with certain *GLA* missense variants termed amenable mutations, but they represent a large fraction of the total number of missense variants of this gene[58]. Some of these amenable variants, e.g., A143T, N215S, and R363C, are particularly common[14,59,60]. Amicus Therapeutics, Inc. defined amenability in the *in vitro* HEK-293 cell assay as a ≥ 1.20-fold increase in α-galactosidase A activity over baseline in the presence of 10 μmol/L of migalastat, with an absolute increase of ≥ 3.0% of wild-type α-galactosidase A activity[4]. It is important to note that the degree of amenability is highly variable[61]. The most amenable *GLA* variants are those that increase the enzymatic activity level from below the disease threshold of 30% of mean normal to above this threshold. Examples include M296I, L300P, R301Q, A143T and N215S[4]. Such variants can be called *super-amenable* because the presence of migalastat corrects the metabolic abnormality. The second group of amenable variants is associated with a marked increase in enzyme activity, though still below the disease threshold. These include G104V, R112H, D136E, and L166G[4]. This group can be termed *good amenable* variants that show an increased enzyme activity that is likely to be clinically meaningful. Patients with variants of these two groups, particularly the *super-amenable*, are best treated with migalastat rather than with ERT. The third group consists of the *poorly amenable GLA* variants that show a very small increase in enzyme activity in the presence of migalastat. Examples include M42V, A20P and Y207S[4]. Patients with these variants will probably not benefit clinically from migalastat. A fourth group of amenable *GLA* variants are those not associated with Fabry disease because their baseline α-galactosidase A activity is above the disease threshold of 30% of mean normal[4]. These are sometimes imprecisely called *pseudo-deficient* variants.

There are three main caveats to the *in vitro* HEK-293 cell assay. First, enzyme activity in HEK-293 assay may be different from the one found in male patients' white blood cells assay[61]. Second, enzyme levels in other cell types and organs may be different from the activity in peripheral blood white cells, both at baseline and on migalastat[62]. Since we do not assay α-galactosidase A activity in affected organs, it is important to carefully monitor organ function over time in treated patients. Third, enzyme activity in some *GLA* gene variants such as the A143T mutation may vary from patient to patient or from family to family, and therefore may fall into either *super-amenable* group in some individuals or into the *pseudo-deficient variant* group in others. In any case, it is always important to measure baseline and on-drug α-galactosidase A activity in the patients' peripheral blood white cells[57]. This assay will confirm, in males only, the degree of amenability of patient cells *in vivo* and can also serve to assess patient compliance with migalastat therapy. From the same publication and from others, we see that some patients treated with migalastat have a paradoxical increase of plasma lyso-Gb3[57]. Such patients must be followed carefully, and therapy must be adjusted or changed as needed. Nevertheless, changes in lyso-Gb3 are not associated with clinical outcomes[63].

Two additional therapeutic modalities are being tested for Fabry disease. The first is substrate synthesis reduction or SRT. Its goal is to decrease the synthesis of glycosphingolipids at the step of glucosylceramide by inhibiting its synthetic enzyme UDP-glucose: *N*-acylsphingosine glucosyltransferase[64]. Two compounds have been tried in Fabry disease - venglustat and Lucerastat[64,65]. In clinical trials of both drugs, virtually all participants had the classic form of Fabry disease (no residual α-galactosidase A activity); a pharmacodynamic effect was observed with a reduction in circulating globotriaosylceramide and lysosomal inclusions in skin biopsies, but no clinical benefit was found[64,66]. The negative clinical findings can be explained mainly by the absence of residual enzyme activity in adult study participants. The presence of sufficient residual α-galactosidase A activity may be central for the restoration of metabolic homeostasis that allows the clearance of excess substrate by the deficient enzyme activity. That residual enzyme activity is central to the efficacy of SRT in Gaucher disease, in which all patients have some residual enzyme (acid beta-glucosidase) activity[67]. Therefore, since all patients with later onset Fabry disease have residual α-galactosidase A activity, clinical trials with SRT should focus on this group of patients, with a particular emphasis on their heart disease - the most significant unmet need in Fabry disease[1]. The second possible reason for the lack of efficacy of SRT in Fabry disease is the likely existence of α-galactosidase A substrates that are not glycosphingolipids. Evidence for that was found in the study of the dorsal root ganglia in the Fabry knockout mouse model[68]. This mouse displays well the small-fiber neuropathy of Fabry disease[69]. Plant lectin isolectin B4 (IB4)-positive neurons in the dorsal root ganglion express surface carbohydrates α-D-galactose groups conjugates, have small size cell bodies, and primarily give rise to unmyelinated fibers and small myelinated fibers, many of which are nociceptive and thought to be involved in a small-fiber neuropathy such as the one affecting patients with Fabry disease[68,70]. IB4 positive neurons stain was stronger in the Fabry mouse compared to wild type, but almost all these cells were negative for globotriaosylceramide, suggesting that these cells have a different α-D-galactose substrate[68].

Gene therapy for Fabry disease is also being developed. The main effort currently involves the use of adeno-associated virus (AAV)-based gene supplementation[71]. The approach that seems most frequently taken is to use the natural affinity of AAV to hepatic cells to transduce hepatocytes with the *GLA* cDNA[72,73]. Transduced liver cells produce large amounts of α-galactosidase A that is then excreted into the circulation to be taken up by cells and organs, akin to a continuous form of ERT. Using this liver-targeting approach, STAAR, a Phase 1/2 study of Isaralgagene civaparvovec (ST 920) gene therapy is the program furthest in its development. ST-920 is based on the hybrid AAV vector AAV2/6, in which hybrid AAV vectors have been engineered using the capsid protein of AAV6 serotype and the genome of AAV serotype 2[74,75]. This program has had no safety concerns, and patients do not routinely take immune modulators such as corticosteroids. Circulating α-galactosidase A activity has been relatively stable for up to two years, but plasma lyso-Gb3 tended to increase upon stopping ERT[75]. Reduction of lysosomal inclusions in peritubular endothelial cells was seen in one of two patients tested, and reduced podocyturia was found in two patients[75]. The maximal dose of $5X10^{13}$ vector genomes/kg was tested in this trial phase, and it is this dose that was chosen for further clinical evaluation, including a phase 3 trial[75]. Other biotechnology companies such as UniQure and Spark Therapeutics have expressed their intention to use a similar gene therapy approach to treat Fabry disease. Another interesting application of AAV gene transfer is to deliver the *GLA*-cDNA via recombinant AAV6 (rAAV6) transduction to peripheral blood human B cells *ex vivo*[76]. Here the AAV delivers the gene to a safe harbor locus in the patient's B cells using a CRISPR system by the process of nucleofection. The *ex vivo* gene-engineered and expanded B cells are then re-infused into patients in the hope of providing a consistent source of the deficient enzyme[76].

Since ERT has not adequately addressed the cardiovascular manifestations of Fabry disease and circulating α-galactosidase A is not taken up efficiently by cardiomyocytes, direct transduction of the affected organs,

and especially the heart, is an important approach to investigate. A program has been developed (4D-310) that uses a synthetic cardiotropic AAV vector invented through directed evolution to deliver a human *GLA* transgene to cardiomyocytes and other affected organs following intravenous administration[77]. The transgene expression is driven by a ubiquitous promoter. Preliminary results of cardiac activity at 12 months post-4D-310 administration showed improvement in left ventricular contractility, exercise capacity, and quality of life in the first four patients in this study[77]. Confirmed transgene delivery and expression in cardiomyocytes was found on cardiac biopsy in one patient[77]. Because of the propensity of the 4D-310 capsid to activate complement with sometimes secondary atypical hemolytic uremic syndrome in some patients, this study is at the time of this writing in FDA clinical hold. The company (4D Molecular Therapeutics) has agreed with the FDA on a plan that will allow the resumption of enrollment.

An approach using lentiviral-mediated gene transfer into hematopoietic stem cells was tested for several years with some pharmacodynamic success, but this program was eventually discontinued[78].

SUMMARY AND CONCLUSIONS

α-Galactosidase A residual activity is the central prognostic indicator of disease severity. As a result, every *GLA* variant, particularly a missense mutation, is typically categorized as associated with classic or late-onset disease. Some *GLA* variants are benign because they lead to a residual enzyme activity that is above the disease threshold. We and others recently found that some missense variants, such as A143T, are often benign but, in other cases, are associated with low α-galactosidase A activity, leading in many cases to the phenotype of late-onset Fabry disease. This finding has epidemiologic implications since the A143T variant is quite common, and when included in the calculation of disease incidence, it results in a significantly higher prevalence of Fabry disease than previously thought[14,79].The mechanism of Fabry disease is still incompletely understood, but one must appreciate that some abnormalities may be adaptive in nature and therefore should not be selectively reversed.

We can help patients with Fabry disease by using non-Fabry-specific therapy and Fabry-specific therapy such as ERT. The latter has shown some efficacy, but in most patients on ERT, the disease continues to progress. Pharmacological chaperone migalastat has significant theoretical advantages. Migalastat completely corrects the metabolic abnormality in patients with the most common amenable missense mutations and should certainly be the preferred Fabry-specific therapy in such patients. SRT and gene therapy are being developed for Fabry disease, but both approaches have pitfalls that must be overcome.

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Conflicts of interest

Raphael Schiffmann is a consultant for Amicus Therapeutics, Protalix Biotherapeutics, Chiesi Pharmaceuticals, and Walking Fish Therapeutics.

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