Universidade de Lisboa

Faculdade de Farmácia

IMMUNOGENICITY IN FABRY DISEASE: THE ROLE OF ENZYME REPLACEMENT THERAPY

Célia Isabel dos Santos Pedro

Dissertation supervised by Professor Doutor João Gonçalves

Master Degree in Biopharmaceutical Sciences

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ABBREVIATIONS

α-Gal A	α galactosidase A
α-NAGA	α-N-acetylgalactosaminidase
AAA	Anti-Agalsidase antibodies
ACEi	Angiotensin Converting Enzyme Inhibitors
ADA	Neutralizing Antidrug Antibodie
ARB	Angiotensin II Receptor Blockers
BSA	Bovine Serum Albumin
CRIM	Crossreactive Immunologic Material
DMSO	Dimethyl Sulfoxide
EDTA	EthyleneDiamineTetraacetic Acid
ERT	Enzyme Replacement Therapy
ELISA	Enzyme-linked Immunosorbent assay
ECL	Electrochemiluminescence
EMA	European Medicines Agency
eGFR	Estimated Glomerular Filtration Rate
FBS	Fetal Bovine Serum
FD	Fabry disease
FDA	Food and Drug Administration
FIPI	Fabry International Prognostic Index
GL-3, Gb3	Globotriaosylceramide
Gal2Cer	Digalactosylceramide
HCl	Hydrochloric acid
IAR	Infusion-Associated Reaction
IC	Immuno-Complexes
Ig	Immunoglobulin
IgE	Immunoglobulin E
IgG	Immunoglobulin G
LOD	Limit of detection
LSD	Lysosomal Storage Disease
LVM	Left Ventricular Mass
Lyso-GL-3	Globotriaosylsphingosine

M6P	Mannose-6-phosphate
MHC	Major Histocompatibility Complex
mL	Milliliter
MRI	Magnetic Resonance Imaging
MSSI	Mainz Score Severity Index
NBS	Newborn Screening
ng	Nanogram
OD	Optical Density
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
rpm	Rotations Per Minute
SD	Standard Deviation
VUS	Variants of Unknown Significance
WML	White Matter Lesions
WHO	World Health Organization
µL	Microliter
µg	Microgram

ABSTRACT

Fabry disease (FD) is a X-linked lysosomal storage disorder (LSD) caused by mutations on the *GLA* gene encoding for α -galactosidase A. This leads to a progressive accumulation of globotriaosylceramide (GL-3, Gb3) in tissues throughout the body. Cardiac, renal and neurological manifestations are common and life expectancy is significantly reduced relative to the general population.

Management of FD involves the administration of intravenous enzyme replacement therapy (ERT). Two forms, agalsidase alfa and agalsidase beta, have been licensed in certain jurisdictions and are generally well tolerated. Although ERT has been demonstrated to improve patient outcomes and disease course, especially when initiated early in the course of the disease, the intravenous infusion of the recombinant proteins can trigger a humoral immune response, resulting in infusion-associated reactions (IARs), and/or the development of neutralizing antidrug antibodies (ADAs), which seem to attenuate therapy efficacy in patients, mediating disease progression despite ERT [1-3].

Immunoglobulin (Ig) G antibodies have frequently been reported in patients with FD receiving ERT. Immunoglobulin G (IgG) responses are reported in a greater proportion of patients receiving agalsidase beta than in patients receiving agalsidase alfa. Immunoglobulin E (IgE) antibodies are less common than IgG antibodies and have not been observed in patients receiving agalsidase alfa. The clinical impact of the development of IgG antibodies to ERT in patients with FD remains unclear, due to lack of data and to the marked heterogeneity of patients both in terms of disease manifestations and response to therapy [4].

Further studies that examine the development of antibodies in patients with FD and the potential impact of such antibodies on the outcome of ERT are necessary.

The general purpose of this project is to study the immunogenicity of a court of Fabry patients and see the impact of immunogenicity on the effectiveness and safety profile of ERT. For this, we will screnn for antibodies IgG1, IgG4 and IgE against agalsidase alfa and beta therapy, pre and pos ERT infusion, by ELISA; and dissociation of Immuno-Complexes (IC) associated to ERT and screening of IgG released from IC through ELISA, in order to identify false negatives.

We will relate the results obtained with the safety data, and clinical and functional cardiac and renal data of patients, to verify the impact of the presence of ADA against agalsidase alfa and beta, on the effectiveness and safety of ERT, which will allow us to still make a direct correlation between the two ERT therapies, agalsidase alpha and beta.

In our study we verified than, IgG1 antibodies were the most frequently observed against both therapies, agalsidase alfa and beta, followed by IgG4 antibodies and lastly the least frequent IgE antibodies, but also present in patients treated with agalsidase alfa therapy and in women, contrary to what some studies demonstrate.

Similar to other studies, a greater development of ADA was observed in patients treated with agalsidase beta therapy versus agalsidase alfa therapy. Also, there is a greater development and in greater amounts of ADA in men versus women.

When comparing the levels of IgG and IgE antibodies against both therapies, we only observed statistically significant differences for agalsidase beta therapy.

In relation to IC, the presence of IC was observed in the pre and post infusion, however there were no statistically significant differences, possibly because the 30 minutes after the infusion of ERT, were not enough for the production of IC to occur.

However, when analyzing the signal differences between samples treated and untreated with acid, we found that the acid treatment allows the dissociation of Anti-Agalsidase antibodies (AAA) from IC. This is of paramount importance for immunogenicity measurement and characterization and shows the presence of stable IC between drug and antibodies with different affinity profiles circulating in blood´s patients.

Due to the fact that we were unable to access the safety data, and clinical and functional cardiac and renal data of patients, we were unable to measure and correlate of the presence of ADA against agalsidase alfa and beta, on the effectiveness and safety of ERT and with the appearance of IARs.

Key Words: Lysosomal storage disorder, Fabry disease, α galactosidase A, Enzyme replacement therapy, Infusion-associated reactions, Neutralizing antidrug antibodies

RESUMO

A doença de Fabry (DF) é uma doença de sobrecarga lisossomal (DAL) ligada ao cromossoma X causada por mutações no gene *GLA* que codifica a α-galactosidase A. Isto, leva a um acúmulo progressivo de globotriaosilceramida (GL-3, Gb3) em todos os tecidos do corpo. As manifestações cardíacas, renais e neurológicas são comuns e a esperança de vida é significativamente reduzida em relação à população em geral.

O controle da DF envolve a administração de terapia de reposição enzimática intravenosa (TRE). Duas formas, agalsidase alfa e agalsidase beta, foram aprovadas em vários países e são geralmente bem toleradas. Embora a TRE tenha demonstrado melhorar a evolução do doente e o curso da doença, especialmente quando iniciada no início da doença, a infusão intravenosa das proteínas recombinantes pode desencadear uma resposta humoral imune, resultando em reações associadas à infusão (RAIs), e/ou o desenvolvimento de anticorpos antifármaco neutralizantes (ADAs), que parecem atenuar a eficácia da terapia em doentes, mediando a progressão da doença apesar da TRE [1-3].

Anticorpos imunoglobulina (Ig) G têm sido frequentemente descritos em doentes com DF recebendo TRE. As respostas de IgG são relatadas numa proporção maior de doentes que recebem agalsidase beta do que em doentes que recebem agalsidase alfa. Os anticorpos IgE são menos comuns do que os anticorpos IgG e não foram observados em doentes recebendo agalsidase alfa. O impacto clínico do desenvolvimento de anticorpos IgG para TRE em doentes com DF permanece obscuro, devido à falta de dados e à acentuada heterogeneidade dos pacientes em termos de manifestações da doença e resposta à terapia [4].

Outros estudos que examinam o desenvolvimento de anticorpos em doentes com DF e o potencial impacto de tais anticorpos na eficácia da TRE são necessários.

O objetivo principal deste projeto é estudar a imunogenicidade de um conjunto de doentes com DF e verificar o impacto da imunogenicidade na eficácia e no perfil de segurança da TRE. Para isso, fizemos o screning de anticorpos IgG1, IgG4 e IgE contra as terapias agalsidase alfa e beta, pré e pós infusão da TRE, através ELISA; e dissociamos os IC associados da TRE e fizemos o screning de IgG libertados dos IC, através ELISA, para identificarmos os falsos negativos.

Relacionamos os resultados obtidos com os dados de segurança, dados clínicos e funcionais cardíacos e renais do doentes, para verificarmos o impacto da presença de ADA contra agalsidase alfa e beta, na eficácia e segurança da TRE, o que nos permitirá, ainda, fazem uma correlação direta entre as duas terapias TRE, agalsidase alfa e beta.

No nosso estudo verificamos que os anticorpos IgG1 foram os mais frequentemente observados contra ambas as terapias, agalsidase alfa e beta, seguidos pelos anticorpos IgG4 e por último os anticorpos IgE menos frequentes, mas também presentes em doentes tratados com terapia com agalsidase alfa e em mulheres, ao contrário de o que alguns estudos demonstram.

À semelhança de outros estudos, um maior desenvolvimento de ADA foi observado em doentes tratados com terapia com agalsidase beta versus terapia com agalsidase alfa. E também maior desenvolvimento, e em maiores quantidades de ADA em homens versus mulheres.

Ao comparar os níveis de anticorpos IgG e IgE contra ambas as terapias, observamos apenas diferenças estatisticamente significativas para a terapia com agalsidase beta.

Em relação aos IC, foi observada a presença de IC no pré e pós-infusão, porém não houve diferenças estatisticamente significativas, possivelmente porque os 30 minutos após a infusão da TRE não foram suficientes para que ocorresse a produção de IC.

No entanto, ao analisar as diferenças de sinal entre as amostras tratadas e não tratadas com ácido, verificamos que o tratamento com ácido permite a dissociação de AAA dos IC. Isso é de suma importância para a medição e caracterização da imunogenicidade e mostra a presença de IC estáveis entre a droga e os anticorpos com diferentes perfis de afinidade circulando no sangue.

Devido ao fato de não nos ter sido possível aceder os dados de segurança e dados clínicos e funcionais cardíacos e renais dos doentes, não fomos capazes de aferir e correlacionar a presença de ADA contra agalsidase alfa e beta, na eficácia e segurança da TRE e com o aparecimento de RAIs.

Palavras-chave: Doenças do armazenamento lisossomal, Doença de Fabry, α galactosidase A, Terapia de reposição enzimática, Reacções associadas à infusão, Anticorpos antidrogas neutralizantes

1. BACKGROUND AND SIGNIFICANCE

1.1. Lysosomal Storage Disease

LSD is the name given to a group of approximately 50 genetic diseases caused by deficiencies in lysosomal and non-lysosomal resident proteins; such deficiencies trigger the accumulation of disease specific substrates on lysosomes and other cellular locations. Thus, the most characteristic histological feature of LSD is the presence of enlarged lysosomes filled with undigested material. Depending on the enzymatic activity impaired in each disease type, LSD have been subdivided into mucopolysaccharidoses, sphingolipidoses and glycoproteinoses [5-7].

Initially described by Christian de Duve, lysosomes are key mediators of protein, complex lipids and polyssacharides degradation with a pivotal role in the coordination of cellular metabolism and intracellular signaling. Lysosomes participate in many biological processes, including antigen presentation, plasma membrane repair, exosome release, cellular adhesion and migration, apoptosis, gene regulation, tumor invasion and metastasis [4].

Nowadays, it is clear that the spectrum of LSD must also include other genetic alterations that disturb the synthesis and/or transport of lysosomal proteins and cargo and that are causal for the characteristically enlarged structures. Independently of its origin, the accumulation of nondegradable material within lysosomes has a profound impact on the organelle's physiology, size, trafficking, and overall degradative capacity [5,9].

Although the characteristics of the different diseases are very diverse, four pathophysiological hallmarks are found in common among several LSD: inflammation, altered calcium homeostasis, lysolipid accumulation and impaired autophagosome maturation [10].

The past 25 years have been characterized by remarkable progress in the treatment of these diseases and by the development of multiple therapeutic approaches. These approaches include strategies aimed at restoring the residual activity of a missing enzyme (ERT), hematopoietic stem cell transplantation, pharmacological chaperone therapy and gene therapy) and approaches based on reducing the flux of substrates to lysosomes. As knowledge has improved about the pathophysiology of LSD, novel targets for therapy have been identified, and innovative treatment approaches are being developed [6].

1.2. Fabry Disease

Fabry Disease was first described in 1898 by two dermatologists, William Anderson [11] and Johannes Fabry, is an X-linked lysosomal storage disorder caused by mutations in the *GLA* gene [12]. Roscoe Brady then showed that the lysosomal enzyme, α galactosidase A (α-Gal A), was deficient in these patients [1]. Sixtyfive years later, Sweeley and Klionsky discovered that markedly reduced or absent activity of the enzyme α-Gal A results in progressive accumulation of glycolipids, primarily Gb3 and its deacylated form, globotriaosylsphingosine (lyso-GL-3), in plasma and in a wide range of cells throughout the body. This includes those particularly relevant to disease pathology (e.g., vascular endothelial cells, podocytes, cardiomyocytes, arterial smooth muscle cells) and other cell types in the kidneys, nervous system, and other organs [2,3].

Since that time, much has been learned about the clinical manifestations and molecular features of FD, now known to be the most common LSD.

Fabry Disease was initially described in male patients with a severe clinical phenotype, now known as “classic” FD. These patients are characterized by absent or severely reduced (<1% of mean normal) α-Gal A activity, marked Gb3 accumulation in vascular endothelial cells, cardiomyocytes, smooth muscle cells, and podocytes, and child hood or adolescent onset of symptoms followed by progressive multi-organ failure, and eventually death. However, a larger group of patients, “non classic” FD, has later-onset phenotypes with varying levels of residual α-Gal A activity, age of onset, and manifestations [3]. Newborn screening (NBS) studies revealed frequencies of the classic and later on set phenotypes of up to 1 in 22,570 males and 1 in 1390 males, respectively [13]. The spectrum of disease severity in heterozygous female patients ranges from asymptomatic to a severe phenotype that resembles that observed in male patients with the classic phenotype and is in part dependent on the mutation and the X chromosome inactivation (Lyonization) profile [3,14,15]. Severe clinical manifestations have been reported in at least 43% of obligate carrier women [16]. Numerous GLA mutations have been reported and efforts are underway to correlate GLA mutations with the major phenotypic subtypes [17].

The past decade has witnessed an increased understanding of the pathogenesis, natural history, and prevalence of FD, and the effectiveness and limitations of ERT. The advances have changed our approach to disease monitoring and therapeutic intervention, necessitating an appraisal and update of monitoring and treatment guidelines for the multisystemic involvement in adult patients with FD published in 2006. The present document complements specific documents that have addressed controversial areas (KDIGO [18]) or aspects of diagnosis and management usually focused around individual organs. Furthermore, these up dated recommendations underline the importance of early

treatment initiation in both males and females, and stress the importance of patient-specific care and a multidisciplinary approach to disease management. Recommendations for the cessation of treatment have not been included here as the clinical consequences of treatment cessation, compared with ERT continuation, remain to be clarified [19-23].

The development of these recommendations was initiated in July 2014 at a meeting of an international panel of FD experts from seven subspecialties, including nephrology, cardiology, neurology, genetics, genetic counseling, pediatrics, and metabolic disorders convened in Atlanta, GA, USA, to review existing treatment guidelines for adults with FD [19]. Subsequent discussions were held during a panel meeting in February 2015 in Orlando, FL, USA. Treatment of pediatric patients was not part of the discussions; recommendations for the monitoring and management of pediatric/adolescent patients were being developed by a panel of experts in pediatric FD and have recently been published. Based on these face-to-face panel discussions, an independent coordinator prepared a draft set of updated recommendations for clinical management of adult patients with FD. Each member of the panel amended the recommendations based on his/her long clinical experience and indepth knowledge of the literature; therefore, no systematic review of the literature on clinical outcome was performed, and the recommendations were not graded. Several revision rounds were performed until a consensus was reached by all panelists, taking important newly published data and perspectives into account [24].

1.2.1. Disease Manifestation

The clinical picture of FD is characterized by progressive signs and symptoms that impact multiple organ systems (Table 1). The “classic” presentation of patients with no or minimal residual α-Gal A activity will be described. However, it is important to note that the presentation is often heterogeneous, with a subset of patients experiencing phenotypes in only one (e.g., cardiac variant) or few organ systems. As FD is X-linked, males present with the classical manifestations more frequently than females [25].

In childhood, patients develop acroparesthesia, which consists of neuropathic pain in their distal extremities. Diffuse pain attacks and crises can also occur, lasting from minutes to days and are often precipitated by rising body temperature due to exercise, fever, or warm ambient environments [26]. Compounding this problem, patients frequently have sweating abnormalities, with the most frequent being anhidrosis or hypohydrosis. Ophthalmologic opacities, such as cornea verticillata and cataracts, are detected in childhood, but many patients retain intact vision [27].

During their teenage years, patients develop angiokeratomas in the "bathing trunk" region. Proteinuria, a sign of kidney disease, may be detected at this time. Additionally, gastrointestinal distress, such as frequent and painful bowel movements, begins to affect patients. In adulthood, patients are at significant risk of end-stage renal disease, heart dysfunction (e.g., hypertrophic cardiomyopathy, cardiac arrhythmias, valvular disease), andcerebrovascular events (e.g., transient ischemic attacks, ischemic strokes). Patients may also develop osteopenia or osteoporosis [28,29]. With nephropathy being a major complication of FD, frequent dialysis treatments become a necessity. Neuropathic pain subsides in some adult patients, but many adults continue to live with debilitating pain [3]. Some adult patients display a unique neuropsychiatric phenotype, characterized by subtle movement impairment and depression [30]. Together, these numerous signs and symptoms significantly reducequality of life [31].

Fabry Disease is considered an attenuated LSD because patients survive into adulthood. However, patients lacking α-Gal A activity exhibit an ~10–20 year shortened life span: male patients with FD have a median survival of 57 years, and the female median survival is 72 years [32]. Many patients with FD do not develop symptoms until their teenage or adulthood years. Moreover, as neuropathic pain is a prominent symptom, patients are frequently misdiagnosed with more common diseases, such as fibromyalgia or rheumatologic pain diseases. Although cornea verticillata and angiokeratomas are useful clinical signs, unfortunately many patients experience what they describe as a "diagnostic odyssey" in that while symptoms are prevalent and debilitating, diagnosis is delayed for long periods of time [33].

The prevalence of FD was once believed to be rare: approximately 1:50,000 [34]. However, recent NBS efforts reveal that the incidence is much more common. In Italy, an incidence of 1:3100 was found [35] and in Taiwan, an incidence as high as 1:1250 was documented [36]. Studies in the United States report incidences of 1:5495 and 1:8454 in Washington and Illinois, respectively. The overall discrepancy in estimated prevalence and measured NBS incidence is likely caused by the heterogeneity of clinical presentation, suggesting that NBS would improve the diagnosis and treatment of patients with FD. Given these findings, the overall patient population will increase as more states and countries include FD in their NBS panels [37].

In Portugal, until the end of 2014, 133 patients with FD were diagnosed at the Unidade de Bioquímica Genética of the Centro de Genética Médica Jacinto Magalhães, from all over the country (unpublished data). On that date, 48 patients were undergoing ERT according to information from the Coordinating Committee for the Treatment of Lysosomal Overload Diseases (Paula Garcia. Lisossomal diseases, the Portuguese landscape. 1st Fabry Iberia KOL meeting) [38].

Table 1 - Clinical manifestations of classic Fabry Disease [25].

Organ System	Characteristics	Usual age at onset (decade)
Peripheral Nervous System	Neuropathic pain (formerly called "acroparesthesia") Hearing loss, tinnitus; dizziness, vertigo	1st Begins in 3rd and increases with age
Dermatological	Angiokeratomas	1st/2nd
Gastrointestinal	Nausea, vomiting, intermittent diarrhea and constipation; abdominal pain and/or bloating; difficulty gaining weight in childhood	1st
Ophthalmological	Cornea verticillata; conjunctival and retinal vasculopathy, cataract, central retinal artery occlusion (rarely), reduced tear secretion	1st/2nd (usually present from birth)
Renal	Pathological albuminuria/proteinuria Decreased glomerular filtration rate progressing to kidney failure	1st/2nd Mean age at kidney failure: 40 years
Cardiac	Cardiomyopathy; reduced exercise tolerance; syncope; cardiac fibrosis; heart failure. Bradycardia – chronotropic incompetence; atrial fibrillation, ventricular tachycardia; sudden cardiac death	4th/5th (usually asymptomatic until well into adulthood)
Vascular	Aortic stiffness	Unknown
Cerebrovascular	TIA; ischemic stroke and (less frequently) hemorrhagic stroke; cerebral venous thrombosis; cervical carotid dissection	3rd and 4th
Neuro-psychological	Common: depression; anxiety; panic attacks; social adaptive function difficulties. Rarely: cognitive decline and dementia	3rd and 4th
Pulmonary	Dyspnea, wheezing; dry cough; sleep-disordered breathing	Unknown
Lymphatic	Lymphedema in all or part of a limb (also below eyes), pitting edema	4th
Skeletal	Osteopenia, osteoporosis	2nd and 3rd
Other	Mild facial dysmorphism	Unknown

1.2.2. Genetics

Fabry Disease is caused by deficiency of α-Gal A, a lysosomal enzyme encoded by the *GLA* gene on the X-chromosome (region Xq22.1). Currently, there are 967 different *GLA* mutations, including 671 missense/nonsense mutations, listed on the Human Gene Mutation Database [39-41]. Missense, nonsense, consensus splice site, cryptic splicing, and frameshift mutations (small and large deletions and insertions) cause FD. In general, nonsense, consensus splice site, and most frameshift mutations result in little or no α-Gal A enzyme activity, and are associated with the classic phenotype. In contrast, a proportion of the missense mutations and rare cryptic splicing mutations can encode enzymes with residual α-Gal A activity, which may explain the later-onset phenotypes. Except in the most recent publications, general registries and clinical studies have not stratified Fabry patients by genotype [42].

Having only one X-chromosome, males that are hemizygous for a pathogenic *GLA* mutation usually develop signs and symptoms, and males with completely deficient α-Gal A activity are impacted most severely, resulting in the "classic" presentation of symptoms. However, unlike other X-linked disorders, heterozygous females may experience significant disease symptoms depending on their residual α-Gal A activity [43]. Females homozygous for *GLA* mutations are especially rare but have been reported [44].

Most of the pathogenic *GLA* mutations are private, occurring in a single or few families; intra-familial phenotypic variability has been observed, complicating the study of genotype–phenotype correlations [3].

Disease manifestations in patients with the same gene mutation, even males from the same family, may vary, making counseling difficult. Factors that will likely alter the impact of a given gene mutation include the presence of additional deleterious *GLA* variants or variants of unknown significance (VUS; either on the *GLA* allele in cis male and female patients, or on the other *GLA* allele in trans female patients), the genetic background of the patient, concomitant diseases, and environmental modifiers.

Through hydrolysis, α-Gal A removes terminal α1,3- and α1,4 linked galactosyl residues from various glycoconjugates within lysosomes. The primary substrate is Gb3, which accumulates to a major extent when α-Gal A is deficient. Minor α-Gal A substrates include deacylated lyso-Gb-3, digalactosylceramide (Gal2Cer), and blood group B and P1 glycosphingolipids. While lyso-Gb-3 accumulates to a lesser extent compared to Gb3, it serves as a biomarker for monitoring therapeutic efficacy and contributes to the pathology of disease [45].

The crystal structure of α-Gal A was solved in 2004 by Scott Garman's group. α-Gal A exists as a dimer, and each monomeric unit of α-Gal A is composed of two domains: an N-terminal (β/α) domain and a C-terminal β-domain. The active site of α-Gal A is located in the N-terminal domain and requires two key aspartate residues, D170 and D231, for hydrolysis of terminal α-linked galactose residues from the glycosphingolipid substrates. Three N-linked glycosylation sites are also present on each α-Gal A monomer, and two of these glycans are predominantly modified with mannose 6-phosphate, which is essential for transport to lysosomes by mannose 6 phosphate receptors [46,47].

There may be mechanisms independent of α-Gal A capable of clearing α-galactosyl glycoconjugates. One example is the lysosomal enzyme, α-N-acetylgalactosaminidase (α-NAGA). In addition to hydrolyzing α-N-acetylgalactosamine from glycoconjugates, α-NAGA also contains α-galactosidase activity. In fact, α-Gal A and α-NAGA are the only enzymes with α-galactosidase activity known to exist in humans. Thus, endogenous α-NAGA may be able to partially compensate for α-Gal A deficiency. Supporting this possibility is the observation that α-NAGA is able to hydrolyze Gb3 in vitro. Further, GSL loading experiments demonstrate that Fabry patient fibroblasts have a ~50% capacity to digest blood group B and a ~15% capacity to digest Gb3, which is probably accomplished by α-NAGA. Although α-NAGA may partially compensate for α-Gal A, this putative compensation I sclearly overwhelmed with agein patients with FD [48,49].

Gb3 is a glycosphingolipid that is known by several names, including ceramide trihexoside, CD77, Pk blood group antigen, and Burkitt lymphoma antigen. Synthesis of Gb3 occurs in the Golgi with the addition of α-galactose to lactosylceramide by Gb3 synthase. This enzyme is also known as lactosylceramide 4-α-galactosyltransferase (encoded by A4GALT). Once synthesized, Gb3 is localized to the outer leaflet of the plasma membrane, where it is clustered in lipid rafts with its glycan portion facing the extracellular environment. Upon endocytosis and delivery to lysosomes, the glycan portion faces the lysosomal lumen, which contains the exoglycosidases, including α-Gal A, that are essential for turnover of this glycosphingolipid. Humans deficient in Gb3 synthase are phenotypically healthy but have an increased risk of miscarriage, suggesting that Gb3 may play a role in embryogenesis. Gb3 synthase mRNA is highly abundant in human heart and kidney, which explains, in part, why patients with FD experience dysfunction of these organs as they contain high levels of this glycosphingolipid. Despite its potential importance in FD treatment, no structures have been reported for Gb3 synthase [50,51].

1.2.3. Mechanisms of Pathogenesis

While much is known about the molecular defects and clinical sequelae of FD, the mechanisms by which substrate accumulation leads to cellular dysfunction remain less defined. Because Gb3 is turned over in lysosomes, the inability to digest Gb3 results in its lysosomal accumulation. However, the impact of α-Gal A substrate accumulation on the development of disease symptoms has increasingly been shown to involve cellular structures beyond strictly the lysosome (Fig. 1). Downstream effects, such as fibrosis, inflammation, and the generation of reactive oxygen species, also seem to play key roles in pathogenesis [52-54].

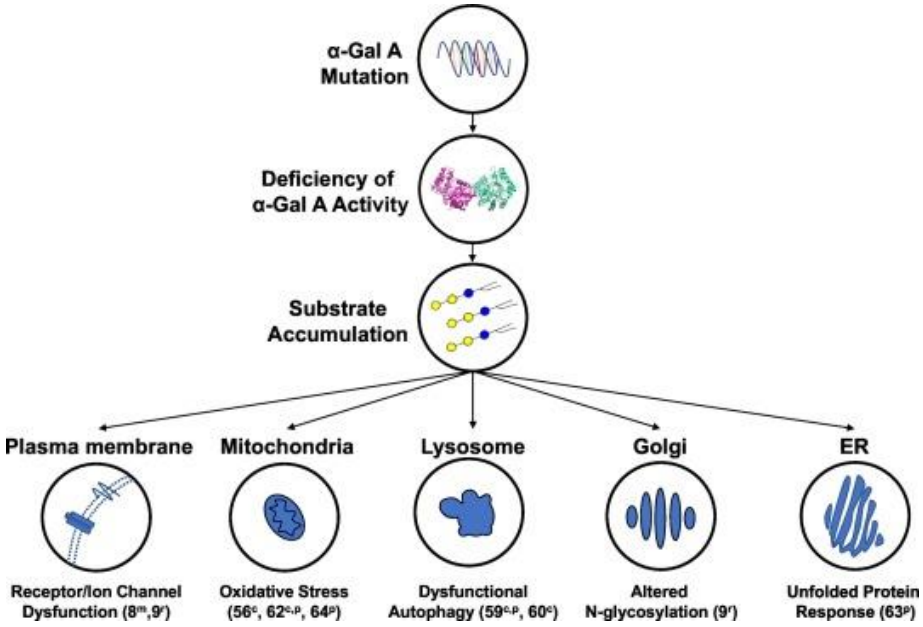

Figure 1. Cellular mechanisms of Fabry Disease pathogenesis [52].

α-Gal A deficiency may lead to the dysfunction of multiple cellular components. Gb3 accumulation has been observed in the plasma membrane of α-Gal A-deficient cells [55]. An increase in plasma membrane Gb3 has the potential to alter the activities of various membrane proteins and channels, likely impacting cellular function in a tissue dependent manner. For example, Gb3 accumulation correlated with increased levels of transient receptor potential vanilloid 1 and altered neuronal Ih and Nav 1.7 currents, suggesting that alteration of these ion channels contributes to the development of pain sensitivity [56]. Additionally, altered activity of cation channel transient receptor potential ankyrin

1 has been reported in Fabry sensory neurons [57]. As in many other LSD, autophagy impairment is also observed in Fabry patient cells. Because autophagy is highly dependent on the formation and fusion of membrane-rich autophagosomes with lysosomes, the alteration of autophagic membranes resulting from α-Gal A substrate accumulation may contribute to the impairment of autophagic flux. In addition, mitochondrial dysfunction may be a pathogenic feature as the activities of respiratory chain enzymes are decreased in fibroblasts from Fabry patients. This raises the possibility that Gb3 accumulation affects mitochondrial function, either directly through accumulation within the mitochondrial membrane or indirectly by preventing mitophagy. Finally, induction of the unfolded protein response has been implicated in FD, which suggests dysfunction of the endoplasmic reticulum [58,59].

Substrate accumulation and organelle damage ultimately results in oxidative stress, inflammation, and apoptosis. The induction of oxidative stress results in part by decoupling eNOS, which increases the generation of reactive oxygen species [60]. Gb3 accumulation also correlates with an increase in the release of the inflammatory cytokines IL-1β, IL-6, and TNF-α in patient plasma, with TNF-α accumulation being especially pronounced in patients experiencing pain crises. Together, the increased oxidant burden and proinflammatory state associated with substrate accumulation likely contributes to symptom development through multiple mechanisms. Proinflammatory cytokines have been implicated as a predisposing factor for thrombosis, stimulating the release of soluble prothrombotic markers, such as von Willebrand factor, and upregulating the expression of endothelial adhesion molecules. In fact, both increases in von Willebrand factor and the expression of endothelial adhesion molecules have been observed in patients and mouse models of the disease. Gb3 accumulation also promotes cell death pathways. In cell types with limited regenerative abilities (e.g., neurons, podocytes), the Gb3-mediated stimulation of apoptotic pathways may contribute to the characteristic symptoms (e.g., pain, podocyte disease) experienced by patients. As mesenchymal stem cells in particular have increased rates of apoptosis and senescence, Gb3 accumulation in the bone marrow could also alter normal hematopoiesis [61-64].

The Gb3 metabolite, lyso-Gb3, has been shown to play important roles in disease pathology. Lyso-Gb3 is formed by the deacylation of Gb3 by acid ceramidase. The accumulation of lyso-Gb3 exacerbates disease pathology as it both inhibits α-Gal A activity and promotes the proliferation of smooth muscle cells, a factor that likely contributes to the increased intima-media thickness observed in Fabry patients. Lyso-Gb3 has been shown to promote inflammatory signaling in cultured podocytes and may also directly sensitize nociceptors, contributing to the renal disease and neuropathic pain experienced by patients, respectively. Overall, the deleterious mechanisms of α-Gal A substrate storage are likely both substrate and cell-type specific, complicating understanding of the molecular

pathways by which Gb3 storage initiates and maintains the signs and symptoms observed in patients [65,66].

1.2.4. Diagnostic

Although FD is an X-linked condition, females can also be severely affected, likely due to a skewed X-inactivation. However, onset of first symptoms in females is generally 5–10 years later than in males. In male patients, a definitive diagnosis of FD involves demonstrating a GLA deficiency of ,5% of wild-type activity in leukocytes and the presence of a GLA mutation, confirmation of the disease-causing GLA mutation is important to help establish the disease phenotype, rule out benign polymorphisms that cause reduced levels of α-Gal A activity, and it permits the testing of at-risk family members [3,13,14,67,68].

In female patients, demonstration of the presence of a disease-causing mutation in the GLA gene is required as the plasma enzyme activity is often found within the normal range, although leukocyte α-Gal A activity may be low. Enzymatic activity is usually measured in plasma, leukocytes, or dried blood spots. For patients with a GLA VUS, clinical, biochemical, or histopathological evidence of FD is required to determine the pathogenic nature of the mutation. In addition, family history can predict pathogenicity for a GLA VUS. The "gold standard" to clarify if a novel mutation is pathogenic or likely benign includes in vitro GLA mutation expression assays (only available at specialized research laboratories). Characteristic clinical features of FD (neuropathic pain, cardiomyopathy, renal insufficiency) should be assessed. The finding of increased plasma and/or urinary GL-3, or plasma lyso-GL-3 and its analogues in the evaluation of male or female patients with a VUS and normal (in female patients) or lowered α-Gal A activity provides additional diagnostic information, but the role of biomarkers in such patients still requires validation. Male patients with a VUS and normal α-Gal A activity do not have FD [3,13,14,67,68].

In patients with an uncertain diagnosis, evidence of lysosomal GL-3 accumulation in renal or cardiac biopsies, although invasive, may be required when interpretation of genetic GLA mutation is challenging, particularly when the clinical signs are nonspecific, alternative or additional diagnoses are under consideration, or in cases in which there is uncertainty over whether ERT should be started. Therefore, the advice of an expert in genetics and management of FD should be sought for interpretation of the pathogenicity of any VUS [69-71].

In individuals with an uncertain diagnosis of FD and no renal, cardiac, or cerebral manifestations, characteristic patterns of neuropathic pain, angiokeratomas, and/or cornea verticillata can support a

diagnosis. FD can be confirmed in the presence of small fiber neuropathy characterized by pain in the hands and feet starting at childhood and increasing with heat/fever, angiokeratomas localizations clustered in the bathing trunk area, umbilicus and/or perioral region, or cornea verticillata. Of note, cornea verticillata is observed in most male and female patients with classic FD and demonstration of cornea verticillata by slit-lamp examination supports the diagnosis. However, cornea verticillata is also known to be associated with the use of various drugs (e.g., amiodarone, chloroquine) [3,70,72].

1.2.5. Therapies

Currently, two therapeutic modalities are available clinically for the treatment of FD: ERT and chaperone therapy (migalastat). The latter, used in patients with certain amenable mutations, facilitates cellular clearance of Gb3 and an overall improvement of disease burden. Other strategies, such as substrate reduction therapy, mRNA based therapy, and gene therapy are in development. ERT, which consists of systemic α-Gal A infusion, was the first approved treatment for FD. There are two available pharmaceutical preparations of recombinant human α-Gal A: 1) agalsidase alfa (Replagal by Shire) is produced by overexpression in human fibroblasts, and 2) agalsidase beta (Fabrazyme by Sanofi Genzyme) is produced by overexpression in hamster ovary cells. Both preparations have similar glycosylation patterns, specific activities, and enzyme kinetics [73], and both have been shown to be clinically efficacious. However, there is a 5-fold dose discrepancy as agalsidase alfa is approved at 0.2 mg/kg biweekly, andagalsidase beta is approved at 1 mg/ kg biweekly. Only agalsidase beta is currently available in the United States, initially approved by the Food and Drug Administration (FDA) in 2003. Agalsidase alfa is available in several locations outside of the United States, such as the European Union, Canada, Australia, Mexico, and South American countries. Currently, pegunigalsidase alfa (PRX-102; a covalently crosslinked, PEGylated form of α-Gal A), is being evaluated for safety and efficacy in clinical trials. There remains an ongoing debate in the field concerning the optimal age of ERT initiation, but it is suggested that earlier treatment results in better outcomes. Additionally, a recent 5-year study of male patients aged 5–18 years supports the efficacy of agalsidase beta at the FDA approved 1 mg/kg biweekly treatment rather than at a reduced dosage [74-76].

Both agalsidase- alfa and agalsidase-beta have been demonstrated to benefit patients in the short term and, in observational studies, patients treated for up to 10 years showed stabilization of estimated glomerular filtration rate (eGFR) or a slowing of the progression of eGFR decline. The natural course

of disease is associated with a rapid decline of renal function (eGFR decline up 12 ml/min per 1.83 m^2 per year) and left ventricular hypertrophy [77-80].

In principal, most studies suggest that initiating early treatment is superior to late initiation, resulting in a more pronounced long-termim provement. In more advanced stages of the disease, however, patients benefit less from ERT. Early ERT has been demonstrated to lead to Gb3 depletion in renal cells in children and adults in a dosage-and frequency-dependent manner. Furthermore, ERT has been reported to stabilize renal function in terms of eGFR, whereas ERT's effect on proteinuria and albuminuria is inconsistent. If ERT is started before myocardial fibrosis has developed, a long-term improvement of myocardial morphology, function, and exercise capacity can be achieved. It also leads to a reduction of cerebrovascular and thromboembolic events and significant improvement in pain-related quality of life. Moreover, ERT is associated with a reduction of gastrointestinal symptoms such as abdominal pain and diarrhea and a reduction of lyso-Gb3 levels, a marker of disease load. Beneficial effects of ERT can be measured by different disease severity scores and tools, such as the Mainz Severity Score Index, the Disease Severity Scoring System, and the Fabry Stabilization indEX. In addition to ERT, provision of concomitant nephroprotective and cardioprotective medication (on the basis of angiotensin-converting enzyme and angiotensin II receptor blockers (ARB)) is highly warranted to preserve renal and cardiac function [81-89].

Despite the availability of ERT, challenges remain for its use in FD. ERT is time consuming (i.e., hours required for infusion) and expensive (~ $200,000 per patient annually), placing a significant burden on patients and the healthcare system. Further, ERT can lead to IARs in females and males, and is not effective in all patients, such as those with end-stage organ disease, as well as the formation of ADAs in about 40% of all ERT-treated males, leading to an attenuation of therapy efficacy. Therefore, the development of improved treatment options is an important goal for many researchers in academia and industry [90-93].

Molecular chaperones are another promising therapeutic avenue for FD treatment. Some patients have single point mutations that result in misfolded α-Gal A. While the mutated enzyme may possess some residual activity, it may be prematurely destroyed by ER-associated protein degradation. Thus, promoting enzyme stability, such as by a small molecule chaperone, may serve as a treatment. The concept is that a ligand (i.e., molecular chaperone) of α-Gal A may occupy its active site, there by promoting enzyme folding and stability. Once the αGal A-chaperone complex enters the lysosome, the chaperone is dissociated from the enzyme due to pH-sensitive conformational changes and α-Gal A is free to act on glycosphingolipid substrates. In order for chaperone therapy to be effective, patients must have amenable mutations (i.e., non-null α-Gal A activity that can be improved by the chaperone). Currently, the chaperone, migalastat (Galafold by Amicus Therapeutics) was approved

in Europe in May of 2016 for long-term treatment of FD in adults and adolescents 16 years or older with an amenable mutation, and was recently approved for use in the United States. The recommended dose is one capsule (123 mg) every other day, and food should not be consumed for at least 2 hours before and 2 hours after taking it. The drug is not currently recommended for use in patients with an eGFR, 30 ml/min per 1.73 m^2 [94,95].

Substrate reduction therapy is another option currently under investigation. Two small molecules are being or are about to be tested in clinical trials: ibiglustat and lucerastat. Both ibiglustat and lucerastat inhibit glucosylceramide synthase, the enzyme that adds glucose to ceramide. Because glucosylceramide is a common precursor in the synthesis of many glycosphingolipids (e.g., globosides, gangliosides, lactosides, sulfatides), glucosylceramide synthase inhibition results in decreased Gb3 synthesis [96].

Glucosylceramide synthase inhibitors have been tested preclinically in Fabry cell and mouse models and in clinical trials for patients with Gaucher disease (glucosylceramidase deficiency). In patient-derived lymphoblasts, glucosylceramide synthase inhibitors depleted Gb3 by 70–80% and reduced Gb3 levels below those of controls in α-Gal A deficient mice. Clinical studies with miglustat, an inhibitor of glucosylceramide synthase, reported significant adverse events, such as weight loss, diarrhea, poor appetite, and tremor. These side effects are probably due to the fact that miglustat more potently inhibits other enzymes, such as lysosomal and non-lysosomal β-glucosylceramidase and intestinal disaccharidases. Recent clinical trials using the more selective glucosylceramide synthase inhibitor, eliglustat, provide support for the safety and efficacy of substrate reduction using more specific inhibitors; however, some patients experienced mild-to-moderate abdominal pain, diarrhea, and abnormal nerve conduction studies. As gastrointestinal distress and peripheral neuropathy are dominant symptoms in FD, the effects of glucosylceramide synthase inhibition need to be further evaluated in patients with FD [97-100].

There are several other FD therapies on the horizon. Gene therapy will likely be a future therapeutic option for patients with FD. In two trials, CD34+ stem cells are obtained from a patient and are engineered to express α-Gal A using a lentivirus vector. The transduced cells are then transplanted back into the same patient (i.e., autologous stem cell transplantation) with the goal that secreted α-Gal A will be taken up by the patient's other cells. Adeno-associated virus capsids are also being evaluated for gene therapy, and novel capsids are in development to improve α-Gal A expression in kidney, heart, and brain. Moving forward, gene therapy for FD has the potential to systemically express α-Gal A in a manner that would be effective for the large number of genetic mutations that cause FD [101,102].

mRNA-based therapies are also likely to become available for FD as studies in Fabry mice and non-human primates were recently reported [103]. This is an attractive option because the translated α-Gal A would express native post-translational modifications, such as mannose 6-phosphate on N-glycans for lysosomal targeting. With new therapeutic options becoming available, we may discover that combination therapies provide maximal benefit. For example, recent studies show that chaperone therapy coupled with ERT is more efficacious than either option alone [104,105].

1.2.5.1. Immune response to ERT

Because ERT is on the basis of the intravenous infusion of a mostly foreign recombinant protein, a humoral response is common, at least in males with classic FD, who lack endogenous enzyme. Such individuals are also classified as "crossreactive immunologic material" (CRIM) negative. CRIM-negative status means that there is not even a mutated or truncated form of endogenous GLA that can be recognized by the immune system as an innate molecule. In these patients, infused enzyme might be recognized as foreign, triggering an immune response. Several studies analyzing patients with LSDs demonstrate that CRIM-negative patients have a high risk for developing immune responses after ERT initiation. Because of this, it is important when treating patients with FD to distinguish an immune response caused by continuous exposure to ERT (leading to the formation of neutralizing ADAs) from IARs [74,92,93,106].

IARs frequently occur in ERT-naïve patients with FD after ERT initiation, mainly in males with nonsense or null mutations (CRIM negative). Symptoms of IARs are mostly limited to fever and chills, necessitating premedication with antihistamines and steroids, as well as prolongation of infusion times (i.e., decreased infusion rates). However, severe life-threatening IARs also have been reported. According to the manufacturer's instructions, 24% of patients treated with agalsidase- alfa present with IARs. In contrast, 67% of patients treated with agalsidase-beta experience this adverse effect [74,107,108].

The nature of IARs is yet not completely understood but they are probably the result of anaphylactoid reactions (i.e., chemical compound–mediated) and not often anaphylactic (i.e., IgE-mediated type 1 hypersensitivity), because direct identification of IgE antibodies has been made in only a few male patients to date. In one report, IgEs were identified in one patient with severe IARs, including pain; other reports described one patient with generalized urticaria during infusions and another with anaphylaxis. In addition, an agalsidase-beta open-label safety study reported seven patients with IgEs or a positive skin test. Furthermore, Wilcox et al. reported three patients with IgEs

identified by direct IgE measurement or a skin test. However, the risk of IARs seems to be higher in patients with an anti-agalsidase IgG antibody– positive status. The humoral response includes crosstalk between antigen-presenting cells, CD41 T-helper cells, and B cells [93,107,109-111].

In patients with IgEs, the following general mechanisms might apply: After antigen presentation, an early anaphylactic response includes the formation of IgE antibodies, triggering mast cell activation and histamine release, which could explain the observed severe IARs. With ongoing antigen presentation this process is commonly followed by IgG4 isotype antibody production against similar epitopes. Because these antibodies compete with IgEs but do not lead to complement activation, immunization will be achieved and IARs may be attenuated or stop over time. Currently, the literature lacks comprehensive studies analyzing the presence of IgE and IgG antibodies in patients with FD [112].

IgG antibodies seem to develop within 3-6 months of starting ERT, but frequency and severity of IARs in affected patients may be attenuated. Smid et al. reported that most IARs were observed during the first 13 infusions. Although awareness of ERT-related IARs emerged in the first ERT clinical trial, to date, no standardized assays or protocols have been developed to determine IgG levels. Most trial studies and reports analyzing an early IgG response were performed by the ERT-manufacturing companies using Enzyme-linked Immunosorbent assays (ELISAs) and their own antibodies. However, Linthorst et al. demonstrated that IgG antibodies (measured by ELISA) mediate an easily measurable neutralizing activity that can be used to classify neutralizing antibody–positive and –negative patients. This neutralizing activity was confirmed by several other studies, and the isotype of drug-neutralizing antibodies in patients with infusion-related ADAs recently was identified as IgG4, mainly [92,107,111,113-115].

Recent studies have found that, once neutralizing ADAs occur, their formation seems to be irreversible, and that the majority of affected patients stay neutralizing ADA–positive over 10 years. About 73% of patients treated with agalsidase-beta and 24% of patients treated with agalsidase-alfa reportedly develop ADAs. This difference might be explained by the different dosages of both drugs and the different cell lines producing them, as well as the CRIM status of the treated patients, which has not been taken in to account thus far. Recent data from Arends et al. confirmed an increased risk for formation of neutralizing ADAs in patients treated with agalsidase-beta. Although the individual CRIM status of the treated patients was not determined, their study excluded patients with non classic FD. However, Rombach et al. and later Smid et al. reported no significant differences in ADA formation when using the same dosage (0,2 mg/kg every 2 weeks) for both drugs at ERT initiation. Of note, the CRIM status of patients in these studies was also not determined in detail. To analyze this topic adequately, head-to-head studies with comparable dosages and a predefined CRIM status

of participating patients are needed. At this point, it seems that the dosage is the most important trigger for immune response [80,92,93,107].

Although the presence of neutralizing ADAs was first described very early after ERT became available, the effect of ADA son FD-specific biomarkers has been a topic of controversy. High ADA titers (measured by ELISA) have been associated with increased Gb3 deposition in endothelial cells, but with unclear effects on plasma Gb3 levels. In contrast, by using serum-mediated inhibition assays, Rombach et al. demonstrated that plasma lyso-Gb3 levels were significantly higher over time in patients with neutralizing ADAs, and that urinary Gb3 decreased only in patients without ADAs. This group also confirmed that ADAs are associated with a poorer clearance of plasma lyso-Gb3. Although some previous reports have suggested that ADAs appeared to have no effects on such parameters as eGFR slopes and cardiac mass, there is evidence that patients with ADAs exhibit harmful clinical outcomes, including increased left ventricular mass (LVM) and progressive loss of renal function. These differences in study outcomes might be due to an ERT dosage–dependent effect on ADA titers. Rombach et al. observed that in patients with ADAs who had been switched from an agalsidase-beta dose of 0.2 to 1.0 mg/kg every 2 weeks, plasma lyso-Gb3 and Gb3 significantly declined after 1 year; this might be the result of a supersaturation of antibodies due to increased ERT dosages during infusion [92,106,113,116].

A model of ERT and neutralizing ADAs during infusion, supported by findings in the literature, can potentially explain varying therapy efficiency during infusions. According to this model, during infusions in patients without ADAs, ERT enters the cell and lysosomes via the mannose-6-phosphate (M6P) receptor and results in clearance of Gb3. If neutralizing ADAs are present, ERT is directly inactivated (neutralized) by ADAs in the plasma. In addition, binding of ADAs to ERTalso leads to an activation of macrophages that internalize ERT-ADA complexes, leading to a decrease in the cellular uptake of free ERT. If the ERT dose sur mounts the antibody titer, more ERTcan enter the lysosomes of target cells, resulting in an appropriate Gb3 clearance with subsequent therapeutic benefits. This hypothesis is supported by recent outcomes by Arends et al. demonstrating an improved biochemical response - decreasing lyso-Gb3 - in ADA-positive patients treated with higher doses of agalsidase, such as agalsidase-beta (1.0 mg/kg) compared with agalsidase-alfa (0.2 mg/kg). Whether the binding of ADAs to ERT further affects the cellular uptake (i.e., transport) via the M6P receptor, or independent of M6P by megalin and sortilin, still remains unclear and should be investigated in future studies [80,113,114,117].

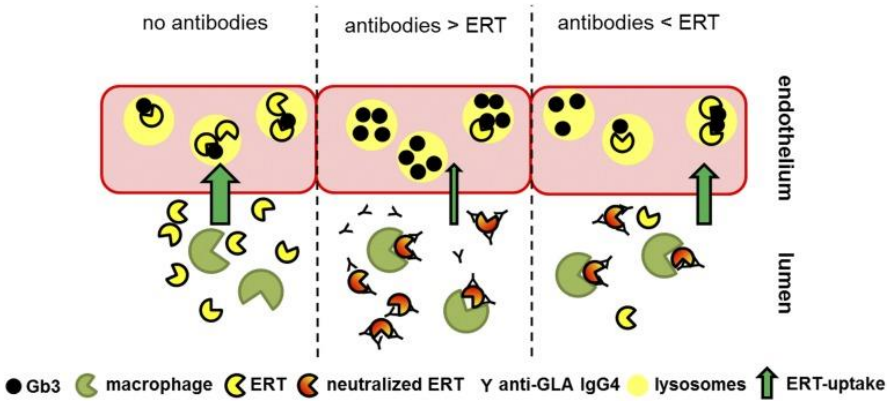

Figure 2. Neutralizing Antidrug Antibodie (ADA) titers affect cellular lysosomal Gb3 clearance. Current literaturebased model of ERT and neutralizing ADAs during infusion. If no antibodies are present, ERT enters cells (here, endothelial cells) via the M6P receptor, leading to Gb3 clearance from lysosomes (left). If antibodies are present, they neutralize ERT activity by binding the enzyme. In addition, IgG-tagged ERT molecules will be internalized and digested by macrophages. If more antibodies than ERT are present, this results in a decreased cellular Gb3 clearance (middle). If the ERT dose exceeds antibody titers, more ERT can enter the lysosomes of target cells, resulting in increased Gb3 clearance (right) [80].

1.3. Immunogenicity

Biopharmaceuticals, including biosimilars, have the potential to elicit an immunogenic response in treated individuals (immunogenicity), which may have an impact on the efficacy and safety profiles of the drug. Thus, it is critical that immunogenicity is evaluated throughout the various phases of clinical development and during postmarketing surveillance. Although rigorous evaluation of biopharmaceutical immunogenicity is required by regulatory authorities, there is a lack of uniform standards for the type, quantity, and quality of evidence, and for guidance on experimental design for immunogenicity assays or criteria to compare immunogenicity of biopharmaceuticals. Moreover, substantial technological advances in methods to assess immune responses have yielded higher immunogenicity rates with modern assays, and limit comparison of immunogenicity of biopharmaceuticals outside of head-tohead clinical trials. Accordingly, research programs, regulatory agencies, and clinicians need to keep pace with continuously evolving analyses of immunogenicity. Prevailing concepts and methods to detect immunogenicity have evolved over the past two decades [118,119].

Immunogenicity is characterized by the presence of ADAs detected in the circulation of either animals or humans after administration of a biopharmaceutical. ADAs that bind to the active site of a biopharmaceutical and may inhibit its activity are termed neutralizing antibodies. Non-neutralizing

antibodies do not bind to the active site but may still produce important clinical consequences, such as reduced therapeutic efficacy by compromising bioavailability. The generation of ADAs (neutralizing or non-neutralizing) is increasingly recognized as a mechanism explaining the reduced efficacy or therapeutic failure of some biopharmaceuticals. This can occur by altered drug pharmacokinetics or, in other instances, the presence of neutralizing antibodies bound to active sites, there by reducing drug activity [120,121].

A multitude of factors can influence immunogenicity, which may be classified into three major categories: treatment-, patient-, and drug property-associated factors. For example, treatment-associated factors include the route of administration (subcutaneous vs. intramuscular vs. intravenous), duration of therapy (short-term vs. long-term), and frequency of administration (intermittent vs. continuous), all of which may affect the likelihood of an immune response. Patient-associated factors include immune system function, which, when compromised, may lower the probability of mounting antibodies, disease state, and polymorphisms in major histocompatibility complex (MHC), which can affect the magnitude of T cell-dependent immune responses. Drug property-associated factors include the degree to which a biopharmaceutical is humanized, glycosylation patterns, and removal or concealment of MHC epitopes by design, as well as issues that arise during the manufacturing process, such as the presence of impurities, aggregates, and contaminants [122-125].

Table 2 - Factors influencing immunogenicity of biopharmaceuticals [125].

Category	Example
Treatment-associated	Mechanism of action Route of administration Frequency of administration Duration of therapy
Patient-associated	Disease type Disease status Immune system function Genetic factors Concomitant disease Concomitant medications Prior exposure Prior sensitization
Drug property-associated	Recombinant expression system Post-translational protein modifications Impurities Contaminants Aggregates

1.3.1. Immunogenicity Assessments

Immunogenicity of biopharmaceuticals in clinical studies is evaluated using a stepwise approach. First, a screening assay is employed to detect the presence of ADAs in treated patients. This is followed by confirmatory assays to determine the specificity of ADAs for the biopharmaceutical and eliminate false positives. For ADA positive samples, characterization assays are conducted to determine the titer and type of ADAs, and bioassays or ligand-binding assays are used to identify neutralizing antibodies. To evaluate the potential clinical impact of ADAs, immunogenicity assessments are conducted in conjunction with pharmacokinetic, safety, and efficacy assessments, with the totality of data considered [126].

A variety of techniques have been used to screen for ADAs in biopharmaceuticals, and methodologies have advanced considerably in the past two decades. Commonly used assays include enzyme-linked immunosorbent assays (ELISAs), which may be conducted using a direct, indirect, or capture format; electrochemiluminescence (ECL) assays and antigen-binding tests, such as radioimmunoassays. Some of these assays can be modified to render them ‘‘drug tolerant’’. Because each format has relative strengths and weaknesses, there is no single assay appropriate for assessing the immunogenicity of all biopharmaceuticals. Selecting the optimal assay for ADA screening is a key consideration in biopharmaceutical development and must take into account the properties of the therapeutic to be tested [127-129].

Because of their convenience, ease, and high-throughput capability, ELISAs are frequently utilized for immunogenicity screening. In a direct ELISA, ADAs from patient samples are captured by the biopharmaceutical, which has been immobilized on a plastic plate; the plate is washed several times and ADAs are then detected spectrophotometrically with a colorimetric labeled antiimmunoglobulin reagent. A limitation of the direct ELISA is that fixation of the biopharmaceutical to a plastic surface may alter its conformation and conceal epitopes, resulting in underestimation of ADAs. The indirect ELISA format circumvents this complication by first immobilizing antibodies on the plate to orient the biopharmaceutical. Disadvantages of the direct and indirect ELISA formats include false positives and high background noise due to non-specific binding as well as potential loss of low-affinity ADAs during washes. In a capture ELISA, ADAs are captured by immobilized biopharmaceutical and then detected using a conjugated version of the biopharmaceutical. This version is more selective and specific than the direct or indirect formats, but the possibility of losing low-affinity ADAs remains [129,130].

Regardless of the technique utilized to screen for ADAs, proper validation of the methods is essential to ensure reproducible, consistent, and definitive results. This step should occur early during the course of clinical development of a biopharmaceutical and may require ongoing monitoring and modification during the pre-approval processes. Validation parameters should include cut-points, sensitivity, drug tolerance, specificity, precision, dilution, and reproducibility. Due to a lack of agreement on the use of reference standards, the experimental systems for assessment of immunogenicity cannot be calibrated, and the assays are merely quasi-quantitative. Therefore, assays must include positive controls (e.g., samples of purified ADAs from a patient with characterized immunoglobulin levels) and negative controls (e.g., serum samples from untreated healthy individuals) [128,129,131].

1.3.2. Regulatory Guidelines

Evaluating the immunogenicity of biopharmaceuticals is mandatory for regulatory approval. Specific guidance for evaluating immunogenicity of therapeutic protein products has been provided by the FDA and European Medicines Agency (EMA) [132-134].

Recommendations include development of assays to adequately detect and confirm the presence of ADAs and techniques to discern neutralizing and non-neutralizing antibodies, systematic collection of data from patients in clinical studies, and standardization of methods across studies for a givenbiopharmaceutical. Both the FDA and the EMA advise that data from immunogenicity assessments in animals are not necessarily indicative of immune responses in humans but can supplement the information obtained from preclinical toxicology studies [132,134].

Because of strict eligibility criteria and short-term followup, results from immunogenicity analyses in clinical trials of biopharmaceuticals may not reflect real-world clinical experience. Indeed, it is necessary to continue the assessment of safety (including immunogenicity) following the approval of all biopharmaceuticals [134,135].

Thus, ongoing monitoring of immunogenicity post approval is extremely valuable, and observational clinical and laboratory test databases from patients treated with biopharmaceuticals in routine clinical practice may provide further insight into the immunogenicity of these drugs. Guidelines from the FDA, EMA, and World Health Organization (WHO) recommend immunogenicity be considered in pharmacovigilance and risk management plans for all biopharmaceuticals, including biosimilars; however, only the EMA guidelines stipulate how immunogenicity should be addressed in post-approval surveillance strategies [132-134].

2. HYPOTHESIS AND AIMS

Biopharmaceuticals are therapeutics produced by a living organism, most often made by genetically engineering living bacterial, animal, or plant cells. Biopharmaceuticals have the potential to elicit an immunogenic response in treated individuals (immunogenicity), which may have an impact on the efficacy and safety profiles of the drug. Thus, it is critical that immunogenicity is evaluated throughout the various phases of clinical development and during postmarketing surveillance.

Immunogenicity is characterized by the presence of ADAs detected in the circulation of either animals or humans after administration of a biopharmaceutical. ADAs that bind to the active site of a biopharmaceutical and may inhibit its activity are termed neutralizing antibodies. Non-neutralizing antibodies do not bind to the active site but may still produce important clinical consequences, such as reduced therapeutic efficacy by compromising bioavailability. The generation of ADAs (neutralizing or non-neutralizing) is increasingly recognized as a mechanism explaining the reduced efficacy or therapeutic failure of some biopharmaceuticals. This can occur by altered drug pharmacokinetics or, in other instances, the presence of neutralizing antibodies bound to active sites, there by reducing drug activity.

ADA formation can also contribute to the development of IARs, as well as the formation of circulating immune complexes that activate the complement system, resulting in a general inflammatory response and cytokine release. Therefore, it is important, when treating patients with FD, to distinguish a response caused by continuous exposure to ERT (leading to the formation of neutralizing ADAs) from IARs. Although ADA have been identified, few studies have investigated the clinical impact of these antibodies.

The general purpose of this project is to study the immunogenicity of a court of Fabry patients and see the impact of immunogenicity on the effectiveness and safety profile of ERT.

The frist specific aim of this project is to screening and confirmation of antibodies IgG1 and IgG4 against agalsidase alfa and beta therapy, pre and pos ERT infusion, through by Indirect ELISA; screening of IgE antibodies against agalsidase alfa and beta therapy, pre and pos ERT infusion, through by Sandwich ELISA; and dissociation of IC associated to ERT and screening of IgG released from IC through by Indirect ELISA, in order to identify false negatives.

The second specific aim of this project is to verify the impact of the presence of ADA against agalsidase alfa and beta, on the effectiveness and safety of ERT, through analyze and correlate the results obtained with the safety data, and clinical and functional cardiac and renal data of patients, such as: Age (Y, Months), gender, age diagnosed (Y), ERT duration, type of ERT alfa/beta/switch

alfa-beta, leucocyte α galactosidase A activity, gene GLA mutation, Classic/Non Classic phenotype, plasma levels of Gb3 and Lyso-GL-3, eGFR (range) (mL/min/1.73 m^2), LVM (range) (g/m2.7), mainz score total, laboratory tests of renal function (creatinine and proteinura 24h), clinical manifestations and IARs. Also, make a direct correlation between the two ERT therapies, agalsidase alpha and beta.

Most trial studies and reports analyzing an early ADA response were performed by the ERT manufacturing companies using ELISAs and their own antibodies. These are studies with methodological differences that include differences in cutoff points, definitions of seropositivity, dilution of the sample, timing of the test and different dosages of agalsidase.

Our results may allow to make a direct correlation, of our clinical results, and their respective correlation with the safety data, and clinical and functional cardiac and renal data of patients, between the different ERT therapies, agalsidase alfa and beta.

In our study, we will not have the disadvantage of having methodological differences. Cutoff points, definitions of seropositivity, dilution of the sample, timing of the test, controls, and dosages of agalsidase, established in our study are the same for both ERT therapies.

3. RESEARCH DESIGN AND METHODS

3.1. Study design and patients

For our study, cross-sectional prospective, 51 patients, 40 men (mean age = 55,9 years (between 20 e 77 years)) and 11 women (mean age = 54 years (between 39 e 73 years)), were recruited from of Hospital Senhora da Oliveira, Centro Hospitalar Vale do Ave (Guimarães, Portugal), in between October-November 2018. These patients were recruited in cardiology appointment, by the doctor Olga Azevedo, cardiologist, and following patients with hypertrophic cardiomyopathy, FD using the diagnostic criteria described by international medical guidelines. Clinical diagnosis was carried out using the medical criteria established by the guidelines. Laboratory diagnostic criteria included genotyping in women and determining the activity of alpha galactosidase A in leukocytes of male patients.

The Ethical approval of participating centres and patients' informed consent to collect and store the samples has been obtained. This study involves only human research, respecting the premises of the Helsinki Declaration.

Inclusion criteria of the study were: diagnosis of FD according to the international recommendations; age of 18 years or more; FD patients submitted to ERT; ERT submission time.

The exclusion criteria were: severe renal condition requiring dialysis or transplantation and/or severe cardiovascular condition requiring intervention for pacemaker placement or transplantation, namely: moderate or severe valvular heart disease, previous septal myectomy or alcohol ablation, ventricular pacemaker implanted in the previous 6 months, surgery or major trauma within the previous 6 months, active cancer, glomerular diseases (nephritic or nephrotic syndrome), immunoglobulin A nephropathy, diabetes, systemic vasculitis, systemic lupus erythematosus, hepatitis C, amyloidosis and multiple myeloma, and Migalastat therapy (Amicus Therapeutics®) were excluded due to the potent enzymatic inhibition by this compound observed in vitro.

For each FD patient, routine follow-up data will also be collected, namely: gender, age at samples collection, age at dianoss plasma α-galactosidase A activity, GLA gene mutation, current medication (angiotensin converting enzyme inhibitors (ACEi), ARB and β-blockers), data about current and, if applicable, previous ERT (product, dose and duration), clinical manifestations (in order to calculate the disease severity indexes – Mainz Score Severity Index (MSSI) 53 and Fabry International Prognostic Index (FIPI) 54), echocardiogram data (indexed LVM) and laboratorial results (kidney

function tests (creatinine and 24 hours proteinuria) and plasma lyso-Gb3). Yearly clinical, echocardiographic and kidney function data will also be collected up to 5 years in retrospective.

Clinical evaluations were performed during the recruited period and consisted of neurological, cardiac, and renal evaluations, namely, calculation of the eGFR, calculation of LVM assessed by echocardiography and presence of cerebral White Matter Lesions (WML) determined with Magnetic Resonance Imaging (MRI).

Regarding safety issues related to ERT, data about premedication (current and ever required) and IAR reported in patients' files (current and ever reported) will be collected.

Blood samples were taken by venipuncture, 30 minutes before (corresponds to 15 days after the last drug administration) and 30 minutes after infusion of ERT, and collected to K2EDTA tubes, and stored at 4 ºC until the next procedure. For plasma separation, EthyleneDiamineTetraacetic Acid (EDTA) blood samples were equilibrated at room temperature and centrifuged at 2000 xg for 10 minutes. The plasma fractions were then aliquoted and frozen at -80 ºC in sterile and LPS free criotubes.

After, peripheral blood mononuclear cell (PBMC) were separated by density gradient with ficoll-paque plus™ (GE healthcare, USA) as described elsewhere. Briefly, K2EDTA blood samples were dropped over a layer of ficoll-paque previous warmed at 37 ºC. Samples were carefully transferred and centrifuged at 2000 rpm for 20 minutes at room temperature with brake off. The PBMC were washed twice with 2% Fetal Bovine Serum (FBS)/2mM EDTA in Phosphate Buffered Saline (PBS)1x, pH 7.4 (wash buffer), counted and ressupended in frozen culture medium (10% Dimethyl Sulfoxide (DMSO) in FBS). Aliquoted and transferred to -80 ºC (24 h-72h) and finally to liquid N2.

Table 3 - Demographic and Clinical data.

Sample ID	Gender	Age (Years, Months)
1FD	Male	60
2FD	Female	RI
3FD	Male	64
4FD	Male	71
5FD	Male	63
6FD	Male	50
7FD	Male	48
8FD	Male	77
9FD	Female	39
10FD	Male	58
11FD	Male	65
12FD	Male	76
13FD	Male	52
14FD	Female	48

15FD	Male	34
16FD	Male	58
17FD	Male	63
18FD	Male	58
19FD	Male	35
20FD	Male	22
21FD	Male	29
22FD	Male	65
23FD	Male	68
24FD	Male	62
25FD	Male	61
26FD	Male	63
27FD	Male	51
28FD	Male	51
29FD	Female	52
30FD	Female	49
31FD	Male	73
32FD	Male	52
33FD	Male	69
34FD	Male	75
35FD	Male	49
36FD	Male	33
37FD	Female	58
38FD	Male	RI
39FD	Male	58
40FD	Female	73
41FD	Male	56
42FD	Male	54
43FD	Male	56
44FD	Male	20
45FD	Female	49
46FD	Female	52
47FD	Female	62
48FD	Female	58
49FD	Male	71
50FD	Male	57
51FD	Male	RI

3.2. Screening and confirmation of antibodies IgG1 and IgG4 anti-Agalsidase alfa and beta

This task aims to screening and confirmation of antibodies IgG1 and IgG4 against agalsidase alfa and beta therapy, through by Indirect ELISA.

The ELISA technique requires initially optimization of several conditions and reagent concentrations. For instance, optimal concentration for the coating, serum samples and antibody detector, requires for each reagent a graded dilution from high concentration down to low concentration into each well. A chessboard approach uses two dimensions, rows vs columns, ensuring titration of two reagents at the same time. As illustrated in figure 3, we performed titration for

agalsidase alfa across columns against biotinylated rabbit anti-human IgG1 antibody across rows, and used a fixed dilution 1/10 of two serum samples: one, AAA- and other AAA+ to test the assay response.

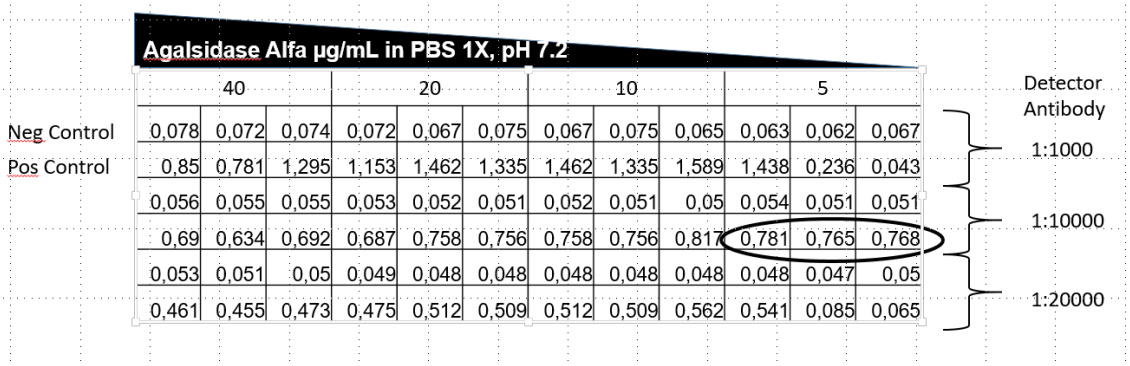

Figure 3. Schematic ELISA layout showing chessboard approach for optimization and selection of suitable concentrations either for Agalsidase alfa (Replagal®) coated as well as biotinylated anti-human IgG1 detector antibody.

When ELISA optimization was concluded, a standard has always been used to determine the assay´s sensitivity as well as sample quantification. A native purified human IgG1 was used as standard and diluted in PBS 1x, pH 7.2 in a range between 5-4 logs of amplitude response. A serial dilution was performed with 8 to 9 points in duplicated, starting from 10 µg/ml down to 10 ηg/mL and coated to a 96 well microplate, distributed fifty µl to each well and incubated overnight at 4 ºC. Blocking was performed with 1% casein in PBS-0.1% Tween 20 (casein blocking buffer), distributed 50 µl to each well and incubated for 1 hour at 37 ºC. After 3x washed, plate was incubated with biotinylated rabbit anti-human IgG1 antibody diluted 1/10000 in diluent. The following steps are described in screening ELISA protocol. Finally the mean optical density (OD) values were plotted in a semi logarithm scale.

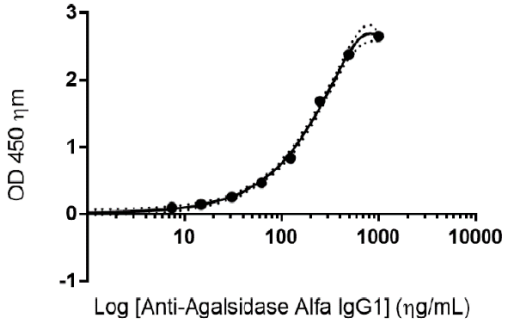

Figure 4. Graphic with human IgG1 standard curve plotted by Graphpad Prism Software analysis.

A high binding polystyrene 96 well microplates (#3690, Corning inc, NY USA) were coated with Agalsidase alfa, Replagal® (Shire Human Genetic Therapies) at 5µg/mL in coating buffer distributed 50 µl to each well in whole plate overnight at 4 ºC. Unbound sites were blocked with casein blocking buffer, distributed 50 µl to each well for 1 hour At 37 ºC. After 3x washing step with PBS-0.05% tween® 20 (wash buffer), serum, standards and control samples were diluted in 1% Bovine Serum Albumin (BSA) in PBS-0.05% Tween® 20 (diluent). Sera samples were used in triplicated, diluted at least 1/10 then incubated for 1 hour at 24 ºC, 50 µl on each well. After incubation with samples, plates were then washed 5x with wash buffer and incubated with biotinylated rabbit anti-human IgG1 (H&L) (Abcam #201485, MA USA) (lote:GR3194366-2) diluted 1/10000 in diluent buffer, distributed 50 µl to each well then incubated for 1 hour at 24 ºC. After 5x washing, streptavidin conjugated with horseradish peroxidase (SAHRP) was diluted 1/10000 in diluent and distributed 50 µl to each well, incubated 1 hour at 24 ºC. Finally, plates were washed 5x, then incubated with substrate 3,3',5,5'-Tetramethylbenzidine (TMB) (Millipore, MA, USA) distributed 50 µl to each well, incubated 30 minutes at 24 ºC and protected from light. After this step, reactions were stopped with 50 µl of 0.5M sulfuric acid. Plates were measured at 450 ηm in a Multiscan FC microplate reader (Thermofisher Scientific, MA USA).

We determine of the screening cut-point and sensitivity. Initially, it was established a negative control group originated from Fabry treatment naïve samples at least 30 (in the absence / non-availability at the moment, of a group of ERT-naive sample (negative control, and recommended by FDA and EMA guidelines), samples of ERT-treated patients with lower response or signal were used, compared to the essay background). A statistical approach was applied to remove outliers due most likely with immunogenicity related factors (reumathoid factor, pre-existing ADA, disease state). The cut-point and sensitivity were calculated as outlined below:

$$\boldsymbol{Cut-Point} = \text{Mean (AVG) Negative Background Samples (NBS)} + 1.645\text{X Standard Deviation (SD)}$$

$$\boldsymbol{Sensitivity}\ (\boldsymbol{LOD}) = \text{Mean background} + 3\text{X SD}$$

FDA recommends that screening and confirmatory ADA assays achieve a sensitivity of at least 100 nanograms per milliliter (ng/mL). Although traditionally FDA has recommended sensitivity of at least 250–500 ng/mL, LOD - Limit of detection.

3.3. Screening of IgE antibodies against α-Gal A alfa and beta therapy

This task aims to screening of IgE antibodies against agalsidase alfa and beta therapy, through by Sandwich ELISA.

Total and anti-agalsidase alfa IgE antibodies were detected by a validated sandwich ELISA according to manufacturer´s protocol from human IgE ELISA kit (Stem Cell Technologies, Grenoble, France). Nevertheless, a small modification was performed and consisted in replacing the detector, biotinylated anti-human IgE antibody by a biotinylated agalsidase alfa used at 20 µg/mL. All pre- and pos-infusion samples were diluted at 1:10 and tested in duplicated. IgE assay results were reported as positive or negative for anti-drug IgE using a cut-point described elsewhere. Because a purified drug-specific positive control was not available, positivity was determined using a heterologous calibrator curve generated with Human IgE antibody from the kit. The sensitivity of the assay is 0.28 ηg/mL.

3.4. Dissociation of immune complexes (IC) associated to ERT

This task aims to a method based on acid treatment of serum samples. The acid reagent enables dissociation of IC and therefore can release antigens and antibodies which were not able to be detected before by standard ELISA methods.

3.4.1. Setting up a positive control for immune complex dissociation

To test if the technique was working properly, we set a positive control based on the interaction between Agalsidase and high titre AAA from a Fabry serum patient (sample 21FD).

This positive control is used to ensure that acid treatment is acting as a dissociation reagent. A high titre ADA positive serum sample against Agalsidase α diluted at 1:10 and 1:40 (dilution made in 1% BSA in [PBS-Tween (0.05%)] was mixed with different concentrations of Agalsidase ranging from 0, 1, 10, 20 and 50 µg/mL (agalsidase diluted in PBS 1x, pH 7.2). Briefly, IC samples were rocked overnight at 4 ºC. On next day, to equilibrate the solutions, incubation time was extended for more 30 minutes at 24 ºC.

3.4.2. Screening of IgG released from IC

For each IC sample, one volume of 100 µl was mixed with an equal volume of acid (glycine-Hydrochloric acid (HCl) reagent (1.5 M glycine, pH1.85, adjusted by concentrated HCl)), vortexed and incubated for 45 minutes at 24 ºC. The remaining IC samples, untreated, were left at 4 ºC, waiting for treatment over. After the incubation time, acidified samples were mixed with 1/3 volume of neutralizing buffer pH 9 (Tris-HCl 1.5M), vortexed and incubated for more 30 minutes at 24 ºC. Finally, IC treated samples were centrifuged at room temperature at 17000 xg for 10 minutes. This step showed that acid treatment could dissociate the AAA from IC lattice. Consequently, an Agalsidase: AAA+ sample ratio condition was selected and further used as a positive IC control.

Treated and untreated samples were distributed in triplicate side by side in previous coated plates with Agalsidase at 5µg/mL and blocked with blocking buffer (3% BSA). The following steps are described in screening indirect ELISA protocol. After collecting and analysing the data, we have seen differences in signals between treated and untreated samples with the same ratio concentrations.

3.5. Data analysis

The laboratory results in FD patients will be compared between the pre specified subgroups and correlated with the aforementioned safety, clinical and cardiac and renal functional parameters in order to evaluate the influence of immunogenicity in these factors.

Statistical analysis will be performed with SPSS® (Statistical Package for the Social Sciences, version 21) software. If not stated otherwise, continuous variables will be expressed as mean with standard deviation, or as median (range). Categorical data will be expressed as numbers and relative frequencies as percentages. Differences between subgroups will be analyzed with the unpaired t test or Mann–Whitney U test for continuous data, and the Fisher's exact test for categorical data. Statistical significance will be considered at a two-sided $P<0.05$. Multivariate logistic regression analysis will be used to determine the influence of immunogenicity in clinical outcome parameters. Results will be reported with their respective 95% confidence intervals or SD.

4. RESULTS

4.1. Screening of antibodies IgG1, IgG4 and IgE anti-Agalsidase alfa and beta

For our study, cross-sectional prospective were recruited between October and November 2018, 51 patients FD, 40 men (mean age = 55,9 years (between 20 e 77 years)) and 11 women (mean age = 54 years (between 39 e 73 years)) from of Hospital Senhora da Oliveira, Centro Hospitalar Vale do Ave (Guimarães, Portugal). These patients were prospectively screened for IgG1, IgG4 and IgE against agalsidase alfa therapy and against agalsidase beta therapy, with homemade immunoenzymatic assays (ELISA).

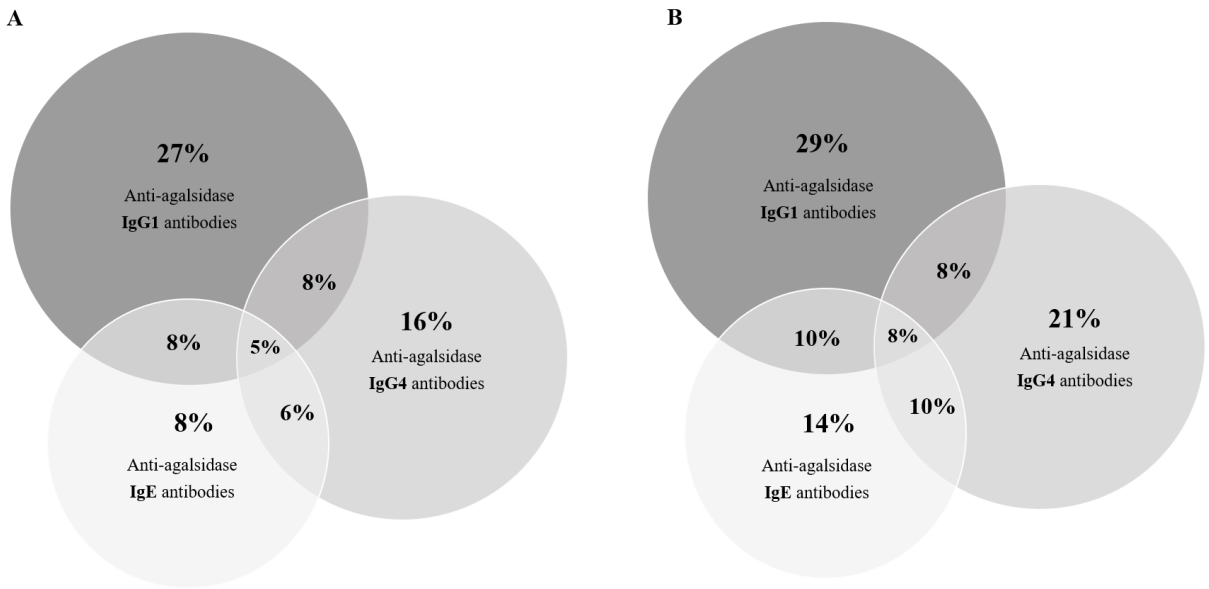

Figure 5. Neutralizing Antidrug Antibodie (ADA) incidence in FD patients.

In (A), proportion (%) of patients tested positive for IgG1, IgG4 and IgE against agalsidase alfa therapy, whereas in (B) the proportion of patients also tested positive for the same classes of antibodies but instead are reactive to agalsidase beta therapy. The percentage (%) values indicated within overlap areas correspond to samples which are double positive to IgG1 and IgG4, IgG1 and IgE and IgG4 and IgE as well as triple positive for IgG1, IgG4 and IgE.

Of the 51 patients, tested positive against agalsidase alfa therapy, 27% for IgG1 antibodies, 16% for IgG4 antibodies and 8% for IgE antibodies. Still 8% tested double positive for IgG1 and IgG4,

8% for IgG1 and IgE and 6% for IgG4 and IgE. In addition, 5% tested triple positives for IgG1, IgG4 and IgE. 30% of men (n = 12/40) had positive reactivity (IgG1, IgG4 or IgE) against agalsidase alfa therapy, versus 18% of women (n = 2/11).

Against agalsidase beta therapy they tested positive, 29% for IgG1 antibodies, 21% for IgG4 antibodies and 14% for IgE antibodies. Still 8% tested double positive for IgG1 and IgG4, 14% for IgG1 and IgE and 10% for IgG4 and IgE. Furthermore, 8% tested triple positives for IgG1, IgG4 and IgE. 45% of men (n = 18/40) had positive reactivity (IgG1, IgG4 or IgE) against agalsidase beta therapy, versus 46% of women (n = 5/11).

IgG1 antibodies were the most frequently observed against both therapies, agalsidase alfa and beta, followed by IgG4 antibodies and lastly the least frequent IgE antibodies, but also present in patients treated with agalsidase alfa therapy, contrary to what some studies demonstrate, and were also detected in women, contrary to what some studies also demonstrate.

Similar to other studies, a greater development of ADA was observed in patients treated with agalsidase beta therapy versus agalsidase alfa therapy. Also, there is greater development of ADA in men versus women.

4.2. Screening of antibodies IgG and IgE anti-Agalsidase alfa and beta pre- and pos-infusion ERT

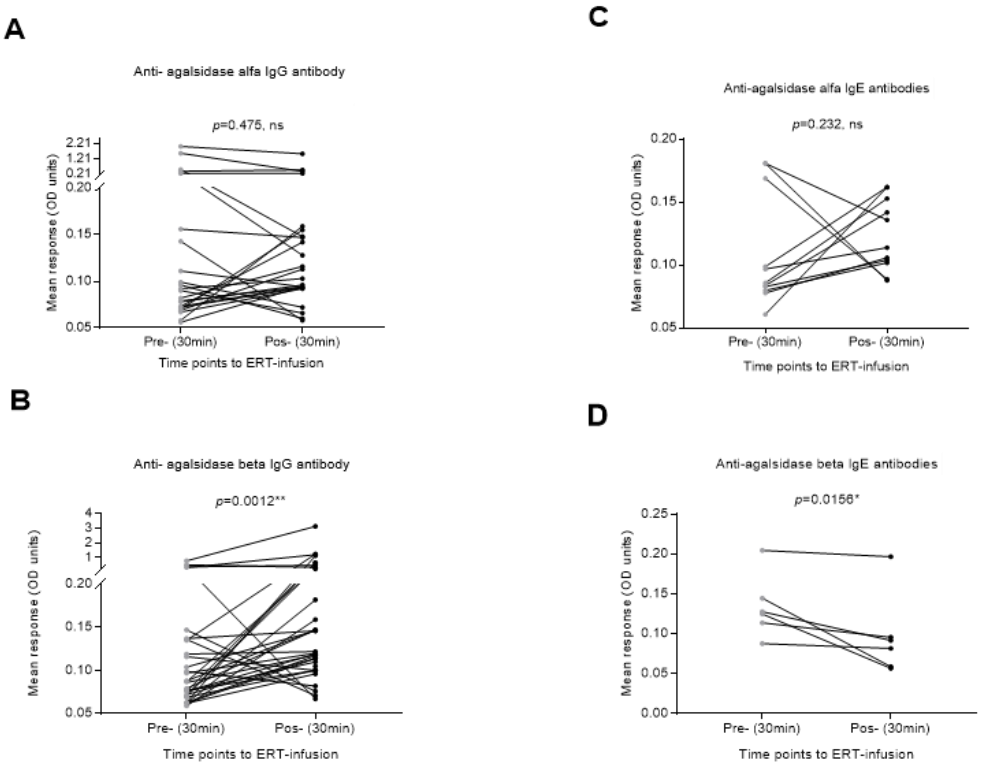

Figure 6. Enzyme Replacement Therapy (ERT) associated immunogenicity in Fabry Disease for both agalsidase® alfa and beta therapies.

ERT treated serum samples, were screened for anti-agalsidase isotype antibodies, alfa and beta, before the infusion and 30 minutes after the infusion, in order to verify ERT associated immunogenicity in FD for both agalsidase alfa and beta therapies. Differences between pre- vs pos-infusion sample data were tested by a Wilcoxon ranked test (non-parametric) with a p-value < 0.05.

Pre- and pos- infusion IgG antibody samples against agalsidase alfa preparation are depicted in figure 6A, there were no statistically significant differences in IgG production between pre- vs pos-infusion of agalsidase alfa.

Pre- and pos-infusion IgG antibody samples against agalsidase beta preparation are depicted in figure 6B, there were statistically significant differences in IgG production between pre- vs pos-infusion of agalsidase beta.

Pre- and pos-infusion IgE antibody samples against agalsidase alfa preparation are depicted in figure 6C, there were no statistically significant differences in IgE production between pre- vs pos-infusion of agalsidase alfa.

Pre- and pos-infusion IgE antibody samples against agalsidase beta preparation are depicted in figure 6D, there were statistically significant differences in IgE production between pre- vs pos-infusion of agalsidase beta.

4.3. Dissociation of immune complexes (IC) associated to ERT and screening of IgG released from IC

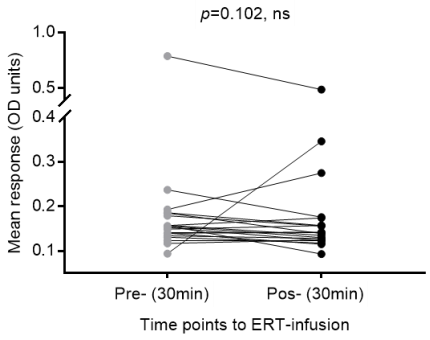

Figure 7. Detection of immune complexes against Enzyme Replacement Therapy (ERT) (agalsidase alfa) in Fabry Disease patients.

In figure 7, samples pre- versus pos-infusion, were analyzed relative to differences in IC levels in plasma by a Wilcoxon rank test ($p<0.05$).

We found that 55% of the samples (n = 28/51) in the pre-infusion of ERT had the presence of IC. While 43% of the samples (n = 22/51) in the post-infusion of ERT had the presence of IC.

Pre- infusion plasma samples are collected 15 days after the last ERT administration, and post-infusion plasma samples are collected 30 minutes after ERT administration. There were no statistically significant differences in IC levels in plasma between pre- vs pos-infusion of ERT, agalsidase alfa.

We also analyzed, differences in signals between treated and untreated samples with the same ratio concentrations in order to confirm if the acid treatment could dissociate the AAA from IC lattice (Table 6).

Therefore, in the untreated conditions we assisted a decreased in OD signal when compared with treated condition. This result meant that on treated condition the acid could dissociated the AAA from lattice whereas on untreated the IC remained stable. This is of paramount importance for immunogenicity measurement and characterization and shows the presence of stable immune-complexes between drug and antibodies with different affinity profiles circulating in blood´s patients.

5. DISCUSSION

Biopharmaceuticals have the potential to raise an immunogenic response in treated individuals, which may impact the efficacy and safety profile of these drugs. As a result, it is essential to evaluate immunogenicity throughout the different phases of the clinical development of a biopharmaceutical, including post-marketing surveillance.

The current management of FD involves the administration of intravenous ERT to supplement the deficiency of α-galactosidase A. There are two available pharmaceutical preparations of recombinant human α-Gal A: agalsidase alfa (Replagal by Shire) and agalsidase beta (Fabrazyme by Sanofi Genzyme). Both preparations have similar glycosylation patterns, specific activities, and enzyme kinetics, and both have been shown to be clinically efficacious.

Both agalsidase- alfa and agalsidase-beta have been demonstrated to benefit patients in the short term and, in observational studies, patients treated for up to 10 years showed stabilization of eGFR or a slowing of the progression of eGFR decline. ERT lead to Gb3 depletion in renal cells in children and adults in a dosage-and frequency-dependent manner. Furthermore, ERT has been reported to stabilize renal function in terms of eGFR, whereas ERT's effect on proteinuria and albuminuria is inconsistent. It also leads to a reduction of cerebrovascular and thromboembolic events and significant improvement in pain-related quality of life. Moreover, ERT is associated with a reduction of gastrointestinal symptoms such as abdominal pain and diarrhea and a reduction of lyso-Gb3 levels, a marker of disease load.

Since ERT is based on the intravenous infusion of a mostly foreign recombinant protein, a humoral response is common. In these patients, the infused enzyme can be recognized as foreign, triggering an immune response after the start of ERT, and it is important that we consider its role in the safety and efficacy of therapy.

The generation of an antibody response to infused therapeutic proteins may reduce both the safety and impair the effectiveness of treatment by modifying tissue distribution, metabolism, receptor binding, subcellular trafficking or catalytic activity of the infused protein. Such antibodies are known as neutralizing ADAs.

In about 40% of men treated with ERT, the formation of ADAs is observed, leading to a reduction in the effectiveness of the therapy. The formation of neutralizing ADAs appears to be irreversible, with the majority of affected patients remaining neutralizing ADA-positive for more than 10 years. About 73% of patients treated with agalsidase-beta and 24% of patients treated with agalsidase-alfa develop ADAs. This difference might be explained by the different dosages of both drugs and the

different cell lines producing them, as well as the CRIM status of the treated patients, which has not been taken in to account thus far.

Although the presence of neutralizing ADAs was first described very early after ERT became available, the effect of ADA son FD-specific biomarkers has been a topic of controversy.

It is assumed that, during infusions in patients without ADAs, ERT enters the cell and lysosomes via the M6P receptor and results in clearance of Gb3. If neutralizing ADAs are present, ERT is directly inactivated (neutralized) by ADAs in the plasma. In addition, binding of ADAs to ERTalso leads to an activation of macrophages that internalize ERT-ADA complexes, leading to a decrease in the cellular uptake of free ERT.

High ADA titers (measured by ELISA) have been associated with increased Gb3 deposition in endothelial cells, but with unclear effects on plasma Gb3 levels. A number of studies have also shown that demonstrated that plasma lyso-Gb3 levels were significantly higher over time in patients with neutralizing ADAs, and that urinary Gb3 decreased only in patients without ADAs. It has also been confirmed that ADAs are associated with a poorer clearance of plasma lyso-Gb3. Although some previous reports have suggested that ADAs appeared to have no effects on such parameters as eGFR slopes and cardiac mass, there is evidence that patients with ADAs exhibit harmful clinical outcomes, including increased LVM and progressive loss of renal function.

In our study, through the screening of IgG1 and IgG4 anti-agalsidase alfa and beta antibodies, we found that IgG1 antibodies were the most frequently observed against both therapies, agalsidase alfa and beta, compared to IgG4 antibodies.

Similar to other studies, a greater development of ADA was observed in patients treated with agalsidase beta therapy versus agalsidase alfa therapy. Also, there were greater amounts of ADA in men versus women. These data confirm the data already published in relation to the ADA of the IgG subtype.

When comparing differences in levels of IgG antibody against agalsidase alfa and beta, in the pre- and post- infusion, the differences are only statistically significant for agalsidase beta.

We aimed to correlate IgG results against both therapies with agalsidase, alfa and beta, with the safety data, and clinical, and functional cardiac and renal data of patients, to measure how IgG levels contribute to the loss of efficacy and safety of ERT, and how the levels of IgG are different depending on the demographic and clinical data of patients, such as phenotype. Also, how IgG levels contribute to the pathogenesis and evolution of FD, through changes in cardiac and renal functional and clinical data. Unfortunately, as we did not have access to clinical, demographic and security data, we were unable to make this analysis and correlation.

Antibody formation can also contribute to the development of IARs, as well as the formation of circulating immune complexes that activate the complement system, resulting in a general inflammatory response and cytokine release.

Therefore, it is important, when treating patients with FD, to distinguish a response caused by continuous exposure to ERT (leading to the formation of neutralizing ADAs) from IARs.

IARs frequently occur in ERT-naïve patients with FD after ERT initiation. Symptoms of IARs are mostly limited to fever and chills, necessitating premedication with antihistamines and steroids, as well as prolongation of infusion times (i.e., decreased infusion rates). However, severe life-threatening IARs can also occur.

To date, studies of antibody formation to agalsidase alfa and agalsidase beta show an apparent difference between the two licensed ERT preparations in terms of the proportion of patients who develop antibodies.

According to the manufacturer's instructions, 24% of patients treated with agalsidase alfa present with IARs versus, 67% of patients treated with agalsidase beta.

The nature of IARs is yet not completely understood but they are probably the result of anaphylactoid reactions (i.e., chemical compound–mediated) and not often anaphylactic (i.e., IgE-mediated type 1 hypersensitivity). In patients with IgEs, the following general mechanisms might apply: After antigen presentation, an early anaphylactic response includes the formation of IgE antibodies, triggering mast cell activation and histamine release, which could explain the observed severe IARs. With ongoing antigen presentation this process is commonly followed by IgG4 isotype antibody production against similar epitopes. Because these antibodies compete with IgEs but do not lead to complement activation, immunization will be achieved and IARs may be attenuated or stop over time.

To date, few studies have demonstrated and studied the presence of IgE in DF and correlated them with the appearance of IARs.

In our study, we verified the presence of IgE against both therapies with agalsidase, alfa and beta, with a greater proportion for therapy with agalsidase beta (27% vs 42%). This data contradicts the data presented in other studies that indicate that there is no presence of IgE against agalsidase alfa therapy. We also verified the presence of IgE in man and women, also contradicting studies that indicate that there is no presence of IgE in women.

When comparing differences in levels of IgE antibody against agalsidase alfa and beta, in the pre- and post- infusion, the differences are only statistically significant for agalsidase beta.

We aimed to correlate IgE levels against both therapies with agalsidase, alfa and beta, with the appearance of IARs, but unfortunately we did not have access to the clinical and safety data (IARs) of our patients, preventing us from doing this correlation.

In our study, we also verified the presence of IC before and after the infusion of ERT (agalsidase alfa), 55% versus 43%. The difference found is not statistically significant possibly because the 30 minutes after the infusion of ERT were not enough for the production of IC to occur. In the future, it is intended to develop a study to verify the production of IC at different times post-infusion of ERT.

We also analyzed differences in signals between treated and untreated samples with the same ratio concentrations, in order to confirm if the acid treatment could dissociate the AAA from IC lattice. The acid reagent enables dissociation of immuno-complexes and therefore can release antigens and antibodies which were not able to be detected before by standard ELISA methods.

Therefore, in the untreated conditions we assisted a decreased in OD signal when compared with treated condition. This result meant that on treated condition the acid could dissociated the AAA from lattice whereas on untreated the IC remained stable. This is of paramount importance for immunogenicity measurement and characterization and shows the presence of stable immune-complexes between drug and antibodies with different affinity profiles circulating in blood´s patients.

The main limitation of this study was we do not have access to clinical, demographic and safety data so that we can correlate the results obtained, and measure how the ADA contribute to the loss of efficacy and safety of ERT.

Also, antibodies are known to develop within the first six months of ERT [136] and then disappear in some tolerant patients[92,136], we studied only a single time point and thus could not comment on immunotolerance (either natural or induced by immunosuppressants).

It was not possible for us, due to a limited time in the study, to carry out the screening of antibodies IgG4 anti-agalsidase alfa and beta, pre and post- infusion for all samples involved in the study, and still identify the presence of IC before and after the infusion of agalsidase beta.

6. CONCLUSION

Antibody (IgG1, IgG4 and IgE) formation appears to be common during treatment with recombinant α-galactosidase A, as with other human proteins. Although both agalsidase alfa and agalsidase beta have been associated with IgG and IgE formation, the reported incidence of antibodies has generally been higher for agalsidase beta. Nevertheless, interpretation of the effects of antibody formation on the safety and efficacy of the two preparations wasn't possible for us, due to the absence of clinical, demographic and safety data. Hence the extreme importance of continuing this study, because immunogenicity can compromise the effectiveness and safety of ERT. And ERT is extremely important for Fabry patients, as it mitigates symptoms, improves the course and course of the disease, allowing for a better quality of life and longevity for patients.

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8. ANNEXES

	Anti-Agalsidase alfa IgG1 Screening (Pre-ERT infusion)						Anti-Agalsidase alfa IgG1 Screening (Pos-ERT infusion)					
	OD 450ηm (Triplicate)			Mean OD 450ηm	SD	Screening Cut-Point ≥1.071 ng/mL	OD 450ηm (Triplicate)			Mean OD 450ηm	SD	Screening Cut-Point ≥1.071 ng/mL
1FD	0,060	0,060	0,060	0,060	0,0000	0,703	0,057	0,054	0,059	0,057	0,0025	0,6659
2FD	0,062	0,056	0,053	0,057	0,0046	0,666	0,055	0,061	0,052	0,056	0,0046	0,6536
3FD	0,056	0,058	0,059	0,058	0,0015	0,678	0,154	0,168	0,155	0,159	0,0078	2,0481
4FD	0,268	0,294	0,275	0,279	0,0135	4,087	0,130	0,128	0,125	0,128	0,0025	1,5989
5FD	0,231	0,251	0,242	0,241	0,0100	3,384	0,257	0,250	0,246	0,251	0,0056	3,5631
6FD	0,121	0,108	0,104	0,111	0,0089	1,364	0,095	0,094	0,094	0,094	0,0006	1,1365
7FD	0,071	0,077	0,072	0,073	0,0032	0,865	0,080	0,081	0,120	0,094	0,0228	1,1365
8FD	0,138	0,150	0,142	0,143	0,0061	1,813	0,062	0,059	0,052	0,058	0,0051	0,6782
9FD	0,188	0,135	0,146	0,156	0,0280	2,003	0,182	0,127	0,132	0,147	0,0304	1,8710
10FD	0,498	0,502	0,512	0,504	0,0072	9,944	0,147	0,147	0,149	0,148	0,0012	1,8856
11FD	0,056	0,053	0,076	0,062	0,0125	0,728	0,053	0,052	0,059	0,055	0,0038	0,6414
12FD	0,054	0,055	0,058	0,056	0,0021	0,654	0,053	0,175	0,055	0,094	0,0699	1,1365
13FD	0,050	0,052	0,052	0,051	0,0012	0,593	0,130	0,050	0,053	0,078	0,0453	0,9290
14FD	0,052	0,057	0,053	0,054	0,0026	0,629	0,054	0,051	0,058	0,054	0,0035	0,6291
15FD	0,115	0,079	0,075	0,090	0,0220	1,084	0,066	0,065	0,068	0,066	0,0015	0,7774
16FD	0,070	0,069	0,070	0,070	0,0006	0,828	0,070	0,069	0,070	0,070	0,0006	0,8276
17FD	0,097	0,082	0,078	0,086	0,0100	1,032	0,065	0,064	0,069	0,066	0,0026	0,7774
18FD	0,085	0,078	0,077	0,080	0,0044	0,955	0,067	0,070	0,072	0,070	0,0025	0,8276
19FD	0,074	0,072	0,069	0,072	0,0025	0,853	0,075	0,095	0,108	0,093	0,0166	1,1234
20FD	1,547	1,537	1,615	1,566	0,0424		0,383	0,385	0,386	0,385	0,0015	6,4191
21FD	2,036	2,016	2,017	2,023	0,0113		1,450	1,560	1,606	1,539	0,0802	
22FD	1,055	1,028	1,021	1,035	0,0180	112,463						
23FD	0,070	0,070	0,066	0,069	0,0023	0,815	0,070	0,078	0,190	0,113	0,0671	1,3912
24FD	0,104	0,073	0,057	0,078	0,0239	0,929	0,117	0,115	0,056	0,096	0,0347	1,1629
25FD	0,103	0,088	0,096	0,096	0,0075	1,163	0,059	0,058	0,063	0,060	0,0026	0,7029
26FD	0,057	0,057	0,055	0,056	0,0012	0,654	0,044	0,048	0,048	0,047	0,0023	0,5441
27FD	0,048	0,056	0,051	0,052	0,0040	0,605	0,052	0,047	0,051	0,050	0,0026	0,5804
28FD	0,056	0,061	0,062	0,060	0,0032	0,703	0,061	0,059	0,056	0,059	0,0025	0,6905
29FD	0,052	0,053	0,053	0,053	0,0006	0,617	0,064	0,068	0,071	0,068	0,0035	0,8025
30FD	0,052	0,049	0,053	0,051	0,0021	0,593	0,066	0,076	0,088	0,077	0,0110	0,9163
31FD	0,091	0,090	0,095	0,092	0,0026	1,110	0,101	0,095	0,112	0,103	0,0086	1,2560
32FD	0,081	0,068	0,079	0,076	0,0070	0,904	0,078	0,080	0,098	0,085	0,0110	1,0190
33FD	0,075	0,083	0,088	0,082	0,0066	0,980	0,109	0,104	0,135	0,116	0,0166	1,4323
34FD	0,077	0,077	0,083	0,079	0,0035	0,942	0,082	0,086	0,113	0,094	0,0169	1,1365
35FD	0,072	0,070	0,077	0,073	0,0036	0,865	0,120	0,133	0,174	0,142	0,0282	1,7984
36FD	0,068	0,070	0,071	0,070	0,0015	0,828	0,141	0,142	0,181	0,155	0,0228	1,9886
37FD	0,070	0,078	0,068	0,072	0,0053	0,853	0,070	0,077	0,083	0,077	0,0065	0,9163
38FD	0,391	0,387	0,397	0,392	0,0050	6,596	0,433	0,492	0,507	0,477	0,0391	9,0357
39FD	0,068	0,062	0,060	0,063	0,0042	0,740	0,056	0,053	0,053	0,054	0,0017	0,6291
40FD	0,067	0,067	0,062	0,065	0,0029	0,765	0,064	0,059	0,058	0,060	0,0032	0,7029
41FD	0,072	0,074	0,068	0,071	0,0031	0,840	0,062	0,060	0,059	0,060	0,0015	0,7029
42FD	0,059	0,058	0,053	0,057	0,0032	0,666	0,054	0,052	0,050	0,052	0,0020	0,6047
43FD	0,055	0,060	0,056	0,057	0,0026	0,666	0,050	0,050	0,050	0,050	0,0000	0,5804
44FD	0,063	0,062	0,063	0,063	0,0006	0,740	0,060	0,060	0,061	0,060	0,0006	0,7029
45FD	0,057	0,056	0,057	0,057	0,0006	0,666	0,066	0,062	0,079	0,069	0,0089	0,8150
46FD	0,082	0,076	0,068	0,075	0,0070	0,891	0,069	0,070	0,072	0,070	0,0015	0,8276
47FD	0,145	0,078	0,073	0,099	0,0402	1,203	0,070	0,074	0,073	0,072	0,0021	0,8528
48FD	0,071	0,065	0,066	0,067	0,0032	0,790	0,073	0,087	0,063	0,074	0,0121	0,8781
49FD	0,080	0,072	0,073	0,075	0,0044	0,891	0,070	0,071	0,069	0,070	0,0010	0,8276
50FD	0,065	0,060	0,059	0,061	0,0032	0,715	0,075	0,061	0,064	0,067	0,0074	0,7899
51FD	0,067	0,063	0,071	0,067	0,0040	0,790	0,075	0,077	0,124	0,092	0,0277	1,1102

Table 4 - Results of screening Anti-Agalsidase alfa IgG1 pre- and pos ERT infusion.

	Anti-Agalsidas beta IgG1 Screening (Pre-ERT infusion)							Anti-Agalsidase beta IgG1 Screening (Pos-ERT infusion)						
	OD 450ηm (Triplicate)			Mean OD 450ηm	SD	Interpolating Three order polynomial Eq ηg/mL R2 =0.9977	Cut-point ≥ 8.19 ηg/mL	OD 450ηm (Triplicate)			Mean OD 450ηm	SD	Interpolating Three order polynomial Eq ηg/mL R2 =0.9977	Cut-point ≥ 8.19 ηg/mL
1FD	0,078	0,075	0,074	0,076	0,002	7,69	-	0,087	0,085	0,082	0,085	0,002		
2FD	0,066	0,068	0,061	0,065	0,003	6,32	-	0,079	0,082	0,082	0,081	0,001		
3FD	0,061	0,063	0,064	0,063	0,001	6,07	-	1,069	1,156	1,178	1,134	0,047		
4FD	0,121	0,116	0,120	0,119	0,002	13,08	+	0,120	0,123	0,123	0,122	0,001		
5FD	0,526	0,505	0,508	0,513	0,009	65,42	+	0,368	0,313	0,326	0,336	0,023		
6FD	0,137	0,138	0,137	0,137	0,000	15,35	+	0,150	0,141	0,146	0,146	0,004		
7FD	0,060	0,061	0,056	0,059	0,002	5,57	-	0,189	0,148	0,140	0,159	0,021		
8FD	0,323	0,335	0,358	0,339	0,015	41,62	+	0,067	0,070	0,063	0,067	0,003		
9FD	0,119	0,116	0,113	0,116	0,002	12,70	+	0,099	0,099	0,103	0,100	0,002		
10FD	0,077	0,076	0,074	0,076	0,001	7,69	-	0,636	0,670	0,642	0,649	0,015		
11FD	0,072	0,084	0,079	0,078	0,005	7,94	-	0,073	0,069	0,074	0,072	0,002		
12FD	0,064	0,063	0,062	0,063	0,001	6,07	-	0,115	0,119	0,118	0,117	0,002		
13FD	0,058	0,057	0,056	0,057	0,001	5,32	-	0,070	0,069	0,067	0,069	0,001		
14FD	0,066	0,068	0,065	0,066	0,001	6,44	-	0,069	0,069	0,071	0,070	0,001		
15FD	0,093	0,083	0,087	0,088	0,004	9,19	+	0,143	0,150	0,149	0,147	0,003		
16FD	0,086	0,086	0,089	0,087	0,001	9,06	+	0,061	0,055	0,056	0,057	0,003		
17FD	0,074	0,080	0,082	0,079	0,003	8,06	-	0,147	0,146	0,149	0,147	0,001		
18FD	0,071	0,070	0,073	0,071	0,001	7,06	-	0,087	0,085	0,087	0,086	0,001		
19FD	0,060	0,057	0,071	0,063	0,006	6,07	-	0,077	0,079	0,076	0,077	0,001		
20FD	0,144	0,129	0,132	0,135	0,006	15,10	+	1,281	1,264	1,224	1,256	0,024		
21FD	0,749	0,789	0,805	0,781	0,024	104,59	+	3,245	3,099	3,073	3,139	0,076		
22FD	0,071	0,076	0,073	0,073	0,002	7,31	-	NA	NA	NA				
23FD	0,062	0,076	0,073	0,070	0,006	6,94	-	0,679	0,639	0,598	0,639	0,033		
24FD	0,284	0,307	0,350	0,314	0,027	38,29	+	1,201	1,208	1,213	1,207	0,005		
25FD	0,094	0,084	0,083	0,087	0,005	9,06	+	0,229	0,243	0,241	0,238	0,006		
26FD	0,064	0,087	0,063	0,071	0,011	7,06	-	0,045	0,043	0,045	0,044	0,001		
27FD	0,090	0,057	0,057	0,068	0,016	6,69	-	0,059	0,060	0,060	0,060	0,000		
28FD	0,108	0,060	0,063	0,062	0,023	5,94	-	0,100	0,092	0,096	0,096	0,003		
29FD	0,075	0,082	0,108	0,088	0,014	9,19	+	0,080	0,076	0,074	0,077	0,002		
30FD	0,136	0,141	0,131	0,136	0,004	15,23	+	0,071	0,072	0,069	0,071	0,001		
31FD	0,107	0,093	0,112	0,104	0,008	11,19	+	0,152	0,144	0,142	0,146	0,004		
32FD	0,083	0,102	0,107	0,097	0,010	10,31	+	0,117	0,121	0,122	0,120	0,002		
33FD	0,087	0,089	0,085	0,087	0,002	9,06	+	0,078	0,077	0,080	0,078	0,001		
34FD	0,071	0,075	0,071	0,072	0,002	7,19	-	0,306	0,295	0,311	0,304	0,007		

35FD	0,073	0,071	0,073	0,072	0,001	7,19	-	0,121	0,112	0,110	0,114	0,005		
36FD	0,064	0,059	0,064	0,062	0,002	5,94	-	0,141	0,121	0,081	0,114	0,025		
37FD	0,069	0,070	0,067	0,069	0,001	6,82	-	0,107	0,108	0,085	0,100	0,011		
38FD	0,378	0,369	0,374	0,374	0,004	46,31	+	0,488	0,480	0,476	0,481	0,005		
39FD	0,079	0,071	0,061	0,070	0,007	6,94	-	0,089	0,088	0,087	0,088	0,001		
40FD	0,250	0,166	0,195	0,204	0,035	23,91	+	0,101	0,108	0,107	0,105	0,003		
41FD	0,068	0,060	0,057	0,062	0,005	5,94	-	0,173	0,183	0,191	0,182	0,007		
42FD	0,137	0,064	0,065	0,065	0,036	6,32	-	0,076	0,084	0,078	0,079	0,003		
43FD	0,086	0,063	0,057	0,069	0,012	6,82	-	0,068	0,068	0,069	0,068	0,000		
44FD	0,113	0,060	0,059	0,077	0,025	7,81	-	0,094	0,104	0,102	0,100	0,004		
45FD	0,113	0,097	0,088	0,099	0,010	10,57		0,078	0,090	0,078	0,082	0,006		
46FD	0,081	0,092	0,055	0,076	0,016	7,69	-	0,117	0,118	0,123	0,119	0,003		
47FD	0,103	0,087	0,068	0,086	0,014	8,94	+	0,116	0,129	0,109	0,118	0,008		
48FD	0,156	0,183	0,101	0,147	0,034	16,62	+	0,077	0,075	0,077	0,076	0,001		
49FD	0,075	0,079	0,074	0,076	0,002	7,69	-	0,109	0,112	0,110	0,110	0,001		
50FD	0,053	0,051	0,051	0,052	0,001	4,70	-	0,076	0,075	0,072	0,074	0,002		
51FD	0,054	0,055	0,058	0,056	0,002	5,20	-	0,086	0,075	0,085	0,082	0,005		

Table 5 - Results of screening Anti-Agalsidase beta IgG1 pre- and pos ERT infusion.

	Identification of false negatives (acid dissociation IC)									
	OD 450ηm (Triplicate)			Mean OD450 (Pre-ERT)	SD	OD 450ηm (Triplicate)			Mean OD450 (Pos-ERT)	SD
1FD	0,132	0,139	0,132	0,134	0,003	0,131	0,127	0,127	0,128	0,002
2FD	0,116	0,118		0,117	0,001	0,100	0,103	0,105	0,103	0,002
3FD	0,094	0,098	0,091	0,094	0,003	0,250	0,528	0,259	0,346	0,129
4FD	0,116	0,127	0,121	0,121	0,004	0,109	0,112	0,110	0,110	0,001
5FD	0,124	0,126	0,129	0,126	0,002	0,149	0,114	0,117	0,127	0,016
6FD	0,155	0,156	0,152	0,154	0,002	0,145	0,136	0,133	0,138	0,005
7FD	0,090	0,094	0,105	0,096	0,006	0,089	0,089	0,091	0,090	0,001
8FD	0,122	0,126	0,170	0,139	0,022	0,100	0,110	0,108	0,106	0,004
9FD	0,144	0,128	0,114	0,129	0,012	0,119	0,118	0,121	0,119	0,001
10FD	0,790	0,793	0,783	0,789	0,004	0,512	0,485	0,468	0,488	0,018
11FD	0,106	0,097	0,090	0,098	0,007	0,101	0,100	0,099	0,100	0,001
12FD	0,100	0,094	0,094	0,096	0,003	0,091	0,095	0,099	0,095	0,003
13FD	0,071	0,068	0,072	0,070	0,002	0,065	0,066	0,066	0,066	0,000
14FD	0,092	0,092	0,091	0,092	0,000	0,087	0,084	0,085	0,085	0,001
15FD	0,150	0,137	0,134	0,140	0,007	0,119	0,111	0,114	0,115	0,003
16FD	0,204	0,191	0,183	0,193	0,009	0,277	0,272	0,276	0,275	0,002
17FD	0,244	0,238	0,230	0,237	0,006	0,177	0,170	0,180	0,176	0,004
18FD	0,152	0,157	0,151	0,153	0,003	0,122	0,123	0,125	0,123	0,001
19FD	0,160	0,158	0,150	0,156	0,004	0,131	0,128	0,132	0,130	0,002
20FD	1,012	1,000	0,983	0,998	0,012	0,386	0,371	0,372	0,376	0,007
21FD	1,169	1,186	1,136	1,164	0,021	0,809	0,815	0,820	0,815	0,004
22FD	0,445	0,444	0,455	0,448	0,005					
23FD	0,097	0,092	0,097	0,095	0,002	0,079	0,085	0,076	0,080	0,004
24FD	0,098	0,100	0,099	0,099	0,001	0,095	0,093	0,108	0,099	0,007
25FD	0,099	0,097	0,098	0,098	0,001	0,098	0,094	0,103	0,098	0,004
26FD	0,085	0,085	0,085	0,085	0,000	0,044	0,048	0,043	0,045	0,002
27FD	0,066	0,066	0,070	0,067	0,002	0,069	0,067	0,070	0,069	0,001
28FD	0,144	0,142	0,137	0,141	0,003	0,089	0,088	0,089	0,089	0,000
29FD	0,080	0,080	0,080	0,080	0,000	0,082	0,085	0,090	0,086	0,003
30FD	0,070	0,071	0,077	0,073	0,003	0,082	0,095	0,094	0,090	0,006
31FD	0,154	0,150	0,144	0,149	0,004	0,154	0,155	0,161	0,157	0,003
32FD	0,123	0,117	0,110	0,117	0,005	0,124	0,131	0,120	0,125	0,005
33FD	0,145	0,134	0,144	0,141	0,005	0,136	0,142	0,147	0,142	0,004
34FD	0,108	0,101	0,110	0,106	0,004	0,100	0,105	0,102	0,102	0,002
35FD	0,145	0,138	0,136	0,140	0,004	0,127	0,145	0,151	0,141	0,010
36FD	0,122	0,118	0,117	0,119	0,002	0,136	0,149	0,157	0,147	0,009
37FD	0,153	0,157	0,163	0,158	0,004	0,128	0,139	0,138	0,135	0,005
38FD	0,133	0,134	0,139	0,135	0,003	0,186	0,204	0,209	0,200	0,010
39FD	0,126	0,120	0,118	0,121	0,003	0,101	0,103	0,103	0,102	0,001
40FD	0,119	0,114	0,115	0,116	0,002	0,094	0,096	0,094	0,095	0,001
41FD	0,124	0,117	0,111	0,117	0,005	0,099	0,099	0,099	0,099	0,000
42FD	0,159	0,164	0,149	0,157	0,006	0,097	0,092	0,091	0,093	0,003
43FD	0,102	0,106	0,113	0,107	0,005	0,085	0,085	0,087	0,086	0,001
44FD	0,119	0,118	0,129	0,122	0,005	0,103	0,103	0,104	0,103	0,000
45FD	0,108	0,117		0,113	0,005	0,091	0,091	0,098	0,093	0,003
46FD	0,197	0,192	0,165	0,185	0,014	0,142	0,139	0,137	0,139	0,002
47FD	0,186	0,200	0,171	0,186	0,012	0,161	0,154	0,156	0,157	0,003
48FD	0,137	0,135	0,120	0,131	0,008	0,112	0,119	0,124	0,118	0,005
49FD	0,192	0,179	0,166	0,179	0,011	0,155	0,157	0,155	0,156	0,001
50FD	0,124	0,126	0,119	0,123	0,003	0,119	0,127	0,124	0,123	0,003
51FD	0,161	0,158	0,152	0,157	0,004	0,167	0,168	0,187	0,174	0,009

Table 6 - Results of dissociation of immune complexes (IC) associated to ERT and screening of IgG released from IC.

	Anti-Agalsidase alfa IgE (Pre-infusion)				Anti-Agalsidase alfa IgE (Pos-infusion)				Anti-Agalsidase beta IgE (Pre-infusion)				Anti-Agalsidase beta IgE (Pos-infusion)			
	anti-agalsidase alfa Mean OD 450ηm	SD	Sigmoidal, 4PL, X is log(concentration) R^2 =0.9995	Cut-Point ≥ 1.5ηg/mL	anti-agalsidase alfa Mean OD 450ηm	SD	Sigmoidal, 4PL, X is log(concentration)	Cut-Point ≥ 1.5ηg/mL	anti-agalsidase alfa Mean OD 450ηm	SD	Sigmoidal, 4PL, X is log(concentration)	Cut-Point ≥ 1.5ηg/mL	anti-agalsidase alfa Mean OD 450ηm	SD	Sigmoidal, 4PL, X is log(concentration)	Cut-Point ≥ 1.5ηg/mL
1FD	0,087	0,013	1,58	-	0,074	0,000	1,33	-	0,044	0,000			0,045	0,003		
2FD	0,080	0,008	1,48	-	0,071	0,001	1,16	-	0,049	0,001			0,047	0,003		
3FD	0,066	0,003	1,29	-	0,060	0,000	0,82	-	0,050	0,004			0,058	0,005		
4FD	0,063	0,001	1,25	-	0,061	0,000	0,74	-	0,049	0,000			0,047	0,000		
5FD	0,083	0,000	1,52	-	0,069	0,001	1,23	-	0,050	0,004			0,053	0,006		
6FD	0,061	0,002	1,23	-	0,067	0,003	0,69	-	0,049	0,004			0,045	0,007		
7FD	0,062	0,007	1,24	-	0,054	0,001	0,72	-	0,042	0,003			0,046	0,004		
8FD	0,072	0,005	1,37	-	0,061	0,000	0,97	-	0,038	0,005			0,043	0,003		
9FD	0,063	0,000	1,25	-	0,081	0,001	0,74	-	0,045	0,002			0,046	0,002		
10FD	0,169	0,003	2,69	+	0,089	0,001	3,20	+	0,125	0,007			0,057	0,002		
11FD	0,076	0,001	1,43	-	0,061	0,003	1,06	-	0,053	0,001			0,054	0,001		
12FD	0,070	0,000	1,35	-	0,065	0,002	0,92	-	0,045	0,000			0,048	0,002		
13FD	0,062	0,001	1,24	-	0,064	0,000	0,72	-	0,047	0,001			0,050	0,001		
14FD	0,067	0,001	1,31	-	0,065	0,002	0,84	-	0,045	0,005			0,040	0,006		
15FD	0,071	0,003	1,36	-	0,067	0,001	0,94	-	0,040	0,004			0,044	0,002		
16FD	0,061	0,000	1,23	-	0,162	0,001	0,69	-	0,041	0,000			0,044	0,005		
17FD	0,072	0,000	1,37	-	0,075	0,001	0,97	-	0,056	0,001			0,052	0,003		
18FD	0,069	0,001	1,33	-	0,070	0,001	0,89	-	0,052	0,003			0,046	0,000		
19FD	0,067	0,002	1,31	-	0,073	0,002	0,84	-	0,046	0,001			0,044	0,001		
20FD	0,181	0,002	2,85	+	0,088	0,000	3,46	+	0,145	0,009			0,059	0,000		
21FD	0,181	0,001	2,85	+	0,136	0,000	3,46	+	0,128	0,010			0,092	0,001		
22FD	0,137	0,005	2,25	+	NA	0,001	2,50	+	0,080	0,002			NA	0,001		
23FD	0,062	0,000	1,24	-	0,069	0,001	0,72	-	0,048	0,001			0,048	0,000		
24FD	0,081	0,001	1,49	-	0,070	0,002	1,19	-	0,205	0,017			0,197	0,001		
25FD	0,081	0,006	1,49	-	0,087	0,004	1,19	-	0,047	0,020			0,047	0,019		
26FD	0,086	0,004	1,56	-	0,153	0,001	1,31	-	0,038	0,017			0,037	0,015		
27FD	0,068	0,002	1,32	-	0,081	0,003	0,87	-	0,037	0,016			0,037	0,015		
28FD	0,099	0,001	1,74	+	0,162	0,002	1,62	+	0,058	0,028			0,060	0,026		
29FD	0,076	0,001	1,43	-	0,095	0,002	1,06	-	0,062	0,034			0,056	0,028		
30FD	0,084	0,000	1,54	-	0,142	0,001	1,26	-	0,043	0,019			0,042	0,017		
31FD	0,080	0,003	1,48	-	0,102	0,002	1,16	-	0,039	0,018			0,039	0,016		
32FD	0,096	0,006	1,70	+	0,093	0,001	1,55	+	0,114	0,084			0,096	0,071		
33FD	0,067	0,003	1,31	-	0,076	0,001	0,84	-	0,051	0,023			0,054	0,025		
34FD	0,083	0,002	1,52	-	0,104	0,003	1,23	-	0,037	0,014			0,036	0,014		

35FD	0,089	0,004	1,60	-	0,083	0,001	1,38	-	0,037	0,014			0,037	0,014		
36FD	0,100	0,001	1,75	+	0,096	0,004	1,64	+	0,055	0,024			0,053	0,022		
37FD	0,097	0,001	1,71	+	0,114	0,003	1,57	+	0,053	0,025			0,050	0,023		
38FD	0,085	0,000	1,55	-	0,084	0,005	1,28	-	0,042	0,016			0,041	0,016		
39FD	0,078	0,003	1,45	-	0,106	0,001	1,11	-	0,038	0,015			0,037	0,014		
40FD	0,080	0,002	1,48	-	0,078	0,003	1,16	-	0,088	0,063			0,082	0,057		
41FD	0,078	0,002	1,45	-	0,079	0,003	1,11	-	0,054	0,003			0,052	0,003		
42FD	0,076	0,001	1,43	-	0,078	0,002	1,06	-	0,064	0,006			0,051	0,002		
43FD	0,075	0,000	1,41	-	0,087	0,003	1,04	-	0,053	0,001			0,051	0,001		
44FD	0,085	0,001	1,55	-	0,088	0,004	1,28	-	0,057	0,005			0,060	0,000		
45FD	0,080	0,003	1,48	-	0,079	0,002	1,16	-	0,053	0,001			0,054	0,004		
46FD	0,069	0,000	1,33	-	0,084	0,003	0,89	-	0,052	0,001			0,056	0,001		
47FD	0,066	0,002	1,29	-	0,074	0,001	0,82	-	0,052	0,000			0,059	0,003		
48FD	0,067	0,000	1,31	-	0,068	0,000	0,84	-	0,052	0,004			0,056	0,010		
49FD	0,077	0,001	1,44	-	0,079	0,002	1,09	-	0,056	0,003			0,049	0,000		
50FD	0,084	0,001	1,54	-	0,082	0,002	1,26	-	0,054	0,001			0,055	0,001		
51FD	0,092	0,001	1,64	-	0,084	0,003	1,45	-	0,064	0,002			0,062	0,002		

Table 7 - Results of screening Anti-Agalsidase alfa and beta IgE pre- and pos ERT infusion.