Olga Esmeralda Padrão Azevedo da Silva

Fabry Disease and the Heart

UMinho | 2021

Universidade do Minho
Escola de Medicina

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Tese de Doutoramento
Doutoramento em Medicina

Trabalho efetuado sob a orientação do
Professor Doutor Damião Cunha
e do
Professor Doutor Nuno Sousa

outubro de 2021

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AGRADECIMENTOS

Ao Professor Doutor Damião Cunha e ao Professor Doutor Nuno Sousa, por todo o apoio e sábias orientações.

Ao Hospital Senhora da Oliveira – Guimarães, por providenciar as condições para a realização deste projeto.

Aos colegas do Centro de Referência de Doenças Lisossomais de Sobrecarga do Hospital Senhora da Oliveira - Guimarães, pelo espírito de equipa, amizade e trabalho fundamental que realizaram nesta investigação.

Aos colegas do Serviço de Cardiologia do Hospital Senhora da Oliveira – Guimarães, por todo o apoio que sempre deram a este projeto.

Aos historiadores da região de Guimarães, pelo conhecimento fundamental que trouxeram a esta investigação.

Aos colegas de Cardiologia que a nível nacional colaboraram nos estudos desta tese, pelo trabalho, amizade e entusiasmo com que abraçaram este projeto, permitindo a sua concretização.

À minha família e amigos, por todo o apoio que me deram no decurso desta longa caminhada.

STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

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DOENÇA DE FABRY E O CORAÇÃO

A doença de Fabry (DF) é uma causa de miocardiopatia hipertrófica (MCH), mas a sua prevalência na MCH não está esclarecida. O nosso rastreio enzimático e genético de DF em 150 doentes adultos com MCH da região Portuguesa de Guimarães revelou 25 casos de DF (16.6%) e a mutação *GLA* c.337T>C (p.F113L) em 21 deles. O nosso estudo genealógico por historiadores revelou uma árvore familiar com 12 gerações e um ancestral comum aos doentes p.F113L, demonstrando um efeito fundador de DF com 400 anos. O rastreio familiar revelou disseminação desta mutação por muitos países no mundo.

A história natural dos fenótipos tardios de DF não é completamente conhecida. Nos nossos estudos, um protocolo diagnóstico foi realizado no momento basal em 120 doentes consecutivos para caraterização do fenótipo associado à mutação p.F113L e, subsequentemente, em 203 doentes (79 homens) (idade 46±18 anos) para caraterização da sua história natural. O envolvimento cardíaco teve o maior impacto prognóstico, incluindo hipertrofia ventricular esquerda (HVE) (40.8%), taquicardia ventricular não sustentada (TVNS) (12.5%), bloqueio auriculoventricular (AV) (18.3%) e bloqueio bifascicular (13.4%). Foi implantado pacemaker em 5.8%. Nos homens, a HVE e o realce tardio (RT) surgiram a partir dos 30 anos; a insuficiência cardíaca, a TVNS e as perturbações da condução cardíaca a partir dos 40 anos; e os bloqueios bifascicular e AV completo a partir dos 50 anos. Nas mulheres, as manifestações cardíacas ocorreram menos frequentemente e 1-2 décadas depois. A frequência e a gravidade das manifestações cardíacas aumentaram com a idade. As manifestações extracardíacas incluíram albuminuria aumentada (36.1%), lesões da substância branca cerebral (54.4%) e surdez neurossensorial (44.5%) e surgiram antes dos 30 anos em ambos os géneros na ausência de comorbilidades. Foram encontradas inclusões lisossómicas em podócitos de doentes jovens p.F113L com proteinuria, mas a doença renal crónica estadio ≥3 ocorreu em 7.6% (≈ população geral). Os eventos cerebrovasculares foram raros (3.3%).

O nosso rastreio multicêntrico de 780 doentes adultos com MCH revelou uma prevalência de DF, ajustada para o efeito fundador, de 0.9%. O bloqueio bifascicular (OR 10.909, CI95% 2.377-50.059) e o RT inferolateral basal (OR 10.677, CI95% 3.633-31.380) foram os preditores mais fortes de DF na MCH. Na sua ausência, segundo o score ID FABRY-HCM, a DF é uma causa menos provável de MCH. Novas variantes *GLA* c.286A>G (p.M96V) e c.607G>T (p.E203X) foram associadas à DF clássica.

Desde a nossa primeira descrição de não-compactação do ventrículo esquerdo (NCVE) num doente de Fabry, permanece por esclarecer se a NCVE é uma manifestação de DF. O nosso rastreio multicêntrico de DF em 78 doentes com NCVE não encontrou casos adicionais, argumentando contra esta hipótese.

PALAVRAS-CHAVE: doença de Fabry, F113L, fenótipo tardio, miocardiopatia hipertrófica, não compactação

FABRY DISEASE AND THE HEART

Fabry disease (FD) is a cause of hypertrophic cardiomyopathy (HCM), but its prevalence in HCM is not clearly established. Our enzymatic and genetic screening of FD in 150 adult patients with HCM from the Portuguese region of Guimarães found 25 Fabry cases (16.6%) and the *GLA* mutation c.337T>C (p.F113L) in 21 of them. Our genealogy study by historians revealed a 12-generation family tree with a common ancestor to p.F113L patients, demonstrating a founder effect of FD with 400 years. Family screening revealed that this mutation has been spread to many countries around the world.

The natural history of late-onset phenotypes of FD is not completely known. In our studies, a diagnostic protocol was performed at baseline in 120 consecutive patients for characterization of the phenotype associated to the p.F113L mutation and, subsequently, in 203 patients (79 males) (age 46±18 years) for characterization of its natural history. Cardiac involvement carried the highest prognostic impact, including left ventricular hypertrophy (LVH) (40.8%), non-sustained ventricular tachycardia (NSVT) (12.5%), atrioventricular (AV) block (18.3%) and bifascicular block (13.4%). Pacemaker implantation occurred in 5.8%. In males, LVH and late gadolinium enhancement (LGE) arose over 30 years; heart failure, NSVT and cardiac conduction disorders over 40 years; and bifascicular block and complete AV block beyond 50 years of age. In females, cardiac manifestations occurred less commonly and 1-2 decades later. The frequency and severity of cardiac manifestations increased with age. Extracardiac manifestations included increased albuminuria (36.1%), brain white matter lesions (54.4%) and sensorineural deafness (44.5%) and arose before 30 years of age in both genders in the absence of comorbidities. Lysosomal inclusions were found on podocytes of young p.F113L patients with proteinuria, but chronic kidney disease stage ≥3 occurred in only 7.6% (≈ general population). Cerebrovascular events were rare (3.3%).

Our multicentre FD screening in 780 adult patients with HCM revealed a FD prevalence, adjusted for the founder effect, of 0.9%. Bifascicular block (OR 10.909, CI95% 2.377-50.059) and basal inferolateral LGE (OR 10.677, CI95% 3.633-31.380) were the most powerful predictors of FD in HCM. In their absence, according to the score ID FABRY-HCM, FD is a less probable cause of HCM. Novel *GLA* variants c.286A>G (p.M96V) and c.607G>T (p.E203X) were shown to be associated with a FD classical phenotype.

Since our first report of left ventricular noncompaction (LVNC) in a FD patient, it remains to be clarified whether LVNC is a manifestation of FD. Our multicentre FD screening in 78 patients diagnosed with LVNC found no additional cases, arguing against this hypothesis.

KEYWORDS: F113L, Fabry disease, hypertrophic cardiomyopathy, late-onset phenotype, noncompaction

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LIST OF ABBREVIATIONS AND ACRONYMS

^{18}F-FDG – ^{18}F-fluorodeoxyglucose

BPI – Brief Pain Inventory

CKD – chronic kidney disease

CKD-EPI – Chronic Kidney Disease Epidemiology Collaboration

DNA – deoxyribonucleic acid

DS3 – Fabry disease severity scoring system

ECG - electrocardiogram

eGFR – estimated glomerular filtration rate

ELISA – enzyme-linked immunoassay

eow – every other week

ERAD – endoplasmic reticulum associated degradation

ERT – enzyme replacement therapy

ESC – European Society of Cardiology

FD – Fabry disease

GB3 – globotriaosylceramide

GCS – glucosylceramide synthetase

GFR – glomerular filtration rate

GLS – global longitudinal strain

GVUS – genetic variants of unknown significance

HCM – hypertrophic cardiomyopathy

ICD – implantable cardioverter-defibrillator

IQR – interquartile range

IVS - interventricular septum

LA – left atrium

LGE – late gadolinium enhancement

LV – left ventricular

LVH – left ventricular hypertrophy

LVNC – left ventricular noncompaction

LVOT – left ventricular outflow tract

Lyso-GB3 – globotriaosylsphingosine

M6P – mannose 6-phosphate

MESA – Multi-Ethnic Study of Atherosclerosis

MIBG – meta-iodobenzylguanidine

MMP-9 – matrix metalloproteinase 9

MRI – magnetic resonance imaging

mRNA – messenger ribonucleic acid

MSSI – Mainz severity score index

NT-proBNP – N-terminal prohormone of brain natriuretic peptide

NYHA – New York Heart Association

PCR – polymerase chain reaction

PEG – polyethylene glycol

PET – positron emission tomography

PET/CT - Positron Emission Tomography-Computed Tomography

PICP – carboxyterminal propeptide of procollagen type I

PIIINP – procollagen III N-terminal propeptide

Pro-BNP – prohormone of brain natriuretic peptide

PW – posterior wall

RV – right ventricular

SCD – sudden cardiac death

SF-36 – Short Form 36 Health Survey Questionnaire

TDI – tissue Döppler imaging

USA – United States of America

VF – ventricular fibrillation

VT – ventricular tachycardia

WML – white matter lesions

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Table 2: Summary of previous studies on the natural history of the late-onset phenotype of FD with predominant cardiac involvement caused by the *GLA* mutation IVS4+919G>A

Table 3: Summary of studies on Fabry disease prevalence in patients with HCM or LVH, published after our study

CHAPTER 1 – INTRODUCTION

1.1 Fabry disease overview

Fabry disease (FD) (OMIM 301500), also known as Anderson-Fabry disease [1,2], is a lysosomal storage disorder caused by mutations in the *GLA* gene, leading to deficiency of the enzymatic activity of α-galactosidase A. Globotriaosylceramide (GB3, also known as ceramide trihexoside) and other neutral glycosphingolipids consequently accumulate in body fluids and lysosomes of cells throughout the body, including in those that are particularly relevant for the disease, such as in the heart (cardiomyocytes, conduction system cells, vascular endothelial and smooth muscle cells and fibroblasts), kidney (podocytes, tubular, glomerular, mesangial and interstitial cells), nervous system (neurons in autonomic and posterior root ganglia) and vascular endothelium and smooth muscle [3-7]. Substrate accumulation starts *in utero* [8-12], but the mechanisms by which it leads to cellular dysfunction and subsequent multiorgan damage remain less defined [13].

There are more than 1000 reported mutations of the *GLA* gene, including missense or nonsense mutations, frameshift mutations, deletions, and insertions [14].

GLA mutations causing a virtually null enzymatic activity (<5% of the normal mean) are associated with severe and early onset classical phenotypes, which are characterized by the development of clinical manifestations in childhood or adolescence, such as acroparesthesias, neuropathic pain, hypohydrosis, heat, cold and exercise intolerance, cornea *verticillata*, angiokeratomas, gastrointestinal symptoms and proteinuria. In adulthood, patients may also suffer from cardiomyopathy, heart failure, dysrhythmias, cardiac conduction blocks, renal failure, brain white matter lesions (WML), cerebrovascular events and sensorineural deafness. In contrast, *GLA* mutations leading to a residual enzymatic activity are associated to attenuated and late-onset phenotypes, which are characterized by the development of cardiac, renal and/or cerebrovascular manifestations in adulthood and may be dominated by the involvement of an organ, such as the heart or the kidneys [6,15,16]. Ultimately, FD leads to a reduction of quality of life and survival [17-19].

In this X-linked disorder, heterozygote females are not merely carriers and their clinical spectrum widely ranges from asymptomatic to full-blown disease as severe as in affected males [20,21], partly due to X-chromosome inactivation [22]. Still, clinical manifestations are usually milder, arise later and progress more slowly and survival is longer in females [19,23-25].

FD is a rare condition, but its prevalence has been largely underestimated in the past as nearly 1: 117000 individuals [26]. Later, newborn screening studies came to reveal much higher prevalences of 1: 3100 hemizygote males in Italy, mainly due to the high frequency of late-onset variants [27]; and 1: 3859 newborns in Austria [28]. More recently, pilot newborn screening programs in the United States of America (USA) have reported FD prevalences ranging from 1: 2913 in Missouri to 1: 5495 in Washington, 1: 8454 in Illinois and 1: 9372 in New York [29-32]. In Japan, FD prevalence on newborn screening was 1: 11854 [33]. In Taiwan, newborn screening has revealed an even higher FD prevalence of 1: 1250 males due to a founder effect [34].

Despite increasing awareness, FD remains a late diagnosis (median diagnosis delay in adults 10.5 years, 95% confidence interval 8.0-13.0) [35]. During the diagnostic odyssey, misdiagnoses are also common, such as fibromyalgia, rheumatic fever and multiple sclerosis [23,36-38].

FD diagnosis in males is based on the biochemical determination of the enzymatic activity of α-galactosidase A [39,40], but confirmation of the disease-causing *GLA* mutation is important to understand the disease phenotype, rule out benign polymorphisms associated to reduced levels of enzymatic activity and screen at-risk family members [41]. In females, the enzymatic activity of α-galactosidase A may be normal [39] as well as the disease biomarkers, such as plasma and urinary GB3 [42,43] and plasma globotriaosylsphingosine (lyso-GB3) [44], so FD diagnosis requires the molecular analysis of the *GLA* gene [45].

Enzyme replacement therapy (ERT) with recombinant α-galactosidase A has been approved for clinical use since 2001. There are two commercially available preparations, agalsidase alfa, produced from human fibroblasts, and agalsidase beta, produced in Chinese hamster ovary cells, both administered intravenously every other week (eow) at the doses of 0.2 mg/kg and 1 mg/kg of body weight, respectively [46]. Despite the large body of evidence supporting its efficacy and safety, ERT presents several limitations, such as a limited tissue penetration, the inability to cross the blood-brain barrier, the induction of infusion adverse reactions and antidrug antibodies and the inconvenience of lifelong biweekly intravenous administrations. Moreover, some issues remain unclear, such as the optimal timing to start therapy, the optimal dose, and the clinical impact of antidrug antibodies [46].

Migalastat has been approved by the European Medicines Agency for the treatment of FD patients aged ≥16 years, with estimated glomerular filtration rate (eGFR) ≥30 mL/min/1.73 m^2 and amenable *GLA* mutations [46,47]. Migalastat is a first-in-class pharmacological chaperone therapy for FD [46,47]. It is a low molecular weight iminosugar analogue of the terminal galactose residue of GB3 that selectively and

reversibly binds to the active site of amenable mutant forms of α-galactosidase A, thereby stabilizing the enzyme, preventing its retention and degradation in the endoplasmic reticulum, and facilitating its trafficking to lysosomes. Once in lysosomes, migalastat dissociates from α-galactosidase A, due to the more acidic pH and higher concentration of substrates, allowing the enzyme to exert its activity on GB3 [48-50]. It is an oral non-immunogenic small molecule, with likely enhanced cellular and tissue distribution, thereby overcoming some of the limitations of ERT, but it only constitutes a therapeutic option to patients with amenable *GLA* mutations, i.e., 35-50% of Fabry patients [46].

However, further evidence is needed on the efficacy of FD therapies in hard-endpoints, such as mortality or major clinical events, and specifically in late-onset phenotypes.

Despite the emergence of FD therapy, life expectancy is lower than in the general population of USA, both in males (58.2 vs. 74.7 years) and females (75.4 vs. 80.0 years). The main cause of death is cardiovascular (40% of deaths in males and 41.7% in females), followed by cerebrovascular (9.3% in males and 8.3% in females) and renal (8.0% in males and 8.3% in females) [19].

1.2 Fabry disease and the Heart

In the heart, GB3 deposits are found in virtually all cells [51-54], representing 1-2% of the cardiac mass. Nevertheless, in cardiac variants, lysosomal inclusions are only found in cardiomyocytes [55]. In women, a mosaic pattern of normal and vacuolated cells caused by random X-chromosome inactivation is observed [56].

GB3 accumulation in lysosomes leads to autophagy impairment, preventing degradation of dysfunctional cell components [57,58]. GB3 accumulation may affect mitochondrial function, either directly through accumulation within the mitochondrial membrane or indirectly by preventing mitophagy [59], likely contributing to the reduction of the activities of the respiratory chain enzymes, as shown in fibroblasts [60]. Substrate accumulation and organelle damage have also been shown to induce oxidative stress [59,61]. In the heart, increased myocardial nitric oxide production may cause oxidative damage of cardiomyocyte myofilaments and deoxyribonucleic acid (DNA), causing cell dysfunction and death [62]. GB3 has also been demonstrated to promote a higher proinflammatory cytokine production and expression [63], to mediate apoptosis [63-65] and to induce endothelial dysfunction [66]. Lyso-GB3, a deacylation product of GB3 [67], has also been shown to inhibit α-galactosidase A activity and to promote the proliferation of smooth muscle cells [68], likely contributing to the increased intima-media thickness. Sphingosine-1 phosphate, another growth-promoting factor present in the plasma of FD patients, has been

shown to induce hypertrophy of cardiomyocytes *in vitro* and to correlate with common carotid artery intima-media thickness and left-ventricular mass index *in vivo* [69].

Anatomopathological analyses of hearts of FD patients have shown hypertrophy, apoptosis and necrosis of the cardiomyocytes, inflammatory infiltrate, replacement and interstitial fibrosis, valve thickening and vascular intima and media thickening with vascular narrowing [51,54]. Progressive cardiomyocyte hypertrophy ultimately ends in cell death of enlarged substrate-engorged cardiomyocytes, either by apoptosis or necrosis, which presumably leads to fibrosis. Accordingly, the cardiomyocyte diameter, the lysosomal glycosphingolipid area and the extent of apoptosis, necrosis and fibrosis are all positively correlated with disease severity and age [56].

Endomyocardial biopsies have shown myocarditis in 56% of FD patients. Myocarditis has been detected even before the occurrence of left ventricular hypertrophy (LVH) or late gadolinium enhancement (LGE). Its frequency has been shown to correlate with maximal LV wall thickness. It is immune-mediated, as positive antiheart and antimyosin antibodies were found in all FD patients with myocarditis [70].

Cardiac manifestations of FD include ventricular hypertrophy and fibrosis, valve thickening or regurgitation, heart failure, angina, dysrhythmias, cardiac conduction abnormalities and sudden death [7,71].

Hypertrophic cardiomyopathy (HCM) is the main cardiac manifestation of FD [7,71], occurring in 43% of males and 26% of females and arising earlier and progressing more rapidly in males than in females (mean age of onset: 39±10 vs. 50±11 years) [16,25]. Left ventricular (LV) mass index was found to increase with age [24,72] and to correlate inversely with eGFR [24].

Intramyocardial LGE on the basal inferolateral LV segment is typically seen on cardiac magnetic resonance imaging (MRI) in approximately 50% of Fabry patients [73], which correlates histologically with focal replacement fibrosis [74]. The reason for the preferential involvement of the basal inferolateral segment is unclear, but it was hypothesized to be due to increased stress at the interface of the fibrous skeleton of the mitral annulus and the LV midwall [75]. LGE may develop before LVH and a study has reported that 50% of females would not have been identified with FD cardiomyopathy without the aid of LGE imaging [76]. Strain imaging by tissue Döppler imaging (TDI) [77] or speckle tracking [78] can identify LGE with high sensitivity and specificity.

In FD patients with basal inferolateral LGE, troponin levels and T2 values are increased, suggesting that LGE also represents inflammation [79]. LGE in the basal inferolateral wall was associated to high global and basal inferolateral wall T2. High basal inferolateral wall T2 was in turn associated with increased troponin and N-terminal prohormone of brain natriuretic peptide (NT-proBNP), global longitudinal strain

(GLS) impairment and electrocardiographic abnormalities (long PR, complete bundle branch block, LVH voltage criteria, long QTc, and T-wave inversion) and predicted clinical worsening after 1 year (Fabry stabilization index >20%) [80]. LGE regions also showed focal ^{18}F-fluorodeoxyglucose (^{18}F-FDG) uptake, further supporting the notion that LGE represents inflammation [81]. Focal ^{18}F-FDG uptake was shown in 50% of the patients before LVH, being associated with lower LV GLS [82]. Increased inflammatory markers, such as interleukin-6 and tumor necrosis factor, were also associated to increased disease burden (LVH and fibrosis) [83].

FD has also been associated with low native T1 time on cardiac MRI, which has been attributed to intracellular glycosphingolipid accumulation [84]. In FD patients without LVH, low T1 was found in 40% of the cases [84,85], being associated with electrocardiographic abnormalities and worse LV wall thickness and mass [85,86], left atrium (LA) size [86,87], E/E' ratio, LV GLS and inferolateral longitudinal strain [87], LV GLS by feature tracking [88], LGE [85] and worse Mainz severity score index (MSSI) and clinical worsening (Fabry Stabilization Index >20%) [86]. In FD with LVH, T1 correlated inversely with LV mass [84]. Pseudonormalization or elevation of T1 in LV inferolateral wall was correlated with the presence of LGE [84]. In FD patients with right ventricular (RV) hypertrophy, low T1 was also found in the right ventricle [89].

Nordin et al. proposed three stages of cardiac involvement in FD: (1) storage stage with normal or low native T1 times without LVH; (2) inflammation and myocyte hypertrophy stage with low native T1 times, inflammation, LGE, chronic troponin elevation, with LVH in males and without LVH in females; and (3) fibrosis and impairment stage with pseudonormalization of native T1 times, extensive LGE, LVH, troponin and NT-proBNP elevation, LV dysfunction and heart failure [90].

In advanced stages of FD, there is thinning and akinesia of the basal posterior wall (PW), which may result in asymmetrical LVH [7,71] and correlates histologically with fibrosis [55]. This finding has been reported to significantly precede severe heart failure [New York Heart Association (NYHA) class III] and cardiac death [91].

Diastolic function is abnormal in 63% of those with LGE [92]. Diastolic dysfunction occurs more commonly as an abnormal relaxation or a pseudonormal pattern [7,71] and it has been associated with the presence of LGE [93] and to correlate with NT-proBNP [94]. LV systolic dysfunction with reduction of ejection fraction is rare (6.7%) [95], occurring in late stages of advanced Fabry cardiomyopathy [7,55,71]. LV systolic and diastolic dysfunction can, however, be detected before the development of LVH. LV longitudinal [96], circumferential and radial strain [97] were found to be reduced in the pre-hypertrophic stage; and the

strain rate during isovolumic relaxation and the ratio of transmitral E-wave velocity to the strain rate during isovolumic relaxation have shown to differentiate FD patients from controls regardless of LVH [98].

Obstruction at the LV outflow tract (LVOT) may occur, but massive LVH involving the papillary muscles has also been reported to cause mid-ventricular obstruction [99]. Obstruction at rest is rare, but it may be elicited by exercise in 43% of the patients [100], also contributing to heart failure.

RV hypertrophy occurs in 31-71% of the patients [101,102]. RV systolic function in patients with Fabry cardiomyopathy tends to be preserved [102]. Nevertheless, RV global and free wall systolic strain may be reduced and RV systolic dysfunction has been associated to RV wall thickness and fibrosis [103].

Glycolipid deposition in the atria [52] may ultimately cause atrial dilation, which occurs more commonly in patients with LVH [104] and fibrosis [78]. LA systolic and early diastolic strain rate were found preferentially reduced in patients with LVH, but LA systolic strain was decreased even before LVH [105]. Peak atrial longitudinal strain was inversely associated to Fazekas score of brain WML, even after adjusting for LV mass index [106]. Atrial dilation is associated to the occurrence of atrial fibrillation [107], which also contributes to heart failure.

Ventricular hypertrophy and fibrosis result in diastolic and systolic dysfunction, which together with dysrhythmias and conduction disorders, valve disease and myocardial ischemia, contribute to heart failure [7,71]. Dyspnoea or heart failure has been reported in 19.7% of untreated females and 19.4% of untreated males [24] and its prevalence increases with age [108]. Severe heart failure (NYHA class ≥III) was found in 10% of the Fabry patients [109]. The annual incidence of severe heart failure was 1.62 per 100 person-years and age and MSSI were independent predictors of its development [109]. NT-proBNP levels were correlated to symptom class, LV mass, E/E' ratio and LA size, reaching higher values in patients with LVH, diastolic dysfunction and LGE [85,110].

FD involvement of the autonomous nervous system may result in a significantly reduced heart rate variability in paediatric male patients, reflecting a reduction in parasympathetic stimulation of the heart [111].

GB3 accumulation in the cardiac conduction system cells [53] leads in an early stage to accelerated atrioventricular conduction, which manifests as a short PR interval [112,113], a characteristic but uncommon finding in FD (14%) [114]. As FD progresses, GB3 accumulation and fibrosis lead to the development of atrioventricular and bundle-branch blocks [55,115,116] and sinus node dysfunction [117], which may require a pacemaker. Bradycardia at rest is common (72%) [118]. Chronotropic incompetence with exercise due to autonomic nervous dysfunction may also occur and contribute to heart

failure [118,119]. PR interval duration >200 ms was reported in 3% and QRS interval duration >120 ms in 9%. The duration of PR and QRS intervals were shown to increase with age and were identified as independent predictors of need of a pacemaker [117]. A positive correlation was found between LV mass on cardiac MRI and QRS duration [120].

GB3 deposition in the atria [121] and subsequent fibrosis, together with LVH and diastolic dysfunction and atrial dilation, are the proposed mechanisms of development of atrial fibrillation [107]. Persistent atrial fibrillation was reported in 3.9% and paroxysmal atrial fibrillation in 13.3% [107]. The annual incidence of atrial fibrillation was 1 per 100 person-years and age and LV mass were independent predictors of its development [109].

The major mechanism of sustained ventricular tachycardia (VT) in FD appears to be re-entry related to myocardial fibrosis [122]. Indeed, the annual increase in fibrosis was identified as an independent predictor of malignant ventricular arrhythmias [123]. Moreover, sympathetic nerve damage, demonstrated by meta-iodobenzylguanidine (MIBG) defects in the inferolateral wall, occurs in FD patients without LGE and, in patients with LGE, MIBG defects are larger than the LGE areas, suggesting that sympathetic nerve damage precedes myocardial fibrosis [124].

Non-sustained VT was reported in 21%, while sustained VT in only 1% and bradycardia requiring device in 6% of Fabry patients during a median follow up 3.6 years, suggesting a higher impact of bradyarrhythmic clinical events [125]. A systematic review of the literature estimated the occurrence of VT in 15.3% of the patients and reported that 75% of the deaths were due to cardiac disease and 62% due to sudden cardiac death (SCD), ranging the incidence of SCD events from 0.34 to 1.4% per annum. Age, male gender, LVH, LGE and non-sustained VT were identified as risk factors associated with SCD events [126].

The annual incidence of cardiac device implantation for the treatment of bradycardia was estimated as 1.07 per 100 person-years and age and QRS duration were independent predictors of device implantation [109]. Patients with devices were older and had greater LV mass, more scar tissue and larger atrial size [127].

GB3 accumulation results in hypertrophy and proliferation of smooth muscle and endothelial cells and widespread narrowing of intramural coronary arteries [54,128]. FD patients with angina present myocardial ischemia on exercise stress test and myocardial perfusion defects on single-photon emission computed tomography [128]. On positron emission tomography (PET), myocardial blood flow is reduced in patients without LVH, both males and females, although it is more reduced in patients with LVH [129]. On cardiac MRI including vasodilator stress perfusion mapping, the reduction of stress myocardial blood flow is more pronounced in the subendocardium and in patients with LVH [130]. LGE and low T1 were

identified as independent predictors of stress global myocardial blood flow [130]. On angiography, epicardial coronary arteries have been described as structurally normal, but presenting slow flow; and small vessel disease correlated with coronary slow flow and extent of fibrosis [128].

Besides coronary microvascular dysfunction, coronary vasospasm may also contribute to angina [131,132] and may precipitate dysrhythmias and sudden death [133]. Moreover, FD patients commonly present cardiovascular risk factors, although it is not clear if FD may increase the risk of accelerated atherosclerosis [134]. Oxygen supply to myocardium may also be impaired by LVOT obstruction and increased LV diastolic filling pressure, which reduce blood perfusion of the subendocardial layer of myocardium. Taken together with an increased demand of oxygen by the hypertrophied myocardium, the result is myocardial ischemia, which may be asymptomatic or manifest as angina and myocardial infarct or contribute to dysrhythmias and heart failure [135,136].

Angina has been reported in 22% of males and 23% of females with FD [135]. Mean age of onset was reported to be 42±5 and 49±13 years in males and females, respectively [16]. It may be the first manifestation of FD cardiomyopathy, preceding the development of LVH [137]. However, myocardial infarct is rare, occurring in 2.7% of males and 1.5% of females [138]. FD remains a rare cause of chest pain in patients without obstructive coronary artery disease (0.15%) [139].

GB3 accumulation in valve fibroblasts may ultimately lead to valve thickening, fibrosis [51,52] and regurgitation [53,72], which are usually mild/moderate and rarely require intervention [71,72]. Aortic valve was reported to be affected in 47% of the cases and mitral valve in 57% [72]. Mitral valve prolapse has also been described [140], although now known to be less prevalent than originally reported [72]. Aortic root dilation may also occur in late stages of the disease, thereby contributing to aortic regurgitation [72].

Degenerative changes in the aortic media from glycolipid deposition [52] can lead to aortic dilatation at the sinuses of Valsalva and ascending aorta in 32.7% and 29.6% of males and 5.6% and 21.1% of females, respectively [141]. An aortic aneurysm, defined as an aortic diameter exceeding 1.5 times the upper limit of normal at the sinuses of Valsalva, was found in 9.6% of male and 1.9% of female patients [141].

Agalsidase alfa has been demonstrated to decrease/stabilize LV mass and wall thickness, both in males [142-144] and females [144-146], and to improve exercise capacity in females [146]. However, myocardial GB3 content did not significantly decrease on endomyocardial biopsies taken at 6 months of treatment with agalsidase alfa [142].

Agalsidase beta has been shown to reduce/stabilize LV mass and wall thickness, both in males [147-150] and females [148,150]. Complete clearance of GB3 deposits has been reported in interstitial capillary endothelial cells from 72% of the patients treated with agalsidase beta for 5 months; and this benefit seemed to be sustained up to 60 months. However, no clearance of GB3 was observed in the cardiomyocytes [151]. Conversely, in another study, there was no improvement of GB3 deposits in endothelial and smooth muscle cells with agalsidase beta for ≥12 months [128].

Migalastat has consistently shown to decrease LV mass [152-158]. However, no data has been reported on its effect in cardiac GB3 deposits.

The incidence of adverse cardiac events (composite endpoint of VT, bradycardia requiring device implantation, severe heart failure, and cardiac death) was 7.6% per year; LVH and LGE were predictors of adverse cardiac events and patients with extensive LGE (≥15% of LV mass) were at highest risk [125]. Age, eGFR, high-sensitivity troponin I, NT-proBNP, LV mass index, E/E' ratio and GLS have shown incremental value in the prediction of adverse cardiac events (defined as composite of cardiac death, malignant VT, atrial fibrillation and severe heart failure) [159].

Cardiac events (defined as myocardial infarction, arrhythmia, angina pectoris, congestive heart failure or significant cardiac procedures, such as pacemaker placement, coronary bypass, stent placement, valve replacement) were reported in 69.9% of males and 81.6% of females. Age at first cardiac event was 41.7 (5.3–80.4) in males and 49.8 (17.3–78.2) in females [19].

Ultimately, heart disease is the main cause of death of Fabry patients (40% in males and 41.7% in females) [19]. The annual incidence of cardiac death was 0.52 per 100 person years and the only independent predictor was LV mass [109]. Cardiac death was reported to occur at a median age of 55.5 years in males and 66.0 years in females [19].

1.3 Fabry disease and the Heart: Main issues under research in this thesis

1.3.1 Fabry disease in the Portuguese region of Guimarães

Preliminary observations showed that FD prevalence in HCM in the Portuguese region of Guimarães seemed to be higher than the prevalence reported in the literature [160-176] and that the *GLA* mutation p.F113L was responsible for most of the cases [177]. Considering that FD is a rare condition [27,29-33], characterized by a high genetic heterogeneity [14] and that *GLA* mutations tend to be private to each family [178,179], the finding of several apparently unrelated patients with FD due to the same *GLA*

mutation in a particular geographic region raised the suspicion of a founder effect of the disease in this Portuguese region [177].

FD is a chronic, progressive and multisystem disease that carries significant morbidity, decreased quality of life and premature mortality [6,16-19]. Moreover, its management requires not only the symptomatic treatment of disease complications by a multidisciplinary team of health professionals, but also the specific treatment with expensive orphan drugs [46], both translating into high healthcare related-costs.

Recognizing the health problem that FD due to this *GLA* mutation could constitute in the local community of the Portuguese region of Guimarães and its potential impact beyond this region, this doctoral thesis aimed to determine the prevalence of FD in patients with HCM in the region of Guimarães, to investigate the existence of a founder effect of the disease in this region, to evaluate its global diffusion worldwide and to characterize the phenotype associated to this mutation.

1.3.2 Natural history of late-onset phenotypes of Fabry disease with predominant cardiac involvement

Late-onset phenotypes are much more prevalent than classic phenotypes of FD [27]. However, they were significantly underdiagnosed in the past [180], essentially because they are characterized by the later onset of clinical manifestations; the absence of some distinctive manifestations of FD, such as acroparesthesias, neuropathic pain, hypohydrosis, heat, cold and exercise intolerance, gastrointestinal symptoms, angiokeratomas and cornea *verticillata* [181-186]; and the frequent dominant involvement of a single organ [15,181,183,187]. As most studies on FD natural history were conducted in the pre-ERT era [180], they probably better reflect the natural history of classical phenotypes.

The increased detection of late-onset phenotypes after the emergence of therapeutic options for FD has made knowledge of their natural history extremely relevant for making evidence-based clinical decisions and accurately evaluating the efficacy of available therapies. Still, knowledge on their natural history has been hampered by the ethical issues related to the follow-up of untreated patients in a time when therapeutic options are available. Additionally, their slower disease course, inherently biased by older age and common comorbidities [181,186,188-191], further increases the difficulty of assessing the natural history of a disease already characterized by a high clinical heterogeneity even within the same genotype [181].

Cardiac involvement may be the predominant feature in some late-onset phenotypes [7]. These phenotypes, commonly designated by some authors as “cardiac variants” of FD, have been described in association to the *GLA* mutations p.F113L [192], p.N215S [181,187,193,194], IVS4+919G>A

[183,185,186,195], p.A20P [160], p.I91T [192], p.N139S [196], p.I232T [197], p.I239M [198], p.Q279E [199,200], p.M296I [160,201], p.M296V [202], p.R301Q [199,200,203], and p.G328R [204]. Some of these mutations are very common and, despite their widespread distribution around the world, they have been described in large clusters in specific geographical areas, such as the IVS4+919G>A mutation in Taiwan [34,205-207] and the p.N215S mutation in the United Kingdom [181,182].

These *GLA* mutations are associated with residual enzymatic activity of α-galactosidase A [181,183]. Plasma lyso-GB3 levels are lower than in patients with classic phenotypes in both genders, and normal or near-normal values are usually found in females [181,182,184,185,188,190,208]. Cardiac deposits of GB3 are only detected in myocardial cells [55,188,189,208,209]. Nevertheless, the severity of cardiac involvement has been suggested to be the same or greater than in classic phenotypes [186,188].

However, current knowledge on the natural history of late-onset phenotypes with predominant cardiac involvement is frequently based on small cohorts [182,184,188,210,211] or registries [187,210,212] that are commonly biased by the overrepresentation of older, male, symptomatic and more severely affected patients. Female underrepresentation is a major issue in most previous studies [181,183,184,186-189,209-212], with a female/male ratio far below 2, ranging from 0.3 to 1.3. Comorbidities and concomitant medication are commonly not taken into account for the phenotype [181,183-185,187,188,209-212]. Finally, the clinical description of cardiac predominant phenotypes is commonly focused on LVH and evidence gaps exist on the natural history of other cardiac and extracardiac manifestations.

The *GLA* missense mutation c.337T>C (p.F113L) is a known pathogenic mutation [192,213]. The mutant enzyme was shown to be unstable and to assume a misfolded conformation at the neutral pH of the endoplasmic reticulum, where it is consequently targeted to endoplasmic reticulum associated degradation (ERAD) [213]. Although it has been associated to a late-onset phenotype with predominant cardiac involvement [27,192], the natural history of FD associated with this mutation is not completely known, as it was also reported in a 46-year-old male presenting severe renal involvement and sensorineural deafness in the absence of cardiac involvement [214] and in a family presenting renal phenotype [215].

For these reasons, the in-depth characterization of the natural history of the late-onset phenotype associated to the *GLA* mutation p.F113L was set as an objective of this doctoral thesis.

1.3.3 Fabry disease prevalence and predictors in Hypertrophic Cardiomyopathy

The prevalence of FD in patients with HCM has widely varied from 0 to 12% in previous studies, due to different inclusion criteria, study designs and screening methods, although the latest studies, based on larger cohorts of patients and improved screening methods, have suggested a narrower prevalence range of 0-4% [160-176]. Moreover, genetic variants of unknown significance (GVUS) are commonly found in screenings of FD [216] and as growing knowledge on Genetics comes to establish the pathogenic or benign nature of these variants, so the prevalence results of these studies must be continuously re-evaluated and eventually changed accordingly.

For these reasons, FD prevalence in patients with HCM was identified as an unclear issue and its further clarification was set as an objective of this doctoral thesis.

On the other hand, FD is a treatable cause of HCM [142-150,152-158] and early diagnosis and treatment are crucial, as early treatment achieves better outcomes [149,217,218].

Many diagnostic red-flags of FD have been described in the literature, which may raise suspicion and enable the early diagnosis of FD in patients with HCM [7,219].

Concentric and symmetric LVH is the most common pattern in FD [25,71], but this pattern may be found in other phenocopies (amyloidosis, mitochondrial disorders, Danon disease) [7,219]. In addition, other LVH patterns can occur in FD [71,220]. A binary appearance of the LV endocardial border, known as the "binary sign", was correlated with a characteristic pattern of glycosphingolipid compartmentalization on histological examination and once thought to represent a hallmark feature of Fabry cardiomyopathy [221]. However, it was later found to be an unreliable marker of Fabry cardiomyopathy, with an estimated sensitivity of 28.0% and a specificity of 80.0%, occurring more commonly in patients with LVH and in a more advanced stage of the disease [222]. Prominent papillary muscles are a characteristic feature of FD [71,223] and the papillary muscle contribution to LV mass was found to be significantly increased in Fabry patients, both with and without LVH, compared to controls [224]. Nevertheless, papillary muscles quantification is not routinely performed or reported in imaging exams. An apical sparing pattern on longitudinal strain has been described in FD; however, it is also found in amyloidosis [225,226]. The loss of the normal base-to-apex circumferential strain gradient was suggested to be a specific LV deformation pattern of Fabry cardiomyopathy, as opposed to non-obstructive HCM that has been associated to a higher global circumferential strain and a normal base-to-apex gradient [227]; however, this parameter needs further validation in other studies.

Intramyocardial LGE in the basal inferolateral LV segment is a typical finding [73], but it is not exclusive of FD [228-230], and, as disease progresses, LGE may involve other LV segments [75]. A low native T1

time accurately differentiates FD from other causes of LVH [84], as it is only otherwise noted in iron overload [231]. However, a pseudonormalization or elevation of T1 may occur with disease progression [84]. Furthermore, local expertise and resources are necessary to perform T1 mapping, so it is not routinely performed in clinical practice in many centres. Finally, some Fabry patients present disease complications that constitute contraindications to cardiac MRI, such as renal failure or nonconditional pacemakers.

Short PR interval, although a characteristic finding in early stages of FD [112,113], has been estimated to occur in only 14% of Fabry patients [114]. Atrioventricular and bundle-branch blocks develop as FD progresses [55,115,116], but they are also common findings with ageing independently of FD.

Although extracardiac manifestations, such as acroparesthesias, angiokeratomas and cornea *verticillata*, are red-flags to FD diagnosis [219], they are not commonly searched by cardiologists during the diagnostic study of HCM. Besides, they are frequently absent in heterozygous females and late-onset phenotypes [6]. Renal or cerebrovascular disease may also be absent in heterozygous females and in some late-onset phenotypes with a predominant organ involvement [6], which limits their utility in the etiological study of HCM. Sensorineural deafness is also a common finding with ageing and may not be adequately valued by cardiologists in the etiological study of HCM.

Hence, despite increasing awareness on the diagnostic red-flags [219], FD remains a late diagnosis [35]. Therefore, it is essential to accurately determine independent predictors of FD in patients with HCM that could lead to the early diagnosis of this treatable disease.

For these reasons, this doctoral thesis aimed to find the independent predictors that could be useful in the clinical practice for the early diagnosis of FD in patients with HCM.

1.3.4 Left ventricular noncompaction and Fabry disease

Left ventricular noncompaction (LVNC) is morphologically characterized by prominent LV trabeculations and deep intertrabecular recesses communicating with the LV cavity, predominantly located in the apical and lateral segments of the left ventricle, distally to the papillary muscles. The myocardium has a two-layer appearance, composed of a thin compacted epicardial layer and a thick spongy noncompacted endocardial layer [232].

LVNC has been found in 0.045% of adult individuals referred for echocardiography [233] and 0.029% of children admitted for heart failure due to cardiomyopathy [234]. It predominantly affects males [233] and individuals of African origin [235,236].

Several criteria have been proposed for the diagnosis of LVNC based on echocardiography or cardiac MRI [237-246]. Besides hypertrabeculation, patients may also develop LV diastolic and systolic dysfunction, fibrosis and dilation [247]. RV noncompaction has been reported in 9-18% and RV dilation/dysfunction/LGE in 16–26% of the cases with LVNC [248].

Patients with LVNC may be asymptomatic or develop complications such as heart failure, thromboembolism or ventricular dysrhythmias and sudden death [249]. A recent systematic review and meta-analysis of 28 studies enrolling 2501 LVNC patients with a median follow-up of 2.9 years reported the following incidences of events: all-cause mortality 2.16, cardiovascular mortality 1.92, heart failure admission 3.53, cardiac transplantation 1.24, ventricular arrhythmias 2.17, cardiac device implantation 2.66, stroke and systemic emboli 1.54 per 100 person-years [250].

The etiology and pathogenesis of LVNC is not clear. LVNC was originally thought to result from a disturbance of the compaction process of the LV myocardium during early development [251]. However, contrarily to previous assumptions, it is now recognized that normal myocardial compaction does not result from coalescence of pre-existing embryonic trabeculations and noncompaction does not result from a failure of coalescence of these trabeculations, as the excessive trabeculations in noncompaction do not have an embryonic identity. In the embryo, the ventricular trabeculations strongly express atrial natriuretic factor (ANF/NPPA), whereas the compact wall does not, nor do the trabeculations of both foetal healthy and noncompaction hearts [252]. Instead, recent studies have shown that proliferation of the compact zone of the embryonic myocardium into the trabeculated zone, with formation of an intermediate hybrid zone, seems essential to the normal myocardial compaction process and that inhibition of this phenomenon could be one of the responsible mechanisms leading to LVNC [253]. Moreover, LVNC has been described in association to mutations in genes involved in heart development, such as *MIB1*, *MIB2*, *NKX2*-5, *NOTCH1*, *NSD1*, *PTPN11*, *TBX20*, or *TBX5* [254]. *MIB1* mutations have already been shown to reduce the NOTCH signalling pathway and prevent trabecular maturation and compaction, resulting in myocardial noncompaction [255].

However, not all cases are congenital and LVNC may be acquired at any stage of life after birth [232]. Furthermore, LVNC has been described in association with mutations in more than 110 genes, including genes that code for proteins in the sarcomere (beta-myosin heavy chain, cardiac myosin-binding protein C, alpha-cardiac actin, cardiac troponin T, titin), Z line (ZASP/Cypher), nuclear lamina (laminA/C), cytoskeleton (alpha-dystrobrevin) and mitochondria (tafazzins) [232,251]. Family history of LVNC has been reported in 27% of the cases [254] and approximately one-third of the patients with LVNC were found to carry a pathogenic mutation [256-259]. Mutations in genes affecting sarcomere function represented

more than half of the known genetic causes, particularly in adults [254]. The most frequently involved genes are *MYH7*, *MYBPC3* and *TTN* [254,256,257,259,260]. Still, the causal relationship between these mutations and LVNC remains to be established in almost all cases [251].

Haemodynamic factors may also contribute to the development of LVNC. It has been hypothesized that LVNC may develop in compensation of a failing myocardium in response to a cardiac stress. The enlargement of the endocardial surface and myocardial volume could increase the stroke volume, which is thought to be attained by the upregulation of genes responsible for trabeculation [251]. This hypothesis is supported by the finding of acquired forms of LVNC in patients diagnosed with other cardiomyopathies [247], pregnant women [261], professional athletes [236,262,263] and healthy individuals who practice vigorous physical activity [264]. The resolution of LVNC after cardiac resynchronization therapy [265], pregnancy [261] and sport discontinuation [251], further supports this hemodynamic hypothesis.

Notably, it remains however to be clarified if LVNC is a distinct cardiomyopathy or a morphological trait shared by different diseases. Indeed, LVNC has been reported not only as an isolated condition, but also in patients with other cardiomyopathies, including dilated, hypertrophic, arrhythmogenic and restrictive cardiomyopathies; congenital cardiac abnormalities, such as LVOT abnormalities, Ebstein anomaly, tetralogy of Fallot and double outlet right ventricle; chromosomal defects, such as trisomy 13, Cornelia de Lange, DiGeorge, Turner and 5qminus syndromes; and neuromuscular disorders, such as mitochondrial myopathies, Barth syndrome, zaspopathy, myotonic dystrophy type 1, dystrobrevinopathy, and Emery-Dreifuss muscular dystrophy [232,247]. LVNC has also been reported in patients with storage disorders such as glycogen storage disease types Ib and IV [266,267], Danon disease [268] and amyloidosis [269].

In 2011, we reported the first case of LVNC in a FD patient [270]. This was a young asymptomatic female, who was diagnosed with FD due to the p.F113L mutation following family screening and later found, during clinical investigation, to fulfil the Jenni echocardiographic criteria and the Petersen cardiac MRI criteria for LVNC, in the absence of any known cardiac or extracardiac manifestations of FD and clinical complications or family history of LVNC. In this report, we pointed out the limitations of the current diagnostic criteria of LVNC and raised the question whether this was an overdiagnosis of LVNC, a coincidental finding of LVNC and FD, or if indeed the two conditions were clinically related [270,271]. Later, two additional cases of LVNC in FD patients have been reported [272-275], both in young females, who fulfilled the Petersen cardiac MRI criteria. Of note, one of these patients was referred to ERT based on LVNC and the finding of lysosomal inclusions in cardiomyocytes on endomyocardial biopsy, despite the absence of known clinical indications to start FD therapy [272,273].

The only study investigating FD prevalence in patients with LVNC was a small study by Stöllberger *et al.* including 26 male patients who fulfilled the Stöllberger echocardiographic criteria for LVNC. FD screening was based on the enzymatic assay of α-galactosidase A and found no patients with the disease [276].

Taken together, it remained essential to clarify whether LVNC could be a cardiac manifestation of FD, given the implications for therapeutic decisions on whether to start FD therapy, particularly in young asymptomatic females with no other known clinical manifestations of FD that would constitute an indication to start therapy.

This thesis aimed to contribute to this clarification through the determination of the prevalence of FD in a larger cohort of patients with LVNC.

1.3.5 Novel pathogenic *GLA* variants

The *GLA* gene is located on the q22.1 region of the X chromosome and its coding part consists of 1290 base pairs divided into seven exons [178,179]. This gene codes α-galactosidase A, a polypeptide of 429 amino acids with five disulphide bonds (Cys52-Cys94, Cys56-Cys63, Cys142-Cys172, Cys202-Cys223, Cys378-Cys382) and four possible N-glycosylation sites (Asn139, Asn192, Asn215, Asn408) [178].

Nonsense and frameshift *GLA* mutations, which cause a premature termination of protein synthesis, are associated with classic FD. Several missense mutations involving catalytic sites, dimerization sites, or protein folding also correlate with classic FD. Other missense mutations that cause protein instability due to improper protein folding or alter the glycosylation site are associated to late-onset phenotypes [178]. Notably, some *GLA* mutations have been associated to both late-onset and classic phenotypes (e.g. R112H, R301Q, G328R, and R404del) [179].

Genotype-phenotype correlations are difficult to establish in FD by several reasons. First, there are more than 1000 documented mutations of the *GLA* gene, including missense or nonsense mutations, frameshift mutations, deletions, and insertions [14]. Second, mutations are frequently private to each family [178,179]. Third, there is a high phenotypic heterogeneity associated to each *GLA* mutation, even within the same family [277].

Novel *GLA* variants of uncertain significance are frequently encountered in FD screenings of newborns or high-risk groups [216]. Interpretation of their clinical significance may be challenging, particularly in the case of missense mutations in heterozygous females, because change is limited to one nucleotide, enzymatic activity and disease biomarkers may be within normal range in females, some FD

manifestations are unspecific and family history may be inconclusive [216,278]. In these cases, histological evidence of lysosomal inclusions may be required to demonstrate their pathogenicity [278].

However, the correct interpretation of GVUS is mandatory for an accurate estimate of the epidemiology of the disease and understanding of its natural history and response to therapy. For this thesis research, the correct interpretation of GVUS was considered crucial for the accurate estimate of the prevalence of FD in HCM or LVNC as well as the finding of FD predictors in patients with HCM.

Therefore, this doctoral thesis set as an objective the demonstration of the pathogenicity of novel pathogenic *GLA* mutations that could be encountered during the screening studies of this investigation.

CHAPTER 2 – OBJECTIVES

This doctoral thesis aimed to accomplish the following specific goals:

1) To determine the prevalence of FD in patients with HCM in the Portuguese region of Guimarães, to demonstrate a founder effect of FD due to the *GLA* gene mutation p.F113L in this region, to describe its global diffusion worldwide and to characterize the phenotype associated to this mutation;
2) To describe the natural history of FD associated to the founder *GLA* gene mutation p.F113L;
3) To determine the overall prevalence and the predictors of FD in patients with HCM;
4) To determine the prevalence of FD in patients with LVNC;
5) To report the novel *GLA* gene pathogenic mutations encountered during FD screening in patients with HCM and to demonstrate their pathogenicity.

CHAPTER 3 – METHODS

The research methodology that was used to achieve each of the objectives of this doctoral thesis is described in the present chapter. This investigation was conducted in accordance with the Declaration of Helsinki [279] and approved by the hospital ethics committee. All patients provided signed informed consent.

3.1 Fabry disease in the Portuguese region of Guimarães

3.1.1 Fabry disease prevalence in patients with Hypertrophic Cardiomyopathy in the Portuguese region of Guimarães

Between January 2008 and December 2016, 150 consecutive patients were recruited from the Cardiomyopathies consultation of the Hospital Senhora da Oliveira – Guimarães, Portugal. The inclusion criteria were adult patients (≥18 years) presenting the diagnostic criteria of HCM according to the European Society of Cardiology (ESC) [280], i. e., LVH with LV wall thickness (≥15 mm), measured by echocardiography, that was not explained by hypertension, valve disease or other cardiac overload conditions.

Enzymatic activity of α-galactosidase A was measured in all patients in dried blood spot samples, as described by Gaspar *et al.* [281]. The molecular analysis of the *GLA* gene was performed in males with reduced activity (< 0.3 nmol/h/spot) and in all females, by polymerase chain reaction (PCR) and direct sequencing of all exons and flanking intronic regions, as described by Shabbeer *et al.* [282]. FD diagnosis was established by deficient enzymatic activity in males and a pathogenic *GLA* gene mutation in females [283].

3.1.2 Founder effect of Fabry disease due to the *GLA* mutation p.F113L in the region of Guimarães

Family pedigrees were elaborated and places of birth were recorded for all the probands with the same pathogenic *GLA* mutation (p.F113L).

A genealogy study was performed aiming to find the genealogical links and the common ancestor to all the families with the p.F113L mutation. The genealogy research was performed by historians of the region of Guimarães, who searched and analyzed birth, marriage and death certificates, the most recent in the

Citizen Records and the most remote, dating to the 17th century, in the Parish Records that are currently filed in the City Archives.

We genotyped one patient from each of the nuclear families (the male probands or, whenever possible, the sons of the female probands) for four DNA polymorphisms with loci in the immediate vicinity of the *GLA* locus. The genomic region between the most centromeric (DXS990; 93 Mb) and the most telomeric (DXS1191; 103.3 Mb) genotyped loci spans about 10 Mb. Accordingly, and given the fact that the *GLA* locus does not map to a highly recombinogenic part of the X chromosome, we assumed a recombination frequency of about 0.10 between DXS990 and DXS1191. Comparing the individual genotypes and assuming the least number of recombinations necessary to explain the individual variants, we constructed likely haplotypes with the *GLA* locus being in the centre (100.6 Mb) and flanked by two of the marker loci.

3.1.3 Fabry disease due to the p.F113L mutation: From Guimarães to other parts of the world

Pedigree analysis and family screening were performed in all index patients who were diagnosed with FD due to the p.F113L mutation in the region of Guimarães. The measurement of the enzymatic activity of α-galactosidase A and the molecular analysis of the *GLA* gene were performed in all the screened relatives, as described in section 3.1.1.

At the time of the screening, some of the relatives were living in other Portuguese regions or were emigrants in other countries. Some of these relatives were screened and diagnosed with FD in our centre during their holidays or vacations in Guimarães, while others were referred to Reference Centres in the proximity of their migration places, being subsequently diagnosed with FD at these centres. Other patients already diagnosed with FD in Guimarães subsequently emigrated to other countries during their follow-up. The migration places of all these FD patients were recorded in order to document the spread of the disease to other parts of the world.

3.1.4 Characterization of the Fabry disease phenotype associated to the p.F113L mutation

Pedigree analysis was extensively performed and a total of 120 consecutive adult patients (≥18 years), including probands and relatives, were diagnosed with FD based on the presence of the p.F113L mutation. These patients were evaluated in the Reference Centre of Lysosomal Storage Disorders of Hospital Senhora da Oliveira – Guimarães. At baseline, all patients were submitted to an extensive predefined diagnostic protocol with characterization of the multiorgan involvement by FD. The clinical protocol included systematic multidisciplinary assessment (Cardiology, Internal Medicine, Neurology, Psychiatry,

Ophthalmology, Otorhinolaryngology, Dermatology), blood and urine analysis with quantification of serum creatinine, albuminuria on 24 h-urine and albumin to creatinine ratio on random urine and eGFR by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula, electrocardiogram (ECG), echocardiogram, cardiac MRI, 24 h-Holter, exercise stress test, brain MRI (by neuroradiologist), electromyography, audiometry and lung spirometry. The enzymatic activity of α-galactosidase A in plasma and leukocytes, the plasma and urinary GB3 and the plasma lyso-GB3 were measured according to previously described methods [40,68,284].

The phenotype of FD patients with the mutation p.F113L was described based on the statistical analysis of the overall cohort. Stratified analysis by gender and by gender and age categories (<40 years and ≥40 years) were also performed in order to explore the differences between the groups. Categorical variables were expressed as percentages and compared between groups of patients by the Chi-square test. Continuous variables were expressed as means and standard deviations and compared between groups of patients by the Student's t-test or Mann-Whitney test, as appropriate. Statistical significance was considered for $p < 0.05$.

3.2 Natural history of late-onset Fabry disease associated to the founder *GLA* mutation p.F113L

Between January 2008 and December 2018, 203 consecutive adult patients (≥18 years), from 34 different family pedigrees, were diagnosed with FD due to the p.F113L mutation, in the Reference Centre of Lysosomal Storage Disorders of Hospital Senhora da Oliveira – Guimarães. FD diagnosis was performed as previously described in section 3.1.1.

At baseline, all patients were submitted to a predefined diagnostic protocol, as described in section 3.1.4. Kidney biopsy was performed in Fabry patients presenting proteinuria ≥500 mg on 24 h-urine.

The occurrence of key clinical manifestations of FD was evaluated by gender and age category (≤30; 31–40; 41–50; 51–60; 61–70; 71–80; >80 years). Descriptive statistical analysis was performed as described in section 3.1.4. Polynomial regression (quadratic function) was used to best describe the relationship between age and LV mass, interventricular septum (IVS) and PW thickness and QRS interval duration. An exponential model was used to best describe the relationship between age and prohormone of brain natriuretic peptide (pro-BNP) levels. Kaplan-Meier curves were performed to estimate survival free from heart failure and pacemaker.

3.3 Fabry disease prevalence and predictors in patients with Hypertrophic Cardiomyopathy

Between January 2008 and March 2018, 780 consecutive patients with HCM were recruited from the Cardiology consultation of 12 Portuguese hospital centres and underwent FD screening. The inclusion criteria and method of FD screening were the same as described in section 3.1.1.

We collected clinical, electrocardiographic, cardiac imaging, laboratory and genetic data. Categorical variables were expressed as percentage. According to the Kolmogorov-Smirnov test, continuous variables were all non-normally distributed and therefore expressed as median with interquartile range (IQR). Comparison of variables between Fabry and non-Fabry patients was performed by Chi-square test for categorical variables and Mann-Whitney test for continuous variables. Patients with *GLA* variants of uncertain clinical significance were excluded from this statistical analysis. In order to identify independent predictors of FD among patients with HCM, we performed a multivariate binary logistic regression analysis, including the cardiac categorical variables that were readily available on ECG, echocardiogram and cardiac MRI and showed statistically significant differences between FD and non-FD patients.

We performed a Discriminant Function Analysis for FD in patients with HCM. We used the Wilk's lambda to calculate the unstandardized coefficients for each of the FD predictors that have been previously identified by multivariate regression analysis and then determined a discriminant score based on the weighted combination of the independent predictors of FD. We calculated the mean discriminant scores for Fabry and non-Fabry patients and determined the best discriminant cut-off for the score. We used the maximum likelihood technique to assign each case to a group according to the specified cut-off, in order to determine the sensitivity, specificity and positive and negative predictive values of the score in the prediction of FD in patients with HCM. Statistical significance was set at $p<0.05$.

3.4 Fabry disease prevalence in patients with Left ventricular noncompaction

Between October 2015 and October 2017, 78 consecutive patients with LVNC were recruited from the cardiology consultation of eight Portuguese hospital centres. The inclusion criteria were adult patients (aged ≥18 years) diagnosed with LVNC based on the presence of any of the current diagnostic criteria of LVNC, by either echocardiography or cardiac MRI. All patients fulfilling the inclusion criteria underwent FD screening.

FD screening was performed at a specialized laboratory (Centogene AG, Rostock, Germany). The enzymatic activity of α-galactosidase A was measured in dried blood spot samples by fluorometry. The molecular analysis of the *GLA* gene was performed in males with reduced enzymatic activity of α-

galactosidase A, i.e., below the normal reference range (<15.3 μmol/l/h), and in all females, independently of the enzymatic activity of α-galactosidase A. The molecular analysis was performed by classical bidirectional Sanger sequencing of the *GLA* gene and included all exons, exon/intron boundaries and the promoter region.

3.5 Novel *GLA* pathogenic mutations and demonstration of their pathogenicity

During FD screening in patients with HCM, the molecular analysis of the *GLA* gene revealed novel variants that were not previously described in the literature or in genetic databases.

To clarify their clinical significance, the following procedures were performed with as much detail as possible: (i) Pedigree analysis and family screening; and (ii) Clinical and biochemical description of the phenotype in the index patient and their relatives. The biopsy of the skin or affected organ of a patient with the novel *GLA* variant was performed whenever possible and considered necessary. In silico methods (Polyphen-2, SIFT, MutationTaster, Provean) were also used in the interpretation of novel *GLA* variants when considered necessary.

Evidence of their pathogenicity was based on the presence of a clinical and biochemical phenotype compatible with FD in the index patient and their affected relatives, demonstration of cosegregation of the genotype and the phenotype in the family and histological evidence of lysosomal inclusions (if available).

CHAPTER 4 – RESULTS

The results of this doctoral thesis are presented in the form of articles that were published in indexed peer-reviewed medical journals.

Chapter 4.1

Azevedo O, Gal A, Faria R, Gaspar P, Miltenberger-Miltenyi G, Gago MF, Dias F, Martins A, Rodrigues J, Reimão P, Pereira O, Simões S, Lopes E, Guimarães MJ, Sousa N, Cunha D.

Founder effect of Fabry disease due to p.F113L mutation: Clinical profile of a late-onset phenotype.

Mol Genet Metab 2020;129:150-160. doi: 10.1016/j.ymgme.2019.07.012. Epub 2019 Jul 24.

Molecular Genetics and Metabolism 129 (2020) 150–160

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism

journal homepage: www.elsevier.com/locate/ymgme

Regular Article

Founder effect of Fabry disease due to p.F113L mutation: Clinical profile of a late-onset phenotype

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ARTICLE INFO

Keywords:
Fabry disease
Founder effect
Late-onset
F113L
Portugal
Cardiomyopathy

ABSTRACT

Background: Knowledge on clinical profiles of late-onset phenotypes of Fabry disease (FD) is essential to better define their natural history. Our study aims to demonstrate a founder effect of FD due to the *GLA* gene mutation c.337T > C (p.F113L) in the Portuguese region of Guimarães; and to characterize the clinical profile of this late-onset phenotype in a large cohort of genetically related adult patients, living in the same region.

Methods and Results: FD screening was performed in 150 adult patients with hypertrophic cardiomyopathy (HCM) and found 25 Fabry patients (16.6%). The p.F113L mutation was found in 21 of them, leading to a genealogy study and haplotype analysis of the p.F113L patients. Genealogy research revealed a 12-generation family tree with a common ancestor to p.F113L patients, suggesting a founder effect that was supported by haplotype findings. Pedigree analysis was performed and 120 consecutive p.F113L patients underwent a pre-defined diagnostic evaluation of FD multiorgan involvement. This late-onset phenotype was characterized by common and/or potentially severe cardiac manifestations (left ventricular hypertrophy 40.8%, atrial fibrillation 5%, non-sustained ventricular tachycardia 12.5%, atrioventricular block 18.3%, bifascicular block 13.4%). Extracardiac manifestations included albuminuria > 30 mg/24 h 36.1%, chronic kidney disease ≥ G3 7.6%, brain white matter lesions 54.4%, stroke 3.3%, sensorineural deafness 44.5%, cornea *verticillata* 13.9%. Plasma lyso-GB3 was undetectable in females, regardless of clinical manifestations.

Conclusion: A founder effect of FD due to p.F113L mutation was documented by genealogy and genetics in a Portuguese region. In this late-onset phenotype, although cardiac manifestations carry the highest prognostic impact, extracardiac involvement is common.

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https://doi.org/10.1016/j.ymgme.2019.07.012
Received 29 May 2019; Received in revised form 18 July 2019; Accepted 18 July 2019
Available online 24 July 2019
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1. Introduction

Fabry disease (FD) (OMIM 301500) is an X-linked lysosomal storage disorder caused by mutations in the *GLA* gene leading to deficiency of the enzymatic activity of α-galactosidase A. Subsequent lysosomal accumulation of globotriaosylceramide (GB3) and other related glycosphingolipids may cause multiorgan damage, being cardiac disease the main cause of death [1].

Mutations causing a virtually null enzymatic activity are associated to a severe and early onset classical phenotype. In childhood or adolescence, patients may develop acroparesthesias, neuropathic pain, hypohydrosis, heat, cold and exercise intolerance, cornea *verticillata*, angiokeratomas, gastrointestinal symptoms and proteinuria. In adulthood, patients suffer from sensorineural deafness and cardiac, renal and cerebrovascular manifestations. On the other hand, some missense mutations may be associated to a residual enzymatic activity and to the development of attenuated and late-onset phenotypes in which the manifestations arise in adulthood and include cardiac, renal and cerebrovascular manifestations [1].

FD prevalence was reported as 1 in each 3100 hemizygote males, with a ratio of 11 late-onset variants for each classical phenotype [2]. Therapeutic decisions are more complex in patients with late-onset phenotypes, because, despite being more prevalent, their natural history is not fully established. First, most studies on FD natural history were conducted in the pre-enzyme replacement therapy (ERT) era [3] and probably better reflect the natural history of the classical phenotype due to the considerable underdiagnosis of late-onset phenotypes. Second, FD is characterized by high clinical heterogeneity, even within members of the same family [4]. Finally, patients with late-onset phenotypes are frequently older and present other diseases and comorbidities [5], making it difficult to fully attribute the clinical manifestations to FD.

The missense mutation c.337T > C (p.F113L) is a known pathogenic mutation that results in a misfolded enzyme, unstable at the neutral pH of the endoplasmic reticulum, where it is consequently degraded, thereby leading to a late-onset phenotype of FD [6]. Although it has been associated to a predominant cardiac involvement [2,7], the natural history of FD associated with this mutation is not completely known, as it was also reported in a 46-year old male presenting severe renal involvement and sensorineural deafness in the absence of cardiac involvement [8] and in a family presenting renal phenotype [9].

Herein, we report an unexpectedly high prevalence of FD among patients with hypertrophic cardiomyopathy (HCM) in the region of Guimarães, Portugal. Genetic and genealogical investigations subsequently supported the assumption of a founder effect of the disease due to the *GLA* gene mutation p.F113L. Pedigree analysis of p.F113L probands led to the diagnosis of a large number of patients with this mutation in this Portuguese region, but also in many parts of the world. This study provides a detailed description of the overall clinical profile of a late-onset phenotype of FD with predominant cardiac manifestations in a large cohort of genetically related adult patients, living in this Portuguese region and sharing common environmental factors. Our study describes the cardiac as well as the extracardiac involvement of this late-onset phenotype, clearly demonstrating that the designation of "cardiac variant" underestimates the systemic involvement by FD.

2. Subjects and methods

2.1. FD screening

Between January 2008 and December 2016, 150 consecutive patients were recruited from the Cardiomyopathies consultation of the Hospital Senhora da Oliveira – Guimarães, Portugal. The inclusion criteria were adult patients (≥18 years) presenting the diagnostic criteria of HCM according to the European Society of Cardiology (ESC) [10], i. e., left ventricular hypertrophy (LVH) with increased left ventricular (LV) wall thickness (≥15 mm), measured by echocardiography, that was not explained by hypertension, valve disease or other cardiac overload conditions.

Enzymatic activity of α-galactosidase A was measured in all patients in dried blood spot samples, as described by Gaspar et al. [11]. The molecular analysis of the *GLA* gene was performed by PCR sequencing of all exons and flanking intronic regions, as described by Shabbeer et al. [12], in males with reduced activity (< 0.3 nmol/h/spot) and in all females. FD diagnosis was established by deficient enzymatic activity in males and a pathogenic *GLA* gene mutation in females [1].

2.2. Genealogy and genetic research

Family pedigrees were elaborated and places of birth were recorded for all the probands with the same pathogenic *GLA* gene mutation (p.F113L). A genealogy study was performed aiming to find the genealogical links and the common ancestor to all the families with the p.F113L mutation. The genealogy research was performed by historians of the region of Guimarães, who searched for and analyzed birth, marriage and death certificates, the most recent in the Citizen Records and the most remote, dating to the 17th century, in the Parish Records currently filed in the City Archives.

We genotyped one patient from each of the 21 nuclear families (the male probands or, whenever possible, the sons of the female probands) for four DNA polymorphisms with loci in the immediate vicinity of the *GLA* locus (Supplementary Table 1). The genomic region between the most centromeric (*DXS990*; 93 Mb) and the most telomeric (*DXS1191*; 103.3 Mb) genotyped loci spans about 10 Mb. Accordingly, and given the fact that the *GLA* locus does not map to a highly recombinogenic part of the X chromosome, we assumed a recombination frequency of about 0.10 between *DXS990* and *DXS1191*. Comparing the individual genotypes and assuming the least number of recombinations necessary to explain the individual variants, we constructed likely haplotypes with the *GLA* locus being in the centre (100.6 Mb) and flanked by two of the marker loci.

2.3. Pedigree analysis and clinical evaluation of Fabry patients

Pedigree analysis was extensively performed and a total of 120 adult patients, including probands and relatives, were diagnosed with FD based on the presence of the p.F113L mutation. These patients were evaluated in the Reference Center of Lysosomal Storage Disorders of Hospital Senhora da Oliveira – Guimarães. At baseline, all patients were submitted to an extensive predefined diagnostic protocol with characterization of the multiorgan involvement by FD. The clinical protocol included systematic multidisciplinary assessment (Cardiology, Internal Medicine, Neurology, Psychiatry, Ophthalmology, Otorhinolaryngology, Dermatology), blood and urine analysis with quantification of serum creatinine, albuminuria on 24 h-urine and albumin to creatinine ratio on random urine and estimation of glomerular filtration rate (eGFR) by the CKD-EPI formula, electrocardiogram, echocardiogram, cardiac MRI, 24 h-Holter, exercise stress test, brain MRI (by neuroradiologist), electromyography, audiometry and lung spirometry. The enzymatic activity of α-galactosidase A in plasma and leukocytes, the plasma and urinary GB3 and the plasma lyso-GB3 were measured according to previously described methods [13–15].

2.4. Statistical analysis

Categorical variables were expressed as percentages and continuous variables as means and standard deviations. Comparisons between groups of patients were performed by Chi-square test for categorical variables and by Student's *t*-Test for continuous variables (or Mann-Whitney test, if the variable distribution was not normal among the groups). Statistical significance was considered for $p < 0.05$.

Table 1
Characteristics of the non-p.F113L Fabry probands.

Gender, age	*GLA* gene mutation	Enzymatic activity of α-GAL A in plasma (nmol/h/mL)	Enzymatic activity of α-GAL A in leukocytes (nmol/h/mg)	Urinary GB3 (μg/mmol creatinine)	Plasma GB3 (nmol/mL)	Plasma Lyso-GB3 (ng/mL)	IVS/PW thickness (mm)/LV mass (g/m^2)	LVH characteristics	Other manifestations
F, 43	p.M290I	14.0	6.0	N/A	n.d.	n.d.	23/12/212	Asymmetrical Non-obstructive EF 78% DD grade II LGE on inferior and inferoseptal walls, apical segments and basal inferolateral segment	Proteinuria Brain white matter lesions
F, 56	p.E203X	6.7	7.1	81	12.86	11.64	20/12/196	Asymmetrical Non-obstructive EF 68% DD grade I LGE on inferolateral wall	Renal insufficiency Proteinuria Stroke Brain white matter lesions Deafness Cornea *verticillata*
F, 50	p.G183V	3.9	21	76	n.d.	4.34	9/15/98	PW LVH Non-obstructive EF 80% LGE on basal inferolateral segment	Short PR interval Brain white matter lesions
M, 50	p.C94S	1	0	N/A	17.77	107.19	21/18/277	Symmetrical Non-obstructive EF 58% DD grade II	Non-sustained VT Renal insufficiency Proteinuria Stroke Deafness Acroparesthesias Anhidrosis Cornea *verticillata* Angiokeratomas

α-GAL A, α-galactosidase A; AV, atrioventricular; DD, diastolic dysfunction; EF, ejection fraction; IVS, interventricular septum; LGE, late gadolinium enhancement; LV, left ventricular; LVH, left ventricular hypertrophy; N/A, non-available; n.d., non-detectable (below lower limit of quantitation); PW, posterior wall; VT, ventricular tachycardia.
Reference values: enzymatic activity of α-galactosidase A on plasma 6–19 nmol/h/mL, and on leukocytes 36–80 nmol/h/mg; urinary GB3 0.87–13 μg/mmol creatinine; plasma GB3 0.8–4.52 nmol/mL; plasma Lyso-GB3 0–1.9 ng/mL

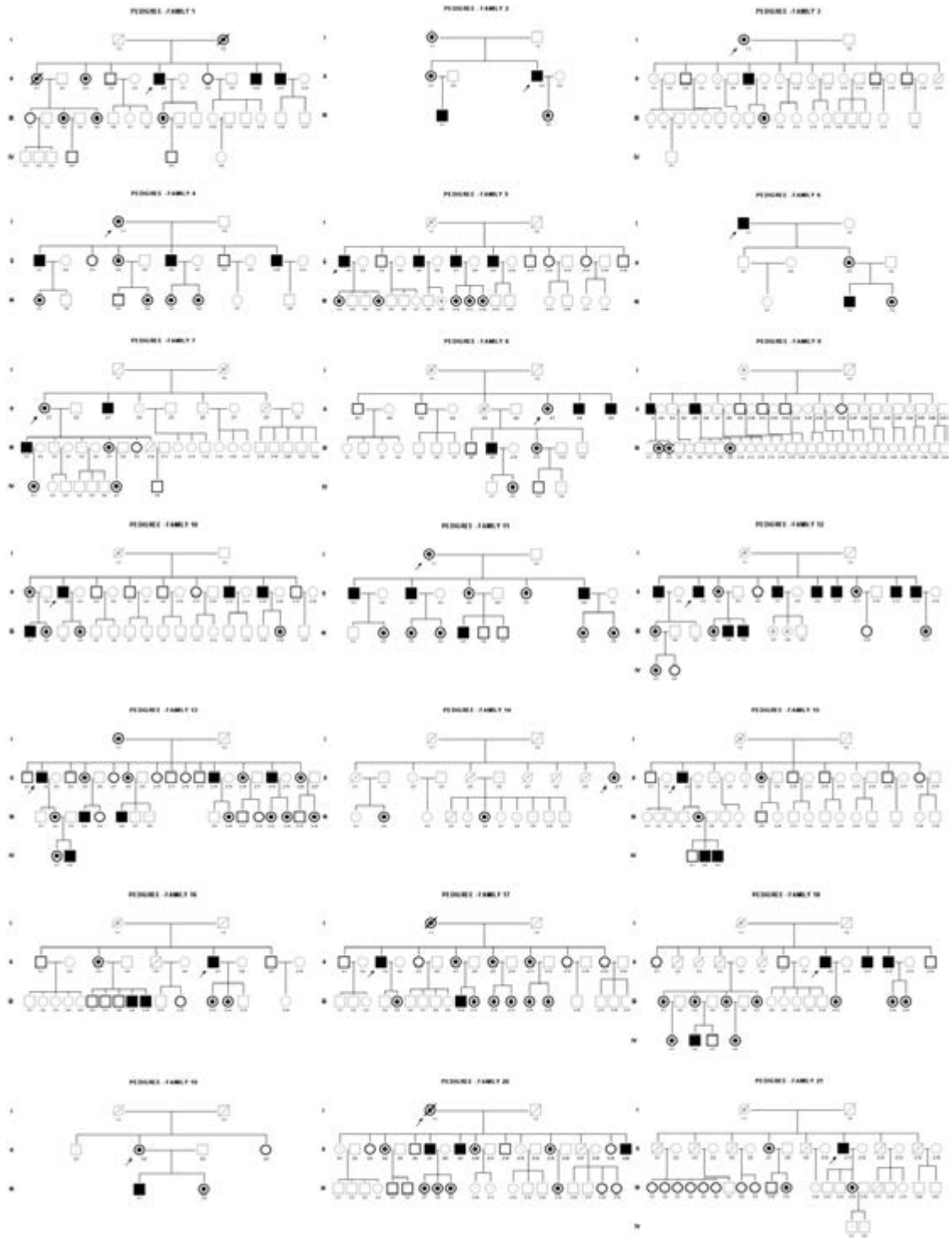


Fig. 1. Pedigrees of the 21 Fabry families with the p.F113L mutation

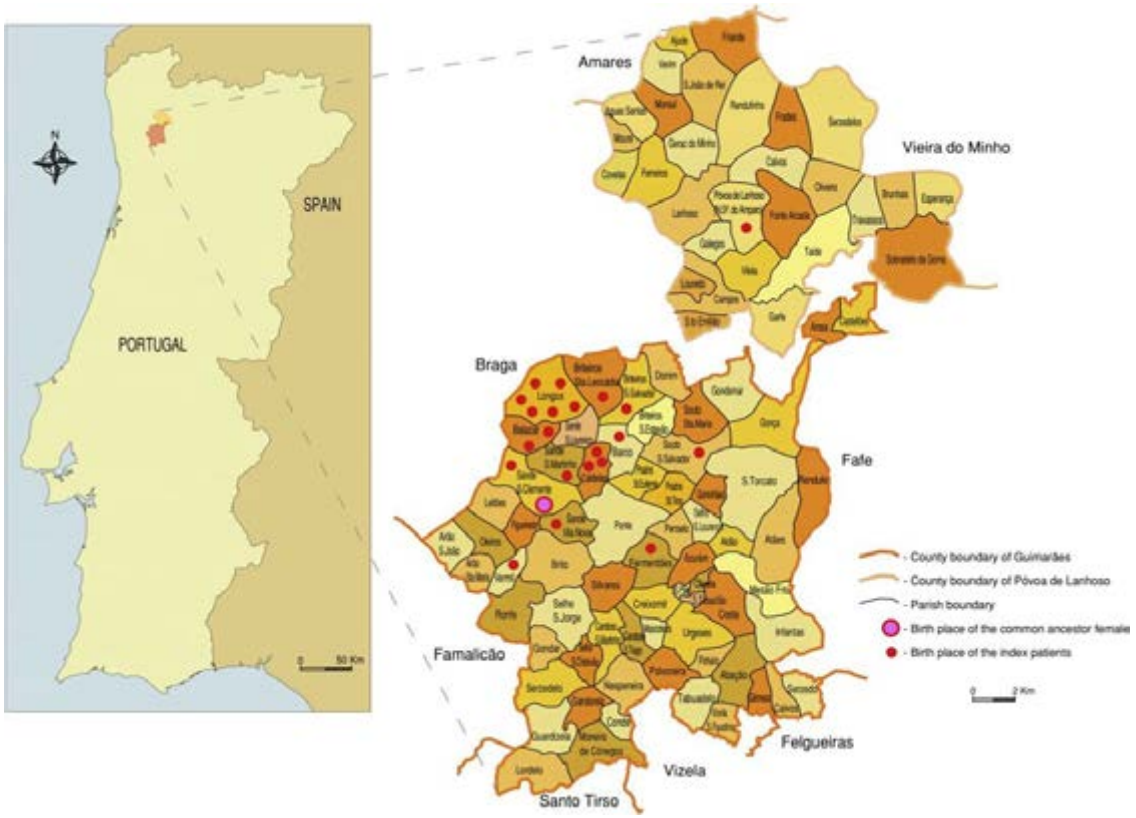


Fig. 2. Map of Portugal, highlighting the region of Guimarães, where a founder effect of FD due to the p.F113L mutation was documented. The red spots represent the birth places of the 21 probands with this mutation. The larger red-pink spot represents the birth place of the female identified by genealogy as the common ancestor to 17 of the 21 families with this mutation.

2.5. Ethical issues

This research was approved by the hospital ethics committee and all patients provided written informed consent.

3. Results

3.1. Prevalence of FD in patients with HCM in the region of Guimarães

We assessed 150 patients with HCM from the Portuguese region of Guimarães, predominantly males (62.7%), with a mean age of 64.4 ± 14.1 years. LVH was asymmetrical in 57.3%. Mean interventricular septum thickness was 18.0 ± 4.0 mm (Supplementary Table 2).

Screening of these 150 patients with HCM led to the diagnosis of FD in 25 patients (16.6%). Twenty-one of the 25 patients carried the same *GLA* gene mutation (c.337T > C; p.F113L). The remaining four Fabry patients carried different *GLA* gene variants: c.870G > C (p.M290I); c.607G > T (p.E203X); c.548G > T (p.G183V); and c.281G > C (p.C94S). Their clinical characteristics are detailed in Table 1.

Further etiological investigation of HCM revealed sarcomere mutations in 38.3% of the patients who were tested with a panel of sarcomere genes, most commonly in the *MYH7* (13.3%), *MYBPC3* (10.0%) and *TNNT2* (5.0%) genes. The diagnosis of senile amyloidosis was finally established in six cases and mitochondrial disease in one patient.

3.2. Genealogy findings

Pedigree analysis at the first clinical consultation did not reveal any apparent family connection between the 21 probands with the p.F113L mutation (Fig. 1). Nevertheless, all the 21 probands were originated from a particular region of Guimarães (Fig. 2), raising the suspicion of a founder effect of the disease. The genealogy research led to the identification of a female who lived in the 17th century in this region and was the common ancestor of 17 of the 21 families with the p.F113L mutation (Fig. 3). It was not possible to find the connection of the families 2, 5, 12 and 19 to the genealogical tree, mainly due to unknown paternity cases.

3.3. Genetic findings

The family structure derived from the historical registries consists of at least 12 generations. From the 93 affected individuals identified throughout these 12 generations, 61 are females and 32 are males. A gender ratio of 2:1 is in line with the X-chromosomal inheritance of the trait (Fig. 3).

The haplotypes are in line with the putative historical family structure. Based on the haplotype results, we hypothesized that female I-1 carried the mutant *GLA* allele on her X chromosome with the haplotype 1(*DXS990*)-1(*DXS8020*)-2(*DXS8096*)-2(*DXS1191*). Indeed, this "founder" haplotype was inherited through at least 12 generations and

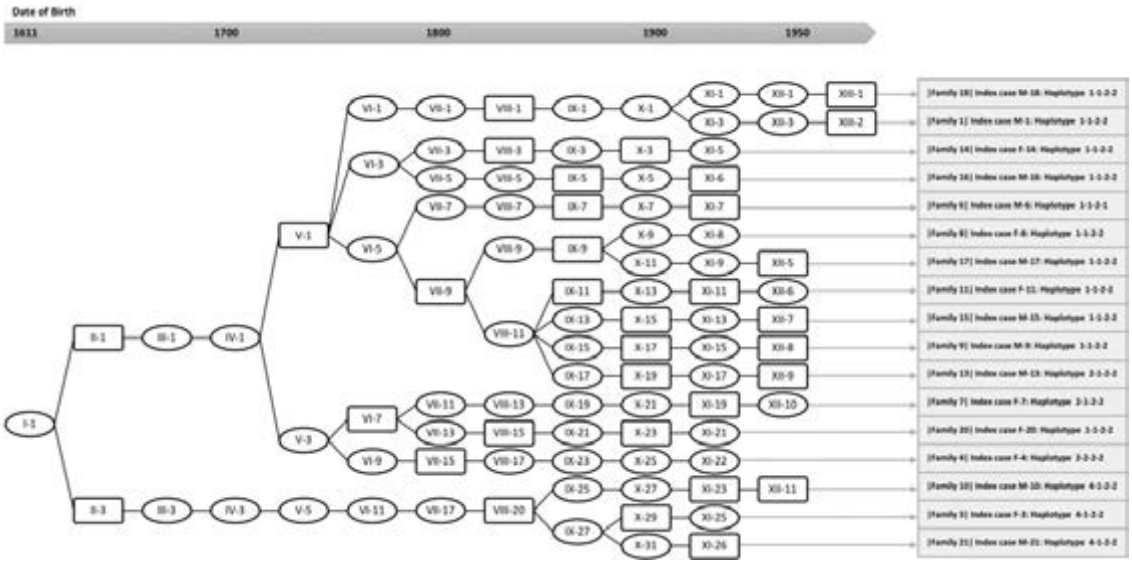


Fig. 3. Family tree provided by the historians research, demonstrating the genealogical connection of 17 Fabry families with the p.F113L mutation to a common ancestor who was born in 1611 in the region of Guimarães. The individuals are coded by Generation number in roman numerals-Individual number within the generation. Males are represented as squares and females as circles. For simplification, only Fabry patients are depicted and respective spouses were removed from the pedigree. Fabry probands were coded as M for male or F for female-Family number according to the order that families were discovered. Genetic testing of patients of these 17 families revealed the haplotypes provided at the right side of each family.

appeared obviously unchanged in patients M-1, M-18, F-8, M-17, M-9, F-11, M-15, M-16, F-14 and F-20.

Patients M-10, M-21, and F-3 carry the haplotype 4-1-2-2, suggesting the occurrence of a recombination changing the ancient *DXS990* allele in a patient from generations III to VII. In patient M-13, the recombination changing the haplotype 1-1-2-2 to 2-1-2-2 likely occurred in his mother or great-grandmother. Patients F-7 and F-4 carry respectively the haplotypes 2-1-2-2 and 2-2-2-2, suggesting recombinations after generation V. Patient M-6 carries the haplotype 1-1-2-1, suggesting a recombination changing the ancient *DXS1191* allele on generations VI, VII, VIII or X.

The haplotypes of patients not connected to the genealogical map [M-2 (4-1-2-2); M-5 (4-1-2-1); M-12 (2-1-2-2); F-19 (1-1-2-2)] suggest that families 2 and 5 may be closely related to families 10, 21 and 3; family 12 may be related to family 7 or 13; and family 19 to one of the families currently carrying the historical haplotype.

3.4. Family screening of FD

Pedigree analysis of the 21 probands with the p.F113L mutation has led to the diagnosis of 120 adult Fabry patients in the region of Guimarães, but also many other patients in other regions of Portugal and other countries around the world (Spain, France, Monaco, United Kingdom, Luxembourg, Switzerland, Germany, Angola, Colombia).

3.5. Clinical phenotype of late-onset FD due to the p.F113L mutation

Herein, we present an overview of the clinical phenotype presented, at the baseline, by 120 adult consecutive patients, 47 males and 73 females (female/male ratio 1.6), with FD due to the p.F113L mutation that live in the Portuguese region of Guimarães. Mean age at diagnosis of FD was 46 ± 18 years and similar in both genders. Median age at diagnosis was 45 years [52 years in males (18–76) and 42 years in females (18–87)].

LVH (LV wall thickness > 12 mm on echocardiogram) was found in 49 patients (40.8%), predominantly in males (70.2% vs. 21.9%). LVH prevalence was 6.5% in patients under 40 years-old (20.0% in males and 0% in females) and 61.6% in patients aged ≥ 40 years-old (93.8% in males and 36.6% in females). It was mostly symmetrical (75.5%). LV diastolic dysfunction occurred in 29.2%. LV ejection fraction was 66.6 ± 7.1%. In patients with LVH, mean LV mass was 145.0 ± 46.0 g/m^2, without gender difference, and LV diastolic dysfunction occurred in 69.4%, with a trend for a female predominance, and usually as an abnormal LV relaxation pattern (58.8%). Cardiac MRI was performed in 104 patients (86.7%) and 38 patients with LVH (77.6%). LGE was detected in 16 patients with LVH (42.1%), without gender dominance. Two females and one male presented LGE without LVH.

Atrioventricular block was detected in 18.3%. Complete atrioventricular block occurred in 5%, exclusively in males (12.8%). Bifascicular block was found in 13.4%, mostly in males (26.1% vs. 5.5%). Pacemaker was implanted in 7 patients (5.8%), mainly in males (12.8% vs. 1.4%), due to pre-syncope and bifascicular block (one male and one female), but the majority in the emergency setting of complete atrioventricular block. Atrial fibrillation was found in 5%. Non-sustained ventricular tachycardia (VT) was detected in 12.5%, with a trend for a male predominance. One male and one female had implanted cardioverter-defibrillators for primary prevention of sudden cardiac death due to severe LVH with diffuse LGE and frequent episodes of non-sustained VT. One male (0.8%) suffered a myocardial ischemic event attributed to FD.

In patients with LVH, the severity of cardiac manifestations was similar in both genders, except for a higher prevalence of heart failure in females (75.0% vs. 36.4%).

Extracardiac involvement by FD due to the p.F113L mutation was common. Albuminuria ≥ 30 mg/24 h was found in 36.1%, more commonly in males (55.3% vs. 23.6%). Although hypertension and diabetes were generally well controlled in this cohort, albuminuria ≥ 30 mg/24 h was also found in 24.1% of the normotensive non-diabetic patients, with a trend for a male predominance (35.7% vs. 17.6%,

Table 2
Clinical phenotype of patients with late-onset FD due to the p.F113L mutation.

Clinical manifestations	Fabry patients	Fabry males	Fabry females	p
	(n = 120)	(n = 47)	(n = 73)	
Age at Fabry disease diagnosis (years) (mean ± SD)	46 ± 18	47 ± 16	45 ± 19	0.273
α-GAL A activity (mean ± SD)				
Plasma (nmol/h/mL)	5.1 ± 4.7	0.53 ± 1.8	7.9 ± 3.6	< **0.001**
Leukocytes (nmol/h/mg)	17.2 ± 17.7	2.4 ± 4.6	26.5 ± 16.4	< **0.001**
Disease biomarkers (mean ± SD)				
Plasma GB3 (nmol/mL)	1.7 ± 3.4	1.9 ± 3.0	1.6 ± 3.6	0.473
Urinary GB3 (μg/mmol creatinine)	81.5 ± 166.7	166.3 ± 250.1	30.7 ± 26.0	< **0.001**
Plasma Lyso-GB3 (ng/mL)	-	8.0 ± 3.4	< 2	-
Cardiac manifestations				
Left ventricular hypertrophy (%)	49 (40.8%)	33 (70.2%)	16 (21.9%)	< **0.001**
IVS thickness (mm) (mean ± SD)	11.6 ± 4.4	13.8 ± 3.9	10.2 ± 4.1	< **0.001**
PW thickness (mm) (mean ± SD)	10.4 ± 3.5	12.3 ± 3.3	9.1 ± 3.0	< **0.001**
LV mass (g/m^2) (mean ± SD)	99.6 ± 49.2	127.9 ± 51.5	81.4 ± 38.0	< **0.001**
LV ejection fraction (%) (mean ± SD)	66.6 ± 7.1	67.2 ± 7.5	66.2 ± 6.8	0.27
LV diastolic dysfunction (%)	35 (29.2%)	20 (42.6%)	15 (20.5%)	**0.01**
Abnormal relaxation pattern (%)	21 (60.0%)	10 (50.0%)	11 (73.3%)	
Pseudonormal pattern (%)	13 (37.1%)	9 (45.0%)	4 (26.7%)	0.316
Restrictive pattern (%)	1 (2.9%)	1 (5.0%)	0 (0.0%)	
Late gadolinium enhancement (%)	19 (18.3%)	12 (30.0%)	7 (10.9%)	**0.014**
Heart failure (%)	24 (20.0%)	12 (25.5%)	12 (16.4%)	0.224
Atrial fibrillation (%)	6 (5.0%)	4 (8.5%)	2 (2.7%)	0.157
Atrial flutter (%)	2 (1.7%)	1 (2.1%)	1 (1.4%)	0.752
Non-sustained ventricular tachycardia (%)	15 (12.5%)	9 (19.1%)	6 (8.2%)	0.077
Atrioventricular block (%)	21 (18.3%)	12 (26.7%)	9 (12.9%)	0.061
1st degree (%)	14 (12.6%)	6 (14.6%)	8 (11.4%)	0.623
2nd degree (%)	6 (5.0%)	4 (8.5%)	2 (2.7%)	0.157
3rd degree (%)	6 (5.0%)	6 (12.8%)	0 (0.0%)	**0.002**
Right bundle branch block (%)	21 (18.6%)	16 (38.1%)	5 (7.0%)	< **0.001**
Left anterior fascicular block (%)	25 (22.3%)	18 (43.9%)	7 (9.9%)	< **0.001**
Left bundle branch block (%)	1 (0.9%)	0 (0.0%)	1 (1.4%)	0.457
Bifascicular block (%)	16 (13.4%)	12 (26.1%)	4 (5.5%)	**0.001**
Pacemaker (%)	7 (5.8%)	6 (12.8%)	1 (1.4%)	**0.009**
Implantable cardioverter-defibrillator (%)	2 (1.7%)	1 (2.1%)	1 (1.4%)	0.752
Myocardial ischemic events (%)	1 (0.8%)	1 (2.1%)	0 (0.0%)	0.211
Cardiac manifestations in patients with LVH				
IVS thickness (mm) (mean ± SD)	16.2 ± 3.0	15.9 ± 2.7	16.8 ± 3.6	0.443
PW thickness (mm) (mean ± SD)	13.7 ± 2.9	13.8 ± 2.6	13.5 ± 3.5	0.601
LV mass (g/m^2) (mean ± SD)	145.0 ± 46.0	149.2 ± 46.6	136.5 ± 45.0	0.348
LV diastolic dysfunction (%)	34 (69.4%)	20 (60.6%)	14 (87.5%)	0.055
Abnormal relaxation pattern (%)	20 (58.8%)	10 (50.0%)	10 (7.4%)	
Pseudonormal pattern (%)	13 (38.2%)	9 (45.0%)	4 (28.6%)	0.382
Restrictive pattern (%)	1 (2.9%)	1 (5.0%)	0 (0.0%)	
Late gadolinium enhancement (%)	16 (42.1%)	11 (40.7%)	5 (45.5%)	0.79
Heart failure (%)	24 (49.0%)	12 (36.4%)	12 (75.0%)	**0.011**
Atrial fibrillation (%)	6 (12.2%)	4 (12.1%)	2 (12.5%)	0.97
Atrial flutter (%)	2 (4.1%)	1 (3.0%)	1 (6.3%)	0.593
Non-sustained ventricular tachycardia (%)	14 (28.6%)	8 (24.2%)	6 (37.5%)	0.335
Atrioventricular block (%)	14 (30.4%)	10 (31.2%)	4 (28.6%)	0.856
1st degree (%)	9 (21.4%)	5 (17.9%)	4 (28.6%)	0.425
2nd degree (%)	2 (4.1%)	2 (6.1%)	0 (0.0%)	0.315
3rd degree (%)	6 (12.2%)	6 (18.2%)	0 (0.0%)	0.069
Right bundle branch block (%)	19 (43.2%)	15 (51.7%)	4 (26.7%)	0.112
Left anterior fascicular block (%)	22 (51.2%)	17 (60.7%)	5 (33.3%)	0.087
Left bundle branch block (%)	1 (2.4%)	0 (0.0%)	1 (6.7%)	0.183
Bifascicular block (%)	14 (29.2%)	11 (34.4%)	3 (18.8%)	0.262
Pacemaker (%)	7 (14.3%)	6 (18.2%)	1 (6.3%)	0.263
Implantable cardioverter-defibrillator (%)	2 (4.1%)	1 (3.0%)	1 (6.3%)	0.593
Myocardial ischemic events (%)	1 (2.0%)	1 (3.0%)	0 (0.0%)	0.482
Renal manifestations				
Albuminuria A2 (30-300mg/24 h) (%)	31 (26.1%)	20 (42.6%)	11 (15.3%)	**0.001**
Albuminuria A3 (> 300 mg/24 h) (%)	12 (10.1%)	6 (12.8%)	6 (8.3%)	0.432
Albuminuria A2 or A3 (≥ 30 mg/24 h) (%)	43 (36.1%)	26 (55.3%)	17 (23.6%)	< **0.001**
Chronic kidney disease stages				
G1 (eGFR ≥ 90 mL/min/1.73m^2) (%)	79 (66.4%)	26 (56.5%)	53 (72.6%)	0.071
G2 (eGFR 60-89 mL/min/1.73m^2) (%)	31 (26.1%)	16 (36.8%)	15 (20.5%)	0.085
G3a (eGFR 45-59 mL/min/1.73m^2) (%)	6 (5.0%)	3 (6.5%)	3 (4.1%)	0.558
G3b (eGFR 30-44 mL/min/1.73m^2) (%)	1 (0.8%)	0 (0.0%)	1 (1.4%)	0.425
G4 (eGFR 15-29 mL/min/1.73m^2) (%)	1 (0.8%)	1 (2.2%)	0 (0.0%)	0.206
G5 (eGFR < 15 mL/min/1.73 m^2) (%)	1 (0.8%)	0 (0.0%)	1 (1.4%)	0.425
Chronic kidney disease stage ≥ G3 (%)	9 (7.6%)	4 (8.7%)	5 (6.8%)	0.711

(*continued on next page*)

Table 2 (*continued*)

Clinical manifestations	Fabry patients	Fabry males	Fabry females	p
	(n = 120)	(n = 47)	(n = 73)	
Neurological and neuropsychiatric manifestations				
Stroke (%)	4 (3.3%)	2 (4.3%)	2 (2.7%)	0.652
Transient ischemic attack (%)	1 (0.8%)	0 (0.0%)	1 (1.4%)	0.42
Brain white matter lesions (%)	56 (54.4%)	21 (52.5%)	35 (55.6%)	0.762
Brain haemorrhage (%)	2 (1.7%)	2 (4.3%)	0 (0.0%)	0.076
Carpal tunnel syndrome (%)	25 (22.3%)	12 (26.7%)	13 (19.4%)	0.365
Acroparesthesias (%)	28 (23.3%)	3 (6.4%)	25 (34.2%)	**< 0.001**
Hypohidrosis (%)	1 (0.8%)	0 (0.0%)	1 (1.4%)	0.42
Cold, heat or exercise intolerance (%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	**-**
Depression (%)	26 (22.2%)	4 (8.7%)	22 (31.0%)	**0.005**
Anxiety (%)	32 (27.1%)	9 (19.6%)	23 (31.9%)	0.14
Eye manifestations				
Cornea *verticillata* (%)	16 (13.9%)	9 (20.0%)	7 (10.0%)	0.13
Cataracts (%)	14 (12.4%)	5 (11.4%)	9 (13.0%)	0.792
Retinal vessel tortuosity (%)	6 (5.3%)	2 (4.5%)	4 (5.8%)	0.772
Ear manifestations				
Sensorineural deafness (%)	49 (44.5%)	29 (64.4%)	20 (30.8%)	**< 0.001**
Tinnitus (%)	17 (15.2%)	10 (21.7%)	7 (10.6%)	0.106
Dermatological manifestations				
Angiokeratomas (%)	3 (2.6%)	0 (0.0%)	3 (4.3%)	0.156
Pulmonary manifestations				
Lung obstructive disease (%)	27 (26.5%)	14 (34.1%)	13 (21.3%)	0.15
Gastrointestinal manifestations (%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	–
Mainz score (mean ± SD)				
General score	1.4 ± 1.4	1.6 ± 1.5	1.2 ± 1.3	0.106
Neurologic score	2.0 ± 2.1	1.3 ± 1.7	2.5 ± 2.3	**0.003**
Cardiovascular score	5.9 ± 6.9	9.5 ± 6.8	3.6 ± 5.9	**< 0.001**
Renal score	0.9 ± 2.2	0.5 ± 1.3	1.2 ± 2.6	0.349
Total score	10.2 ± 8.9	13.0 ± 8.2	8.4 ± 8.8	**0.005**
FIPI score (mean ± SD)				
Cardiac score	1.5 ± 1.4	1.7 ± 1.4	1.3 ± 1.4	0.098
Renal score	1.5 ± 1.3	2.3 ± 1.0	1.1 ± 1.2	**< 0.001**
Neurologic score	1.9 ± 2.0	2.2 ± 2.1	1.8 ± 1.9	0.205
Death score	2.2 ± 2.4	2.2 ± 2.2	2.1 ± 2.5	0.575
Composite score	2.2 ± 1.9	2.8 ± 2.1	1.8 ± 1.7	**0.008**
Comorbidities				
Hypertension (%)	38 (31.7%)	19 (40.4%)	19 (26.0%)	0.098
Diabetes *mellitus* (%)	15 (12.5%)	8 (17.0%)	7 (9.6%)	0.229
Dyslipidaemia (%)	49 (40.8%)	24 (51.1%)	25 (34.2%)	0.067
Smoking (%)	13 (10.8%)	11 (23.4%)	2 (2.7%)	**< 0.001**

p: Males vs. Females.
Bold means statistically significant i.e. $p < 0.05$.
Valid percentages are shown.
eGFR, estimated glomerular filtration rate; IVS, interventricular septum; LV, left ventricular; LVH, left ventricular hypertrophy; PW, posterior wall.
Reference values: enzymatic activity of α-galactosidase A on plasma 6–19 nmol/h/mL, and on leukocytes 36–80 nmol/h/mg; urinary GB3 0.87–13 μg/mmol creatinine; plasma GB3 0.8–4.52 nmol/mL; plasma Lyso-GB3 0–1.9 ng/mL

p = 0.072). Renal insufficiency (chronic kidney disease stage 3–5, i.e., eGFR < 60 mL/min/1.73 m^2) was rare (7.6%) and there were no cases of renal dialysis or transplant.

Acroparesthesias were present in 23.3%, more commonly in females (34.2% vs. 6.4%). However, heat, cold or exercise intolerance were not found. Carpal tunnel syndrome was diagnosed in 22.3%, without gender dominance, and in 34.6% of the patients with acroparesthesias. In patients with acroparesthesias and concomitant carpal tunnel syndrome, acroparesthesias were exclusively located in the median nerve territory of the hands. Surgery was performed in 8.0% of the patients with carpal tunnel syndrome (one male and one female) with resolution of acroparesthesias. The acroparesthesias of the patients without carpal tunnel syndrome were predominantly mild.

Stroke was a rare event (3.3%) that occurred in a 42-year old male with mild dyslipidemia and history of smoking; a 51-year old male with diabetes, hypertension, dyslipidemia and atrial fibrillation; a 41-year old female with controlled diabetes; and a 71-year old female with controlled hypertension. Transient ischemic attack occurred in a 78-year old female with controlled hypertension and dyslipidemia (0.8%). Brain MRI was performed in 103 patients (85.8%), revealing common brain white matter lesions (WML) (54.4%) in both genders. Although cardiovascular risk factors were generally well controlled in this cohort, brain WML were also present in 44.3% of the patients without these risk factors, again without gender predominance. Depression was diagnosed in 22.2%, more commonly in females, and anxiety disorder in 27.1%, without gender predominance.

Sensorineural deafness was common (44.5%), particularly in males (64.4% vs. 30.8%). Cornea *verticillata* was found in 13.9%. Angiokeratomas were rare (2.6%) and solitary. Gastrointestinal manifestations were absent. Lung obstructive disease was diagnosed in 26.5%, as mild (88.5%) or moderate (11.5%).

Plasma lyso-GB3 was available in 34 males and 63 females, being increased in all males (mean 8.0 ± 3.4, ranging from 2.6 to 17.6 ng/mL) and below the lower limit of quantitation (2 ng/mL) in all females (Table 2).

Most clinical manifestations are more common in patients aged ≥40 years (LVH, diastolic dysfunction, LGE, heart failure, atrioventricular block, right bundle branch block, left anterior fascicular bock, bifascicular block, non-sustained VT, albuminuria > 30 mg/24 h, chronic kidney disease ≥G3, brain WML, sensorineural deafness,

cornea *verticillata*) and, for most of them, both in males and females. Likewise, patients aged ≥ 40 years present higher LV wall thickness and mass and Mainz and FIPI scores (Supplementary Table 3).

4. Discussion

4.1. Founder effect of FD in the Portuguese region of Guimarães

The prevalence of FD in patients with HCM has widely ranged between 0 and 12% in previous studies, essentially due to different inclusion criteria, study designs and screening methods and the common finding of variants of unknown significance in the *GLA* gene [16,17]. In our study, the inclusion criteria were based on the ESC definition of HCM and the baseline characteristics of patients with HCM reflected the literature [10]. Our study included both males and females and used a combined enzymatic and genetic approach that allows an accurate diagnosis of FD in both genders [18]. Genetic variants of unknown significance were not found and all the reported *GLA* gene variants were already described as pathogenic mutations [4,19,20].

The unexpectedly high prevalence of FD among patients with HCM was therefore justified by a founder effect of the disease in the Portuguese region of Guimarães. If we considered the 21 p.F113L families as the same large family, the prevalence of FD among patients with HCM would be 3.3%, which is within the prevalence range of 0–4% of the latest studies [16,21].

Our study provides a detailed genealogical documentation of the founder effect of FD in the Portuguese region of Guimarães. Since the Council of Trent (1545–1563), the Catholic Church made birth and marriage registration obligatory [22], so Parish records constitute a valuable source of genealogical data in Portugal. Nevertheless, it was not possible to find the connection to the genealogical tree of four of the 21 p.F113L families. In the 18th century the paternity registration became non-obligatory, increasing the number of registered births with unknown paternity, which constitute an obstacle to the genealogical research [22]. Still, haplotype analysis came to support the genealogical evidence of this founder effect.

In humans, a founder effect may occur in stable populations with high rate of endogamy and low migration rate. In this countryside region of Guimarães, in the 17th century, marriages occurred within the same socioeconomic stratum. The first couple of this genealogical tree was a stable land contract owner, but the descendants that transmitted the disease did not inherit this property and therefore married with lower social status families. There was a progressive impoverishment of these families until the 19th century, leading individuals to search for temporary work that was warranted in nearby farmers houses, avoiding the need of distant migration. Although worldwide emigration was common in Portugal, it was not generally accessible, until the 19th century, to poor individuals. These socioeconomic factors perpetuated the disease transmission in this region until the contemporary era [23]. More recently, with the generalization of migration, this founder mutation has been spread to many countries around the world.

The documentation of a founder effect of FD in a geographical region may justify specific health policies aiming genetic counselling and earlier diagnosis and treatment, as therapy might delay disease complications [1].

4.2. Late-onset phenotype of FD due to the p F113L mutation

In this late-onset phenotype of FD, LVH remained the main cardiac manifestation, affecting 40.8% of patients. In the cardiac predominant phenotype due to the IVS4 mutation, LVH was reported in 25%, namely in 2.1% of patients under 40 years-old (4% of males and 1.6% of females) and 44.4% of patients aged ≥ 40 years-old (67.4% of males and 32.8% of females). However, the mean/median age of the patients included in the statistical analysis was not reported [24]. In a study including 84 patients with the p.N215S mutation (37 males and 47 females with mean age at diagnosis 51 ± 19 and 38 ± 16 years, respectively), LVH was also reported in 25% [17 males (46%) and 4 females (8.5%)] [25], which suggests a lower prevalence of LVH compared to the p.F113L mutation, particularly if we take into account the higher mean age at diagnosis of males and the lower female to male ratio (1.3). However, like in the study by Hsu et al. [24], LVH was defined based on LV mass indexed to height (not LV wall thickness). If we consider only the patients for whom LV mass index was available, LVH prevalence in p.N215S patients reaches 29.6% [25].

In 26 Fabry patients, half of whom with LVH, Moon et al. found LGE in 50% of the cases, but their phenotypes were not reported [26]. In 100 patients with the IVS4 mutation, 28% of whom with LVH, LGE was reported in 38% of patients and 61% of patients with LVH [24]. In contrast, our study found LGE in 18.3% of the patients and 42.1% of the patients with LVH, thus suggesting a lower frequency or a later development of LGE in the late-onset phenotype due to the p.F113L mutation.

Our study reports for the first time the actual prevalence of each rhythm and conduction disorder in a cardiac predominant late-onset phenotype of FD. Cardiac conduction disorders carried a significant impact on prognosis of patients with the p.F113L mutation, potentially leading to the implantation of a pacemaker in a life-threatening situation. However, no cases of sustained VT or sudden cardiac death due to ventricular dysrhythmias were found, thus suggesting a higher prognostic impact of bradydysrhythmias compared to tachydysrhythmias. Moreover, there was a low rate of cardioverter-defibrillators implantation for primary prevention of sudden cardiac death. Acharya et al., in a small study of 19 patients, with presumably both phenotypes, also reported a low incidence of clinically significant ventricular arrhythmias during a mean follow-up of 53 months, with only one patient suffering ventricular fibrillation in the setting of multiorgan failure [27]. However, in 16 patients with advanced Fabry cardiomyopathy, characterized by severe LVH and LGE, who received implantable loop recorders, Weidemann et al. found five episodes of VT (three sustained and two non-sustained) in four male patients (25%) [28], suggesting a much higher risk of ventricular dysrhythmias and sudden death. In our study, patients did not receive implantable loop recorders, so the real prevalence of VT may be underestimated.

Although the cardiac manifestations carried the highest prognostic impact, the extracardiac manifestations were also common. Albuminuria ≥ 30 mg/24 h, brain WML and sensorineural deafness were, at least, as common as LVH. The finding of albuminuria ≥ 30 mg/24 h and brain WML in patients without cardiovascular risk factors supports the conviction that they are indeed secondary to FD. Hearing impairment was found in only 3/12 (25%) males and 1/7 (14%) females with the p.N215S mutation [29], but these results could have been limited by a small sample size. The high prevalence of sensorineural deafness in our cohort highlights the importance of a systematic otorhinolaryngology evaluation in late-onset phenotypes.

The prognostic impact of extracardiac manifestations, however, seems to be lower, as no cases of renal dialysis or transplant were found, cerebrovascular events were rare and sensorineural deafness was limited to high frequencies in most patients. Moreover, despite increased albuminuria, chronic kidney disease stage 3–5 occurred in only 7.6%, being worthy to note that chronic kidney disease stage 3–5 has been described in 6.1% of the Portuguese adults aged 20–79 years [30]. Finally, the clinical significance of brain WML, namely in terms of cognitive function and neuropsychiatric manifestations, remains to be elucidated [31]. Neuropsychiatric manifestations, such as depression and anxiety disorder, were also common, but it remains to be established to what extent these are due to FD pathogenesis, or just a reaction to the disease burden [31].

To our knowledge, this study is the first to report the prevalence of carpal tunnel syndrome in a late-onset phenotype. Acroparesthesias were reported in 21.6% of males and 14.9% of females with the p.N215S mutation, but no data was reported on the prevalence of carpal

tunnel syndrome or the characteristics of these acroparesthesias [25]. In our cohort, 23.3% of the patients reported acroparesthesias, but these were attributed to carpal tunnel syndrome in 34.6% of the cases. As endothelial GB3 storage is characteristically absent in late-onset phenotypes of FD [32], carpal tunnel syndrome may be caused by GB3 storage on fibroblasts and subsequent increase of extracellular matrix within the carpal tunnel [33]. Repetitive wrist movement related to the high prevalence of factory professionals in our cohort may also have caused or aggravated a FD-related carpal tunnel syndrome. The acroparesthesias of the patients without carpal tunnel syndrome were predominantly mild and non-disabling, unlike the ones typically seen in the small-fiber neuropathy of classical phenotypes, and, given the unspecific nature of acroparesthesias and the high prevalence of factory professionals in our cohort, are probably not related to FD.

Our study also shows that, despite its low prevalence, cornea *verticillata* may occur in both genders with late-onset phenotypes. The angiokeratomas found in females with the p.F113L mutation were rare and solitary and it is uncertain if they are related to FD.

In our cohort, plasma lyso-GB3 values were in agreement with the values reported in the late-onset phenotypes [34], including the cardiac predominant phenotypes due to the p.N215S [25] or the IVS4 mutation [35]. In females, plasma lyso-GB3 was always below the lower limit of quantification, independently of the development of clinical manifestations, being therefore a poor biomarker for predicting the risk of complications in females with the p.F113L mutation.

Compared to previous studies on predominant cardiac phenotypes, our study presents key features that deserve to be highlighted. First, it represents a large cohort of genetically related adult patients that share common environmental factors, due to a demonstrated founder effect, thus allowing an accurate appreciation of FD clinical heterogeneity. Second, this cohort results from an extensive family screening, reflected on a female to male ratio of 1.6, which significantly reduces the bias related to the underrepresentation of females generally observed in previous studies. Third, in this study, patients were systematically submitted to a predefined multidisciplinary diagnostic protocol at baseline, thereby overcoming the bias related to registries or retrospective studies and providing a detailed clinical characterization of cardiac as well as extracardiac manifestations, taking also into account the influence of comorbidities on the disease phenotype.

As limitations of this study, we point out that it did not include the comparison between Fabry patients and age-matched controls to account the effect of age on the development of clinical manifestations, such as sensorineural deafness or brain WML. Also, this study did not include the longitudinal follow-up of these patients. Further longitudinal studies are necessary to better define the natural history and to accurately evaluate the effect of current and future therapies on FD late-onset phenotypes.

In summary, our study on the founder mutation p.F113L in the Portuguese region of Guimarães provides unique information on the clinical profile of the cardiac predominant late-onset phenotypes of FD and demonstrates that the designation of “cardiac variant” underestimates its systemic involvement. Considering the broad dissemination of Fabry patients with the p.F113L mutation around the world, the characterization of this late-onset phenotype has a cross-border impact in the therapeutic decisions of many patients. Longitudinal studies of this and other large cohorts will contribute to achieve more evidence-based clinical decisions and guidelines on FD late-onset phenotypes.

Funding

This research was supported by a research grant from Shire Human Genetic Therapies, Inc.

Shire Human Genetic Therapies, Inc. had no influence on the study design/conduct, data collection/analysis/interpretation or manuscript preparation/review/approval.

Disclosures

Olga Azevedo, Andreas Gal and Gabriel Miltenberger-Miltenyi have received educational and/or research grants from Shire Human Genetic Therapies, Amicus and Sanofi Genzyme. Paulo Gaspar and Jorge Rodrigues have received educational grants from Shire Human Genetic Therapies. Miguel Gago has received educational grants from Shire Human Genetic Therapies and Sanofi Genzyme. Maria José Guimarães has received educational and research grants from Sanofi Genzyme. The remaining authors have no disclosures.

Acknowledgements

The authors thank to the Cardiology Department of Hospital Senhora da Oliveira – Guimarães, particularly to Dr. Francisco Sousa and Dr. António Lourenço, for their important contribution to the patient recruitment into this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymgme.2019.07.012.

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SUPPLEMENTARY DATA

Supplementary Table 1. Position of the tested loci, both on the cytogenetic and physical map of the X chromosome.

Marker	Chromosomal Position	Physical Position (Mb)
DXS990	Xq21.32	93
DXS8020	Xq22.1	99.6
GLA	Xq22.1	100.6
DXS8096	Xq22.2	102.8
DXS1191	Xq22.2	103.3

Supplementary Table 2. Baseline characteristics of patients with HCM

BASELINE CHARACTERISTICS	PATIENTS WITH HCM (n=150)
Gender: Males / Females (%)	94 (62.7%) / 56 (37.3%)
Age (years) (mean ± SD)	64.4 ± 14.1
Family history of LVH (%)	44 (39.6%)
Family history of LVH not excluding X-linked pattern	37 (84.1%)
Symptoms (%)	129 (87.2%)
Dyspnoea (%)	109 (73.6%)
Chest pain (%)	45 (30.4%)
Palpitations (%)	31 (20.9%)
Pre-syncope (%)	26 (18.2%)
Syncope (%)	15 (10.1%)
NYHA class I / II / III /IV (%)	50 (45.9%) / 55 (50.5%) / 4 (3.7%) / -
Echocardiogram (n=150)	
LVH pattern	
Asymmetrical (%)	86 (57.3%)
Symmetrical (%)	52 (34.7%)
Apical (%)	10 (6.7%)
Other (%)	2 (1.3%)
LV outflow tract obstruction at rest (%)	32 (21.6%)
Interventricular septum thickness (mm) (mean ± SD)	18.0 ± 4.0
Posterior wall thickness (mm) (mean ± SD)	12.8 ± 3.1
LV mass (g/m2) (mean ± SD)	165.2 ± 56.1
LV ejection fraction (%) (mean ± SD)	67.3 ± 9.1
LV Diastolic dysfunction (%)	131 (89.7%)
Grade I / II / III (%)	73 (56.6%) / 47 (36.4%) / 9 (7.0%)
Septal Ea (cm/s) (mean ± SD)	5.0 ± 1.8
Lateral Ea (cm/s) (mean ± SD)	7.0 ± 2.7
LA volume (mL/m2) (mean ± SD)	38.7 ± 16.1
Cardiac MRI (n=80)	
Late gadolinium enhancement (%)	59 (73.8%)
Electrocardiogram (n=150)	
Sinus rhythm (%)	132 (88.0%)
Atrial fibrillation (%)	11 (7.3%)
Atrial flutter (%)	4 (2.7%)
Pacemaker rhythm (%)	7 (5.3%)
Short PR interval (%)	4 (2.8%)
Atrioventricular block (%)	20 (14.0%)
Left bundle branch block (%)	13 (9.1%)
Right bundle branch block (%)	7 (4.9%)
Bifascicular block (%)	16 (11.2%)
T wave inversion (%)	73 (53.3%)
ST segment depression (%)	55 (40.9%)
24h Holter (n=108)	
Sinus rhythm (%)	97 (89.8%)
Atrial fibrillation (%)	11 (10.2%)
Atrial flutter (%)	2 (1.9%)
Pacemaker rhythm (%)	6 (5.6%)
Atrioventricular block (%)	14 (13.3%)
Left bundle branch block (%)	8 (7.6%)
Right bundle branch block (%)	3 (2.9%)
Bifascicular block (%)	13 (12.4%)
Supraventricular tachycardia (%)	63 (58.3%)
Non-sustained ventricular tachycardia (%)	28 (25.9%)

Devices	
Pacemaker (%)	14 (9.3%)
Implantable cardioverter-defibrillator (%)	7 (4.7%)
Diagnostic red-flags for Fabry disease	
Stroke (%)	20 (13.4%)
Renal failure (%)	29 (19.6%)
Microalbuminuria >30mg/24h (%)	38 (36.5%)
Proteinuria >300mg/24h (%)	20 (19.2%)
Other comorbidities	
Hypertension (%)	107 (71.3%)
Diabetes mellitus (%)	34 (22.7%)
Dyslipidemia (%)	86 (57.3%)
Obesity (%)	28 (18.7%)
Smoking (%)	20 (13.3%)
Coronary heart disease (%)	13 (8.7%)

Valid percentages are shown

Supplementary Table 3. Clinical phenotype of patients with late-onset FD due to the p.F113L mutation, stratified by gender and age group

CLINICAL MANIFESTATIONS	FABRY PATIENTS (n=120)			FABRY MALES (n=47)			FABRY FEMALES (n=73)			*p4* (M vs. F <40y)	*p5* (M vs. F ≥40y)
	<40 years (n=46)	≥ 40 years (n=74)	*p1*	<40 years (n=15)	≥ 40 years (n=32)	*p2*	<40 years (n=31)	≥ 40 years (n=42)	*p3*		
Age at Fabry disease diagnosis (years) (mean ± SD)	28±8	57±11	**<0.001**	27±9	57±9	**<0.001**	28±9	57±13	**<0.001**	0.972	0.789
α-GAL A activity (mean ± SD)											
Plasma (nmol/h/mL)	5.7±4.7	4.8±4.7	0.435	0.5±1.6	0.5±1.9	0.475	7.7±3.8	8.1±3.4	0.570	**<0.001**	**<0.001**
Leukocytes (nmol/h/mg)	16.7±16.5	17.5±18.4	0.832	1.5±0.5	2.7±5.4	0.811	23.1±15.8	28.8±16.6	0.122	**<0.001**	**<0.001**
Disease biomarkers (mean ± SD)											
Plasma GB3 (nmol/mL)	2.4±3.9	1.3±2.9	0.129	1.8±3.2	1.9±3.0	1.000	2.6±4.2	0.8±2.7	**0.032**	0.652	0.066
Urinary GB3 (μg/mmol creatinine)	52.9±62.5	97.9±202.5	0.119	116.1±87.3	185.1±288.1	0.486	28.2±21.5	32.5±29.0	0.739	**<0.001**	**<0.001**
Plasma Lyso-GB3 (ng/mL)	-	-	-	6.9±2.4	8.5±3.7	0.143	-	-	-	-	-
Cardiac manifestations											
Left ventricular hypertrophy (%)	3 (6.5%)	46 (62.2%)	**<0.001**	3 (20.0%)	30 (93.8%)	**<0.001**	0 (0.0%)	16 (38.1%)	**<0.001**	**0.010**	**<0.001**
IVS thickness (mm) (mean ± SD)	8.6±2.2	13.5±4.3	**<0.001**	10.4±2.8	15.4±3.4	**<0.001**	7.7±1.2	12.1±4.5	**<0.001**	**<0.001**	**<0.001**
PW thickness (mm) (mean ± SD)	8.1±1.6	11.8±3.6	**<0.001**	9.5±1.7	13.5±3.1	**<0.001**	7.4±1.0	10.5±3.4	**<0.001**	**<0.001**	**<0.001**
LV mass (g/m^2) (mean ± SD)	70.0±19.4	118.0±53.0	**<0.001**	88.9±19.7	146.1±51.8	**<0.001**	60.9±10.9	96.6±43.5	**<0.001**	**<0.001**	**<0.001**
LV ejection fraction (%) (mean ± SD)	65.1±5.7	67.5±7.6	**0.017**	66.1±6.4	67.7±7.9	0.154	64.6±5.4	67.4±7.5	0.079	0.632	0.624
LV diastolic dysfunction (%)	1 (2.2%)	34 (45.9%)	**<0.001**	1 (6.7%)	19 (59.4%)	**0.001**	0 (0.0%)	15 (31.7%)	**<0.001**	0.144	**0.043**
Abnormal relaxation pattern (%)	0 (0.0%)	21 (61.8%)		0 (0.0%)	10 (52.6%)		-	11 (73.3%)			
Pseudonormal pattern (%)	1 (100.0%)	12 (35.3%)	0.419	1 (100.0%)	8 (42.1%)	0.526	-	4 (26.7%)	-	-	0.380
Restrictive pattern (%)	0 (0.0%)	1 (2.9%)		0 (0.0%)	1 (5.3%)		-	0 (0.0%)			
Late gadolinium enhancement (%)	1 (2.2%)	18 (29.0%)	**0.001**	1 (7.1%)	11 (42.3%)	**0.021**	0 (0.0%)	7 (19.4%)	**0.013**	0.152	0.050
Heart failure (%)	1 (2.2%)	23 (31.1%)	**<0.001**	1 (6.7%)	11 (34.4%)	**0.042**	0 (0.0%)	12 (28.6%)	**0.001**	0.146	0.593
Atrial fibrillation (%)	0 (0.0%)	6 (8.1%)	**0.048**	0 (0.0%)	4 (12.5%)	0.152	0 (0.0%)	2 (4.8%)	0.218	-	0.227
Atrial flutter (%)	0 (0.0%)	2 (2.7%)	0.261	0 (0.0%)	1 (3.1%)	0.489	0 (0.0%)	1 (2.4%)	0.387	-	0.845
Non-sustained ventricular tachycardia (%)	1 (2.2%)	14 (18.9%)	**0.007**	1 (6.7%)	8 (25.0%)	0.136	0 (0.0%)	6 (14.3%)	**0.028**	0.146	0.244
Atrioventricular block (%)	4 (8.7%)	17 (24.3%)	**0.037**	2 (13.3%)	10 (32.3%)	0.207	2 (6.5%)	7 (17.9%)	0.153	0.393	0.165
1st degree (%)	2 (4.4%)	12 (18.2%)	**0.032**	1 (7.1%)	5 (18.5%)	0.328	1 (3.2%)	7 (17.9%)	0.054	0.555	0.953
2nd degree (%)	3 (6.5%)	3 (4.1%)	0.546	2 (13.3%)	2 (6.3%)	0.417	1 (3.2%)	1 (2.4%)	0.827	0.193	0.403
3rd degree (%)	0 (0.0%)	6 (8.1%)	**0.048**	0 (0.0%)	6 (18.8%)	0.073	0 (0.0%)	0 (0.0%)	-	-	**0.003**
Right bundle branch block (%)	0 (0.0%)	21 (30.9%)	**<0.001**	0 (0.0%)	16 (57.1%)	**<0.001**	0 (0.0%)	5 (12.5%)	**0.041**	-	**<0.001**
Left anterior fascicular block (%)	0 (0.0%)	25 (37.3%)	**<0.001**	0 (0.0%)	18 (66.7%)	**<0.001**	0 (0.0%)	7 (17.5%)	**0.014**	-	**<0.001**
Left bundle branch block (%)	0 (0.0%)	1 (1.5%)	0.403	0 (0.0%)	0 (0.0%)	-	0 (0.0%)	1 (2.5%)	0.375	-	0.426
Bifascicular block (%)	0 (0.0%)	16 (21.9%)	**0.001**	0 (0.0%)	12 (38.7%)	**0.005**	0 (0.0%)	4 (9.5%)	0.077	-	**0.003**
Pacemaker (%)	0 (0.0%)	7 (9.5%)	**0.032**	0 (0.0%)	6 (18.8%)	0.073	0 (0.0%)	1 (2.4%)	0.387	-	**0.017**
Implanted cardio-defibrillator (%)	0 (0.0%)	2 (2.7%)	0.261	0 (0.0%)	1 (3.1%)	0.489	0 (0.0%)	1 (2.4%)	0.387	-	0.845
Coronary heart disease (%)	0 (0.0%)	5 (6.8%)	0.072	0 (0.0%)	5 (15.6%)	0.105	0 (0.0%)	0 (0.0%)	-	-	**0.008**
Cardiac manifestations in patients with LVH											
IVS thickness (mm) (mean ± SD)	15.4±2.3	16.3±3.1	0.768	15.4±2.3	16.0±2.7	0.883	-	16.8±3.6	-	-	0.475
PW thickness (mm) (mean ± SD)	12.4±0.8	13.8±3.0	0.343	12.4±0.8	13.9±2.7	0.188	-	13.5±3.5	-	-	0.541
LV mass (g/m^2) (mean ± SD)	117.4±29.9	146.8±46.6	0.265	117.4±29.9	152.4±47.2	0.188	-	136.5±45.0	-	-	0.249
LV diastolic dysfunction (%)	1 (33.3%)	33 (71.7%)	0.162	1 (33.3%)	19 (63.3%)	0.311	-	14 (87.5%)	-	-	0.083

Abnormal relaxation pattern (%)	0 (0.0%)	20 (60.6%)		0 (0.0%)	10 (52.6%)		-	10 (71.4%)	-		
Pseudonormal pattern (%)	1 (100.0%)	12 (36.4%)	0.435	1 (100.0%)	8 (42.1%)	0.526	-	4 (28.6%)	-	-	0.446
Restrictive pattern (%)	0 (0.0%)	1 (3.0%)		0 (0.0%)	1 (5.3%)		-	0 (0.0%)	-		
Late gadolinium enhancement (%)	1 (33.3%)	15 (42.9%)	0.748	1 (33.3%)	10 (41.7%)	0.782	-	5 (45.5%)	-	-	0.833
Heart failure (%)	1 (33.3%)	23 (50.0%)	0.576	1 (33.3%)	11 (36.7%)	0.909	-	12 (75.0%)	-	-	**0.013**
Atrial fibrillation (%)	0 (0.0%)	6 (13.0%)	0.504	0 (0.0%)	4 (13.3%)	0.500	-	2 (12.5%)	-	-	0.936
Atrial flutter (%)	0 (0.0%)	2 (4.3%)	0.712	0 (0.0%)	1 (3.3%)	0.748	-	1 (6.3%)	-	-	0.644
Non-sustained ventricular tachycardia (%)	1 (33.3%)	13 (28.3%)	0.851	1(33.3%)	7 (23.3%)	0.700	-	6 (37.5%)	-	-	0.309
Atrioventricular block (%)	0 (0.0%)	14 (32.6%)	0.236	0 (0.0%)	10 (34.5%)	0.220	-	4 (28.6%)	-	-	0.698
1st degree (%)	0 (0.0%)	9 (23.1%)	0.348	0 (0.0%)	5 (20.0%)	0.393	-	4 (28.6%)	-	-	0.542
2nd degree (%)	0 (0.0%)	2 (4.3%)	0.712	0 (0.0%)	2 (6.7%)	0.645	-	0 (0.0%)	-	-	0.291
3rd degree (%)	0 (0.0%)	6 (13.0%)	0.504	0 (0.0%)	6 (20.0%)	0.392	-	0 (0.0%)	-	-	0.055
Right bundle branch block (%)	0 (0.0%)	19 (46.3%)	0.118	0 (0.0%)	15 (57.7%)	0.058	-	4 (26.7%)	-	-	0.055
Left anterior fascicular block (%)	0 (0.0%)	22 (55.0%)	0.066	0 (0.0%)	17 (68.0%)	**0.023**	-	5 (33.3%)	-	-	**0.032**
Left bundle branch block (%)	0 (0.0%)	1 (2.6%)	0.776	0 (0.0%)	0 (0.0%)	-	-	1 (6.7%)	-	-	0.210
Bifascicular block (%)	0 (0.0%)	14 (31.1%)	0.251	0 (0.0%)	11 (37.9%)	0.188	-	3 (18.8%)	-	-	0.183
Pacemaker (%)	0 (0.0%)	7 (15.2%)	0.466	0 (0.0%)	6 (20.0%)	0.392	-	1 (6.3%)	-	-	0.216
Implanted cardio-defibrillator (%)	0 (0.0%)	2 (4.3%)	0.712	0 (0.0%)	1 (3.3%)	0.748	-	1 (6.3%)	-	-	0.644
Coronary heart disease (%)	0 (0.0%)	5 (10.9%)	0.547	0 (0.0%)	5 (16.7%)	0.443	-	0 (0.0%)	-	-	0.084
Renal Manifestations											
Albuminuria A2 (30-300mg/24h) (%)	8 (17.8%)	23 (31.1%)	0.109	5 (33.3%)	15 (46.9%)	0.381	3 (10.0%)	8 (9.0%)	0.293	0.054	**0.010**
Albuminuria A3 (>300mg/24h) (%)	2 (4.4%)	10 (13.5%)	0.111	0 (0.0%)	6 (18.8%)	0.073	2 (6.7%)	4 (9.5%)	0.665	0.306	0.250
Albuminuria A2 or A3 (≥30mg/24h) (%)	10 (22.2%)	33 (44.6%)	**0.014**	5 (33.3%)	21 (65.6%)	**0.038**	5 (16.7%)	12 (28.6%)	0.241	0.205	**0.001**
Chronic kidney disease stages											
G1 (eGFR ≥90mL/min/1.73m^2) (%)	42 (91.3%)	37 (50.7%)	**<0.001**	13 (86.7%)	13 (41.9%)	**0.004**	29 (93.5%)	24 (57.1%)	**0.001**	0.437	0.199
G2 (eGFR 60-89mL/min/1.73m^2) (%)	4 (8.7%)	27 (37.0%)	**0.001**	2 (13.3%)	14 (45.2%)	**0.034**	2 (6.5%)	13 (31.0%)	**0.010**	0.437	0.214
G3a (eGFR 45-59mL/min/1.73m^2) (%)	0 (0.0%)	6 (8.2%)	**0.046**	0 (0.0%)	3 (9.7%)	0.213	0 (0.0%)	3 (7.1%)	0.129	-	0.697
G3b (eGFR 30-44mL/min/1.73m^2) (%)	0 (0.0%)	1 (1.4%)	0.425	0 (0.0%)	0 (0.0%)	-	0 (0.0%)	1 (2.4%)	0.389	-	0.387
G4 (eGFR 15-29mL/min/1.73m^2) (%)	0 (0.0%)	1 (1.4%)	0.425	0 (0.0%)	1 (3.2%)	0.482	0 (0.0%)	0 (0.0%)	-	-	0.241
G5 (eGFR <15mL/min/1.73m^2) (%)	0 (0.0%)	1 (1.4%)	0.425	0 (0.0%)	0 (0.0%)	-	0 (0.0%)	1 (2.4%)	0.387	-	0.387
Chronic kidney disease stage ≥ G3 (%)	0 (0.0%)	9 (12.3%)	**0.013**	0 (0.0%)	4 (12.9%)	0.145	0 (0.0%)	5 (11.9%)	**0.047**	-	0.898
Neurological and neuropsychiatric manifestations											
Stroke (%)	0 (0.0%)	4 (5.4%)	0.109	0 (0.0%)	2 (6.3%)	0.322	0 (0.0%)	2 (4.8%)	0.218	-	0.779
Transient ischemic attack (%)	0 (0.0%)	1 (1.4%)	0.429	0 (0.0%)	0 (0.0%)	-	0 (0.0%)	1 (2.4%)	0.387	-	0.379
Brain white matter lesions (%)	14 (33.3%)	42 (68.9%)	**<0.001**	3 (21.4%)	18 (61.2%)	**0.004**	11 (39.3%)	24 (68.6%)	**0.020**	0.247	0.956
Brain haemorrhage (%)	0 (0.0%)	2 (2.7%)	0.261	0 (0.0%)	2 (6.3%)	0.322	0 (0.0%)	0 (0.0%)	-	-	0.100
Carpal tunnel syndrome (%)	1 (2.4%)	24 (33.8%)	**<0.001**	1 (7.7%)	11 (34.4%)	0.067	0 (0.0%)	13 (33.3%)	**0.001**	0.137	0.926
Acroparesthesias (%)	10 (21.7%)	18 (24.3%)	0.745	0 (0.0%)	3 (9.4%)	0.220	10 (32.3%)	15 (35.7%)	0.758	**0.013**	**0.009**
Hypohidrosis (%)	1 (2.2%)	0 (0.0%)	0.203	0 (0.0%)	0 (0.0%)	-	1 (3.2%)	0 (0.0%)	0.241	0.482	-
Cold, heat or exercise intolerance (%)	0 (0.0%)	0 (0.0%)	-	0 (0.0%)	0 (0.0%)	-	0 (0.0%)	0 (0.0%)	-	-	-
Depression (%)	7 (15.2%)	19 (26.8%)	0.142	0 (0.0%)	4 (12.9%)	0.145	7 (22.6%)	15 (37.5%)	0.178	**0.046**	**0.020**
Anxiety (%)	10 (21.7%)	22 (30.6%)	0.293	2 (13.3%)	7 (22.6%)	0.459	8 (25.8%)	15 (36.6%)	0.331	0.336	0.201
Eye manifestations											
Cornea *verticillata* (%)	1 (2.2%)	15 (21.4%)	**0.004**	0 (0.0%)	9 (30.0%)	**0.018**	1 (3.3%)	6 (15.0%)	0.107	0.475	0.130
Cataracts (%)	0 (0.0%)	14 (20.6%)	**0.001**	0 (0.0%)	5 (17.2%)	0.088	0 (0.0%)	9 (23.1%)	**0.005**	-	0.556
Retinal vessel tortuosity (%)	2 (4.4%)	4 (5.9%)	0.739	0 (0.0%)	2 (6.9%)	0.298	2 (6.7%)	2 (5.1%)	0.786	0.306	0.759

Ear manifestations											
Sensorineural deafness (%)	3 (7.5%)	46 (65.7%)	**<0.001**	1 (7.7%)	28 (87.5%)	**<0.001**	2 (7.4%)	18 (47.4%)	**0.001**	0.974	**<0.001**
Tinnitus (%)	4 (9.5%)	13 (18.6%)	0.196	3 (21.9%)	7 (21.9%)	0.973	1 (3.6%)	6 (15.8%)	0.111	0.063	0.514
Dermatological manifestations											
Angiokeratomas (%)	1 (2.3%)	2 (2.9%)	0.849	0 (0.0%)	0 (0.0%)	-	1 (3.3%)	2 (5.1%)	0.717	0.490	0.201
Pulmonary manifestations											
Lung obstructive disease (%)	2 (5.3%)	25 (39.1%)	**<0.001**	1(8.3%)	13 (44.8%)	**0.025**	1 (3.8%)	12 (34.3%)	**0.004**	0.565	0.390
Gastrointestinal manifestations (%)	0 (0.0%)	0 (0.0%)	-	0 (0.0%)	0 (0.0%)	-	0 (0.0%)	0 (0.0%)	-	-	-
Mainz score (mean ± SD)											
General score	0.6±0.7	1.9±1.5	**<0.001**	0.5±0.6	2.2±1.5	**<0.001**	0.7±0.8	1.7±1.5	**0.003**	0.774	0.144
Neurologic score	1.4±2.0	2.4±2.2	**0.001**	0.3±0.6	1.8±1.8	**0.002**	1.9±2.2	2.9±2.3	**0.029**	**0.042**	**0.011**
Cardiovascular score	1.3±3.2	8.8±7.0	**<0.001**	2.3±4.6	12.9±4.0	**<0.001**	0.8±2.1	5.6±6.9	**<0.001**	0.682	**<0.001**
Renal score	0.5±1.4	1.1±2.6	0.390	0.5±1.4	0.5±1.3	0.937	0.5±1.4	1.6±3.2	0.231	0.968	0.210
Total score	3.8±4.8	14.2±8.5	**<0.001**	3.7±5.4	17.4±5.0	**<0.001**	3.8±4.5	11.8±9.7	**<0.001**	0.481	**0.002**
FIPI score (mean ± SD)											
Cardiac score	0.5±0.8	2.1±1.3	**<0.001**	0.3±0.5	2.5±1.1	**<0.001**	0.6±0.8	1.8±1.4	**<0001**	0.180	**0.049**
Renal score	0.9±0.9	1.9±1.3	**<0.001<**	1.7±0.5	2.5±1.1	**0.009**	0.6±0.9	1.4±1.3	**0.003**	**0.001**	**0.017**
Neurologic score	0.6±1.1	2.7±2.0	**0.001<0**	0.2±0.6	3.1±1.9	**<0.001**	0.8±1.3	2.5±2.0	**<0.001**	0.050	0.082
Death score	0.7±1.3	3.1±2.4	**.001<0.**	0.3±0.7	3.1±2.2	**<0.001**	0.8±1.5	3.1±2.6	**<0.001**	0.268	0.896
Composite score	0.6±0.9	3.1±1.8	**001**	0.6±0.8	3.8±1.6	**<0.001**	0.7±0.9	2.6±1.8	**<0.001**	0.968	**0.006**
Comorbidities											
Hypertension (%)	2 (4.3%)	36 (48.6%)	**<0.001**	1 (6.7%)	18 (56.3%)	**0.001**	1 (3.2%)	18 (42.9%)	**<0.001**	0.592	0.253
Diabetes mellitus (%)	0 (0.0%)	15 (20.3%)	**0.001**	0 (0.0%)	8 (25.0%)	**0.034**	0 (0.0%)	7 (16.7%)	**0.017**	-	0.377
Dyslipidaemia (%)	5 (10.9%)	44 (59.5%)	**<0.001**	1 (6.7%)	23 (71.9%)	**<0.001**	4 (12.9%)	21 (50.0%)	**0.001**	0.524	0.058
Smoking (%)	1 (2.2%)	12 (16.2%)	**0.016**	1 (6.7%)	10 (31.3%)	0.064	0 (0.0%)	2 (4.8%)	0.218	0.146	**0.002**

p1: Fabry patients < 40 years vs. Fabry patients ≥ 40 years

p2: Fabry males < 40 years vs. Fabry males ≥ 40 years

p3: Fabry females < 40 years vs. Fabry females ≥ 40 years

p4: Fabry males < 40 years vs. Fabry females < 40 years

p5: Fabry males ≥ 40 years vs. Fabry females ≥ 40 years

Valid percentages are shown

eGFR, estimated glomerular filtration rate; F, females; IVS, interventricular septum; LV, left ventricular; M, males; PW, posterior wall

Reference values: enzymatic activity of α-galactosidase A on plasma 6 – 19 nmol/h/mL, and on leukocytes 36 – 80 nmol/h/mg; urinary GB3 0.87 – 13 µg/mmol creatinine; plasma GB3 0.8 – 4.52 nmol/mL; plasma Lyso-GB3 0 – 1.9 ng/mL.

Chapter 4.2

Azevedo O, Gago MF, Miltenberger-Miltenyi G, Robles AR, Costa MA, Pereira O, Vide AT, Castelo Branco G, Simões S, Guimarães MJ, Salgado A, Sousa N, Cunha D.

Natural history of the late-onset phenotype of Fabry disease due to the p.F113L mutation.

Mol Genet Metab Rep 2020;22:100565. doi: 10.1016/j.ymgmr.2020.100565.

Molecular Genetics and Metabolism Reports 22 (2020) 100565

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism Reports

journal homepage: www.elsevier.com/locate/ymgmr

Natural history of the late-onset phenotype of Fabry disease due to the p.F113L mutation

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ARTICLE INFO

Keywords:
Fabry disease
Late-onset
F113L
Natural history
Cardiac
Phenotype

ABSTRACT

Background: The common *GLA* gene mutation p.F113L causes late-onset phenotype of Fabry disease (FD) with predominant cardiac manifestations. A founder effect of FD due to this mutation was found in the Portuguese region of Guimarães. Our study aims to deepen the knowledge on the natural history of this late-onset variant.

Methods: 203 consecutive adult Fabry patients with p.F113L mutation (79 males; mean age 46 ± 18 years), from this region, were submitted at baseline to a predefined diagnostic protocol. The occurrence of FD manifestations was analyzed in each decade of age in both genders.

Results: In males, left ventricular hypertrophy (40.2%) and late gadolinium enhancement (21.4%) arose over 30 years; heart failure (HF) (21.9%), ventricular tachycardia (8.9%) and conduction disorders over 40 years; and bifascicular (13.1%) and complete atrioventricular blocks (5.9%) beyond 50 years of age. Cardiac manifestations occurred more commonly and 1–2 decades earlier in males; their frequency increased with age. Septum and posterior wall thickness, LV mass, QRS interval duration and pro-BNP levels increased with age in both genders. Mean survival free from HF (64 ± 1 vs. 76 ± 2 years) and pacemaker (71 ± 2 vs. 86 ± 1 years) was higher in females ($p < .001$). Albuminuria A2/A3 (33.7%), brain white matter lesions (50.3%) and sensorineural deafness (44.7%) arose before 30 years of age in both genders, increasing with age. Renal failure and stroke were rare. Lysosomal inclusions were demonstrated in podocytes of patients with proteinuria.

Conclusion: This study improves the knowledge on natural history of late-onset variants of FD, carrying major impact on clinical decisions and guidelines.

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https://doi.org/10.1016/j.ymgmr.2020.100565
Received 15 November 2019; Received in revised form 5 January 2020; Accepted 6 January 2020
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1. Introduction

Fabry disease (FD) (OMIM 301500) is an X-linked lysosomal storage disorder caused by mutations in the *GLA* gene, leading to deficiency of the enzymatic activity of α-galactosidase A and subsequent lysosomal accumulation of globotriaosylceramide (GB3) and other related glycosphingolipids [1]. Multiorgan damage ensues, being death mainly driven by heart disease [2].

Mutations causing a virtually null enzymatic activity are associated to severe and early onset classical phenotypes, while mutations leading to a residual enzymatic activity are associated to attenuated and late-onset phenotypes. Classical phenotypes are characterized by early development, in childhood or adolescence, of acroparesthesias, neuropathic pain, hypohydrosis, heat, cold and exercise intolerance, cornea *verticillata*, angiokeratomas, gastrointestinal symptoms and proteinuria. In adulthood, patients also suffer from sensorineural deafness and cardiac, renal and cerebrovascular manifestations. In contrast, late-onset variants are characterized by the development of cardiac, renal and/or cerebrovascular manifestations in adulthood and the phenotype may be dominated by the involvement of an organ, such as the heart or the kidneys [1,3].

Despite being more prevalent [4], late-onset phenotypes were significantly underdiagnosed in the past [3]. Their increased recognition after the emergence of therapeutic options for FD raised the need to know their natural history, in order to reach evidence-based clinical decisions and accurately evaluate the efficacy of available therapies. Still, knowledge on their natural history has been hampered by the ethical issues related to the follow-up of untreated patients in a time when therapeutic options are available. Additionally, their slower disease course, inherently biased by older age and common comorbidities [5–7], further increases the difficulty of assessing the natural history of a disease already characterized by a high clinical heterogeneity even within the same genotype [5,6].

Current knowledge specifically on predominant cardiac phenotypes is frequently based on small cohorts [8–12] or registries [12–14] that are commonly biased by the overrepresentation of older, male, symptomatic and more severely affected patients. Female underrepresentation is a major issue in most previous studies [5,8–14], with a female/male ratio far below 2, ranging from 0.3 to 1.3. Comorbidities and concomitant medication are commonly not taken into account for the phenotype [8,10–15]. Finally, the clinical description of cardiac predominant phenotypes is commonly focused on left ventricular hypertrophy (LVH) and evidence gaps exist on the natural history of other cardiac and extracardiac manifestations.

The *GLA* gene mutation c.337T > C (p.F113L) results in a misfolded enzyme that is unstable and consequently degraded at the neutral pH of the endoplasmic reticulum, which ultimately causes a late-onset phenotype of FD [6,16]. We have demonstrated since 2013 the existence of a founder effect of FD due to the p.F113L mutation in the Portuguese region of Guimarães, based on genealogy and haplotype analysis [6,17]. We have also reported its spread to many countries in the world due to migration in the last century and described the late-onset phenotype with predominant cardiac manifestations that was presented, at baseline, by a large cohort of patients with this mutation [6]. Aiming to deepen the knowledge on the natural history of late-onset FD, we herein provide a detailed description of the phenotype presented at baseline by a larger cohort of patients with the p.F113L mutation as well as the chronology of the clinical manifestations in both genders decade by decade, thereby obtaining an accurate overview of the natural history of this common late-onset FD variant.

2. Methods

2.1. Subjects

Between January 2008 and December 2018, we diagnosed, as previously described [6], 203 consecutive adult patients (≥18 years) with FD due to the p.F113L mutation, from 34 different family pedigrees, in the Reference Center of Lysosomal Storage Disorders of Hospital Senhora da Oliveira – Guimarães. They shared a common ancestor nearly 400 years ago (Fig. S1) and lived all in the region of Guimarães, thereby also sharing environmental factors.

2.2. Clinical evaluation of Fabry patients

At baseline, all patients were submitted to a predefined diagnostic protocol, including systematic multidisciplinary assessment (Cardiology, Internal Medicine, Neurology, Psychiatry, Ophthalmology, Otorhinolaryngology, Dermatology), blood and urine analysis with quantification of serum creatinine, albuminuria on 24 h-urine and albumin to creatinine ratio on random urine, estimation of glomerular filtration rate (eGFR) by the CKD-EPI formula, electrocardiogram, echocardiogram, cardiac MRI, 24 h-Holter, exercise stress test, brain MRI (by neuroradiologist), electromyography, audiometry and lung spirometry. Kidney biopsy was performed in Fabry patients presenting proteinuria ≥500 mg on 24 h-urine. The enzymatic activity of α-galactosidase A in plasma and leukocytes, the urinary GB3 and the plasma lyso-GB3 were measured as previously described [6].

2.3. Statistical analysis

Categorical variables were expressed as percentages and compared between groups of patients by the Chi-square test. Continuous variables were expressed as means and standard deviations and compared between groups of patients by the Student's *t*-test or Mann-Whitney test. The occurrence of key clinical manifestations of FD was evaluated by gender and age category (≤30; 31–40; 41–50; 51–60; 61–70; 71–80; >80 years). Polynomial regression (quadratic function) was used to best describe the relationship between age and LV mass, interventricular septum (IVS) and posterior wall (PW) thickness and QRS interval duration. An exponential model was used to best describe the relationship between age and pro-BNP levels. Kaplan-Meier curves were performed to estimate survival free from heart failure (HF) and pacemaker. Statistical significance was considered for $p < .05$.

2.4. Ethical issues

This research was approved by the hospital Ethics Committee and all patients provided signed informed consent.

3. Results

We assessed, at baseline, 203 consecutive adult patients with FD due to the p.F113L mutation, 79 males and 124 females (female/male ratio 1.6). Mean age at diagnosis was 46 ± 18 years and higher in males (49 ± 15 vs. 44 ± 19, $p = .021$). Thirty-four patients were index patients and the remaining were diagnosed through family screening (83.3%).

There was no association between the enzymatic activity on plasma or leukocytes and the development of clinical manifestations in females.

3.1. Cardiac manifestations

LVH (LV wall thickness > 12 mm) was found in 40.2% of the patients, more commonly in males (73.1% vs. 19.0%) (Table 1). In patients with LVH, mean LV mass was 147.5 ± 45.1 g/m^2 and diastolic dysfunction was present in 67.1%. The frequency of LVH increased with age, arising in males over 30 years and females over 50 years and affecting all males beyond 60 years and all females beyond 80 years of age. Notably, 33.3% of males aged 31–40 years and 78.6% of males aged 41–50 years already presented LVH, while only 15.4% of females aged 51–60 years had LVH (Fig. 1). LV mass and IVS and PW thickness

Table 1
Clinical phenotype of patients with late-onset FD due to the p.F113L mutation.

Clinical manifestations	Fabry patients (n = 203)	Fabry males (n = 79)	Fabry females (n = 124)	p
Age at FD diagnosis (years) (mean ± SD)	46 ± 18	49 ± 15	44 ± 19	**0.021**
α-GAL A activity (mean ± SD)				
Plasma (nmol/h/mL)	5.1 ± 5.1	0.4 ± 1.4	8.2 ± 4.2	<**0.001**
Leukocytes (nmol/h/mg)	16.6 ± 17.4	2.0 ± 3.6	26.7 ± 15.9	<**0.001**
Disease biomarkers (mean ± SD)				
Urinary GB3 (μg/mmol creatinine)	91.0 ± 152.1	181.7 ± 208.5	30.2 ± 23.1	<**0.001**
Plasma Lyso-GB3 (ng/mL)	-	7.7 ± 3.0	<2[a]	-
Cardiac manifestations				
LVH (%)	80 (40.2%)	57 (73.1%)	23 (19.0%)	<**0.001**
IVS thickness (mm) (mean ± SD)	11.6 ± 4.4	14.3 ± 4.0	9.9 ± 3.6	<**0.001**
PW thickness (mm) (mean ± SD)	10.3 ± 3.5	12.6 ± 3.5	8.9 ± 2.7	<**0.001**
LV mass (g/m^2) (mean ± SD)	100.3 ± 49.4	132.5 ± 51.8	79.6 ± 34.7	<**0.001**
LV ejection fraction (%) (mean ± SD)	66.0 ± 7.0	65.9 ± 7.9	66.0 ± 6.3	0.710
LV diastolic dysfunction (%)	55 (27.8%)	35 (44.9%)	20 (16.7%)	<**0.001**
Abnormal relaxation pattern (%)	32 (58.2%)	17 (48.6%)	15 (75.0%)	
Pseudonormal pattern (%)	22 (40.0%)	17 (48.6%)	5 (25.0%)	0.145
Restrictive pattern (%)	1 (1.8%)	1 (2.9%)	0 (0.0%)	
LVH on cardiac MRI (%)	60 (36.1%)	44 (68.8%)	16 (15.7%)	<**0.001**
LV mass on cardiac MRI (g/m^2)	72.2 ± 29.2	93.8 ± 29.8	58.6 ± 18.8	<**0.001**
Late gadolinium enhancement (%)	36 (21.4%)	25 (39.1%)	11 (10.6%)	<**0.001**
Heart failure (%)	44 (21.9%)	26 (32.9%)	18 (14.8%)	**0.002**
NYHA Class I (%)	34 (77.3%)	23 (88.5%)	11 (61.1%)	
NYHA Class II (%)	9 (20.5%)	2 (7.7%)	7 (38.9%)	**0.034**
NYHA Class III (%)	1 (2.3%)	1 (3.8%)	0 (0.0%)	
NYHA Class IV (%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
Atrial fibrillation (%)	9 (4.4%)	6 (7.6%)	3 (2.4%)	0.081
Atrial flutter (%)	3 (1.5%)	2 (2.5%)	1 (0.8%)	0.321
Non-sustained ventricular tachycardia (%)	18 (8.9%)	11 (14.1%)	7 (5.6%)	**0.040**
Atrioventricular block (%)	30 (14.8%)	16 (20.3%)	14 (11.3%)	0.079
1st degree (%)	19 (10.5%)	8 (12.3%)	11 (9.5%)	0.552
2nd degree (%)	9 (4.5%)	6 (7.6%)	3 (2.4%)	0.083
3rd degree (%)	12 (5.9%)	10 (12.7%)	2 (1.6%)	**0.001**
Right bundle branch block (%)	36 (19.6%)	26 (38.8%)	10 (8.5%)	<**0.001**
Left anterior fascicular block (%)	42 (23.0%)	30 (45.5%)	12 (10.3%)	<**0.001**
Left bundle branch block (%)	2 (1.1%)	1 (1.6%)	1 (0.9%)	0.668
Bifascicular block (%)	26 (13.1%)	19 (25.3%)	7 (5.7%)	<**0.001**
Pacemaker (%)	13 (6.4%)	10 (12.7%)	3 (2.4%)	**0.004**
Implantable cardioverter-defibrillator (%)	2 (1.0%)	1 (1.3%)	1 (0.8%)	0.747
Myocardial ischemic events (%)	10 (4.9%)	10 (12.7%)	0 (0.0%)	<**0.001**
Renal manifestations				
Albuminuria A2 (30-300mg/24 h) (%)	48 (24.6%)	28 (36.8%)	20 (16.8%)	**0.002**
Albuminuria A3 (>300 mg/24 h) (%)	18 (9.2%)	12 (15.8%)	6 (5.0%)	**0.011**
Albuminuria A2 or A3 (≥30 mg/24 h) (%)	66 (33.7%)	40 (52.6%)	26 (21.7%)	<**0.001**
Chronic kidney disease stages				
G1 (eGFR ≥90 mL/min/1.73m^2) (%)	139 (70.6%)	45 (60.0%)	94 (77.0%)	**0.011**
G2 (eGFR 60-89 mL/min/1.73m^2) (%)	45 (22.8%)	23 (30.7%)	22 (18.0%)	**0.040**
G3a (eGFR 45-59 mL/min/1.73m^2) (%)	10 (5.1%)	6 (8.0%)	4 (3.3%)	0.143
G3b (eGFR 30-44 mL/min/1.73m^2) (%)	1 (0.5%)	0 (0.0%)	1 (0.8%)	0.432
G4 (eGFR 15-29 mL/min/1.73m^2) (%)	1 (0.5%)	1 (1.3%)	0 (0.0%)	0.201
G5 (eGFR <15 mL/min/1.73m^2) (%)	1 (0.5%)	0 (0.0%)	1 (0.8%)	0.432
Chronic kidney disease stage ≥ G3 (%)	13 (6.6%)	7 (9.3%)	6 (4.9%)	0.225
Neurological and neuropsychiatric manifestations				
Stroke (%)	6 (3.0%)	3 (3.8%)	3 (2.4%)	0.572
Transient ischemic attack (%)	1 (0.5%)	0 (0.0%)	1 (0.8%)	0.424
Brain white matter lesions (%)	83 (50.3%)	35 (53.8%)	48 (48.0%)	0.463
Brain haemorrhage (%)	2 (1.0%)	2 (2.6%)	(0.0%)	0.073
Carpal tunnel syndrome (%)	36 (19.7%)	17 (23.9%)	19 (17.0%)	0.247
Acroparesthesias (%)	62 (30.7%)	14 (17.9%)	48 (38.7%)	**0.002**
Hypohidrosis (%)	3 (1.5%)	0 (0.0%)	3 (2.4%)	0.166
Cold, heat or exercise intolerance (%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	-
Depression (%)	41 (20.8%)	12 (15.8%)	29 (24.0%)	0.169
Anxiety (%)	58 (29.3%)	22 (28.9%)	36 (29.5%)	0.933
Eye manifestations				
Cornea *verticillata* (%)	19 (10.3%)	11 (15.5%)	8 (7.1%)	0.068
Cataracts (%)	25 (13.5%)	10 (14.3%)	15 (13.0%)	0.811
Retinal vessel tortuosity (%)	6 (3.3%)	2 (2.9%)	4 (3.5%)	0.814
Ear manifestations				
Sensorineural deafness (%)	80 (44.7%)	46 (62.2%)	34 (32.4%)	<**0.001**
Tinnitus (%)	30 (16.3%)	17 (23.0%)	13 (11.8%)	**0.045**
Dermatological manifestations				
Angiokeratomas (%)	4 (2.1%)	0 (0.0%)	4 (3.5%)	0.106
Pulmonary manifestations				
Lung obstructive disease (%)	29 (17.5%)	15 (22.7%)	14 (14.0%)	0.147
Gastrointestinal manifestations (%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	-

(*continued on next page*)

Table 1 (*continued*)

Clinical manifestations	Fabry patients (n = 203)	Fabry males (n = 79)	Fabry females (n = 124)	p
Comorbidities				
Hypertension (%)	66 (32.7%)	30 (38.0%)	36 (29.3%)	0.198
Diabetes mellitus (%)	23 (11.4%)	13 (16.5%)	10 (8.1%)	0.069
Dyslipidemia (%)	85 (42.1%)	46 (58.2%)	39 (31.7%)	**<0.001**
Smoking (%)	21 (10.4%)	18 (22.8%)	3 (2.4%)	**<0.001**
Concomitant medications				
ACEI/ARB (%)	79 (38.9%)	41 (51.9%)	38 (30.6%)	**0.002**

p: Males vs. Females.
$p < .05$ are marked in bold.
Valid percentages are shown.
ACEI, angiotensin-converting-enzyme inhibitors; α-GAL A, α- galactosidase A; ARB, angiotensin II receptor blocker; e-GFR, estimated glomerular filtration rate; FD, Fabry disease; IVS, interventricular septum; LV, left ventricular; LVH, left ventricular hypertrophy; NYHA, New York Heart Association; PW, posterior wall.
Reference values: Enzymatic activity of α-galactosidase A on plasma 6–19 nmol/h/mL, and on leukocytes 36–80 nmol/h/mg; urinary GB3 0.87–13 μg/mmol creatinine; plasma Lyso-GB3 0–1.9 ng/mL
[a] Below lower level of quantitation of plasma lyso-GB3 (2 ng/mL).

increased with age in both genders according to a quadratic model (Fig. 2). No correlation was found between these parameters and plasma lyso-GB3 in males.

HF developed in 21.9%, more commonly in males (32.9% vs. 14.8%) (Table 1). In patients with LVH, it occurred in 55.0%, more commonly in females (78.3% vs. 45.6%, $p = .008$). HF arose in males over 40 years and females over 50 years. It is noteworthy that 28.6% of the males aged 41–50 years already presented HF. Its frequency increased with age, so that 80% of males over 60 years and nearly 70% of females over 70 years suffered from HF (Fig. 1). Most patients were in New York Heart Association (NYHA) class I or II (Table 1). pro-BNP levels increased with age according to an exponential model in males ($R^2 = 0.525$; $p < .001$) and females ($R^2 = 0.464$; $p < .001$). Mean survival free from HF was higher in females (64 ± 1 vs. 76 ± 2 years) (Fig. 3).

Cardiac MRI was performed in 166 patients (81.8%; 64 males and 102 females), and in 59 patients with LVH (73.8%; 44 males and 15 females). Sixty patients (one additional patient compared to echocardiography) fulfilled cardiac MRI criteria of LVH [18] (36.1%). LGE occurred in 4.8% of patients without LVH, without gender difference (5.3% vs. 4.7%, $p = .918$). In patients with LVH, LGE occurred in 52.5%, also without gender dominance (54.5% vs. 46.7%, $p = .598$). LGE arose in males over 30 years and females over 40 years and its frequency increased with age in both genders (Fig. 1). LV mass on MRI was a predictor of LGE ($p < .001$). LGE was associated with ST depression ($p = .001$) and negative T waves ($p < .001$) on the ECG, but still 17.8% of the patients without ST depression and 13.4% without negative T waves presented LGE.

24 h-Holter was performed in 185 patients (91.1%; 70 males and 115 females). Atrial fibrillation occurred in 4.4% (mean age at diagnosis: 70 ± 10 years). Non-sustained ventricular tachycardia (VT) occurred in 8.9%, mainly in males (14.1% vs. 5.6%) (Table 1), arising in males over 40 years and females over 50 years. It is relevant that 16.7% of the males aged 41–50 years already presented non-sustained VT. Its frequency increased with age, so that one third of the patients over 70 years had non-sustained VT (Fig. 1). Nevertheless, only one male and one female needed cardioverter-defibrillators for primary prevention of sudden cardiac death due to severe LVH with diffuse LGE and frequent non-sustained VT.

Left anterior fascicular block (LAFB) was found in 23.0% of the patients, right bundle branch block (RBBB) in 19.6%, bifascicular block (BB) in 13.1% and complete atrioventricular (AV) block in 5.9%, all more commonly in males (Table 1). LAFB developed in both genders since the age of 40 years and RBBB in females over 40 years and males over 50 years. BB occurred in males over 50 years, but only in females over 70 years. Likewise, complete AV block occurred in males since the age of 50 years, but only in females over 80 years. The frequency of all cardiac conduction disturbances increased with age in both genders, as well as the duration of the QRS interval (Figs. 1 and 2). Notably, 59.1% of the males aged 51–60 years already presented LAFB, 57.1% of them had RBBB, 36.4% had BB and 14.8% had already suffered complete AV block and therefore implanted pacemaker (Fig. 1). Pacemaker implantation (6.4%) occurred two decades earlier in males [60 ± 7 (49–71) vs. 81 ± 5 (75–84), years] (Table 2), mainly in the emergency setting of a complete AV block. Kaplan-Meier curve of survival free from pacemaker is shown in Fig. 3.

History of myocardial ischemic events was found exclusively in males ($n = 10$) (Table 1). Myocardial infarction occurred in eight and unstable angina in six males. All patients had significant stenoses/occlusions on coronary angiography, except one who had normal coronary arteries and suffered a type 2 myocardial infarction in the context of severe LVH. Six males underwent percutaneous coronary intervention and two underwent coronary artery bypass graft. Mean age at first myocardial ischemic event was 53 ± 10 years (42–76). All these patients presented cardiovascular risk factors: hypertension (60%), diabetes *mellitus* (10%), dyslipidemia (90%) and smoking (40%).

3.2. Extracardiac manifestations

Albuminuria A2/A3 was found in 33.7%, mainly in males (52.6% vs. 21.7%) (Table 1). Of note, 23.1% of the patients with normoalbuminuria were under angiotensin-converting-enzyme inhibitors (ACEI) or angiotensin II receptor blockers (ARB). Conversely, 51.5% of the patients with albuminuria A2/A3 had concomitant hypertension and 22.7% diabetes, although medically controlled. More importantly, albuminuria A2/A3 was found in 23.8% of the non-hypertensive non-diabetic patients, with a male predominance (39.1% vs. 15.5%, $p = .002$). Kidney biopsy showed lysosomal inclusions in podocytes of young p.F113L patients with increased albuminuria (Fig. 4) and albuminuria A2/A3 was an early manifestation, affecting 22.2% of males and 12.9% of females before the age of 30 years. In males, its frequency increased progressively with age, reaching a peak of 71.4% in the 7th decade. In females, its frequency slowly increased until 40 years of age, when nearly one third of them had developed it (Fig. 5). A similar natural course was seen in the subset of patients without hypertension or diabetes (Fig. S2). Renal insufficiency (eGFR <60 mL/min/1.73m^2) occurred rarely (6.6%), without gender dominance, and there were no cases of renal replacement therapy (RRT).

Brain MRI was performed in 165 patients (81.3%; 65 males and 100 females). Brain white matter lesions (WML) occurred in 50.3%, without gender difference (53.8% vs. 48.0%) (Table 1). They were found in patients with cardiovascular risk factors, despite medically controlled, but also in 38.4% of the patients without these risk factors, without gender predominance. Brain WML were an early manifestation,

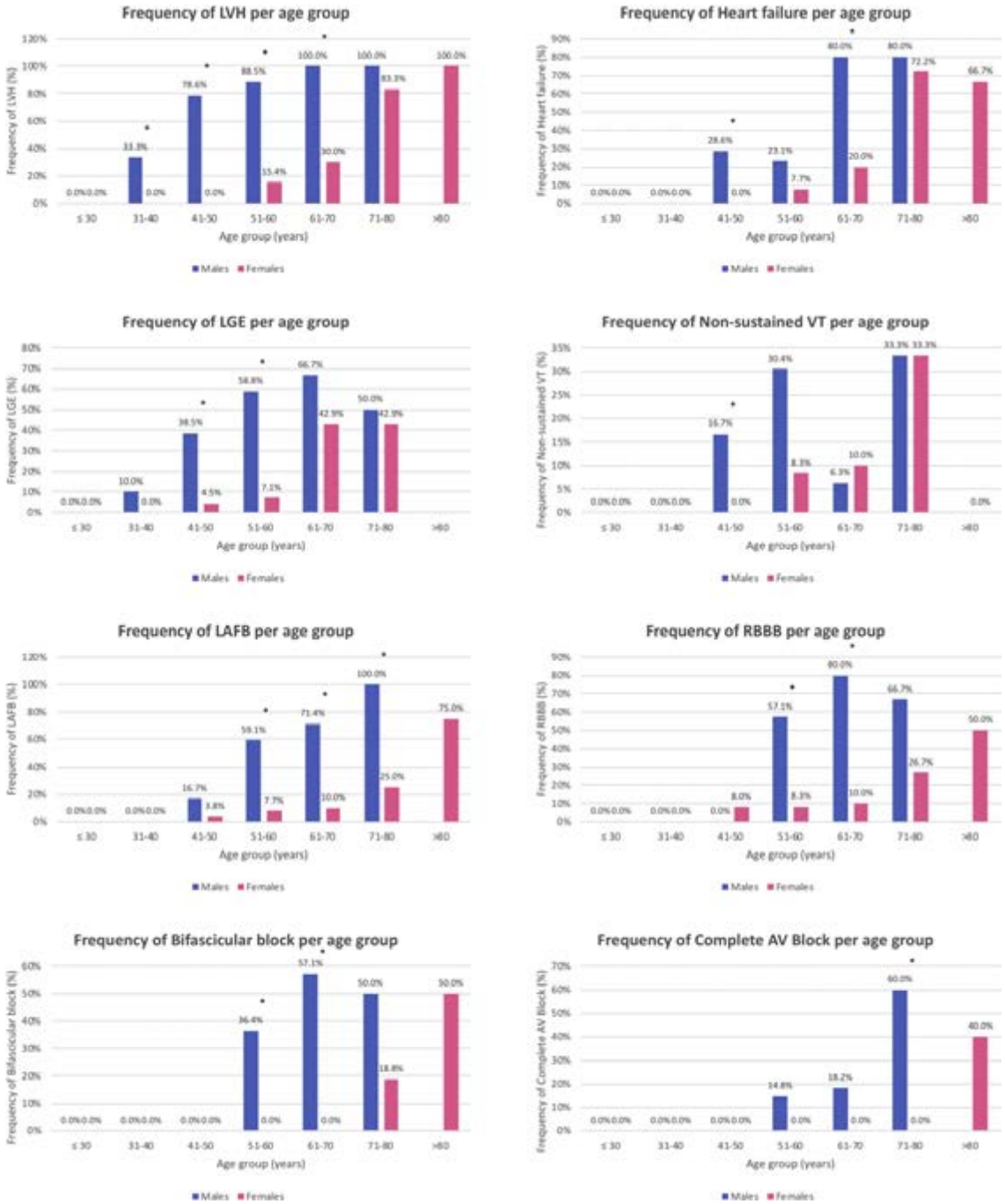
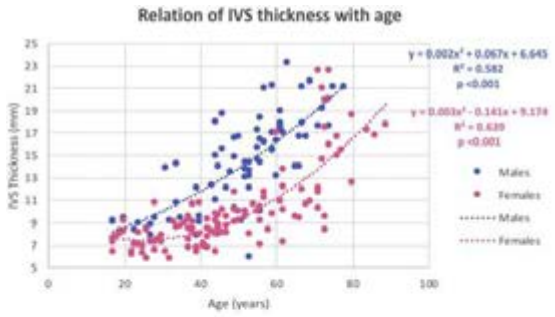
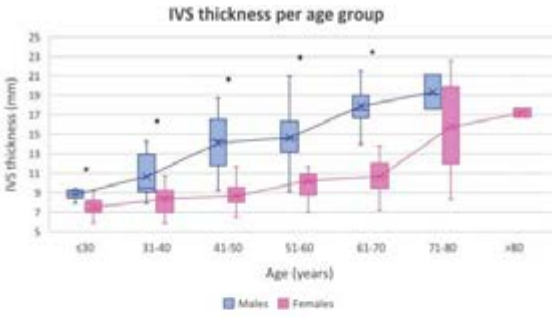
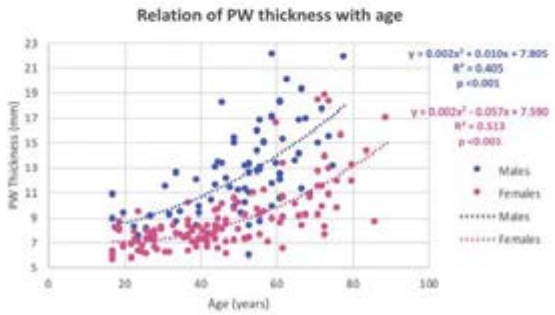
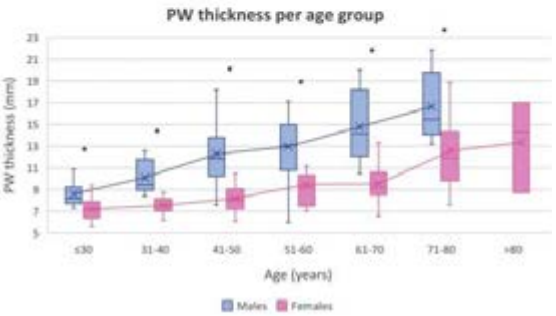


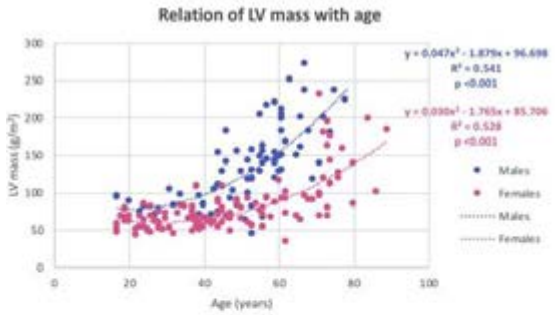
Fig. 1. Frequency of cardiac manifestations according to age category in male and female Fabry patients with the p.F113L mutation (LVH, heart failure, LGE, non-sustained VT, LAFB, RBBB, bifascicular block, complete AV block). * $p < .05$.

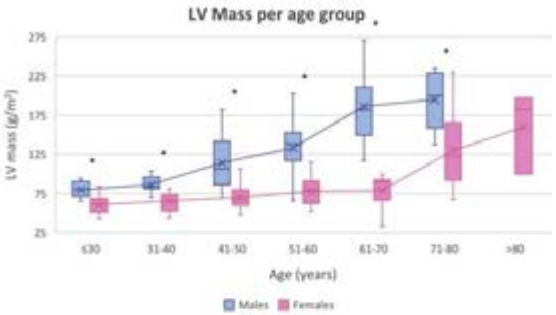


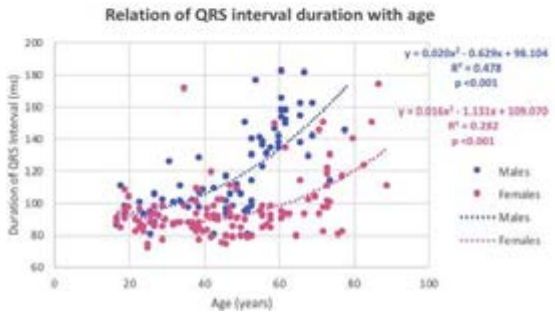












MEAN±SD	IVS THICKNESS (mm)		PW THICKNESS (mm)		LV MASS (g/m²)	
	Males	Females	Males	Females	Males	Females
≤30y	8.8±0.5	7.5±1.0	8.6±1.1	7.2±0.9	79.8±10.1	60.7±11.0
31-40y	10.6±2.3	8.3±1.4	10.1±1.5	7.6±1.0	86.4±10.3	65.2±14.8
41-50y	14.1±2.9	8.7±1.2	12.3±2.7	8.2±1.2	114.2±33.9	70.0±13.7
51-60y	14.6±3.3	10.2±2.4	13.0±3.3	9.5±2.5	133.2±42.4	78.2±17.8
61-70y	17.9±2.5	10.6±1.8	14.8±3.5	9.6±1.8	186.7±43.9	79.1±20.6
71-80y	19.3±1.7	15.8±4.4	16.7±3.3	12.6±3.4	194.9±38.4	130.0±46.1
>80y	-	17.2±0.5	-	13.4±4.2	-	159.9±52.7

Fig. 2. Left panel: Relationship of IVS and PW thickness, LV mass and QRS interval duration with age, depicted by a quadratic model; Right panel: IVS and PW thickness and LV mass per age category in male and female patients with FD due to the p.F113L mutation. The mean line is shown and means are marked on the boxplots with an X. * $p < .05$.

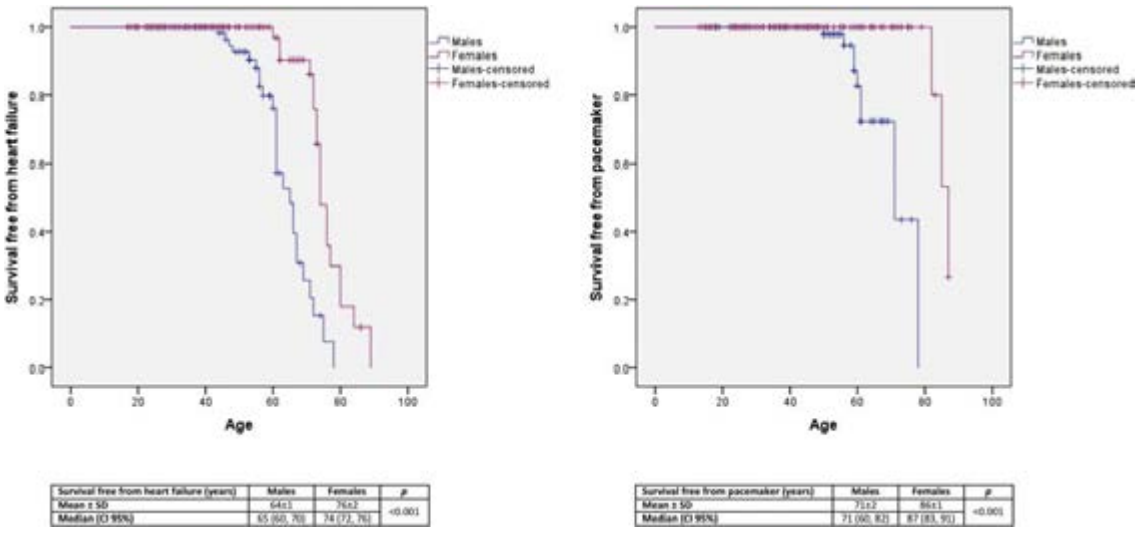


Fig. 3. Kaplan-Meier curves of survival free from heart failure and survival free from pacemaker in male and female Fabry patients with the p.F113L mutation.

Table 2
Mean age at diagnosis of each clinical manifestation of FD in patients with the p.F113L mutation.

Age at diagnosis (years) (Mean ± SD) (Min-Max)	Fabry patients	Males	Females	*p*
LVH	61 ± 12 (31–89)	57 ± 10 (31–78)	73 ± 8 (57–89)	**<0.001**
LGE	60 ± 9 (40–76)	58 ± 8 (40–76)	65 ± 8 (46–74)	**0.013**
Atrial fibrillation	70 ± 10 (52–80)	67 ± 11 (52–78)	77 ± 3 (74–80)	0.262
Non-sustained VT	62 ± 10 (45–77)	57 ± 8 (45–77)	70 ± 6 (60–77)	**0.003**
RBBB	64 ± 9 (51–85)	61 ± 6 (51–78)	76 ± 9 (62–85)	**0.001**
LAFB	63 ± 11 (42–89)	60 ± 7 (47–78)	72 ± 14 (42–89)	**0.003**
BB	66 ± 9 (53–85)	62 ± 6 (53–78)	78 ± 6 (71–85)	**<0.001**
Complete AV block	63 ± 10 (49–83)	60 ± 7 (49–71)	79 ± 6 (75–83)	**0.036**
Pacemaker	65 ± 11 (49–84)	60 ± 7 (49–71)	81 ± 5 (75–84)	**0.009**
Albuminuria A2 or A3	54 ± 14 (18–86)	55 ± 12 (22–78)	51 ± 18 (18–86)	0.222
CKD ≥ G3	70 ± 10 (50–86)	66 ± 10 (50–78)	75 ± 7 (66–86)	0.202
Brain WML	53 ± 15 (18–89)	53 ± 11 (27–75)	52 ± 17 (18–89)	0.912
Stroke	53 ± 12 (41–71)	48 ± 5 (42–51)	59 ± 16 (41–71)	0.700
Sensorineural deafness	58 ± 13 (20–88)	57 ± 12 (20–77)	59 ± 15 (23–88)	0.380
Cornea *verticillata*	60 ± 8 (43–72)	59 ± 7 (46–68)	63 ± 10 (43–72)	0.238

p: Males vs. Females.
$p < .05$ are marked in bold.
AV, atrioventricular; BB, bifascicular block; CKD, chronic kidney disease; LAFB, left anterior fascicular block; LGE, late gadolinium enhancement; LVH, left ventricular hypertrophy; RBBB, right bundle branch block; VT, ventricular tachycardia; WML, white matter lesions.

affecting 11.1% of males and 26.9% of females under 30 years of age. Fig. 4 shows brain WML in a young female patient without comorbidities. Their frequency increased with age, being a universal finding in both genders over 70 years (Fig. 5). Stroke was rare and occurred in males (aged 42–51 years) and females (aged 41–71 years), all with concomitant cardiovascular risk factors and one with atrial fibrillation. Acroparesthesias occurred mainly in females and were predominantly mild (Table 1).

Sensorineural deafness was common (44.7%), particularly in males (62.2% vs.32.4%) (Table 1). It emerged early, affecting 20.0% of males and 3.6% of females under 30 years, and its frequency increased with age in both genders (Fig. 5). It occurred mainly at high frequencies and hearing aids were seldom indicated. Cornea *verticillata* was found in 10.3% of the patients. Notably, this is a milder form of cornea *verticillata* compared to the one that is typically observed in the classic patients. Angiokeratomas were rare (2.1%) and solitary.

4. Discussion

This study provides a detailed description of the natural history of the late-onset phenotype of FD due to one of the most common *GLA* gene mutations, the p.F113L mutation, thereby improving the knowledge on the natural history of nonclassical FD.

4.1. Cardiac manifestations

We have previously shown that, in this late-onset phenotype, cardiac manifestations carry the highest prognostic impact [6]. This study now shows that the first cardiac manifestations are LVH and LGE, which arise in males over 30 years and are followed by HF, non-sustained VT and cardiac conduction disorders, which arise in males over 40 years, culminating with the development of bifascicular block and complete AV block in males beyond the age of 50 years. Cardiac manifestations are more common and arise one to two decades earlier in males and

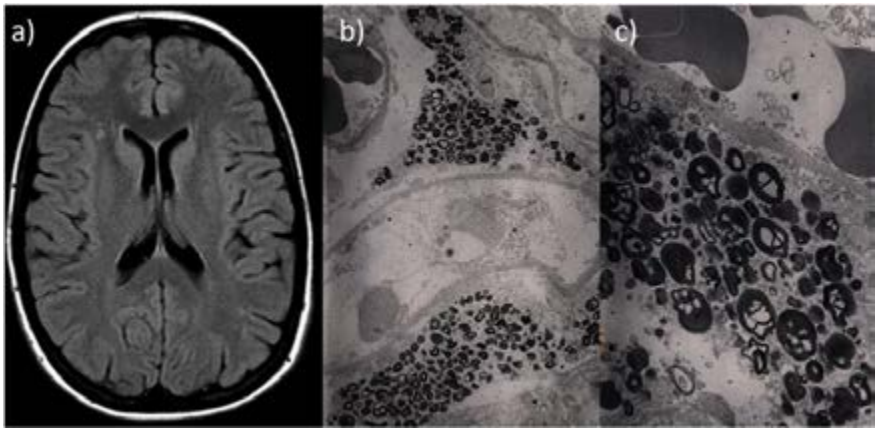


Fig. 4. (a) Brain MRI showing WML lesions in a 29-year old female with FD due to the p.F113L mutation, in the absence of cardiovascular risk factors or other morbidities; (b, c) Electronic microscopy from a kidney biopsy showing lysosomal inclusions in renal podocytes in a 42-year old female with FD due to the p.F113L mutation, who presented proteinuria >1 g/24 h, in the absence of other morbidities.

their frequency increases with age in both genders. Likewise, the severity of LVH and cardiac blocks also increases with age, as demonstrated by the significant increase of LV mass, IVS and PW thickness and QRS interval with age.

LVH prevalence on cardiac predominant phenotypes has ranged from 21-25% in the IVS4 phenotype [15,19] to 29–67% in the p.N215S phenotype [5,8,13], which might be explained by the *GLA* gene mutation, characteristics of the studied patients, but also different LVH definitions. There are several echocardiographic indices of LVH [20]. We have reported LVH in nearly 40% of p.F113L patients [6], based on the LVH definition of LV wall thickness > 12 mm, because this criterion carries a therapeutic impact, as it has been commonly used to establish the presence of LVH and mandate the initiation of specific therapy in Fabry patients [21].

Despite different LVH definitions, and similarly to our findings in the p.F113L phenotype, LVH has been described to arise as early as the third decade of life in patients with the p.N215S mutation [9,13] and after 30 years of age in patients with the IVS4 mutation [19]. Likewise, LVH prevalence increases with age in both genders in the IVS4 [19] and the p.N215S phenotypes [5]. However, IVS and PW thickness has been

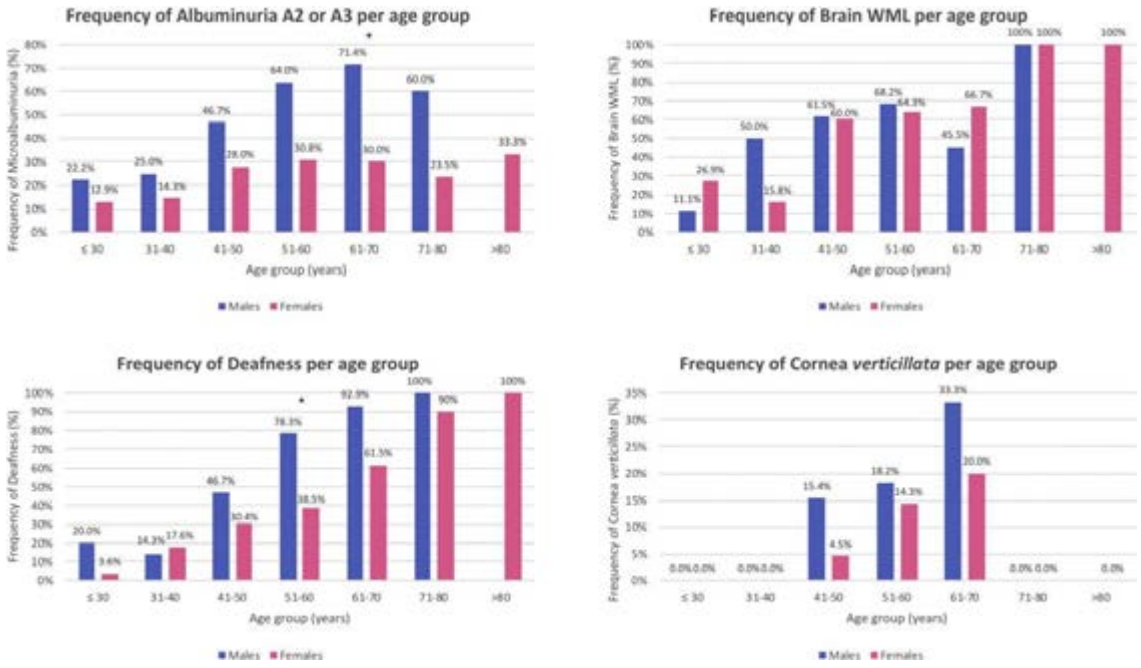


Fig. 5. Frequency of albuminuria A2/A3, brain WML, sensorineural deafness and cornea *verticillata* according to age category in male and female Fabry patients with the p.F113L mutation. * $p < .05$.

reported to increase progressively with age in males, remaining only mildly elevated in females with the p.N215S mutation [13]. In our study, LVH severity increased with age and severe LVH developed both in males and females with the p.F113L mutation.

Cardiac MRI is a valuable tool for the assessment of LVH and cardiac fibrosis [22]. However, most studies on cardiac predominant phenotypes do not provide cardiac MRI data [5,10,13–15]. In the p.N215S phenotype, two small studies have reported LGE, respectively, in 53% (8/15) [8] and 69% (18/26) [9], and as early as the third decade of life [9]. In a larger cohort of 100 IVS4 patients, Hsu et al. reported LVH based on cardiac MRI criteria in 28% and LGE in 38%. In patients without LVH, LGE was found in 38.1% of males and 16.7% of females, suggesting that cardiac fibrosis progressed in silence before the development of LVH. However, in patients with LVH, LGE was found in 61%, exclusively in males (77.3%), being surprisingly absent in females, but the characteristics of those six females were not reported [19]. Our study, based on a larger cohort of 166 patients who underwent cardiac MRI, found cardiac MRI criteria of LVH in 36.1% and an apparently lower prevalence of LGE in the overall cohort (21.4%), in patients with LVH (52.5%) and also in patients without LVH (5.3% of males and 4.6% of females), supporting the hypothesis of a lower frequency or later development of LGE in this late-onset phenotype [6]. Nevertheless, LGE was also an early manifestation in p.F113L phenotype, arising after the 30 years of age. Finally, although LGE was associated with ST depression and negative T waves on ECG, the finding of LGE in 17.8% of the patients without ST depression and in 13.4% without negative T waves argues against the hypothesis raised by Niemann et al. that their absence almost excludes LGE [23].

Most studies on the cardiac predominant phenotypes report on the prevalence of LVH, but data on the specific prevalence or natural course of HF, which is the actual clinical event associated with LVH, are very scarce. We have previously shown that HF occurs in nearly 20% of the p.F113L patients [6] and now we provide the first detailed data on its natural course.

We previously published for the first time the actual frequency of each cardiac rhythm and conduction disorder in a cardiac predominant phenotype [6]. Now, in this study, we provide the first detailed description of the chronology of each of the major cardiac rhythm and conduction disorders that occur and ultimately carry the highest impact on survival in cardiac predominant phenotypes.

In our study, all myocardial ischemic events, except one, were acute coronary syndromes in the setting of severe atherosclerotic coronary heart disease. The only myocardial ischemic event that could eventually be attributed to FD was a type 2 myocardial infarction that occurred in an elderly male with normal coronary arteries and a probable imbalance of myocardial oxygen supply and demand in the context of severe LVH. This finding, together with the typical absence of endothelial GB3 deposits in late-onset FD [24], suggests that myocardial ischemic events secondary to FD are rare in late-onset phenotypes. Some previous studies on late-onset FD reported myocardial ischemic events as Fabry cardiac events [3,13], but no information was given on coronary angiography findings or atherosclerotic risk of the patients. Moreover, percutaneous coronary intervention and coronary artery bypass graft have been considered cardiac events [3], despite the virtually null probability of representing manifestations of late-onset FD. Our study shows that we should cautiously attribute myocardial ischemic events to late-onset FD, because most of them are indeed acute coronary syndromes secondary to atherosclerotic coronary heart disease, which is a common comorbidity with advancing age.

4.2. Extracardiac manifestations

We have previously shown that, in this cardiac predominant phenotype, extracardiac involvement is common [6]. Now, we show that the earlier manifestations are extracardiac, with increased albuminuria, brain WML and sensorineural deafness arising before 30 years of age in both genders and increasing thereafter.

In the p.N215S phenotype, increased albuminuria has been reported, respectively, in 7.7% [9] and 50% of the patients [8] in two small studies and proteinuria in 32.4% of males and 6.4% of females in a larger study [5]. In the IVS4 phenotype, increased albuminuria has been described in 20% [15]. This wide range of results may be explained by different sample sizes and definitions of pathological albuminuria/proteinuria. Nevertheless, like in the p.F113L phenotype, microalbuminuria has been reported as an early manifestation, occurring before 40 years of age in the IVS4 phenotype [15] and as early as the second decade of life in the p.N215S phenotype [5]. Also similar to the p.F113L phenotype, its prevalence increased with age in both genders, but stabilized in middle-aged women with the p.N215S mutation [5]. Chronic kidney disease (CKD) ≥ G3 was reported in 21.6% of males and 2.4% of females with the p.N215S mutation and its prevalence increased with age. End-stage renal failure has been described in two young p.N215S males (25 and 38 years old) (2.4%) with marked persistent proteinuria [5]. In another study, 4% of p.N215S patients (males and females) developed CKD G5 with need for RRT before 65 years of age [13]. In the p.F113L phenotype, CKD ≥ G3 was only found in 9.3% of males and 4.9% of females, CKD G5 in 0.5% and no patients were under RRT.

This study also demonstrates the presence of lysosomal inclusions in renal podocytes of young patients presenting with increased albuminuria, without any known morbidities besides late-onset FD due to the p.F113L mutation, therefore supporting the hypothesis that increased albuminuria in these patients is indeed caused by FD. Likewise, lysosomal inclusions have also been found in renal podocytes of p.N215S patients [8,9].

Our study showed that brain WML are common manifestations (50.3%), which arise early before 30 years of age and increase progressively thereafter, becoming a universal finding in p.F113L males and females above 70 years of age. Small studies reported lower frequencies of brain WML in IVS4 patients (32%) [11,12] and p.N215S patients (21–32%) [8,9]. However, in a larger cohort of p.N215S patients, brain WML were found in 51.4% of males and 27.7% of females, appearing before 40 years of age, increasing progressively thereafter and affecting most elderly patients [5]. In the general population, brain WML have been associated with cerebrovascular risk factors and ageing [25,26]. A previous study reported the following age-related prevalences of brain WML in the general population: 3.5% (<40 years), 22% (41–50 years), 47% (51–60 years), 64% (61–70 years), 84% (71–80 years) and 95% (>81 years) [25]. Compared to the general population, it is striking in our FD cohort the higher prevalence of brain WML in young patients without other comorbidities, which also supports the notion that brain WML are caused by FD and not by ageing or other conditions. As late-onset phenotypes of FD are typically characterized by the absence of endothelial GB3 deposits [24], brain WML, particularly in young patients, are probably mainly caused by GB3 deposits in microglial cells and astrocytes and subsequent pro-inflammatory alterations [27]. Importantly, and in line with the absence of endothelial GB3 deposits, stroke remained a rare event, such as in the p.N215S [5,8,9,13], IVS4 [10–12] and overall late-onset phenotypes [3], occurring in patients with concomitant cardiovascular risk factors.

To our knowledge, this is the first study describing the natural course of sensorineural deafness in a cardiac predominant phenotype of FD. Our study shows for the first time that this common manifestation (44.7%) arises before 30 years of age in males and females with the p.F113L mutation and increases with age, virtually affecting all the elderly individuals.

4.3. Strengths and limitations

This study represents one of the largest cohorts of adult patients with late-onset FD due to the same *GLA* gene mutation, which provides a unique opportunity to perform a gender and age-stratified analysis

and obtain a reliable overview of the natural history of this late-onset variant. Most patients were diagnosed through family screening, which allowed the diagnosis of patients from both genders, all age categories and disease status, capturing the full spectrum of the disease. The female/male ratio was 1.6, closer to the expected ratio in an X-linked disease, considerably reducing the bias related to female underrepresentation of previous studies [5,8–14]. We analyzed baseline data at the time of FD diagnosis, so the disease status of the patients reflects the natural course of the disease until that moment, without the influence of FD specific therapy. We also evaluated concomitant morbidities and medications and accounted for their influence on the phenotype. Patients were systematically submitted to a predefined multidisciplinary diagnostic protocol, which assured a high level of completeness and detail of the data on all organ system manifestations, overcoming common bias of retrospective studies or registries. Finally, p.F113L is one of the most common *GLA* gene mutations causing late-onset FD, so these natural history data will have an impact on the clinical decisions of many patients.

However, the natural history presented in this study does not represent a longitudinal follow-up of these patients, rather it was built based on the baseline data presented by untreated adult patients distributed through the full range of age categories. The longitudinal follow-up of untreated patients in a time where therapies are available raises ethical issues and, given the attenuated and slow course of the disease, a long time of follow-up would be needed to capture its natural history. Nevertheless, considering the large size of our cohort, the fact that it includes all the patients diagnosed with FD within these 34 families and the fact that most of them were diagnosed through family screening and not because of medical attention driven by symptoms, it is reasonable to expect that the data within each age category is a reliable representation of the clinical heterogeneity of the disease at each timeframe of its natural course.

4.4. Future directions

Our study demonstrates a high clinical heterogeneity in both males and females with this late-onset phenotype, with some patients developing an earlier and more severe phenotype than others. Studies specifically addressing the efficacy of therapies on late-onset phenotypes are urgently needed to ascertain if all patients benefit from therapy. If that would not be the case, the holy grail would be, within the high clinical heterogeneity of late-onset phenotypes, to correctly select the patients with higher risk of disease progression and faster disease course who could benefit or benefit the most from therapy.

Moreover, the best timing to start therapy in FD is not clear, particularly in classic females and late-onset phenotypes (males and females). Current guidelines recommend to start therapy in these subsets of patients as soon as there is evidence of target organ damage [28]. Some studies have already shown that early treatment is better than late treatment [29,30]. However, how early should the treatment be started is not yet defined, although from a theoretical point view it would make sense to start treatment before target organ damage occurs.

Therefore it is essential to deepen the study on the natural history of FD, find the determinants of phenotype and determine the predictors of disease progression in both genders, so we could practice Precision Medicine for each individual patient, based on factors much beyond gender, age or *GLA* gene mutation, and accurately determine, for each individual, if he is a good candidate for therapy, the best therapy and the best timing to start it in order to avoid target organ damage.

5. Conclusion

Our study on the natural history of the p.F113L phenotype found many similarities to the natural history of other predominant cardiac phenotypes, suggesting a general common natural course to these FD variants, despite the different *GLA* gene mutations. Nevertheless, this study comes to fill many evidence gaps on the natural history of these FD phenotypes, namely on HF, cardiac rhythm and conduction disturbances, ischemic myocardial events and sensorineural deafness. It also provides cardiac and brain MRI data on a large cohort of patients with late-onset FD. Finally, the demonstration of lysosomal inclusions on renal podocytes of young p.F113L patients with proteinuria as well as of brain WML in young patients without morbidities besides late-onset FD due to the p.F113L mutation supports the hypothesis that these clinical manifestations are caused by FD pathology. In summary, our study improves the knowledge on the natural history of late-onset FD, which is essential to make evidence-based clinical decisions and guidelines and to accurately evaluate the clinical efficacy of FD therapies.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymgmr.2020.100565.

Funding

No funding sources.

Disclosures

Olga Azevedo and Gabriel Miltenberger-Miltenyi have received educational and/or research grants from Shire Human Genetic Therapies, Amicus and Sanofi Genzyme. Miguel Gago has received educational grants from Shire Human Genetic Therapies and Sanofi Genzyme. Maria José Guimarães has received educational and research grants from Sanofi Genzyme. The remaining authors have no disclosures.

Author statement

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Damião Cunha - Supervision; review & editing of manuscript.

Acknowledgements

The authors thank to all the health professionals of the Reference Center on Lysosomal Storage Disorders of Hospital Senhora da Oliveira, Guimarães, Portugal, for their important contribution to the diagnosis, treatment and follow-up of FD patients; to Dr. Pedro Pereira, for the selection of the figures of electronic microscopy of the kidney biopsy; to

the historians Dr. Rui Faria, Dr. Alice Martins and Dr. Fátima Dias, for their continuous collaboration in the genealogical research of Fabry families with the founder mutation p.F113L; and to the patients and families.

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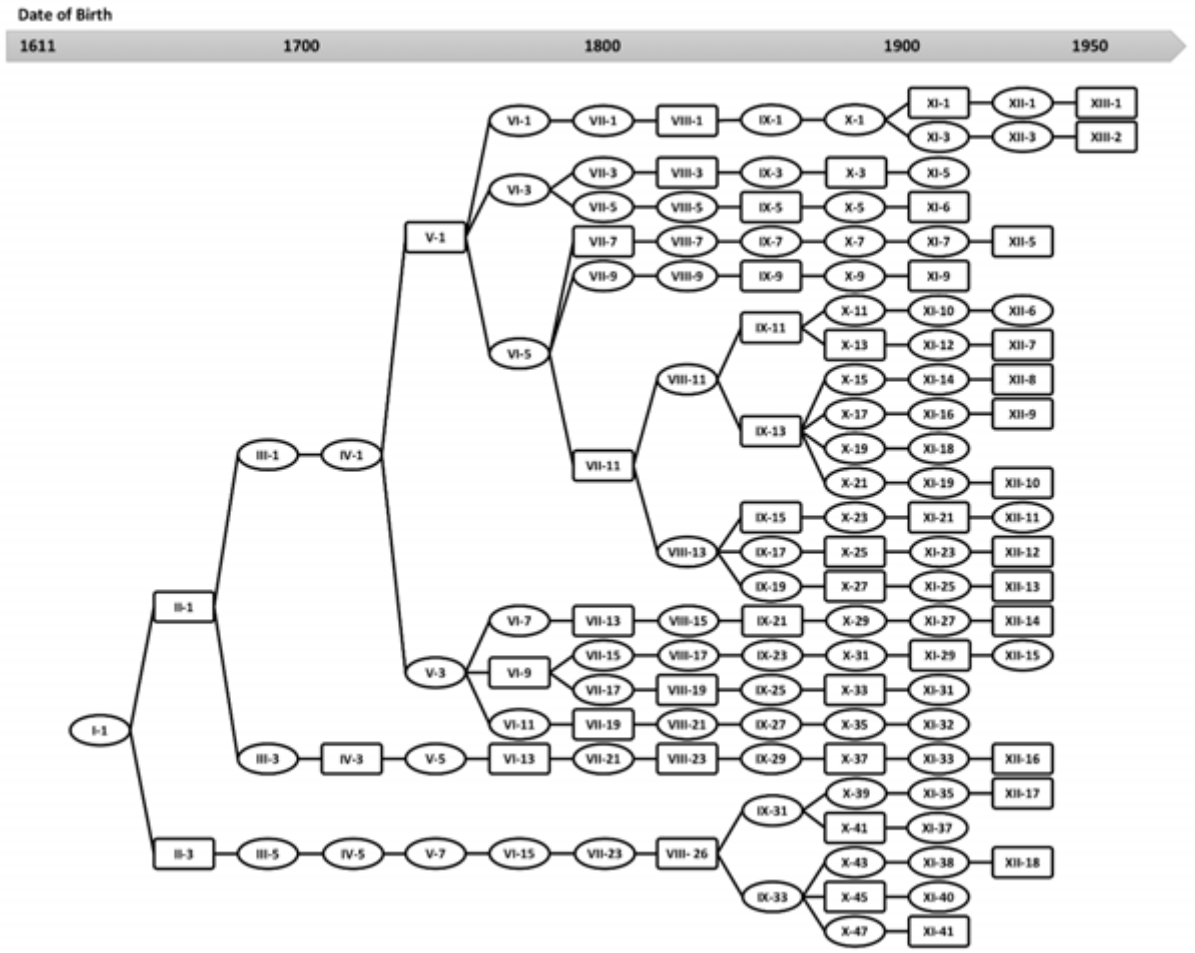
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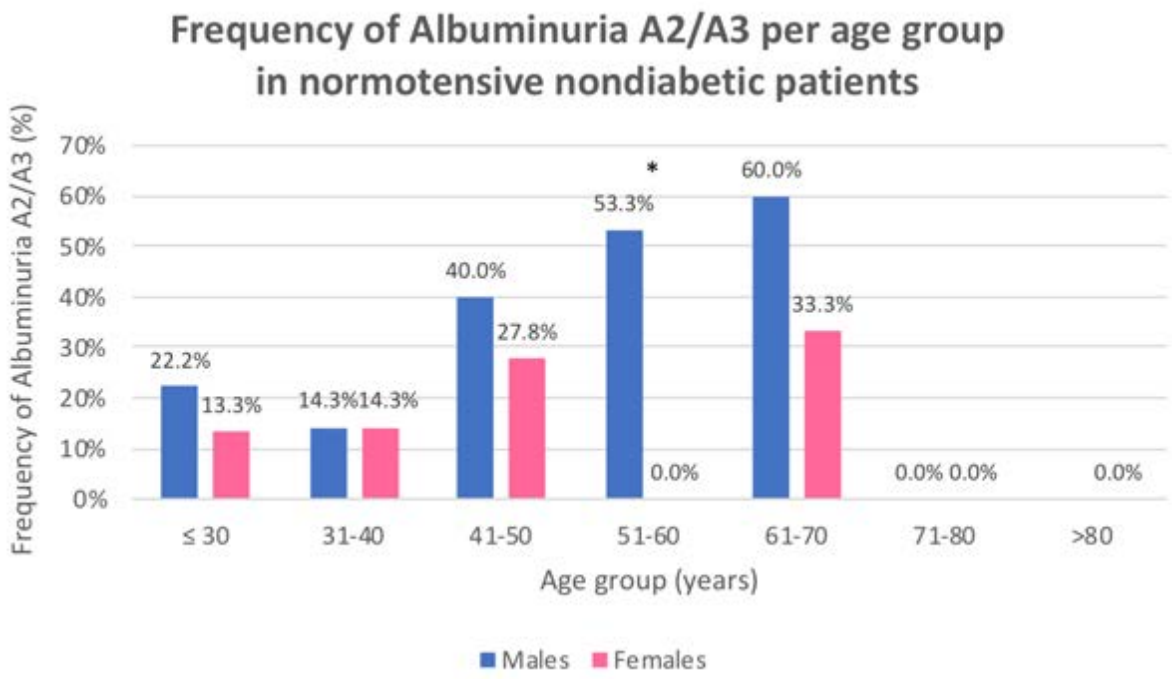
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SUPPLEMENTARY DATA

Supplementary Figure 1. Family tree provided by the historians research, demonstrating the genealogical connection of 25 of 34 FD families with the p.F113L mutation to a common ancestor who was born in 1611 in the region of Guimarães. For simplification, only FD patients are depicted and respective spouses were removed from the pedigree.



Supplementary Figure 2. Frequency of albuminuria A2/A3 per age category in normotensive nondiabetic Fabry patients with the p.F113L mutation. * p<0.05



Chapter 4.3

Azevedo O, Marques N, Reis L, Cruz I, Craveiro N, Antunes H, Lourenço C, Gomes R, Guerreiro RA, Faria R, Sá F, Lima R, Gaspar P, Faria R, Miltenberger-Miltenyi G, Sousa N, Cunha D.

Predictors of Fabry disease in patients with hypertrophic cardiomyopathy: How to guide the diagnostic strategy?

Am Heart J 2020;226:114-126. doi: 10.1016/j.ahj.2020.04.006.

Predictors of Fabry disease in patients with hypertrophic cardiomyopathy: How to guide the diagnostic strategy?

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Background Fabry disease (FD) is a treatable cause of hypertrophic cardiomyopathy (HCM). We aimed to determine the independent predictors of FD and to define a clinically useful strategy to discriminate FD among HCM.

Methods Multicenter study including 780 patients with the ESC definition of HCM. FD screening was performed by enzymatic assay in males and genetic testing in females. Multivariate regression analysis identified independent predictors of FD in HCM. A discriminant function analysis defined a score based on the weighted combination of these predictors.

Results FD was found in 37 of 780 patients with HCM (4.7%): 31 with p.F113L mutation due to a founder effect; and 6 with other variants (p.C94S; p.M96V; p.G183V; p.E203X; p.M290I; p.R356Q/p.G360R). FD prevalence in HCM adjusted for the founder effect was 0.9%. Symmetric HCM (OR 3.464, CI95% 1.151-10.430), basal inferolateral late gadolinium enhancement (LGE) (OR 10.677, CI95% 3.633-31.380), bifascicular block (OR 10.909, CI95% 2.377-50.059) and ST-segment depression (OR 4.401, CI95% 1.431-13.533) were independent predictors of FD in HCM. The score ID FABRY-HCM [*–0.729 + (2.781xBifascicular block) + (0.590xST depression) + (0.831xSymmetric HCM) + (2.130xbasal inferolateral LGE)*] had a negative predictive value of 95.8% for FD, with a cut-off of 1.0, meaning that, in the absence of both bifascicular block and basal inferolateral LGE, FD is a less probable cause of HCM, being more appropriate to perform HCM gene panel than targeted FD screening.

Conclusion FD prevalence in HCM was 0.9%. Bifascicular block and basal inferolateral LGE were the most powerful predictors of FD in HCM. In their absence, HCM gene panel is the most appropriate step in etiological study of HCM. (Am Heart J 2020;226:114-26.)

Fabry disease (FD) (OMIM 301500) is an X-linked lysosomal storage disorder caused by mutations in the *GLA* gene, which codes for the enzyme α-galactosidase A. The deficiency of the enzymatic activity of α-galactosidase A leads to the lysosomal accumulation of globotriaosylceramide (GB3) and other related glycosphingolipids, causing multiorgan damage. Hypertrophic cardiomyopathy (HCM) is the main cardiac manifestation of FD, occurring more commonly in males (43% vs 26%) and nearly one decade later in females (mean age of onset 39 ± 10 vs 50 ± 11 years).[1]

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Submitted August 22, 2019; accepted April 12, 2020.

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https://doi.org/10.1016/j.ahj.2020.04.006

The prevalence of FD in patients with HCM has widely varied from 0% to 12% in previous studies (Supplementary Table I), due to different inclusion criteria, study designs and screening methods, although the latest studies, based on larger cohorts of patients and improved screening methods, have suggested a narrower prevalence range of 0% to 4%.[2-18] Moreover, the common finding of genetic variants of unknown significance (GVUS) in screenings of FD[19] mandates that prevalence results must be continuously re-evaluated as growing knowledge on Genetics comes to establish the pathogenic or benign nature of these variants.

FD is a treatable cause of HCM. Enzyme replacement therapy (ERT) has demonstrated to improve, stabilize or slow the progression of left ventricular (LV) mass,[20-22] wall thickness,[20,21] mid-wall fractional shortening[22] and strain[21] as well as NYHA class[20] and exercise tolerance.[21] However, early diagnosis and treatment are crucial, as the presence of cardiac fibrosis at the timing of ERT initiation may hamper long-term improvement of LV morphology and function and exercise capacity.[21] More recently, migalastat has also shown to improve LV mass and wall thickness.[23]

However, despite increasing awareness on the diagnostic red-flags,[24] FD remains a late diagnosis, being the median delay from symptom onset to diagnosis of 10.5 years (95% CI: 8-13).[25] Therefore, it is essential to accurately determine independent predictors of FD in patients with HCM and to create clinically useful tools that could lead to the early diagnosis of this treatable disease. Herein, we present the prevalence of FD in patients with HCM in a large multicenter study. To the best of our knowledge, this is the first study providing the independent predictors of FD in a large cohort of patients with HCM and a score to identify Fabry cases in patients with HCM.

Methods

FD screening in patients with HCM

Between January 2008 and March 2018, 780 consecutive patients with HCM were recruited from the Cardiology consultation of 12 Portuguese hospital centers (Supplementary Figure 1) and underwent FD screening. The inclusion criteria were adult patients (≥18 years) presenting HCM according to the ESC,[26] ie, left ventricular hypertrophy (LVH) with increased LV wall thickness (≥ 15 mm), by echocardiography, that was not explained by hypertension, valve disease or other cardiac overload conditions.

Enzymatic activity of α-galactosidase A was measured in all patients in dried blood spot (DBS) samples, as described by Gaspar et al.[27] The molecular analysis of the *GLA* gene was performed by PCR sequencing of all exons and their flanking intronic regions, as described by Shabbeer et al,[28] in all females and in males with reduced α-galactosidase A enzymatic activity (<0.3 nmol/h/spot).

Genealogy study

We published in 2013, for the first time, the founder effect of FD due to the mutation p.F113L in the Portuguese region of Guimarães, based on genealogy and haplotype analysis.[29,30] In this study, we also performed a genealogy research of all Fabry index patients that were found with the p.F113L mutation, in order to confirm the familial connection between them and a common ancestor. Historians searched for and analyzed birth, marriage and death certificates, the most recent in the Citizen Records and the most remote, dating to the 17th century, in the Parish Records currently filed in the City Archives.

Predictors of FD in patients with HCM

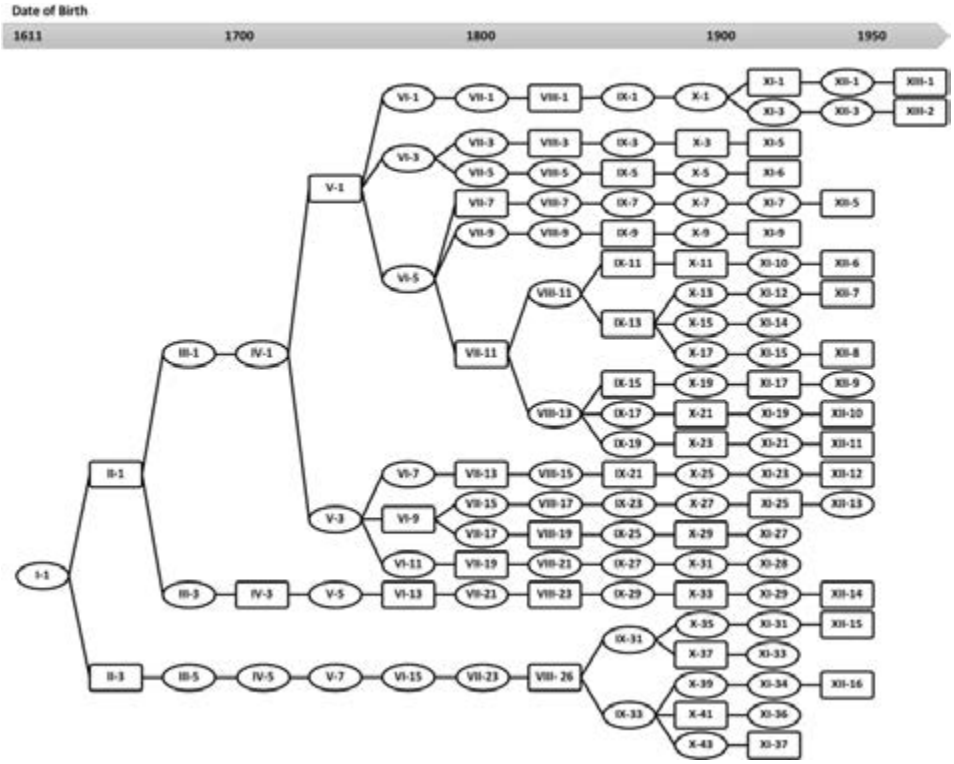
We collected clinical, electrocardiographic, cardiac imaging, laboratory and genetic data. Categorical variables were expressed as percentage. According to the Kolmogorov-Smirnov test, continuous variables were all non-normally distributed and therefore expressed as median with interquartile range (IQR). Comparison of variables between Fabry and non-Fabry patients was performed by χ^2 test for categorical variables and Mann-Whitney U test for continuous variables. Patients with *GLA* variants of uncertain clinical significance were excluded from this statistical analysis. In order to identify independent predictors of FD among patients with HCM, we performed a multivariate binary logistic regression analysis, including the cardiac categorical variables that were readily available on ECG, echocardiogram and cardiac MRI and showed statistically significant differences between FD and non-FD patients. We performed a Discriminant Function Analysis for FD in patients with HCM. We used the Wilk's lambda to calculate the unstandardized coefficients for each of the FD predictors that have been previously identified by multivariate regression analysis and then determined a discriminant score based on the weighted combination of the independent predictors of FD. We calculated the mean discriminant scores for Fabry and non-Fabry patients and determined the best discriminant cut-off for the score. We used the maximum likelihood technique to assign each case to a group according to the specified cut-off, in order to determine the sensitivity, specificity and positive and negative predictive values of the score in the prediction of FD in patients with HCM.

Statistical significance was set at $P < .05$.

Ethical issues

This research project was approved by the Ethics Committee of all the hospital centers included in this study. All patients provided written informed consent.

Figure 1



Family pedigree of FD patients with the mutation p.F113L, demonstrating a genealogical connection to a common ancestor who was born in 1611 in the Portuguese region of Guimarães. The individuals are coded by generation number in roman numerals-Individual number within the generation. Males are represented as squares and females as circles. For simplification, only Fabry patients are depicted and respective spouses were removed from the pedigree. From the 31 p.F113L patients, 27 were found and born in the region of Guimarães, three were found in other regions but were born <10 km from Guimarães and one was found 375 km from Guimarães. The genealogical connection was found for 23 patients (21 from Guimarães and two that were born near Guimarães). The family connection of the remaining p.F113L patients was not found due to lack of archive data.

Results

Prevalence of FD in HCM

The study included 780 patients with HCM, predominantly males (59.7%), with mean age of 66.0 ± 14.3 years, 22.1% of them with family history of HCM. LVH was asymmetrical in 65.0% of the cases. Mean interventricular septum thickness was 17.6 ± 4.3 mm. Mean LV ejection fraction was 64.4 ± 10.1%. LV outflow tract (LVOT) obstruction occurred at rest in 28.3%. Cardiac MRI was performed in 475 patients (60.9%) and revealed LGE in 274 cases (57.7%), mostly intramyocardial (81.5%) and in the septum (basal (50.0%) and mid (48.2%) antero-septal and basal (44.9%) and mid (53.6%) infero-septal segments). 24 h-Holter monitoring was performed in 684 patients (87.7%) and detected ventricular tachycardia (VT) in 15.0% (Supplementary Table II). A sarcomere gene panel had been performed in 497 patients (63.7%). Sarcomeric variants were found in 124 patients (24.9%), mainly in the *MYBPC3* (11.7%), *MYH7* (6.4%), *TNNT2* (2.4%) and *TPM1* (2.0%) genes. Phenocopies were found in 20 patients (2.6%): amyloidosis (15 cases), mitochondrial disease (2 cases), *PRKAG2* cardiomyopathy (1 case), hemochromatosis (1 case) and sarcoglycanopathy (1 case).

FD was diagnosed in 37 of the 780 patients with HCM (4.7%). The pathogenic mutation p.F113L was identified in 31 patients (4.0%) (Supplementary Table III), due to the founder effect previously documented in the Portuguese region of Guimarães[29,30] (Figure 1).

Table I. Characteristics of the patients with non-F113L *GLA* gene variants

Gender, age	*GLA* gene variant	Enzymatic activity of α-GAL A in plasma (nmol/h/mL) / leukocytes (nmol/h/mg)	Urinary GB3 (μg/mmol creatinine) / Plasma GB3 (nmol/mL)	Plasma Lyso-GB3 (ng/mL)	IVS / PW thickness (mm)	LV mass (g/m2)	LVH characteristics	Other clinical manifestations
Pathogenic *GLA* gene variants								
M, 50	p.C94S	1.0 / 0	NA / 17.77	107.19	21 / 18	277	Symmetrical Non-obstructive EF 58% DD grade II	Non-sustained VT Renal failure Microalbuminuria Proteinuria Stroke Deafness Acroparesthesias Anhidrosis Cornea verticillata Angiokeratomas
F, 56	p.M96V	2.2 / 5.6	124 / NA	6.73	22 / 17	167	Symmetrical Non-obstructive EF 70% DD grade II Diffuse LGE	Short PR interval Atrial fibrillation Microalbuminuria Cornea verticillata Deafness
F, 50	p.G183V	3.9 / 21.0	76 / n.d.	4.34	9 / 15	98	PW LVH Non-obstructive EF 80% LGE on basal inferolateral segment	Short PR interval Brain white matter lesions
F, 56	p.E203X	6.7 / 7.1	81 / 12.86	11.64	20 / 12	196	Asymmetrical Non-obstructive EF 68% DD grade I LGE on inferolateral wall	Renal failure Microalbuminuria Proteinuria Stroke Brain white matter lesions Deafness Cornea verticillata
F, 43	p.M290I	14.0 / 6.0	NA / n.d.	n.d.	23 / 12	212	Asymmetrical Non-obstructive EF 78% DD grade II LGE on inferior and inferoseptal walls, apical segments and basal inferolateral segment	Proteinuria Brain white matter lesions
M, 63	p.R356Q p.G360R	1.0 / 0	251 / NA	71.1	20 / 15	129	Symmetrical Non-obstructive DD grade II LGE in the apical segments and mid-septal segments	Myocardial infarct Stroke Brain white matter lesions Acroparesthesias Hypohidrosis Abdominal pain and diarrhea Angiokeratomas Cornea verticillata Deafness
Other *GLA* gene variants								
M, 28	p.A143T	NA / NA	NA / NA	NA	23 / 13	209	Asymmetrical Obstructive DD grade II LGE in the basal septal and mid anteroseptal and inferolateral segments *Pathogenic mutation on the *MYBPC3* gene (p.R943X)	Non-sustained VT

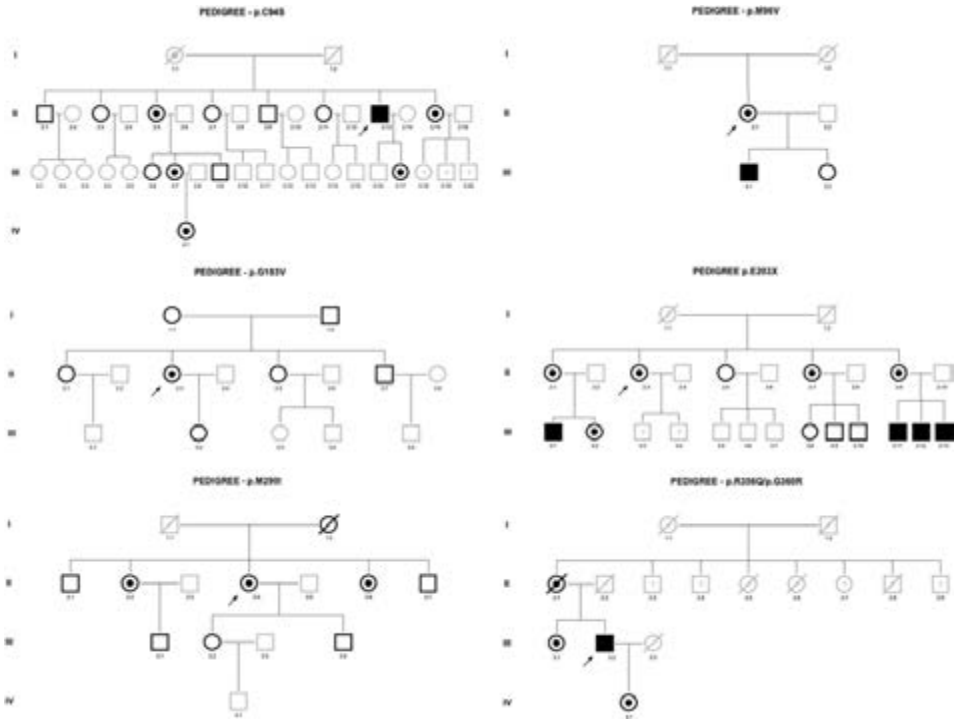
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Table I (*continued*)

Gender, age	*GLA*gene variant	Enzymatic activity of α-GAL A in plasma (nmol/h/mL) / leukocytes (nmol/h/mg)	Urinary GB3 (μg/mmol creatinine) / Plasma GB3 (nmol/mL)	Plasma Lyso-GB3 (ng/mL)	IVS / PW thickness (mm)	LV mass (g/m2)	LVH characteristics	Other clinical manifestations
M, 73	p.R118C	NA / NA	NA / NA	NA	21 / 10	197	Asymmetrical Obstructive DD grade II LGE in septum *GVUS on *MYBPC3* gene (p.D610H)	1st degree AV block Complete RBBB Non-sustained VT
F, 69	p.R118C	NA / NA	NA / NA	NA	11 / 9	214	Apical HCM EF 52% DD grade II No LGE	None
F, 75	p.R118C	NA / NA	NA / NA	NA	15 / 13	133	Symmetrical Non-obstructive EF 70% DD grade I No LGE *GVUS on *MYH7* gene (p.R1781H)	None
M, 36	p.D313Y	NA / NA	NA / NA	1.0	18 / 10	89	Asymmetrical Non-obstructive EF 71% DD grade I LGE in antero-septal segments	None
F, 70	p.D313Y	NA / NA	NA / NA	1.6	20 / 20	234	Symmetrical Obstructive EF 70% DD grade II No LGE	Short PR interval Complete LBBB Paroxysmal atrial fibrillation
F, 70	p.D313Y	NA / NA	NA / NA	1.2	24 / 6	112	Asymmetrical Non-obstructive EF 81% DD grade I No LGE	Atrial fibrillation
F, 76	p.D313Y	NA / NA	NA / NA	NA	15 / 12	177	Symmetrical Non-obstructive DD grade I No LGE	None
F, 83	p.D175E	NA / NA	NA / NA	NA	16 / 10	185	Asymmetrical Non-obstructive EF 68% LGE in the septal and basal inferolateral segments	Renal failure Microalbuminuria Proteinuria Stroke
F, 61	p.V22A p.A73S	9.0 / 31.0	NA / NA	NA	16 / 10	98	Asymmetrical Non-obstructive EF 60% DD grade II LGE on septum *Pathogenic mutation on the *MYBPC3* gene (p.R502W)	1st degree AV block Non-sustained VT Acroparesthesias

α-GAL A, α-galactosidase A; AV, atrioventricular; DD, diastolic dysfunction; EF, ejection fraction; GVUS, genetic variant of unknown significance; IVS, interventricular septum; LBBB, left bundle branch block; LGE, Late gadolinium enhancement; LV, left ventricular; LVH, left ventricular hypertrophy; NA, non-available; n.d., non-detectable; PW, posterior wall; RBBB, right bundle branch block; VT, ventricular tachycardia.
Reference values: Enzymatic activity of α-galactosidase A on plasma 6–19 nmol/h/mL, and on leukocytes 36–80 nmol/h/mg; urinary GB3 0.87–13 μg/mmol creatinine; plasma GB3 0.8–4.52 nmol/mL; plasma Lyso-GB3 0–1.9 ng/mL.

Figure 2



Pedigrees of the Fabry families with non-F113L *GLA* gene variants.

Considering this founder effect, the prevalence of FD in patients with HCM would be 0.9%.

The other six Fabry patients presented respectively the following *GLA* gene variants: p.C94S; p.M96V; p.G183V; p.E203X; p.M290I; and p.R356Q/p.G360R (Table I). Lysosomal inclusions were demonstrated in the myocardium of the p.M290I patient and in the skin of the p. R356Q/p.G360R patient.

Their pedigrees are illustrated in Figure 2. Relevant family history of these *GLA* gene variants include: (i) p. C94S: Affected relatives presented clinical manifestations compatible with FD (renal failure in one sister; and proteinuria and acroparesthesias in one daughter, one niece and one great-niece) and increased plasma lysoGB3; (ii) p.M96V: The affected 19-year old son presented renal failure, proteinuria, cornea *verticillata*, angiokeratomas, acroparesthesias, hypohidrosis, abdominal pain and diarrhea as well as increased urinary GB3 (182 μg/mmol creatinine) and plasma lyso-GB3 (10.82 ng/mL). Skin biopsy showed lysosomal inclusions compatible with FD; (iii) p.G183V: All her relatives tested negative for this *GLA* variant, being impossible to determine if this case results from a sporadic "de novo" mutation or an illegitimate father; (iv) p.E203X: All the affected relatives exhibited a classical phenotype with increased FD biomarkers; (v): p.M290I: Her two affected sisters have been so far asymptomatic; (vi) p.R356Q/p.G360R: His daughter and sister had the same *GLA* gene variants. The daughter has been so far asymptomatic and his sister had HCM and microalbuminuria.

The screening also found two males with residual enzymatic activity of α-galactosidase A, carrying respectively the p.A143T (0.15 nmol/h/spot) and p.R118C (0.23 nmol/h/spot) variants; two females with the p.R118C variant; four patients with the p.D313Y variant; an elderly female with diabetes, hypertension and dyslipidemia, no family history suggestive of FD and the GVUS p.D175E; and a female with the novel variants p.V22A/p.A73S, who presented a pathogenic mutation in the *MYBPC3* gene and in whom pedigree analysis did not support the

Table II. Comparison of baseline characteristics between the patients with HCM secondary to FD vs non-FD

Baseline characteristics	Fabry patients (n = 37)	Non-Fabry patients (n = 733)	*P*
Male gender (%)	24 (64.9%)	439 (59.9%)	.547
Age (years) (median, IQR)	64.0 (57.5, 75.0)	68.0 (57.0, 77.0)	.453
Family history of LVH (%)	23 (65.7%)	134 (19.8%)	<.001
Symptoms (%)	32 (86.5%)	559 (76.5%)	.158
Dyspnea (%)	28 (75.7%)	401 (54.9%)	.013
Chest pain (%)	11 (29.7%)	182 (24.9%)	.509
Palpitations (%)	9 (24.3%)	132 (18.1%)	.339
Pre-syncope (%)	9 (24.3%)	64 (8.8%)	.002
Syncope (%)	4 (10.8%)	92 (12.6%)	.750
NYHA class			
I (%)	18 (62.1%)	197 (38.6%)	
II (%)	11 (37.9%)	265 (52.0%)	.056
III (%)	0 (0.0%)	46 (9.0%)	
IV (%)	0 (0.0%)	2 (0.4%)	
Echocardiogram (n = 38 vs 742)			
LVH pattern			
Asymmetrical (%)	17 (45.9%)	483 (66.0%)	
Symmetrical (%)	20 (54.1%)	145 (19.8%)	<.001
Apical (%)	0 (0.0%)	93 (12.7%)	
Other (%)	0 (0.0%)	11 (1.5%)	
LV outflow tract obstruction at rest (%)	3 (8.1%)	181 (24.8%)	.020
IVS thickness (mm) (median, IQR)	18.0 (17.0, 21.0)	17.0 (15.0, 20.0)	.087
PW thickness (mm) (median, IQR)	13.0 (12.0, 17.0)	11.0 (9.0, 13.0)	<.001
LV end-diastolic diameter (mm) (median, IQR)	43.0 (38.0, 46.5)	47.0 (43.0, 52.0)	<.001
LV mass (g/m^2) (median, IQR)	154.5 (129.0, 195.0)	153.0 (116.0, 194.0)	.422
LV ejection fraction (%) (median, IQR)	67.0 (61.0, 74.0)	65.0 (58.0, 71.0)	.139
Septal Sa (cm/s) (median, IQR)	5.9 (5.0, 6.0)	7.0 (5.0, 9.0)	<.001
Lateral Sa (cm/s) (median, IQR)	6.0 (6.0, 7.0)	8.0 (6.0, 10.0)	<.001
LV Diastolic dysfunction (%)	32 (86.5%)	586 (84.8%)	.781
Grade I (%)	17 (53.1%)	304 (52.3%)	
Grade II (%)	15 (46.9%)	252 (43.4%)	.480
Grade III (%)	0 (0.0%)	25 (4.3%)	
Undetermined grade (%)	0 (0.0%)	5 (0.7%)	
Septal Ea (cm/s) (median, IQR)	5.0 (4.0, 6.0)	5.4 (4.0, 7.0)	.098
Lateral Ea (cm/s) (median, IQR)	6.0 (5.0, 9.0)	7.0 (5.6, 9.0)	.328
LA volume (mL/m2) (median, IQR)	33.0 (28.0, 39.0)	35.4 (27.0, 48.0)	.185
Cardiac MRI (n = 28 vs 442)			
Late gadolinium enhancement (%)	23 (85.2%)	244 (55.8%)	.003
Electrocardiogram (n = 37 vs 732)			
Sinus rhythm (%)	36 (97.3%)	609 (83.2%)	.023
Atrial fibrillation (%)	1 (2.7%)	89 (12.2%)	.081
Atrial flutter (%)	0 (0.0%)	11 (1.5%)	.453
Pacemaker rhythm (%)	2 (5.4%)	41 (5.6%)	.960
Short PR interval (%)	2 (7.1%)	13 (1.9%)	.059
Atrioventricular block (%)	4 (11.4%)	67 (9.3%)	.670
Left bundle branch block (%)	2 (5.7%)	54 (7.5%)	.697
Right bundle branch block (%)	4 (11.4%)	48 (6.7%)	.276
Bifascicular block (%)	13 (37.1%)	25 (3.5%)	<.001
T wave inversion (%)	22 (62.9%)	348 (48.7%)	.103
ST segment depression (%)	19 (54.3%)	197 (27.6%)	.001
Sokolow-Lyon Index (mm) (median, IQR)	30.0 (18.0, 51.0)	32.0 (25.0, 41.0)	.933
24 h Holter (n = 36 vs 638)			
Sinus rhythm (%)	35 (97.2%)	539 (84.4%)	.035
Atrial fibrillation (%)	3 (8.3%)	88 (13.8%)	.353
Atrial flutter (%)	1 (2.8%)	8 (1.3%)	.437
Pacemaker rhythm (%)	3 (8.3%)	32 (5.0%)	.381
Atrioventricular block (%)	4 (11.4%)	51 (8.0%)	.476
Left bundle branch block (%)	2 (5.7%)	35 (5.5%)	.959
Right bundle branch block (%)	5 (14.3%)	29 (4.6%)	.011
Bifascicular block (%)	13 (37.1%)	14 (2.2%)	<.001
Supraventricular tachycardia (%)	18 (50.0%)	157 (24.6%)	.001
Ventricular tachycardia (%)	6 (16.7%)	108 (17.0%)	.964
Devices			

Table II (*continued*)

Baseline characteristics	Fabry patients (n = 37)	Non-Fabry patients (n = 733)	*P*
Pacemaker (%)	5 (13.5%)	54 (7.4%)	.171
Implantable cardioverter-defibrillator (%)	0 (0%)	69 (9.4%)	.050
Diagnostic red-flags for Fabry disease			
Stroke (%)	4 (10.8%)	66 (9.1%)	.730
Renal failure (%)	4 (10.8%)	89 (12.7%)	.739
Microalbuminuria >30 mg/24 h (%) (n = 34 vs 295)	19 (55.9%)	90 (30.9%)	.004
Proteinuria >300 mg/24 h (%) (n = 32 vs 288)	9 (28.1%)	39 (13.7%)	.032
Other comorbidities			
Hypertension (%)	22 (59.5%)	500 (68.2%)	.266
Diabetes mellitus (%)	7 (18.9%)	158 (21.6%)	.694
Dyslipidemia (%)	24 (64.9%)	386 (52.9%)	.154
Obesity (%)	4 (10.8%)	166 (22.6%)	.090
Smoking (%)	5 (13.5%)	89 (12.2%)	.809
Coronary heart disease (%)	5 (13.5%)	62 (8.5%)	.290

pathogenicity of these *GLA* variants (Table I). In fact, this p.V22A/p.A73S female had normal enzymatic activity of alpha-galactosidase A in plasma (9 nmol/h/mL) and mildly reduced in leukocytes (31 nmol/h/mg). She did not have children. Her mother also presented HCM as well as the *MYBPC3* mutation, but her *GLA* status is unclear, as the HCM genetic testing did not include the *GLA* gene. Her brother carried only one of these variants (p.A73S), but had no clinical manifestations and presented normal enzymatic activity of alpha-galactosidase A in plasma (10 nmol/h/mL) and leukocytes (37 nmol/h/mg). He also has no children.

Predictors of FD in patients with HCM

Compared to non-Fabry patients, Fabry patients reported more commonly family history of LVH and complained more often of dyspnea and pre-syncope. LVH was mainly symmetrical, with higher posterior wall thickness and lower LV end-diastolic diameter. LVOT obstruction at rest was less common. Septal and lateral Sa were lower. LGE was more common, particularly in the basal and mid inferolateral segments (63.6% vs 9.6%, $P < .001$; 54.5% vs 8.1%, $P < .001$), basal and mid inferior segments (40.9% vs 9.6%, $P < .001$; 40.9% vs 13.5%, $P < .001$) and apical lateral, anterior and septal segments (31.8% vs 13.5%, $P = .017$; 36.4% vs 18.4%, $P = .037$; 36.4% vs 19.1% $P = .049$). Fabry patients also presented more commonly bifascicular block and ST-segment depression on ECG and microalbuminuria and proteinuria (Table II). Of note, nine of the 37 Fabry patients did not perform cardiac MRI, for the following reasons: nonconditional pacemaker (n = 2), severe renal failure (n = 2), nonconditional pacemaker and severe renal failure (n = 1), other medical device/foreign body incompatible with MRI (n = 3) and patient refusal (n = 1).

On binary logistic multivariate regression analysis, we included the cardiac categorical variables that were readily available from ECG, echocardiogram and cardiac MRI and showed statistically significant differences between FD and non-FD patients (Table II): bifascicular block, ST-segment depression, symmetrical pattern of LVH, LV outflow tract obstruction at rest and basal inferolateral LGE. The following variables were identified as predictors of FD: symmetric LVH (OR 3.464, CI 95% 1.151-10.430, $P = .027$), basal inferolateral LGE (OR 10.677, CI 95% 3.633-31.380, $P < .001$), bifascicular block (OR 10.909, CI 95% 2.377-50.059, $P = .002$) and ST-segment depression on ECG (OR 4.401, CI 95% 1.431-13.533, $P = .032$). The most powerful predictors were bifascicular block and basal inferolateral LGE.

Based on the identified predictors of FD, we created the score ID FABRY-HCM to help to identify FD cases among patients with HCM:

ID FABRY-HCM = *−0.729 + (2.781 x Bifascicular block on ECG) + (0.590 x ST depression on ECG) + (0.831 x Symmetric LVH) + (2.130 x basal inferolateral LGE).*

If these variables are present, its respective value in the score formula will be 1; if they are absent, its value will be 0. In the ID FABRY-HCM score, the cut-off value of 1.0 has a sensitivity of 63.4%, specificity of 97.2%, positive predictive value of 68.3% and a negative predictive value of 95.8%. From the analysis of this score, we can conclude that neither the presence of symmetrical HCM or ST-segment depression alone, or the combination of the two, is enough to obtain a score in favor of FD. However, the presence of either bifascicular block or basal inferolateral LGE alone achieves a favorable score for FD. More importantly, in the absence of both bifascicular block and basal inferolateral LGE, the negative predictive value for FD is 95.8%.

Discussion

This study found a FD prevalence of 0.9% in patients with HCM and identified bifascicular block, basal inferolateral LGE, symmetrical HCM and ST-segment depression as independent predictors of FD among patients with HCM. Moreover, this study provides a score to identify FD cases in patients with HCM. According to this score, HCM in the absence of both bifascicular block and basal inferolateral LGE has a negative predictive value of 95.8% for FD.

Prevalence of FD in HCM

Compared to previous studies on the prevalence of FD in HCM,[2-18] our study presents several strengths: (i) large sample size; (ii) inclusion of males and females; (iii) well-defined diagnostic criteria of HCM; (iv) combined enzymatic and genetic approach as a screening method; (v) solid evidence of the pathogenicity of *GLA* variants that were included for prevalence calculation.

To the best of our knowledge, this is the second largest study on the prevalence of FD in HCM.[2-18] In previous studies, inclusion criteria widely ranged (i) from extremely restrictive (patients submitted to myectomy[4] or endomyocardial biopsy[5]); (ii) to extremely broad (LVH ≥12 mm,[16] ≥13 mm,[3,6,12,13] >13 mm[7] or ≥13 mm without exclusion of hypertension and valve disease[2,14]); and (iii) from more elaborated, combining LV thickness with several factors (no obstruction,[15] negative genetic test for sarcomeric HCM[6,9,18] or exclusion of family history of sudden death in >2 relatives, inheritance pattern inconsistent with X-linked pattern and histology compatible with sarcomeric HCM[18]); (iv) to more straightforward (ESC definition of HCM[8,10,11,17]). Like in these last studies, our study inclusion criteria were based on the ESC definition of HCM and, therefore, as described in the literature, LVH was more commonly asymmetrical with preferential septal involvement and LGE was more commonly found in the septum. LV diastolic dysfunction with preserved ejection fraction was found in most patients. Obstruction at rest (23.8%), atrial fibrillation (11.7%) and VT (15.0%) were, however, less frequent than reported in the literature (33.3%, 22.5% and 25.0%, respectively).[26]

Some of the previous studies included only males and used enzymatic assay as screening method.[2,3,12,13,17] One study included only females and used electron microscopy of endomyocardial biopsies as a screening method.[5] Other studies included both genders and used urinary GB3,[16] electron microscopy of myectomy specimens,[4] the enzymatic assay alone,[10] the genetic testing[6,7,9,11] or a combined enzymatic and genetic approach.[8,14,15,18] Similarly to these last studies, we included males and females and used a combined enzymatic and genetic approach that allows FD diagnosis in both genders.[31]

GVUS are commonly found on FD screenings.[19] In our study, most of the reported *GLA* variants were already described as pathogenic mutations. The mutation c.337 T > C (p.F113L) leads to enzyme misfolding and consequent degradation in the endoplasmic reticulum, causing a late-onset phenotype characterized by predominant cardiac involvement.[30] The mutation c.281G > C (p.C94S) affects an enzyme disulfide bond and causes a severe classical phenotype.[32] The mutation c.548G > T (p.G183V) occurs in a highly conserved residue, buried in the enzyme 3D structure of the catalytic domain, and is predicted to cause enzyme instability and a classical phenotype.[33] The mutation c.607G > T (p.E203X) affects the enzyme active site and causes a classical phenotype of FD.[34] The mutation c.870G > A (p.M290I) leads to enzyme destabilization and misfolding and was associated to a classical phenotype.[33,35] Our patient presents a similar missense mutation (c.870G > C), described as pathogenic in ClinVar database,[36] resulting in the same protein abnormality (p.M290I). The variant c.1067G > A (p.R356Q) is most likely benign, but the mutation c.1078G > C (p.G360R) occurs in the enzyme dimer interface, causing a classical phenotype of FD.[37]

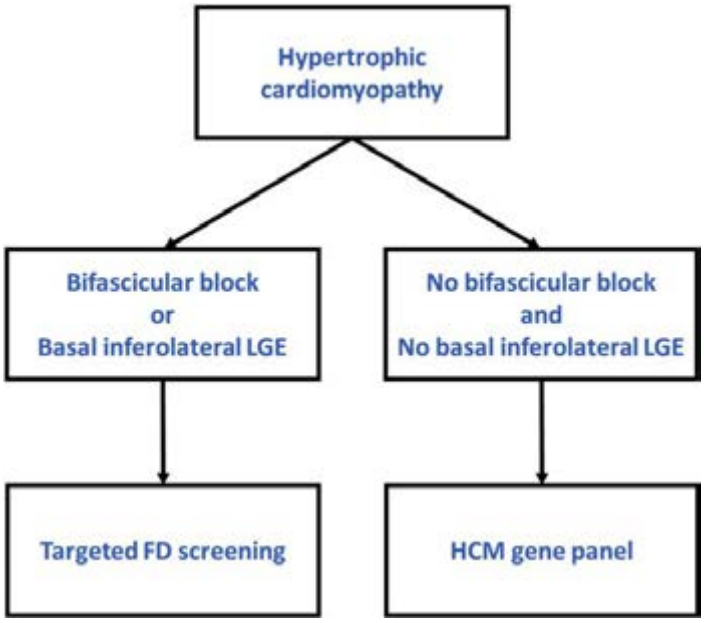
To the best of our knowledge, the *GLA* variants p.V22A and p.A73S were never reported before. According to in-silico models Polyphen-2 and MutationTaster, p.V22A is predicted to be a polymorphism, while p.A73S is predicted to be a disease causing variant.[38,39] The variant p.A73E, which affects the same amino acid as p.A73S, was already reported in a 58-year old female with LVH, end-stage renal disease and stroke and reduced enzymatic activity of α-galactosidase A.[40] Furthermore, p.A73V, another variant at the same codon, was associated to reduced enzymatic activity of α-galactosidase A in a male newborn.[41] However, the documentation of the p.A73S variant in her brother with normal enzymatic activity of alpha-galactosidase A on plasma and leukocytes, together with the finding of a pathogenic mutation on the *MYBPC3* gene, also present in her mother with HCM, made us classify this HCM as sarcomeric and this *GLA* variant as non-disease causing.

To our knowledge, the variant c.286A > G (p.M96V) has also never been reported, but indisputable evidence of its pathogenic role was based on the severe classical phenotype of FD exhibited by the patient and her son, both with increased urinary GB3 and plasma lyso-GB3, together with the demonstration of lysosomal inclusions on skin biopsy.

Patients with the genetic variants p.A143T and p.R118C were not classified as Fabry patients, as the pathogenic role of these variants has recently been questioned.[42,43]

Terryn et al reported p.A143T cases with clinical manifestations that could be attributable to FD, but without GB3 deposits in organ biopsies and in which biopsies revealed alternative diagnoses.[42] Recently, Valtola et al reported elevated plasma lysoGB3 levels

Figure 3



Proposed diagnostic strategy to the identification of Fabry cases during the etiological study of HCM.

and GB3 deposits in p.A143T males with LVH, who had a relatively high residual enzymatic activity (25%-40%) and a negative test for an HCM panel with 59 genes, suggesting that p.A143T is very likely a late-onset FD-causing variant.[44] Nevertheless, our p.A143T patient presented severe HCM at the age of 28 years old, which is too young for an HCM due to late-onset FD, even for a male, which means that even if we considered p.A143T variant as pathogenic, this variant alone could not explain HCM at this age neither its severity. Moreover, he had a documented pathogenic mutation in the *MYBPC3* gene that could fully explain the HCM phenotype. Therefore, we did not classify this HCM case as caused by FD, although we cannot exclude a contributing role of this *GLA* variant to the progression of HCM with advancing age.

Besides the relatively high residual enzymatic activity in leukocytes in p.R118C males (32%-45%), Ferreira et al reported the case of a 56-year old male with this *GLA* variant, who presented mild LVH and myelin figures within rare cardiomyocytes, but had normal levels of plasma lyso-Gb3 and urinary GB3 measured at the age of 64 years and no significant progression of the cardiac disease from the age of 54 to 64 years.[43] In our 73-year-old male with asymmetrical obstructive HCM with LGE in the septum, it is reasonable to hypothesize that it could be a sarcomeric HCM, given the pattern of LGE and LVH which is more typical of a sarcomeric cause than FD, the presence of obstruction which is rare in FD and the fact that the genetic testing, besides revealing a GVUS in the *MYBPC3* gene, included only 11 HCM genes. In our 69-year old female with apical HCM, although genetic testing was not performed, a sarcomeric cause is also possible, as apical HCM is rare in FD. In the 75-year old female with moderate symmetrical non-obstructive HCM with no LGE, besides the GVUS on the *MYH7* gene, we should also consider the possibility of isolated basal septal hypertrophy of the elderly. Although we cannot exclude that the p.R118C variant may be associated to a very attenuated and late-onset FD, the unclear role of this *GLA* variant and the possibility of alternative diagnoses that could explain the HCM phenotype in our p.R118C patients made us exclude these patients from the cohort of patients classified as FD patients.

The variant p.D313Y is associated with an enzymatic pseudodeficiency,[45] as reflected by the normal plasma lyso-Gb3 values both in our female and male patients, and therefore not included in FD prevalence calculation. HCM genetic testing would be appropriate to assess a sarcomeric cause in the 36-year old male with asymmetrical non-obstructive HCM and antero-septal LGE, and in the 70-year old female with asymmetrical non-obstructive HCM and no LGE, despite the absence of a family history

of HCM in both of these p.D313Y cases. HCM genetic testing would also be appropriate to assess a sarcomeric or PRKAG2 cause in the 70-year old female with this *GLA* variant and symmetrical obstructive HCM, short PR interval, left bundle branch block, family history of HCM and no history of hypertension. It should be considered the possibility of isolated basal septal hypertrophy of the elderly in the 76-year old female with the p.D313Y variant and moderate symmetrical non-obstructive LVH, no LGE and no family history of HCM.

Finally, the variant p.D175E, predicted by Polyphen-2 as benign, was already described in a female with normal lyso-GB3 and in-vitro near-normal enzymatic activity.[33] In our patient, sarcomeric HCM and isolated basal septal hypertrophy cannot be fully excluded as causes of LVH, and proteinuria, renal failure and stroke could also be explained by the cardiovascular risk factors. Therefore, we considered p.D175E as a GVUS, probably benign.

Based on these considerations and the founder effect of FD due to the p.F113L mutation in the region of Guimarães,[29,30] FD prevalence in HCM was set at 0.9%. If we considered p.A143T and p.R118C variants to be associated with attenuated and late-onset FD, FD prevalence in HCM would be 1.4% (11/780).

Predictors of FD in patients with HCM

FD is a treatable condition and cardiologists' awareness is paramount to achieve an appropriate and timely diagnosis and treatment.

In this study, the most powerful predictors of FD among HCM patients were bifascicular block and basal inferolateral LGE. The LVH pattern is readily known as soon as the diagnosis of HCM is established and, therefore, is one of the first characteristics that will guide the etiological study of HCM. Although HCM is typically symmetrical in FD,[24] the common occurrence of fibrosis and thinning of the posterior wall with loss of the symmetrical LVH pattern at the HCM stage (≥15 mm)[24,46] make, together with the existence of this LVH pattern in other phenocopies (amyloidosis, mitochondrial disorders, Danon disease),[24] symmetrical HCM a weak predictor of FD, as was demonstrated in this study. Conversely, although basal inferolateral LGE may appear in other conditions[47,48] and LGE may involve other segments with FD progression,[49] the basal inferolateral LGE revealed to be one of the most powerful predictors of FD. Niemann et al also found that when ST segment or T wave alterations are absent, replacement fibrosis is very unlikely in FD, which might explain the finding of ST depression as a predictor of FD in HCM.[50] Cardiac conduction disorders are known cardiac manifestations of FD.[1] However, to our knowledge, this is the first study demonstrating that bifascicular block is the most powerful predictor of FD in patients with HCM.

Although extracardiac manifestations, such as acroparesthesias, angiokeratomas and cornea *verticillata*, are red-flags to FD diagnosis,[24] they are not commonly searched by cardiologists during the diagnostic study of HCM. Besides, they are frequently absent in heterozygous females and late-onset phenotypes.[1] Renal or cerebrovascular disease may also be absent in heterozygous females and in some late-onset phenotypes with a predominant organ involvement,[1] which limits their utility in the etiological study of HCM. Conversely, a score to guide the suspicion of FD based on LVH characteristics that are readily available on ECG, echocardiogram and cardiac MRI appeared to be useful in the clinical practice. However, from the analysis of the score, it became clear that its application was not needed in the clinical practice and that the algorithm to define the most appropriate diagnostic strategy in the etiological study of HCM could be simplified as illustrated in Figure 3. In simple words, in the presence of either bifascicular block or basal inferolateral LGE, targeted FD screening is the most appropriate next step in the etiological study of HCM; and in the absence of both bifascicular block and basal inferolateral LGE, FD is a less probable cause of HCM and a wide HCM gene panel is the most adequate strategy to its etiological study. The positive predictive value of the score (68.3%) is explained by the low prevalence of FD. However, given the cost difference between a targeted FD screening and a wider HCM gene panel, especially in males, it seems reasonable to proceed first with a targeted FD screening in patients with positive scores, i.e., with bifascicular block and/or basal inferolateral LGE, despite the modest positive predictive value. The score sensitivity is 63.4%, which might be explained by the fact that bifascicular block already represent an advanced stage of the disease that may be absent in the milder cases of FD at the HCM stage (≥15 mm). Although less probable, the basal inferolateral LGE may also be absent in these milder cases of Fabry HCM. Moreover, the high negative predictive value for FD (95.8%) that is achieved in patients with HCM in the absence of both bifascicular block and basal inferolateral LGE is known to be less meaningful in the field of rare diseases, as even a low number of false negatives may represent a non-negligible percentage of missed diagnoses in a universe of rare cases.

However, although the score will miss milder Fabry HCM cases and these may represent a non-negligible percentage of FD cases, it is important to emphasize that this algorithm does not intend to limit FD screening in patients with negative scores, i.e. without bifascicular block and basal inferolateral LGE, rather it intends to guide physicians on the most probable cause of HCM and therefore the most appropriate next step in the etiological study of HCM. Given the potential clinical impact of specific FD therapy, it is advisable to systematically screen all patients with unexplained LVH for FD, either by targeted screening or within a wider genetic approach. However, targeted FD screening has a lower cost than a wider gene panel of HCM. In the presence of bifascicular block or basal inferolateral LGE, this

score favors targeted FD screening. In patients with HCM in the absence of both bifascicular block and basal inferolateral LGE, it favors a wider panel of HCM genes that currently also include the *GLA* gene, despite the lower probability of FD in this case (Figure 3). Our study presents some limitations that should be acquainted. The diagnosis of FD was considered according to the current knowledge on genetics that is constantly evolving and may reveal in the future different pathophysiological roles for the *GLA* gene variants that were found in this study. The majority of the Fabry cases presented the same *GLA* gene mutation. This score only applies to adult patients with the ESC definition of HCM and therefore is not applicable to pediatric patients or patients with milder LVH (wall thickness <15 mm), in whom the early diagnosis of FD could potentially carry a higher prognostic impact. Nevertheless, it should be emphasized that the identification of Fabry cases at the HCM stage could enable the identification of several relatives[51] at earlier stages of the disease. Finally, future studies are needed to obtain an external validation of this score in other large cohorts of HCM and in patients with other *GLA* gene mutations.

In summary, this study, based on the ESC definition of HCM, using a combined enzymatic and genetic approach and taking into account the current knowledge on genetics of FD and the existence of a founder effect in a particular Portuguese region, sets FD prevalence among patients with HCM in 0.9%. This study also showed that bifascicular block and basal inferolateral LGE are the most powerful predictors of FD in patients with HCM. In the absence of both bifascicular block and basal inferolateral LGE, FD is a less probable cause of HCM, according to the ID FABRY-HCM score, being more appropriate to perform an HCM gene panel as the next step of the etiological study of HCM.

Funding

This research was supported by Shire Human Genetic Therapies, Inc.

Shire Human Genetic Therapies, Inc. had no role on the study design/conductance, data collection/analysis/interpretation or manuscript preparation/review/approval.

Disclosures

Olga Azevedo and Gabriel Miltenberger-Miltenyi received educational support and/or research grants from Shire Human Genetic Therapies, Inc., Genzyme Corporation and Amicus. Nuno Marques, Hugo Antunes and Liliana Reis received educational support from Shire Human Genetic Therapies, Inc. and Genzyme Corporation. Nuno Craveiro and Rui Azevedo Guerreiro received educational support from Shire Human Genetic Therapies, Inc. The remaining authors have no disclosures.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ahj.2020.04.006.

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SUPPLEMENTARY TABLES

Supplementary Table I: Summary of previous studies on Fabry disease prevalence in patients with HCM or LVH

Authors	Year	Screened population	Screening methods	*GLA* gene variants found	Fabry disease reported prevalence	Comments
Nakao *et al.*	1995	230 consecutive male patients with LVH on echocardiogram (IVS or PW thickness ≥13mm)	α-galactosidase A plasma activity in all patients Genetic analysis (PCR sequencing of *GLA* gene) in the 7 patients with low enzymatic activity (<1.2; N: 4.8-17.6nmol/h/ml)	p. Met296Ile p. Ala20Pro	3.0% (7/230)	2 patients with pathogenic *GLA* gene mutations 5 patients with no identified mutations on *GLA* gene exons but with markedly reduced mRNA, 3 of whom with inclusions on electron microscopy of endomyocardial biopsy Screened population included patients with LVH and other comorbidities such as hypertension and aortic valve disease Study only in males
Sachdev *et al.*	2002	79 male patients with HCM (LV wall thickness ≥13mm) diagnosed at ≥40 years 74 male patients with HCM (LV wall thickness ≥13mm) diagnosed at <40 years	α-galactosidase A plasma activity in all patients Genetic analysis (PCR sequencing of *GLA* gene) in the 6 patients with low enzymatic activity (<0.7 ; 4.8-17.6nmol/h/ml)	p. N215S p. I317T p. N408Ifs p. D313Y	6.3% of patients diagnosed at ≥40 years (5/79) 1.4% of patients diagnosed at <40 years (1/74)	5 patients with pathogenic mutations 1 patient with the variant p.D313Y currently described as an enzymatic pseudodeficiency (it is not clear if this patient was diagnosed with HCM at ≥40 or <40 years) Study only in males

Ommen *et al.*	2003	100 consecutive patients (44 males) with asymmetrical obstructive HCM submitted to septal myectomy	Electron microscopy of myectomy tissue	N/A	0.0%	Screened population presents an HCM pattern more typical of sarcomere-protein mutation
Chimenti *et al.*	2004	34 consecutive females with HCM (unexplained LVH with LV wall thickness ≥13mm) submitted to endomyocardial biopsy	Endomyocardial biopsy (including electron microcopy) α-galactosidase A leukocyte activity in all patients Genetic analysis in the 4 patients with inclusions on endomyocardial biopsy	p. L344-X28-stop p. C378Y p. R227Q p. N215S	11.8% (4/34)	4 patients with pathogenic *GLA* gene mutations All patients with LVH with LV wall thickness ≥13mm that was not explained by hypertension and aortic valve disease underwent endomyocardial biopsy Study only in females
Arad *et al.*	2005	35 consecutive patients with HCM (unexplained LVH with LV wall thickness ≥13mm) with negative genetic test for sarcomere-protein mutations of a cohort of 75 patients with HCM	Genetic analysis of all patients (PCR sequencing of *GLA* gene)	N/A	0.0%	Small sample - Fabry disease screening was only performed in 35 of the 75 patients Sarcomere protein mutations of the 40 remaining patients not provided (presumed to be all pathogenic)
Morita *et al.*	2006	50 patients (41 males) with unexplained LVH (LV wall thickness >13mm)	Genetic analysis of all patients (PCR sequencing of *GLA* gene)	p. D313Y	2.0%	The only patient that was found presented the variant p. D313Y, currently described as an enzymatic pseudodeficiency
Monserrat *et al.*	2007	508 consecutive patients (328 males) with HCM according to WHO/ESC criteria	α-galactosidase A plasma activity Genetic analysis (PCR sequencing of *GLA* gene) in patients with low enzymatic activity (0-30% of the mean of controls in males and 0-50% in females)	p. L89P p. E358del p. S238N p. A143T	1.0%	4 patients with pathogenic mutations; 1 patient with p. A143T, whose pathogenicity has been questioned Genetic analysis was not performed in females with enzymatic activity higher than 50% of the mean of controls

Havndrup *et al.*	2010	59 consecutive patients with HCM with negative genetic test for sarcomere-protein mutations of a cohort of 90 patients with HCM	Genetic analysis (PCR sequencing of *GLA* gene)	p.N139S p.A156T p.G271S	3.3% (3/90)	Small sample - Fabry disease screening was only performed in 59 of the 90 patients Sarcomere protein mutations of the 31 remaining patients not provided (presumed to be all pathogenic) N139S– novel variant with little documentation of its pathogenicity besides LVH
Hagege *et al.*	2011	392 patients (278 males) with HCM (unexplained LVH with LV wall thickness ≥15mm)	α-galactosidase A activity on DBS test α-galactosidase A leukocyte activity and Genetic analysis (PCR sequencing of *GLA* gene) in the 9 patients with low enzymatic activity on DBS test (<40% of the median of controls, i.e, <2.1 mmol/h/l)	p.W162C p.F113L p.N215S	1.0% (4/392)	3 pathogenic mutations Genetic analysis was not performed in females with enzymatic activity higher than 40% of the median of controls
Elliott *et al.*	2011	1386 patients (885 males aged ≥35 years and 501 females aged ≥40 years) with unexplained LVH (LV wall thickness ≥15mm)	Genetic analysis (DHPLC protocol followed by PCR sequencing of all exons exhibiting an anomalous DHPLC profile) Plasma α-galactosidase A activity in all patients with sequence variants of the *GLA* gene	p. N215S p. D244N p. T410A p. A143T p. R118C	0.5% (7/1386)	3 of the 7 patients presented the variants p. R118C and p.A143T, whose pathogenicity has been questioned
Mawatari *et al.*	2013	738 male patients with LVH (LV wall thickness ≥13mm)	α-galactosidase A plasma activity Leukocyte α-galactosidase activity was measured in the 4	p. E66Q	0.0%	All the 3 patients with low plasma and leukocyte enzymatic activity presented the variant p. E66Q, currently considered a polymorphism

			patients with low plasma α-galactosidase activity (<1.5nmol/h/ml) Genetic analysis was performed in the 3 patients with both low plasma and leukocyte enzymatic activity (18–45% of the control mean value)			Study only in males
Palecek *et al.*	2014	100 males with unexplained LVH (LV wall thickness ≥ 13 mm)	α-galactosidase A activity in DBS test (45%) or in plasma (55%) Leukocyte α-galactosidase A activity in patients with low enzymatic activity on DBS (below the range of controls) or plasma (<1.5 nmol/hour/ml) Genetic analysis (PCR sequencing) was performed in the 4 patients with low leukocyte enzymatic activity (<15% of the mean control value of 47.1 nmol/hour/mg protein)	p. N215S p.Y152H c.[801+48 T>G];[0], r.[801_802ins801+1_801+66;801+48U>G]	4% (4/100)	The pathogenicity of the novel variant p. Y152H was supported by the results of in silico analysis using PolyPhen-2 and Mutation Taster (data not shown) The pathogenic effect on *GLA* transcript (insertion of 66 bases from intron 5 and using an alternative donor splice site) was determined by RT/PCR analysis and sequencing. The predicted effect of this mutation is a frame shift from Methionin267 - p.M267fs. Study only in males
Terryn *et al.*	2013	540 consecutive patients (362 males) with LVH (IVS or PW thickness ≥13mm) without excluding hypertension or aortic valve disease	In males α-galactosidase A activity in DBS test and genetic analysis if low enzymatic activity on DBS test (<0.64μmol/l/h) In females genetic analysis (PCR sequencing)	p. A15E p.A143T	0.9% (5/540)	Patients with LVH, without exclusion of hypertension or aortic valve disease 4 patients had the variant p. A143T, whose pathogenicity has been questioned
Favalli *et al.*	2016	473 patients (unspecified gender distribution) with the following inclusion criteria: HCM, concentric	Plasma and leukocyte α-galactosidase A activity in all patients	p. N215S Other unspecified *GLA* gene variants	1.69% (8/473)	The study included 2034 patients referred to screening based on the presence of

		mild LVH, no LV outflow tract obstruction	Genetic analysis: PCR sequencing of exons and intronic flanking regions in all patients + MLPA in males with low enzymatic activity and normal PCR sequencing + analysis of intronic haplotypes potentially associated to decreased enzymatic activity + search for IVS4+919 G>A mutation			diagnostic red-flags, including 473 from the cardiology clinic and the remaining from other medical specialties The study mentions that 5 probands from the cardiology setting carried the mutation p.N215S However, it is not specified in the paper which particular *GLA* gene variants were found in the other 3 identified probands that had been referred from the cardiology clinic Nevertheless, most of the *GLA* variants were already known and pathogenic
Gaggl *et al.*	2016	2596 consecutive patients (1689 males) with IVS thickness of ≥12mm	Urinary GB3 isoforms (GB3-24 and GB3-18) and total urinary GB3 Patients with an elevated GB3-24:18 ratio (GB3-24:18 ratio > 2.3) underwent clinical examination, leukocyte α-galactosidase-A activity and *GLA*-mutation-analysis	p.Q333X 1 patient with unknown *GLA* gene mutation	0.12% (3/2596)	These 3 patients had already Fabry disease diagnosis, previous to this study 1 of these 3 Fabry patients was not detected by urinary GB3 isoforms, so this method may be unreliable for Fabry disease diagnosis in patients with missense mutations and residual enzymatic activity 28 of the 99 patients with elevated GB3 isoform ratio were lost to follow-up and did not underwent leukocyte α-galactosidase-A activity determination and *GLA*-mutation analysis

Kubo *et al.*	2017	177 males with HCM (unexplained LVH with LV wall thickness ≥15mm)	Plasma α-galactosidase A activity in all patients Genetic analysis (PCR sequencing of *GLA* gene and analysis of intron 4 for specific mutation IVS4+919G>A) in patients with low enzymatic activity	p.R112L IVS4+919G>A	1.1% (2/177)	Study only in males
Maron *et al.*	2018	585 patients (71% males) with HCM (excluded patients with histology or genetic testing compatible with sarcomeric HCM, family history of sudden death in >2 relatives, inheritance pattern inconsistent with X-linked pattern)	α-galactosidase A activity in DBS Male patients with low α-galactosidase A activity levels and all females were tested for mutations in the *GLA* gene	p.N215S	0.34% (2/585)	

IVS, interventricular septum; PW, posterior wall; LV, left ventricular; DBS, dried blood spot

Supplementary Table II: Baseline characteristics of patients with HCM

BASELINE CHARACTERISTICS	PATIENTS WITH HCM (n=780)
Gender: Males / Females (%)	466 (59.7%) / 314 (40.3%)
Age (years) (median, IQR)	68.0 (57.0, 77.0)
Family history of LVH (%)	157 (22.1%)
Symptoms (%)	599 (76.8%)
Dyspnoea (%)	434 (55.6%)
Chest pain (%)	198 (25.4%)
Palpitations (%)	143 (18.3%)
Pre-syncope (%)	74 (9.5%)
Syncope (%)	98 (12.6%)
NYHA class I / II / III /IV (%)	119 (27.4%) / 267 (61.5%) / 46 (10.6%) / 2 (0.5%)
Echocardiogram (n=780)	
LVH pattern	
Asymmetrical (%)	507 (65.0%)
Symmetrical (%)	167 (21.4%)
Apical (%)	94 (12.1%)
Other (%)	12 (1.5%)
LV outflow tract obstruction at rest (%)	186 (23.8%)
IVS thickness (mm) (median, IQR)	17.0 (15.0, 20.0)
PW thickness (mm) (median, IQR)	11.0 (9.1, 13.0)
LV end-diastolic diameter (mm) (median, IQR)	47.0 (43.0, 52.0)
LV mass (g/m^2) (median, IQR)	153.7 (117, 194.0)
LV ejection fraction (%) (median, IQR)	65.0 (59.0, 71.0)
Septal Sa (cm/s) (median, IQR)	7.0 (5.0, 8.0)
Lateral Sa (cm/s) (median, IQR)	7.4 (6.0, 9.1)
LV Diastolic dysfunction (%)	626 (80.3%)
Grade I / II / III / Undetermined (%)	324 (41.5%) / 272 (34.9%) / 25 (3.2%) / 5 (0.6%)
Septal Ea (cm/s) (median, IQR)	5.2 (4.0, 7.0)
Lateral Ea (cm/s) (median, IQR)	7.0 (5.3, 9.0)
LA volume (mL/m2) (median, IQR)	35.0 (27.0, 47.0)
Cardiac MRI (n=475)	
Late gadolinium enhancement (%)	274 (57.7%)
Electrocardiogram (n=779)	
Sinus rhythm (%)	653 (83.7%)
Atrial fibrillation (%)	91 (11.7%)
Atrial flutter (%)	11 (1.4%)
Pacemaker rhythm (%)	43 (5.5%)
Short PR interval (%)	16 (2.1%)
Atrioventricular block (%)	73 (9.4%)
Left bundle branch block (%)	57 (7.3%)
Right bundle branch block (%)	53 (6.8%)
Bifascicular block (%)	38 (4.9%)
T wave inversion (%)	377 (48.3%)
ST segment depression (%)	223 (28.6%)
Sokolow-Lyon Index (mm) (mean ± SD)	33.7 ± 13.8
24h Holter (n=684)	
Sinus rhythm (%)	584 (74.9%)
Atrial fibrillation (%)	93 (11.9%)
Atrial flutter (%)	9 (1.2%)
Pacemaker rhythm (%)	35 (4.5%)
Atrioventricular block (%)	57 (7.3%)
Left bundle branch block (%)	38 (4.9%)
Right bundle branch block (%)	35 (4.5%)

Bifascicular block (%)	27 (3.5%)
Supraventricular tachycardia (%)	179 (22.9%)
Ventricular tachycardia (%)	117 (15.0%)
Devices	
Pacemaker (%)	59 (7.6%)
Implantable cardioverter-defibrillator (%)	70 (9.0%)
Diagnostic red-flags for Fabry disease	
Stroke (%)	71 (9.1%)
Renal failure (%)	94 (12.1%)
Microalbuminuria >30mg/24h (%) (n=329)	110 (33.4%)
Proteinuria >300mg/24h (%) (n=320)	49 (15.3%)
Other comorbidities	
Hypertension (%)	528 (67.7%)
Diabetes mellitus (%)	167 (21.4%)
Dyslipidemia (%)	417 (53.5%)
Obesity (%)	173 (22.2%)
Smoking (%)	95 (12.2%)
Coronary heart disease (%)	71 (9.1%)

Valid percentages are shown

HCM, hypertrophic cardiomyopathy; IQR, interquartile range; IVS, interventricular septum; LA, left atrium; LV, left ventricular; LVH left ventricular hypertrophy; MRI, magnetic resonance imaging; NYHA, New York Heart Association; PW, posterior wall

Supplementary Table III: Characteristics of the index patients with Fabry disease due to F113L mutation

BASELINE CHARACTERISTICS	FABRY F113L PATIENTS (n=31)
Male gender (%)	22 (71.0%)
Age (years) (median, IQR)	68.0 (61.0, 75.0)
Family history of LVH (%)	20 (64.5%)
Symptoms (%)	27 (87.1%)
Dyspnoea (%)	23 (74.2%)
Chest pain (%)	10 (32.3%)
Palpitations (%)	6 (19.4%)
Pre-syncope (%)	8 (25.8%)
Syncope (%)	4 (12.9%)
NYHA class	
I (%)	14 (60.9%)
II (%)	9 (39.1%)
III (%)	0 (0.0%)
IV (%)	0 (0.0%)
Echocardiogram (n=31)	
LVH pattern	
Asymmetrical (%)	14 (45.2%)
Symmetrical (%)	17 (54.8%)
Apical (%)	0 (0.0%)
Other (%)	0 (0.0%)
LV outflow tract obstruction at rest (%)	3 (9.7%)
IVS thickness (mm) (median, IQR)	18.0 (17.0, 19.0)
PW thickness (mm) (median, IQR)	13.0 (12.0, 17.0)
LV end-diastolic diameter (mm) (median, IQR)	43.0 (37.0, 46.0)
LV mass (g/m^2) (median, IQR)	153.7 (128.3, 188.3)
LV ejection fraction (%) (median, IQR)	66.0 (61.5, 73.5)
Septal Sa (cm/s) (median, IQR)	5.1 (4.5, 6.5)
Lateral Sa (cm/s) (median, IQR)	6.0 (5.5, 7.0)
LV Diastolic dysfunction (%)	27 (87.1%)
Grade I (%)	16 (59.3%)
Grade II (%)	11 (40.7%)
Grade III (%)	0 (0.0%)
Septal Ea (cm/s) (median, IQR)	5.0 (4.0, 6.0)
Lateral Ea (cm/s) (median, IQR)	6.0 (4.5, 9.0)
LA volume (mL/m2) (median, IQR)	33.0 (27.5, 37.7)
Cardiac MRI (n=23)	
Late gadolinium enhancement (%)	18 (81.8%)
Electrocardiogram (n= 31)	
Sinus rhythm (%)	30 (96.8%)
Atrial fibrillation (%)	1 (3.2%)
Atrial flutter (%)	0 (0.0%)
Pacemaker rhythm (%)	2 (6.5%)
Short PR interval (%)	0 (0.0%)
Atrioventricular block (%)	4 (12.9%)
Left bundle branch block (%)	2 (6.5%)
Right bundle branch block (%)	4 (12.9%)
Bifascicular block (%)	13 (41.9%)
T wave inversion (%)	17 (54.8%)
ST segment depression (%)	14 (45.2%)
24h Holter (n=30)	
Sinus rhythm (%)	29 (93.5%)
Atrial fibrillation (%)	2 (6.5%)
Atrial flutter (%)	1 (3.2%)

Pacemaker rhythm (%)	3 (9.7%)
Atrioventricular block (%)	4 (12.9%)
Left bundle branch block (%)	2 (6.5%)
Right bundle branch block (%)	5 (16.1%)
Bifascicular block (%)	13 (41.9%)
Supraventricular tachycardia (%)	15 (48.4%)
Ventricular tachycardia (%)	5 (16.1%)
Devices	
Pacemaker (%)	5 (16.1%)
Implantable cardioverter-defibrillator (%)	0 (0%)
Diagnostic red-flags for Fabry disease	
Stroke (%)	1 (3.2%)
Renal failure (%)	2 (6.5%)
Microalbuminuria >30mg/24h (%) (n= 28)	16 (57.1%)
Proteinuria >300mg/24h (%) (n= 28)	7 (25.0%)
Brain white matter lesions (%) (n=22)	15 (68.2%)
Acroparesthesias (%) (n=31)	5 (16.1%)
Angiokeratomas (%) (n=31)	1 (3.2%)
Cornea verticillata (%) (n=27)	5 (18.5%)
Deafness (%) (n=29)	24 (82.8%)
Other comorbidities	
Hypertension (%)	19 (61.3%)
Diabetes mellitus (%)	5 (16.1%)
Dyslipidemia (%)	22 (71.0%)
Obesity (%)	3 (9.7%)
Smoking (%)	4 (12.9%)
Coronary heart disease (%)	4 (12.9%)

Valid percentages are shown

IQR, interquartile range; IVS, interventricular septum; LA, left atrium; LV, left ventricular; LVH left ventricular hypertrophy; MRI, magnetic resonance imaging; NYHA, New York Heart Association; PW, posterior wall

Supplementary Figure 1: Map of Portugal showing the geographical distribution of the hospital centers included in this study



Appendix: List of investigators of FD screening in HCM

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Chapter 4.4

Azevedo O, Marques N, Craveiro N, Pereira AR, Antunes H, Reis L, Guerreiro RA, Pontes dos Santos R, Miltenberger-Miltenyi G, Sousa N, Cunha D.

Screening for Fabry disease in patients with left ventricular noncompaction.

Rev Port Cardiol 2019;38:709-716. doi: 10.1016/j.repc.2019.02.014.

Rev Port Cardiol. 2019;38(10):709–716

Revista Portuguesa de
Cardiologia
Portuguese Journal of Cardiology
www.revportcardiol.org



ORIGINAL ARTICLE

Screening for Fabry disease in patients with left ventricular noncompaction

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Received 23 September 2018; accepted 3 February 2019
Available online 1 January 2020

KEYWORDS
Fabry disease;
Noncompaction;
Hypertrabeculation;
Cardiomyopathy;
Screening

Abstract
Introduction and Aim: It is unclear whether left ventricular noncompaction (LVNC) is a distinct cardiomyopathy or a morphologic manifestation of different cardiomyopathies. We previously reported a case of LVNC in a Fabry disease (FD) patient, but it remains to be clarified whether LVNC is a cardiac manifestation of FD, a coincidental finding or an overdiagnosis, which has major therapeutic implications. This study aims to determine the prevalence of FD among patients with LVNC.
Methods: We performed a retrospective study including all patients diagnosed with LVNC in eight hospital centers. Diagnosis of LVNC was based on at least one echocardiographic or cardiac magnetic resonance criterion. FD screening was performed by combined enzyme and genetic testing.
Results: The study included 78 patients diagnosed with LVNC based on the Jenni (84.6%), Stöllberger (46.2%), Chin (21.8%), Petersen (83.8%) and Jacquier (16.2%) criteria. Left ventricular

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https://doi.org/10.1016/j.repc.2019.02.014
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systolic dysfunction was present in 48.7%. Heart failure was found in 60.3%, ventricular dysrhythmias in 21.6% and embolic events in 11.5%. FD screening found no additional cases among patients with LVNC, besides the previously described case.
Conclusion: No additional FD cases were found among patients with LVNC, which argues against the hypothesis that LVNC is a cardiac manifestation of FD.
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PALAVRAS-CHAVE
Doença de Fabry;
Não compactação;
Hipertrabeculação;
Cardiomiopatia;
Rastreio

Rastreio de doença de Fabry em doentes com não compactação do ventrículo esquerdo

Resumo
Introdução e objetivo: Não está esclarecido se a não compactação do ventrículo esquerdo (NCVE) é uma miocardiopatia distinta ou uma manifestação morfológica de várias miocardiopatias. Nós reportamos previamente um caso de NCVE num doente com doença de Fabry (DF), mas permanece por esclarecer se a NCVE é uma manifestação cardíaca de DF, um achado coincidente ou um sobrediagnóstico, o que tem importantes implicações terapêuticas. Este estudo pretende determinar a prevalência de DF em doentes com NCVE.
Métodos: Estudo retrospetivo incluindo todos os doentes diagnosticados com NCVE em oito centros hospitalares. O diagnóstico de NCVE foi baseado em pelo menos um dos critérios de ecocardiografia ou ressonância cardíaca. O rastreio de DF foi realizado por teste enzimático e genético.
Resultados: O estudo incluiu 78 doentes diagnosticados com NCVE com base nos critérios de Jenni (84,6%), Stöllberger (46,2%), Chin (21,8%), Petersen (83,8%) e Jacquier (16,2%). A disfunção sistólica do VE estava presente em 48,7%. A insuficiência cardíaca foi encontrada em 60,3%, as disritmias ventriculares em 21,6% e os eventos embólicos em 11,5%. O rastreio de DF não encontrou casos adicionais nos doentes com NCVE, para além do caso previamente descrito.
Conclusão: Não foram encontrados casos adicionais de DF nos doentes com NCVE, o que argumenta contra a hipótese de a NCVE ser uma manifestação cardíaca de DF.
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Introduction

Fabry disease (OMIM 301500) is an X-linked lysosomal storage disorder caused by mutations in the *GLA* gene, which is located on Xq22.1 and codes for the enzyme alpha-galactosidase A (α-Gal A). Deficiency in the enzyme activity of α-Gal A leads to the lysosomal accumulation of globotriaosylceramide (Gb3) and related glycosphingolipids, causing multiorgan damage.[1]

Fabry disease is a rare condition, estimated to occur in 1 in 3100 hemizygous males.[2] The diagnosis in males is based on biochemical determination of α-Gal A activity. In heterozygous females, α-Gal A activity may be normal, so diagnosis requires molecular analysis of the *GLA* gene.[1] Clinical manifestations may arise early, in childhood or adolescence, and include acroparesthesias, cornea verticillata, angiokeratomas and microalbuminuria or proteinuria. In adulthood, patients may develop sensorineural deafness, renal failure, cerebrovascular events and cardiac manifestations.[1]

Fabry disease affects the heart, causing left ventricular hypertrophy (LVH) and fibrosis, heart failure, angina, valve thickening or regurgitation, dysrhythmias, cardiac conduction abnormalities and sudden death.[1,3] The main cardiac manifestation is hypertrophic cardiomyopathy, which occurs in 43% of male patients (mean age of onset 39±10 years) and 26% of female patients (mean age of onset 50±11 years).[4] LVH in Fabry disease is usually non-obstructive and symmetrical[3] with prominent papillary muscles[5] and, in nearly half of cases, myocardial late gadolinium enhancement is typically observed in the mid-wall of the inferolateral basal segments of the left ventricle.[6]

Left ventricular noncompaction (LVNC) is morphologically characterized by prominent left ventricular (LV) trabeculations and deep intertrabecular recesses communicating with the LV cavity, located in the apical and lateral regions of the left ventricle, distal to the papillary muscles. The myocardium has a two-layer appearance, composed of a thin compacted epicardial layer and a thick spongy noncompacted endocardial layer.[7]

LVNC has been found in 0.045% of adult individuals referred for echocardiography[8] and 0.029% of children admitted for heart failure due to cardiomyopathy.[9] It

Table 1 Diagnostic criteria of left ventricular noncompaction.

Echocardiographic criteria	
Jenni et al.[13]	(1) Numerous and prominent trabeculations and deep intertrabecular recesses that are localized predominantly in the apical and mid-ventricular segments of the inferior and lateral walls of the left ventricle; (2) Two-layered myocardium, with a thin compacted layer that is adjacent to the epicardium and a much thicker noncompacted layer that is near the endocardium, resulting in a maximum end-systolic ratio of the thickness of noncompacted to compacted layer >2 in short-axis parasternal view; (3) Color Döppler evidence of the perfusion of the intertrabecular recesses directly from the LV cavity; (4) Absence of other cardiac abnormalities.
Chin et al.[14]	(1) The quotient between the distance from the epicardial surface to the trough of the intertrabecular recess and the distance from the epicardial surface to the peak of the trabeculation decreases from the level of the papillary muscles to the apex and is $\leq$0.5, at end-diastole, at the level of the LV apex. Measurements are performed in parasternal long-axis view at the level of the mitral valve and papillary muscles and in subxiphoid or apical 4-chamber views at the level of the LV apex.
Stöllberger et al.[15]	(1) More than three trabeculations protruding from the LV wall, apically to the papillary muscles, are visualized in one imaging plane; (2) The intertrabecular recesses are perfused directly from the LV cavity as shown by color Döppler.
Cardiac MRI criteria	
Petersen et al.[16]	The maximal end-diastolic ratio of the thickness of the noncompacted to the compacted layer, measured in any of the three long-axis views, is >2.3.
Jacquier et al.[17]	The LV trabeculated mass is over 20% of the total LV mass (papillary muscles are excluded from the trabeculated mass).
Stacey et al.[18]	The maximal end-systolic ratio of the thickness of the noncompacted to compacted layer in short-axis views is $\geq$2.
Captur et al.[19]	Maximal apical fractal dimension >1.3, measured at end-diastole in short-axis views of the apical third of the left ventricle.

LV: left ventricular; MRI: magnetic resonance imaging.

predominantly affects males[8] and individuals of African origin.[10] First described on autopsy in 1969 by Feldt et al.[11] and on echocardiography by Engberding et al. in 1984,[12] it is most commonly diagnosed by transthoracic echocardiography or cardiac magnetic resonance imaging (MRI). Several diagnostic criteria have been proposed using echocardiography or cardiac MRI[13–19] (Table 1).

Patients with LVNC may be asymptomatic or develop complications such as heart failure, thromboembolism or ventricular dysrhythmias and sudden death.[20]

The etiology and pathogenesis of LVNC are unknown. It has been hypothesized to result from a disturbance of the compaction process of the LV myocardium during early development, but growing evidence suggests that, although the papillary muscles are formed by compaction of pre-existing trabeculations, these trabeculations contribute little, if anything, to the compacted layer of the ventricular walls.[21] Moreover, not all cases are congenital and LVNC may be acquired at any stage of life after birth.[7]

LVNC is characterized by marked genetic heterogeneity. Mutations have been described in genes that code for proteins in the mitochondria (tafazzins), Z line (ZASP/Cypher), cytoskeleton (alpha-dystrobrevin), nuclear lamina (lamin A/C) and sarcomere (beta-myosin heavy chain, alpha-cardiac actin and cardiac troponin T).[7] However, the causal relationship between these mutations and LVNC remains to be established.

LVNC has been reported in patients with congenital cardiac abnormalities, such as LV outflow tract abnormalities, Ebstein anomaly, tetralogy of Fallot and double outlet right ventricle; chromosomal defects, such as trisomy 13, Cornelia de Lange, DiGeorge, Turner and 5q minus syndromes; and neuromuscular disorders, such as mitochondrial myopathies, Barth syndrome, zaspopathy, myotonic dystrophy type 1, dystrobrevinopathy, and Emery-Dreifuss muscular dystrophy.[7] LVNC has also been reported in patients with storage disorders such as glycogen storage disease types Ib and IV,[22,23] Danon disease[24] and amyloidosis.[25]

In 2011, we reported the first case of LVNC in a Fabry disease patient.[26] This was a young asymptomatic female who fulfilled the Jenni echocardiographic criteria and the Petersen cardiac MRI criteria for LVNC. She had been diagnosed with Fabry disease following family screening, and the pathogenic mutation of the *GLA* gene detected was a missense mutation (p.F113L) that is known to be associated with a predominantly cardiac later-onset phenotype. However, thorough diagnostic study revealed none of the known clinical manifestations of Fabry disease, including LVH, late gadolinium enhancement or other cardiac or extracardiac manifestations, nor

did she present LV dilation or dysfunction or any clinical complication of LVNC, such as heart failure, dysrhythmias or thromboembolism. Moreover, all of her relatives with Fabry disease underwent echocardiography and cardiac MRI and none presented LVNC. In this report, we pointed out the limitations of the current diagnostic criteria of LVNC and raised the question whether this was an overdiagnosis of LVNC, a coincidental finding of LVNC and Fabry disease, or if indeed the two conditions were clinically related.[26,27]

More recently, two additional cases of LVNC in Fabry disease patients have been reported,[28–31] also in young females. In the second case, the diagnosis of LVNC was based only on cardiac MRI (Petersen criteria), without clear echocardiographic criteria of LVNC due to insufficient quality of the acoustic window. She had classical Fabry disease due to a nonsense *GLA* gene mutation (p.R220X). As in our case, she also did not present LVH, dilation, dysfunction, late gadolinium enhancement or other cardiac abnormalities, and there was no history of heart failure, dysrhythmias or thromboembolism. There was also no family history of LVNC. She had no clinical manifestations of Fabry disease besides cornea verticillata and angiokeratomas. An endomyocardial biopsy showed deposition of glycosphingolipids in cardiomyocytes and based on this finding the patient was referred for enzyme replacement therapy.[28,29]

In the third case, the diagnosis of LVNC was based on cardiac MRI (Petersen criteria) and echocardiography (although it was not reported which specific criteria were fulfilled). She was diagnosed with Fabry disease based on low levels of α-Gal A, but the pathogenic *GLA* gene mutation was never found. The only clinical manifestations of Fabry disease were angiokeratomas and acroparesthesias. As in the two previous cases, there was no LVH, dilation or dysfunction. Cardiac MRI showed subendocardial late gadolinium enhancement, but its location and extension was not reported. Interestingly, she had Wolff-Parkinson-White syndrome, interpreted by the authors as an unrelated finding, and underwent ablation of a para-Hisian accessory pathway, which was followed by disappearance of the short PR interval and delta waves. However, as in the two previous cases, she had no history of heart failure or thromboembolism. Moreover, there were no available data consistent with a family history of LVNC or Fabry disease.[30,31]

Taken together, it remains essential to clarify whether LVNC could be a cardiac manifestation of Fabry disease, given the implications for therapeutic decisions on whether to start enzyme replacement or chaperone therapy, particularly in young asymptomatic females with no other known clinical manifestations of Fabry disease.

The only published study to date on Fabry disease screening in patients with LVNC included 26 male patients who fulfilled the Stöllberger echocardiographic criteria for LVNC. Fabry disease screening was based on the enzymatic assay of α-Gal A and found no patients with the disease.[32]

Herein, we report the results of Fabry disease screening in a larger cohort of patients with LVNC.

Table 2 Baseline characteristics of patients with left ventricular noncompaction (n=78).

Gender (male/female)	49 (62.8%)/29 (37.2%)
Race (Caucasian/African)	74 (94.9%)/4 (5.1%)
Age at diagnosis of LVNC, years (mean ± SD)	47±17
Age at Fabry disease screening, years (mean ± SD)	53±17
Family history of LVNC	5 (6.4%)
Family history of Fabry disease	1 (1.3%)
Clinical presentation	
Incidental finding	35 (44.9%)
Heart failure	27 (34.6%)
Dysrhythmia	9 (11.5%)
Thromboembolism	4 (5.1%)
Other	8 (10.3%)
Clinical complications in follow-up	
Heart failure	47 (60.3%)
Dysrhythmias	28 (35.9%)
Thromboembolism	9 (11.5%)
Diagnosis of LVNC	
Echocardiography	71/78 (91.0%)
Cardiac MRI	58/68 (85.3%)
Echocardiographic criteria	
Jenni	66 (84.6%)
Stöllberger	36 (46.2%)
Chin	17 (21.8%)
Cardiac MRI criteria	
Petersen	57 (83.8%)
Jacquier	11 (16.2%)
Stacey	0 (0.0%)
Captur	0 (0.0%)
Echocardiography	
LV ejection fraction, % (mean ± SD)	47.2±15.2
LV systolic dysfunction	38 (48.7%)
LV diastolic dysfunction	42 (56.0%)
Mean Sa, cm/s (mean ± SD)	7.7±2.8
Mean Ea, cm/s (mean ± SD)	9.2±4.1
Mean E/Ea ratio	8.9±2.7
IVS/PW thickness, mm (mean ± SD)	9.8±2.9/8.7±1.9
LV mass, g/m^2 (mean ± SD)	121.5±82.3
LA volume, ml/m^2 (mean ± SD)	32.9±13.9
Valve disease	15 (19.2%)
Cardiac MRI	
Ratio of thickness of NC:C layer	3.4±3.9
LV mass, g/m^2 (mean ± SD)	99.5±56.0
LV end-systolic volume, ml/m^2 (mean ± SD)	71.8±50.0
LV end-diastolic volume, ml/m^2 (mean ± SD)	130.1±75.1
LV ejection fraction, % (mean ± SD)	46.2±16.2

Table 2 (*Continued*)

Late gadolinium enhancement	10 (15.4%)
ECG	
Sinus rhythm	70 (89.7%)
Atrial fibrillation	6 (7.7%)
Atrial flutter	2 (2.6%)
AV block	4 (5.1%)
LBBB	10 (12.8%)
RBBB	3 (3.8%)
Bifascicular block	2 (2.6%)
24-hour Holter	
Sinus rhythm	67 (90.5%)
Atrial fibrillation	10 (13.5%)
Atrial flutter	1 (1.4%)
AV block	12 (16.2%)
LBBB	11 (14.9%)
RBBB	3 (4.1%)
Bifascicular block	2 (2.7%)
Supraventricular tachycardia	13 (17.6%)
VT	16 (21.6%)
Non-sustained VT	15 (20.3%)
Sustained VT	1 (1.4%)
Clinical manifestations of Fabry disease	
Renal failure	7 (9.0%)
Microalbuminuria	1 (1.3%)
Proteinuria	0 (0.0%)
Stroke	8 (10.3%)
Acroparesthesias	1 (1.3%)
Deafness	2 (2.6%)

AV: atrioventricular; C: compacted; ECG: electrocardiogram; IVS: interventricular septum; LA: left atrial; LBBB: left bundle branch block; LV: left ventricular; LVNC: left ventricular noncompaction; MRI: magnetic resonance imaging; NC: non-compacted; PW: posterior wall; RBBB: right bundle branch block; VT: ventricular tachycardia.

Methods

Patient population

Between October 2015 and October 2017, 78 consecutive patients with LVNC were recruited from the cardiology consultation of eight Portuguese hospital centers. The inclusion criteria were adult patients (aged ≥18 years) diagnosed with LVNC based on the presence of any of the current diagnostic criteria of LVNC, by either echocardiography or cardiac MRI, as described in Table 1. All patients fulfilling the inclusion criteria underwent Fabry disease screening.

Fabry disease screening

Fabry disease screening was performed at a specialized laboratory (Centogene AG, Rostock, Germany). α-Gal A enzyme activity was measured in dried blood spot samples by fluorometry. Molecular analysis of the *GLA* gene was performed in males with reduced α-Gal A enzyme activity, i.e., below the normal reference range (<15.3 μmol/l/h), and in all females, independently of α-Gal A activity. The molecular analysis was performed by classical bidirectional Sanger sequencing of the *GLA* gene and included all exons, exon/intron boundaries and the promoter region.

Ethical issues

This research project was approved by the ethics committees of all the hospital centers included in this study and all patients provided written informed consent.

Results

Patients with LVNC were predominantly males (62.8%). Only four patients (5.1%) were identified as being of African origin. Mean age at diagnosis of LVNC was 64.4±14.1 years. A family history of LVNC was recorded in five patients (6.4%). The patients did not present concomitant neuromuscular disorders or any other medical conditions reported to be associated with LVNC.

The diagnosis of LVNC was established by echocardiography in 91.0% of the cases, based on the Jenni criteria in 84.6%, the Stöllberger criteria in 46.2%, the Chin criteria in 21.8% and the three echocardiographic criteria in 20.5% of the cases. Nearly half of the patients presented LV systolic dysfunction (48.7%) and mean LV ejection fraction was 47.2±15.2%. Cardiac MRI was performed in 68 patients (87.2%) and the diagnosis of LVNC was established in 58 patients (85.3%), mainly based on the Petersen criteria (83.8%). The trabeculated LV mass was calculated in only 11 patients, all of them fulfilling the Jacquier criteria of LVNC. The presence of the Stacey or Captur criteria was not assessed in any cardiac MRI. The diagnosis of LVNC was established by both echocardiography and cardiac MRI in 53 of the 68 patients who underwent cardiac MRI (77.9%).

LVNC was an incidental finding in 44.9% of the patients. Heart failure was the most common clinical manifestation at the time of diagnosis (34.6%). In the follow-up (53±33 months), heart failure remained the most frequent clinical complication (60.3%), with New York Heart Association functional class ranging from I to IV (I: 8.5%; II: 59.6%; III: 23.4%; IV: 8.5%). Dysrhythmias were also common (35.9%). Twenty-four-hour Holter monitoring was performed in 74 patients (94.9%) and revealed atrial fibrillation in 10 patients (13.5%) and ventricular tachycardia (VT) in 16 patients (21.6%), although only one case exhibited sustained VT. Thromboembolism occurred in nine patients (11.5%), as stroke in eight and as myocardial infarction in one (Table 2).

Twenty-eight patients (35.9%) had undergone genetic study that included a wide range of genes (*MYH7*: 32.1%; *MYBPC3*: 30.8%; *TNNT2*: 28.2%; *TNNI3*: 19.2%; *ACTC1*: 28.2%; *TPM1*: 28.2%; *MYL2*: 11.5%; *MYL3*: 11.5%; *DTNA*: 7.7%; *DMD*: 10.3%; *LDB3*: 28.2%; *TAZ*: 26.9%; *LMNA*: 24.4%; *CSRP3*: 24.4%; *TCAP*: 20.5%; *SGCD*: 21.8%; *PLN*: 23.1%; *TTN*: 2.6%; *SCN5A*: 1.3%). A pathogenic mutation was found in the *MYH7* gene in one patient (c.1003G>C) (unpublished data). Eight genetic variants of uncertain significance were found in six cases: four in *MYBPC3* (c.1505G>A; c.2762A>T; c.1624G>C; c.3676C>T), the last two in the same patient; two in *TTN* (c.16425T>G; c.60259C>G); one in *SCN5A* (c.393-5C>A); and

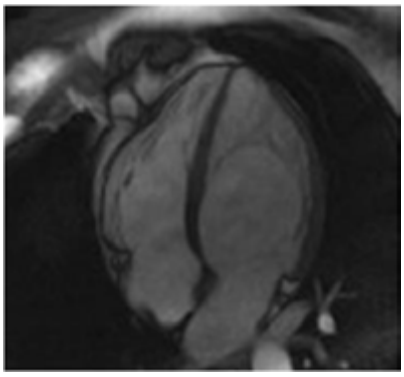


Figure 1 Left ventricular noncompaction in a Fabry disease patient: cardiac magnetic resonance imaging showing hypertrabeculation mostly affecting the left ventricular apical region and lateral wall in apical 4-chamber view.

one in *SGCD* (c.772-774delAAG) in the same patient carrying two genetic variants of the *MYBPC3* gene.

The screening of 78 patients with LVNC revealed no additional cases of Fabry disease besides the one already identified.[26] This Fabry patient was never under enzyme replacement or chaperone therapy. During the eight-year follow-up, this patient has remained asymptomatic and without known clinical manifestation of Fabry disease or complication of LVNC. Moreover, there were no significant changes regarding the location or extension of LVNC or LV chamber size, mass or function during follow-up (Figure 1).

Discussion

This multicenter study of Fabry disease screening in 78 consecutive patients with LVNC revealed no additional Fabry patients besides the single case that had been previously reported by our center, which argues against the hypothesis that LVNC is a clinical manifestation of Fabry disease.

The most likely explanation for the finding of LVNC in this Fabry patient is an overdiagnosis of LVNC. Studies have increasingly exposed the lack of specificity of the current diagnostic criteria for LVNC, potentially leading to overdiagnosis.[10,33,34] Moreover, there is growing evidence that LVNC has no independent prognostic impact compared to conventional risk factors, such as LV dilation, dysfunction or fibrosis, and that patients with LVNC without LV dilation, dysfunction or fibrosis have an excellent prognosis.[34–36]

In a study of 199 patients referred to a heart failure clinic and 60 healthy controls, Kohli et al. found that 23.6% of the patients and 8.3% of the controls fulfilled one or more echocardiographic criteria for LVNC. Among the heart failure patients with LVNC criteria, 29.8% fulfilled all three echocardiographic criteria and 36.2% fulfilled only a single criterion, with the frequency of each criterion ranging from 12.1% to 18.6%.[10] In a large population-based cohort of 1000 subjects from the Multi-Ethnic Study of Atherosclerosis (MESA) registry who underwent cardiac MRI, the Petersen criteria for LVNC were fulfilled in 43% of the subjects without cardiac disease.[33] Another study based on the MESA registry including 2742 subjects undergoing cardiac MRI showed that a greater extent of LV trabeculations in asymptomatic individuals appears to be benign and is not associated with deterioration in LV volumes or function over an almost 10-year period.[36] In a study including 700 patients referred for cardiac MRI, Ivanov et al. found an LVNC prevalence of 39%, 23%, 25% and 3%, according to the Petersen, Stacey, Jacquier and Captur criteria, respectively. In this study, during a median follow-up of seven years, there were no statistically significant differences in the assessed outcomes (death, ischemic stroke, ventricular tachycardia/fibrillation, and heart failure hospitalization) between patients with and without LVNC, irrespective of the criteria applied.[34] Finally, in a study including 113 patients with LVNC according to the Jenni and Petersen criteria, Andreini et al. found that the degree of hypertrabeculation had no prognostic impact over and above LV dilation, dysfunction or late gadolinium enhancement. Indeed, patients without LV dilation, dysfunction or fibrosis had an excellent prognosis at four-year follow-up.[35]

In light of this evidence, the possibility of an overdiagnosis of LVNC, with no associated prognostic impact, must be considered in these three cases of Fabry disease published in the literature. In fact, all three case reports of LVNC occurred in young females with no family history of LVNC in Fabry or non-Fabry relatives, no LV hypertrophy, dilation or dysfunction, no late gadolinium enhancement suggestive of Fabry disease involvement, no or few clinical manifestations of Fabry disease, and no clinical complications of LVNC.[26–31]

Appropriate follow-up would also be required to correctly interpret the significance of LVNC in these Fabry patients. In our case, the patient was never under enzyme replacement or chaperone therapy, because at baseline she did not present any known manifestation of Fabry disease that would constitute a clinical indication to start specific therapy according to the guidelines. During the eight-year follow-up of this patient, no significant change has been documented in the location or extension of LVNC or in LV chamber size, mass or function. Moreover, this patient has remained asymptomatic and with no known clinical manifestation of Fabry disease or complication of LVNC, which supports the hypothesis of an overdiagnosis of LVNC in this Fabry patient.

Another possibility is that LVNC and Fabry disease in these patients are coincidental and unrelated conditions. Although the simultaneous finding of two unrelated rare conditions may seem unlikely, it is now known that LVNC has a much higher prevalence[10,33,34] than the previous estimate of 0.045%[8] and the currently estimated Fabry disease prevalence of 1 in 3100[2] is much higher than the previous estimate of 1 in 117 000.[37]

The finding of LVNC in a wide variety of congenital cardiac abnormalities, chromosomal defects, neuromuscular disorders[7] and storage disorders,[22–24] as well as in elite athletes[38] and pregnant women,[39] has raised the question of whether LVNC is indeed a distinct cardiomyopathy or simply a common epiphenomenon in cardiac overload conditions. In our case, this young woman was not an athlete, but she had been pregnant once, although we have no record of echocardiograms before, during or just after pregnancy. A history of pregnancy or vigorous exercise was not reported in the other two cases.

Finally, there is the possibility that LVNC could be a cardiac manifestation of Fabry disease. However, there is no evidence that Fabry disease causes LVNC by either compensatory or genetic mechanisms. Indeed, although they had Fabry disease, none of these patients had concomitant LVH, dilation or dysfunction that could have led to cardiac overload and hence to LVNC. Furthermore, there is no evidence that the *GLA* gene mutation, the mutated α-Gal A, or the intramyocardial deposition of glycosphingolipids due to Fabry disease are the cause of or are in any way related to the development of LVNC, and any assumption on this point remains speculative. Lysosomal accumulation of Gb3 begins in utero and has been demonstrated as early as the 17th week of gestation in various fetal cell types, such as cardiomyocytes,[40] kidney podocytes,[40–42] hepatocytes,[41,43] corneal epithelial cells[44] and myenteric plexus cells.[42] Although Gb3 storage begins in utero, clinical manifestations only develop several years later. Therefore, Gb3 deposits are expected findings in biopsies, including endomyocardial biopsies, of all Fabry patients independently of the presence of clinical manifestations, and such deposition should not be interpreted as evidence that LVNC is due to Fabry disease.

The only study screening for Fabry disease in patients with LVNC did not reveal any positive case. However, this was a small cohort of 26 male patients and the only criteria for LVNC diagnosis were the Stöllberger echocardiographic criteria.[32] Our study, on the other hand, included a larger cohort of patients and the inclusion criteria allowed (LVNC) diagnosis based on one or more criteria, by echocardiography or cardiac MRI. Our cohort of patients appears to be representative of LVNC and its wide clinical spectrum. Although a significant proportion of cases were incidental findings, there was good agreement between echocardiography and cardiac MRI regarding the diagnosis of LVNC, nearly half of the patients presented LV systolic dysfunction, and in the follow-up most patients developed complications. Even so, no Fabry disease patient was diagnosed besides the single case already reported in the literature. Nevertheless, our study is also limited by its small sample size, which may have prevented the detection of Fabry disease if LVNC was a rare manifestation of this disease. However, the common finding of LVNC in other patient populations, such as heart failure, also argues against the hypothesis of LVNC being a rare clinical manifestation of Fabry disease. The fact that our patient remained stable and did not develop any complications during an eight-year follow-up without specific therapy for Fabry disease also argues against the hypothesis that LVNC is a clinical manifestation or a risk marker for the development of other cardiac manifestations of Fabry disease.

Based on recent evidence exposing the low specificity of the current diagnostic criteria and the increasing rate of overdiagnosis of LVNC,[10,33,34] it is plausible that more cases of LVNC will be diagnosed among Fabry patients, raising concerns about its therapeutic implications. This is particularly important in Fabry females, who, in part due to the inactivation pattern of the X chromosome, present a highly variable prognosis, some of them exhibiting a benign disease course and a lifelong prognosis without significant clinical manifestations in the absence of specific therapy.[45]

Conclusion

In summary, our study found no evidence to support the hypothesis that LVNC is a clinical manifestation of Fabry disease. Taken together with the available evidence, our results do not support the use of the current diagnostic criteria of LVNC for the decision to start specific therapy for Fabry disease, either enzyme replacement or chaperone therapy, in young asymptomatic females presenting LVNC criteria in the absence of other cardiac abnormalities or known manifestations of Fabry disease that would constitute a clinical indication to start therapy according to the current guidelines.

Disclosures

Olga Azevedo, MD, and Gabriel Miltenberger-Miltenyi, PhD, have received educational and/or research grants from Shire Human Genetic Therapies, Inc., Genzyme Corporation and Amicus Nuno Marques, MD, Hugo Antunes, MD, and Liliana Reis, MD, have received educational grants from Shire Human Genetic Therapies, Inc. and Genzyme Corporation. Nuno Craveiro, MD, Rui Azevedo Guerreiro, MD, and Rui Pontes dos Santos, MD, have received educational grants from Shire Human Genetic Therapies, Inc. The other authors have no disclosures.

Funding

This research was supported by a research grant from Shire Human Genetic Therapies, Inc.

Shire Human Genetic Therapies, Inc. had no influence on the design and conduct of the study, in the collection, analysis, and interpretation of the data, or in the preparation, review, or approval of the manuscript.

Conflicts of interest

The authors have no conflicts of interest to declare.

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Chapter 4.5

Azevedo O, Gago M, Miltenberger-Miltenyi G, Gaspar P, Sousa N, Cunha D.

Mild left ventricular hypertrophy unravels a novel nonsense mutation of the *GLA* gene associated with the classical phenotype of Fabry disease.

Cardiology 2017;137:67-73. doi: 10.1159/000455117. Epub 2017 Feb 3.

Novel Insights from Clinical Experience

CARDIOLOGY

Cardiology 2017;137:67–73
DOI: 10.1159/000455117

Received: November 28, 2016
Accepted: November 28, 2016
Published online: February 3, 2017

Mild Left Ventricular Hypertrophy Unravels a Novel Nonsense Mutation of the *GLA* Gene Associated with the Classical Phenotype of Fabry Disease

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Established Facts

- Fabry disease is an X-linked lysosomal storage disorder caused by mutations of the *GLA* gene that codes for α-galactosidase A enzyme. The main cause of death is cardiac disease, and the main cardiac manifestation is left ventricular hypertrophy (LVH).
- In patients with unexplained LVH, a complete evaluation is important to find diagnostic red flags of treatable causes, such as Fabry disease.

Novel Insights

- Fabry disease diagnosis should be considered even in milder forms of unexplained LVH, particularly in females. In this X-linked disorder, females may present a highly variable phenotype, and mild LVH may be the main clinical manifestation.
- The novel nonsense mutation c.607G>T (p.E203X) of the *GLA* gene occurs in the active site of the α-galactosidase A enzyme, causing Fabry disease (classical phenotype).

Keywords
Fabry disease · Nonsense · Mutation · Classical phenotype · *GLA* gene · Left ventricular hypertrophy

Abstract
We report on the clinical, biochemical, and genetic findings of a large family with the classical phenotype of Fabry disease due to the novel nonsense mutation c.607G>T (p.E203X) of the *GLA* gene, which occurs in the active site of the α-galactosidase A enzyme. This report highlights that (i) Fabry disease diagnosis should be considered in all cases of unexplained left ventricular hypertrophy (LVH), even in its milder forms; (ii) a complete evaluation of patients with unexplained LVH is important to find diagnostic red flags of treatable causes of LVH, such as Fabry disease; (iii) cascade family screening is paramount to the earlier diagnosis and treatment of other affected family members; and (iv) the Fabry disease phenotype is highly variable in heterozygote females, even within the same family. © 2017 S Karger AG, Basel

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Introduction

Fabry disease (OMIM 301500) is an X-linked lysosomal storage disorder caused by mutations in the *GLA* gene, which is located on the Xq22.1 region and codes for the α-galactosidase A enzyme. The deficiency of the α-galactosidase A enzymatic activity leads to the lysosomal accumulation of globotriaosylceramide (GB3) and other related glycosphingolipids in several tissues and organs [1].

Fabry disease is a rare condition, but its prevalence has been underestimated. Former studies reported a prevalence of around 1:117,000 individuals [2]. More recently, Spada et al. [3] reported a much higher prevalence of 1:3,100 hemizygote males, mainly due to the finding of later onset variants of Fabry disease in a ratio of 11 for each classical case.

In the classical phenotype the clinical manifestations usually begin in childhood or adolescence with acroparesthesias; episodic "Fabry crises" of acute pain lasting hours to days; characteristic skin lesions (angiokeratomas); cornea verticillata; hypohidrosis; heat, cold, and exercise intolerance; mild proteinuria, and gastrointestinal symptoms. In adulthood, Fabry disease leads to cardiac manifestations, namely left ventricular hypertrophy (LVH), heart failure, angina, dysrhythmias, cardiac conduction abnormalities, and sudden death. Other manifestations include end-stage renal failure; early strokes or transient ischemic attacks; brain white matter lesions; carpal tunnel syndrome, and sensorineural deafness. In the later onset phenotype, the clinical manifestations arise in adulthood and include cardiac, renal, and cerebrovascular manifestations [1]. Fabry disease leads to a reduction of quality of life and survival [1, 4, 5], and cardiac disease is the main cause of death [5].

In this X-linked disorder, heterozygote females are not merely carriers. The clinical manifestations in females range from asymptomatic to full-blown disease that may be as severe as in affected males. Heterozygote females may experience symptoms in childhood and adolescence (such as pain and proteinuria) and adulthood (such as cardiac, cerebrovascular or, more rarely, renal manifestations) [1, 6, 7].

In males, the diagnosis of Fabry disease is based on the biochemical determination of the enzymatic activity of α-galactosidase A [1]. In females, the enzymatic activity of α-galactosidase A may be normal as well as the disease biomarkers, such as plasma GB3 and more rarely urinary GB3 [1, 8]. Plasma Lyso-GB3 levels may also be normal in females, particularly in later onset variants [9]. Therefore, the diagnosis of Fabry disease in females requires the molecular analysis of the *GLA* gene [1].

There are more than 600 documented mutations of the *GLA* gene, including missense or nonsense mutations, frameshift mutations, deletions, and insertions [1, 10], and the mutations are frequently private to each family. A genotype-phenotype correlation is not clearly established. The missense mutations of the *GLA* gene are mainly associated with a residual enzymatic activity and an attenuated/later onset phenotype of Fabry disease [1, 11, 12]. Some clinical features of Fabry disease are unspecific, which makes it difficult to interpret the clinical meaning of newly found missense mutations, particularly in females [13]. On the other hand, nonsense mutations generate a stop codon leading to the synthesis of a truncated protein, which results in a virtually null enzymatic activity in males and the development of the classical phenotype of Fabry disease that is characterized by an earlier onset and most severe clinical manifestations [1, 11, 12].

Herein, we describe a family with the classical phenotype of Fabry disease caused by the novel nonsense mutation c.607G>T (p.E203X) of the *GLA* gene.

Case Report

A 58-year-old female, with an unremarkable medical background besides history of carpal tunnel syndrome, presented with complaints of exercise dyspnea (NYHA class II). Her physical examination was normal. The electrocardiogram showed a sinus rhythm, short PR interval and criteria of LVH. The transthoracic echocardiogram revealed mild LVH (thickness of interventricular septum 12 mm and posterior wall 10 mm; LV mass 108 g/m^2), diastolic dysfunction grade I (abnormal LV relaxation pattern), preserved LV systolic function (LV ejection fraction 71%), normal heart valve structure and function, and no other cardiac abnormalities (Fig. 1a).

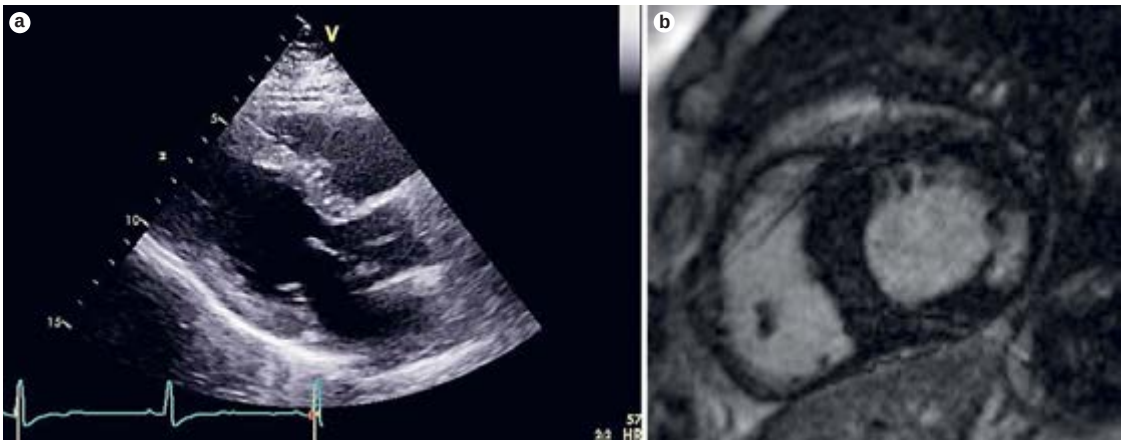
She was referred to a cardiology consultation due to unexplained LVH. A cardiac MRI showed mild LVH (LV mass 82 g/m^2), normal LV systolic function (LV ejection fraction 74%), and intramyocardial late gadolinium enhancement (LGE) on the basal inferolateral LV segment (Fig. 1b). No relevant dysrhythmias were detected on 24-h Holter monitoring. The laboratory analyses were unremarkable, except for microalbuminuria (47 mg/24 h). Creatinine clearance was normal (125 mL/min) and no proteinuria was detected (157 mg/24 h).

Fabry disease screening was performed by a dried blood spot test and revealed reduced enzymatic activity of α-galactosidase A (0.29 nmol/h/spot; normal average value: 0.64). Molecular analysis by PCR sequencing of the exons and flanking intronic regions of the *GLA* gene identified a novel nonsense mutation on exon 4 (c.607G>T; p.E203X).

The enzymatic activity of α-galactosidase A was normal on plasma (8.6 nmol/h/mL; normal: 6–19) and reduced on leukocytes (29 nmol/h/mg; normal: 36–80). Urinary GB3 was increased

Cardiology 2017;137:67–73
DOI: 10.1159/000455117

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Fig. 1. Transthoracic echocardiogram (parasternal long axis view) showing mild LVH (**a**) and cardiac magnetic resonance showing intramyocardial LGE on the basal inferolateral segment of the left ventricle (**b**) in the index patient with Fabry disease.

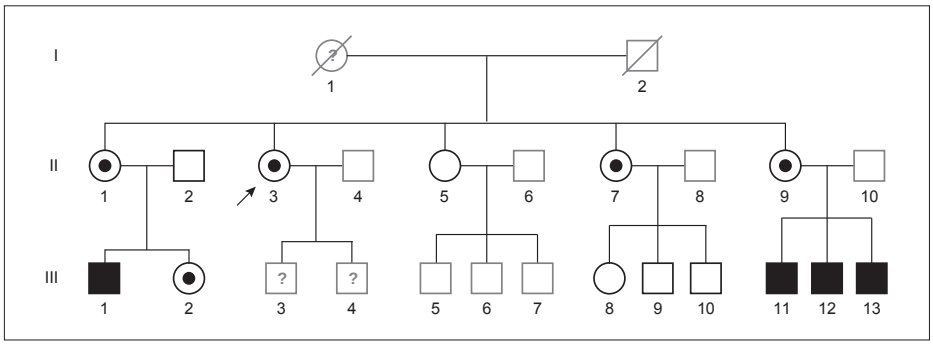


Fig. 2. Family pedigree of Fabry disease. Squares are males and circles are females. The arrow indicates the proband. Tested and untested subjects for Fabry disease are depicted in black and gray, respectively. Untested subjects with a question mark were unwilling to perform the test for Fabry disease. Affected and unaffected subjects are indicated with filled and open symbols, respectively.

(80 μg/mmol creatinine; normal: 0.87–13) as well as plasma Lyso-GB3 (5.06 ng/mL; normal: 0–1.99).

The multidisciplinary clinical evaluation also revealed cornea verticillata, cataracts, and mild airway obstruction. The patient had no history of acroparesthesias, neuropathic pain, hypohidrosis or gastrointestinal manifestations. No angiokeratomas, sensorineural deafness or brain white matter lesions were detected.

The diagnosis of Fabry disease was established and cascade family screening identified 8 family members carrying the same *GLA* gene mutation: 3 sisters, 4 nephews, and 1 niece (Fig. 2). All the family members carrying the *GLA* gene mutation c.607G>T (p.E203X) presented cornea verticillata, which is a very specific finding of Fabry disease, and other clinical manifestations compatible with Fabry disease, namely LVH, renal failure, stroke, brain white matter lesions or sensorineural deafness. From childhood, the male family members also presented manifestations that are particularly characteristic of Fabry disease, such as angiokeratomas, acroparesthesias, neuropathic pain, anhidrosis, unexplained fever crisis, episodes of abdominal pain, and diarrhea. Enzymatic activity of α-galactosidase A was significantly reduced or null in all males carrying the mutation. Urinary GB3 and plasma Lyso-GB3 were increased in all family members carrying the *GLA* gene muta-

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Table 1. Enzymatic activity of α-galactosidase A, biomarker levels, and clinical phenotype of the index patient and her family members diagnosed with Fabry disease through family cascade screening

Fabry patient	Gender/ age, years	Enzymatic activity of α-galactosidase A	Urinary GB3	Plasma Lyso-GB3	Clinical phenotype
II-1	F/59	Normal on plasma (6.7 nmol/h/mL) Decreased on leukocytes (7.1 nmol/h/mg)	Increased (81 μg/mmol creatinine)	Increased (11.64 ng/mL)	Severe LVH, diastolic dysfunction grade I, diffuse transmural LGE on inferolateral LV segments Renal failure (NKF class III – creatinine clearance 39 mL/min), microalbuminuria (2,392 mg/24 h), proteinuria (2,781 mg/24 h) Lacunar stroke, white matter lesions Cornea verticillata Sensorineural deafness Hypohidrosis No angiokeratomas No acroparesthesias, no pain No gastrointestinal manifestations
II-3	F/58	Normal on plasma (8.6 nmol/h/mL) Decreased on leukocytes (29 nmol/h/mg)	Increased (80 μg/mmol creatinine)	Increased (5.06 ng/mL)	Mild LVH, diastolic dysfunction grade I, LGE on basal inferolateral LV segment, short PR interval Microalbuminuria (47 mg/24 h) Carpal tunnel syndrome Cornea verticillata, cataracts Mild airway obstruction No renal failure (creatinine clearance 125 mL/min) or proteinuria (157 mg/24 h) No brain abnormalities No angiokeratomas No acroparesthesias, no pain No hypohidrosis No gastrointestinal manifestations No deafness
II-7	F/55	Normal on plasma (6.8 nmol/h/mL) Decreased on leukocytes (20 nmol/h/mg)	Increased (42 μg/mmol creatinine)	Increased (10.22 ng/mL)	Severe LVH, diastolic dysfunction grade II, no LGE, sinus bradycardia, short PR interval, history of atrial fibrillation White matter lesions Cornea verticillata Sensorineural deafness Hypohidrosis, heat intolerance No renal failure (creatinine clearance 88 mL/min), no microalbuminuria (30 mg/24 h) or proteinuria (<75 mg/24 h) No angiokeratomas No acroparesthesias, no pain No gastrointestinal manifestations
II-9	F/54	Normal on plasma (7.2 nmol/h/mL) Normal on leukocytes (41 nmol/h/mg)	Increased (37 μg/mmol creatinine)	Increased (6.02 ng/mL)	Short PR interval White matter lesions Cornea verticillata Sensorineural deafness Hypohidrosis No LVH, Normal LV function, No LGE No renal failure (creatinine clearance 119 mL/min), no microalbuminuria (12 mg/24 h) or proteinuria (<105 mg/24 h) No angiokeratomas No acroparesthesias, no pain No gastrointestinal manifestations
III-1	M/36	Null on plasma (0 nmol/h/mL) Null on leukocytes (0 nmol/h/mg)	Increased (1,189 μg/mmol creatinine)	Increased (93.17 ng/mL)	Moderate LVH, normal LV function, LGE on inferolateral wall, normal PR interval Microalbuminuria (170 mg/24 h) and Proteinuria (458 mg/24 h) Acroparesthesias and neuropathic pain since early adolescence, precipitated by heat, unresponsive to conventional analgesics Cornea verticillata Sensorineural deafness Anhidrosis No renal failure (creatinine clearance 98 mL/min) No white matter lesions No angiokeratomas No gastrointestinal manifestations

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Table 1 (continued)

Fabry patient	Gender/ age, years	Enzymatic activity of α-galactosidase A	Urinary GB3	Plasma Lyso-GB3	Clinical phenotype
III-2	F/30	Normal on plasma (7.2 nmol/h/mL) Decreased on leukocytes (22 nmol/h/mg)	Increased (14 μg/mmol creatinine)	Increased (4.43 ng/mL)	Microalbuminuria (84 mg/24 h) Acroparesthesias and neuropathic pain at early adult age, unresponsive to conventional analgesics Cornea verticillata Sensorineural deafness Hyperhidrosis No LVH, normal LV function, no LGE, normal PR interval No renal failure (creatinine clearance 168 mL/min), no proteinuria (204 mg/24 h) No white matter lesions No angiokeratomas No gastrointestinal manifestations
III-11	M/32	Null on plasma (0 nmol/h/mL) Null on leukocytes (0 nmol/h/mg)	Increased (396 μg/mmol creatinine)	Increased (101.23 ng/mL)	Moderate LVH, diastolic dysfunction grade II, no LGE, short PR interval Microalbuminuria (179 mg/24 h) Acroparesthesias, severe neuropathic pain on limb extremities since early childhood, unresponsive to conventional analgesics Anhidrosis, extreme heat intolerance, unexplained fever crisis Angiokeratomas Cornea verticillata, retinal vascular tortuosity No renal failure (creatinine clearance 119 mL/min) or proteinuria (253 mg/24 h) No deafness No brain abnormalities No gastrointestinal manifestations
III-12	M/26	Null on plasma (0 nmol/h/mL) Decreased on leukocytes (2 nmol/h/mg)	Increased (341 μg/mmol creatinine)	Increased (133.23 ng/mL)	Sinus bradycardia Microalbuminuria (35 mg/24 h) Acroparesthesias, severe neuropathic pain on limb extremities since early childhood, unresponsive to conventional analgesics Anhidrosis, extreme heat intolerance, unexplained fever crisis Abdominal pain and diarrhea Angiokeratomas Cornea verticillata, retinal vascular tortuosity No LVH, LV dysfunction or LGE No renal failure (creatinine clearance 102 mL/min) or proteinuria (226 mg/24 h) No brain abnormalities No deafness
III-13	M/20	Null on plasma (0 nmol/h/mL) Null on leukocytes (0 nmol/h/mg)	Increased (366 μg/mmol creatinine)	Increased (109.08 ng/mL)	Mild LVH, no LV dysfunction, no LGE Microalbuminuria (174 mg/24 h), proteinuria (422 mg/24 h) Acroparesthesias, severe neuropathic pain on limb extremities since early childhood, unresponsive to conventional analgesics Anhidrosis, extreme heat intolerance, unexplained fever crisis Cornea verticillata, retinal vascular tortuosity No renal failure (creatinine clearance 167 mL/min) No brain abnormalities No angiokeratomas No deafness No gastrointestinal manifestations

Reference values: enzymatic activity of α-galactosidase A on plasma 6–19 nmol/h/mL, and on leukocytes 36–80 nmol/h/mg; urinary GB3 0.87–13 μg/mmol creatinine; plasma Lyso-GB3 0–1.9 ng/mL.

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tion c.607G>T (p.E203X) (Table 1). Individuals not carrying the mutation were asymptomatic and presented normal enzymatic activity of α-galactosidase A.

The index patient is currently under enzyme replacement therapy (ERT) along with 2 sisters and 4 nephews (II-1, II-7, III-1, III-11, III-12, III-13). Her niece (III-2) is also expected to begin ERT soon.

Discussion

To our knowledge, this is the first report of a classical phenotype of Fabry disease caused by the nonsense mutation c.607G>T (p.E203X) of the *GLA* gene. This clinical case illustrates the importance of performing a complete diagnostic evaluation in patients with unexplained LVH, including personal and family clinical history, family pedigree, physical examination, electrocardiogram, echocardiogram, cardiac MRI, Holter monitoring, and laboratory analysis. This diagnostic evaluation can provide clues to the differential diagnosis of the cause of LVH and allow the diagnosis of treatable causes of LVH such as Fabry disease. In this case, the short PR interval, the distribution pattern of the LGE and the microalbuminuria constituted diagnostic red flags for Fabry disease [14–16].

The mild yet unexplained LVH of the index patient highlights that Fabry disease diagnosis should be considered and screening should be performed in all cases of unexplained LVH, including the milder forms of LVH. This report also emphasizes the importance of performing the family pedigree and the cascade family screening to the earlier diagnosis and treatment of other affected family members. According to Laney and Fernhoff [17], family cascade screening leads to the diagnosis of Fabry disease in an average of 5 family members. In this case, family screening led to the diagnosis of Fabry disease in 8 family members, all already presenting a clinical indication to commence ERT.

This study provides a complete clinical and biochemical description of the phenotype of the family members, allowing the characterization of the classical phenotype of Fabry disease associated to the nonsense mutation c.607G>T (p.E203X) of the *GLA* gene. The diagnosis of a classical phenotype of Fabry disease is based on the observation that all male patients carrying the mutation presented a significantly reduced or null enzymatic activity of α-galactosidase A and clinical manifestations, since childhood, that are particularly characteristic and specific for the classical phenotype of Fabry disease. These include angiokeratomas, cornea verticillata, acroparesthesias, neuropathic pain, anhidrosis, unexplained fever crisis, episodes of abdominal pain, and diarrhea [1]. Urinary GB3 and plasma Lyso-GB3 were increased in all family members carrying the *GLA* gene mutation c.607G>T (p.E203X), including the females, although the highest values were encountered in males. This finding is also consistent with a classical phenotype because female patients with later onset variants of Fabry disease usually present normal plasma Lyso-GB3 values [9].

Unlike the male family members, the female patients of this family did not present a history of clinical manifestations in childhood, such as acroparesthesias or neuropathic pain, which is consistent with the milder phenotype that usually characterizes females with this X-linked disorder [6, 7].

Despite carrying a nonsense mutation of the *GLA* gene, the index patient, a 58-year-old female (II-3), presented a mild phenotype compared to her 59-year-old sister (II-1) and her younger 55-year-old sister (II-7), who presented a severe phenotype. Also carrying the same nonsense *GLA* gene mutation, we found that the youngest sister (II-9) presented no evidence of target organ damage. The case of this family illustrates the wide variability of the clinical phenotype of females with Fabry disease harboring the same *GLA* gene mutation [1, 6, 7]. The pattern of X chromosome inactivation has been hypothesized as the major determinant of the wide spectrum of the clinical phenotype presented by heterozygote female patients [18].

Moreover, besides differing in age, the 2 sisters with the severe phenotype also differ in clinical presentation. One has severe LVH with cardiac fibrosis and a significant renal failure, while the other has severe LVH, but does not present cardiac fibrosis or clinical evidence of renal involvement by Fabry disease, which supports the notion that the development of cardiac manifestations is also dependent on age and renal function [19].

This is, to the best of our knowledge, the first report of Fabry disease due to the *GLA* gene mutation c.607G>T (p.E203X). This novel nonsense mutation occurs in the active site of the α-galactosidase A enzyme [1, 20]. This report provides a complete documentation of the cosegregation of this *GLA* gene mutation and the clinical and biochemical findings of Fabry disease in this family, thus establishing the *GLA* gene mutation c.607G>T (p.E203X) as pathogenic and causative of Fabry disease.

In conclusion, this is the first report of a classical phenotype of Fabry disease caused by the novel nonsense mutation c.607G>T (p.E203X) of the *GLA* gene. This paper also highlights that (i) Fabry disease diagnosis should be considered even in milder forms of unexplained LVH;

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(ii) a complete diagnostic evaluation in patients with unexplained LVH is important to find diagnostic red flags of treatable causes of LVH, such as Fabry disease; (iii) cascade family screening is paramount to the earlier diagnosis and treatment of other affected family members; and (iv) the Fabry disease phenotype is highly variable in heterozygote females, even within the same family.

Conflict of Interest

The authors have no conflicts of interest to disclose.

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CHAPTER 5 – DISCUSSION

5.1 Fabry disease in the Portuguese region of Guimarães

5.1.1 Fabry disease prevalence in Hypertrophic cardiomyopathy in the Portuguese region of Guimarães

This investigation has demonstrated a high prevalence of FD in the Portuguese region of Guimarães (16.6%) due to a founder effect of the disease in this region, secondary to the *GLA* mutation p.F113L, which causes a late-onset phenotype with predominant cardiac involvement.

The demonstration of an unexpectedly high prevalence of FD in adult patients with HCM in the Portuguese region of Guimarães (16.6%) was based on a screening study, which had the advantages of including both males and females with the diagnostic criteria of HCM according to the ESC guidelines and using a combined enzymatic and genetic screening method that ensured an accurate diagnosis of FD in both genders.

Therefore, apart from an apparently lower frequency of LVOT obstruction at rest (21.6% vs. 35%) and implantable cardioverter-defibrillator (ICD) insertion (4.7% vs. 13%) when compared to the Portuguese Registry of HCM, the baseline characteristics of the patients with HCM in the study reflected the clinical characteristics associated to HCM in the literature [280,285]. Accordingly, our cohort was composed mainly by males and family history was reported in more than a third of the patients. HCM was mainly asymmetrical, diastolic dysfunction was common, LV ejection fraction was preserved and most patients had heart failure, the majority of whom in NYHA class I-II. LGE was present in nearly 75% of the patients, non-sustained VT in nearly 25%, pacemaker implantation in approximately 9% and syncope was reported in nearly 10%.

Furthermore, GVUS were not found in this screening study and all the *GLA* variants were classified as pathogenic mutations, based on solid clinical, biochemical and/or histological evidence.

The mutation c.281G>C (p.C94S) occurs in a highly conserved residue, affecting an enzyme disulfide bond, and had already been described as a pathogenic mutation and associated to a classical phenotype [286-288]. The "in vitro" enzymatic activity of mutant α-galactosidase A in HEK-293 cells has been reported as below the limit of detection [289]. In-silico methods also predict a damaging effect on protein function (MutationTaster, Polyphen-2) [290,291]. Accordingly, our patient with the p.C94S mutation was a 50-year-old male with near-null/null enzymatic activity on plasma and leukocytes and increased plasma lyso-GB3 in the classical male range, who presented a severe classical phenotype of FD, including severe

acroparesthesias, neuropathic pain, anhidrosis and heat intolerance since childhood, cornea *verticillata* and angiokeratomas, as well as severe HCM, end-stage renal failure, history of stroke and severe deafness. Additionally, a clear genotype-phenotype cosegregation was documented and all affected relatives presented increased plasma lysoGB3 and clinical manifestations compatible with FD (renal failure in one sister; and proteinuria and acroparesthesias in one daughter, one niece and one great-niece).

The mutation c.548G>T (p.G183V) occurs in a highly conserved residue, in a helix of the protein, buried in the enzyme three-dimensional structure of the catalytic domain; it has been predicted to cause enzyme instability and had already been described as pathogenic and associated to a classical phenotype [287,288,292]. The "in vitro" enzymatic activity of mutant α-galactosidase A in HEK-293 cells has been reported as null or below the limit of detection [287,289]. In-silico methods also predict a damaging effect on protein function (MutationTaster, Polyphen-2) [290,291]. Accordingly, our patient with the p.G183V mutation was a 50-year-old female with reduced enzymatic activity on plasma and leukocytes and increased plasma lyso-GB3 within the expected classical female range, who presented HCM, short PR interval and brain WML. Interestingly, all her relatives tested negative for this *GLA* variant, raising the suspicion of a sporadic *"de novo"* mutation or a non-paternity case.

The mutation c.870G>A (p.M290I) occurs at the end of the α7-helix; it has been predicted to cause enzyme destabilization and misfolding and had already been associated to a classical phenotype [287,288,293]. Our patient presented a similar missense mutation (c.870G>C) resulting in the same protein abnormality (p.M290I). This variant allele was found at a frequency of 5.5e-06 in 183451 control chromosomes (gnomAD) [294]. In-silico methods predict a damaging effect on protein function (MutationTaster, Polyphen-2) [290,291]. This *GLA* variant had been previously reported in ClinVar database as pathogenic, although, more recently, it has been reported by another submitter as GVUS possibly pathogenic [295]. This variant has also been reported in two Portuguese heterozygote females with a late-onset phenotype in a Swiss cohort [296]. Our 43-year-old female patient presented severe HCM, proteinuria and brain WML, also compatible to a late-onset phenotype. Her two affected sisters have been reportedly asymptomatic so far. Despite a normal plasma lyso-GB3, as expected for females with late-onset phenotype, our patient had a reduced enzymatic activity on leukocytes (within 7.5-16.6% of normal range), much lower than the one previously described "in vitro" in HEK-293 cells (68.0±3.4%) [289]. The pathogenicity of this *GLA* variant was finally demonstrated by histological evidence of lysosomal inclusions in cardiomyocytes.

The novel *GLA* variant c.607G>T (p.E203X) was detected in the course of this investigation and its pathogenicity and association to the classical phenotype of FD have been demonstrated based on the

biochemical and clinical findings of the affected patient and family relatives. Furthermore, this nonsense mutation occurs on the region of substrate binding [288] and a clear genotype-phenotype cosegregation has also been documented. In-silico methods also predict a damaging effect on protein function (MutationTaster) [290]. Indeed, our 50-year-old female patient with the p.E203X mutation presented reduced enzymatic activity on leukocytes and increased plasma lyso-GB3 within the expected classical female range, along with cornea *verticillata*, severe HCM, renal failure, history of stroke, brain WML and deafness. Moreover, all the affected male relatives presented null/near-null enzymatic activity on plasma and leukocytes; and all the affected male and female relatives exhibited a classical phenotype and increased FD biomarkers within the expected range for classic males and females. Notably, all the affected males presented manifestations that are particularly characteristic of the classical phenotype pf FD, i.e., angiokeratomas, cornea *verticillata*, acroparesthesias, neuropathic pain, extreme heat intolerance, unexplained fever crisis and anhidrosis since early childhood.

The mutation c.337T>C (p.F113L) had already been described as pathogenic [192,213] and associated to a late-onset phenotype with predominant cardiac involvement [27,192]. This variant occurs in a highly conserved residue, in a position of a beta strand of the protein [288] and the mutant enzyme was shown to be unstable and to assume a misfolded conformation at the neutral pH of the endoplasmic reticulum, thereby being subject to ERAD [213]. In-silico methods also predict a damaging effect on protein function (MutationTaster, Polyphen-2) [290,291]. The "in vitro" enzymatic activity of mutant enzyme in HEK-293 cells was estimated as 18.3 ± 0.8% of the activity of the wild-type enzyme [289]. Our research has also extensively documented biochemical and clinical findings compatible with FD in affected probands and relatives and provided histological evidence of lysosomal inclusions in Fabry patients with this mutation. The detection of this *GLA* mutation in most of the identified Fabry index patients in Guimarães and the origin of these patients in the same region of Guimarães constituted important red-flags for a possible founder effect.

5.1.2 Founder effect of Fabry disease in the Portuguese region of Guimarães

The demonstration of a founder effect of FD due to the p.F113L mutation in the Portuguese region of Guimarães was essentially based on detailed genealogical research that identified a large 12-generation family tree and a female born in the 17th century as the common ancestor to most of the Fabry families with this mutation in this region. This genealogical documentation was enabled by the invaluable historical collection contained on the parish records, which, since the Council of Trent (1545-1563), mandatorily included the birth and marriage registrations.

Of note, the genealogical connection to the family tree has not been found for some families, mainly due to cases of unknown paternity, a phenomenon that emerged when the paternity registration became non-obligatory in the 18th century and that currently constitutes one of the main obstacles to genealogical research. Although a more remote connection of these families to the family tree cannot be excluded, haplotype analysis suggested the families within the family tree that were more closely related to the haplotyped unconnected families, hence suggesting a possible connection between them.

Besides providing solid and precise evidence of a founder effect of FD due to the p.F113L mutation in the Portuguese region of Guimarães, this genealogical research also allowed to understand the historical and socioeconomic reasons that favoured the occurrence of a founder effect of the disease in this Portuguese region. In humans, a founder effect may occur in stable populations with high rate of endogamy and low migration rate. The common ancestor was a female born on 20th of May 1611 in the parish of São Clemente de Sande of the county of Guimarães. In this countryside region, in the 17th century, marriages occurred within the same socioeconomic stratum. The first couple of this genealogical tree was a stable land contract owner, but the descendants that transmitted the disease did not inherit this property and therefore married within lower social status families. There was a progressive impoverishment of these families until the 19th century, leading individuals to search for temporary work that was warranted in nearby farmers houses, avoiding the need of distant migration. Although worldwide emigration was common in Portugal, it was not generally accessible, until the 19th century, to poor individuals. These socioeconomic factors perpetuated the disease transmission in this region until the contemporary era. It is also noteworthy that the late-onset phenotype of FD associated to the p.F113L mutation does not affect the reproductive fitness of the patients, therefore enabling the disease propagation throughout generations. More recently, with the generalization of migration, this founder mutation has been spread to many countries around the world.

Indeed, this investigation has shown that this late-onset FD due to the p.F113L mutation has a national and cross-border impact in many countries around the world (Spain, France, Monaco, United Kingdom, Luxembourg, Switzerland, Germany, Angola, Colombia). The extensive family screening of all the index patients diagnosed with FD due to the p.F113L mutation in the Portuguese region of Guimarães has led, along the years, to the diagnosis of many patients living in other regions of Portugal and other countries. Migration of these family relatives to other countries had occurred in the last years or decades, mostly motivated by economic reasons.

Genealogical research may provide solid evidence of a founder effect, precisely identifying individuals and dates and family connections between them; however, genealogical data is not available in some locations

and for the most remote ages. Hence, the existence of a founder effect can also be investigated by genetic research, which may also be used to estimate the age of the founder mutation.

Haplotype analysis of two Italian patients and 10 Portuguese families carrying the p.F113L mutation (eight from neighbouring locations of Guimarães, one from a southern region of Portugal and one from the Madeira Island) has recently shown that these patients shared the same haplotype, thus supporting our finding of a founder effect of FD in the Portuguese region of Guimarães and the disease spread to other Portuguese regions and countries [297].

Another founder effect of FD has been recently documented based on genetic research in Southern China and Taiwan, where a high prevalence of the disease due to the IVS4+919G>A mutation was found [298]. The IVS4+919G>A mutation, which has also been associated to a late-onset phenotype with predominant cardiac involvement, was first reported in a Japanese male with HCM in Kyushu, Japan [195]. Recently, Liang *et al.* reported that the haplotype containing the IVS4+919G>A mutation was shared by 33 males born in Taiwan, Japan, Singapore, Malaysia, Vietnam and China, but not by healthy controls, supporting the existence of a founder effect. The age of this founder mutation was estimated as more than 800 years (first, second and third quartiles were 800.7, 922.6 and 1068.4 years) based on the lengths of the mutation-carrying haplotypes and the linkage-disequilibrium decay theory [298]. It was hypothesized that the common ancestor may have originated in Southern China and it was speculated that the IVS4+919G>A mutation may have been brought to Japan, around 500 years ago, by a chief Chinese pirate who brought many Chinese pirates to the Hirado city, Kyushu, Japan, to establish a small kingdom called Song [298].

In Brazil, a smaller founder effect of FD due to the 30delG mutation has also been described based on genetic research, which showed that the nine patients carrying this mutation shared the same haplotype, unlike healthy controls, suggesting a founder effect [299]. When expanding haplotype analyses to two additional microsatellites markers, four different haplotypes were identified and the most prevalent was interpreted as the ancestral haplotype. The mutation was estimated to have occurred between the years of 1710 and 1740 (between 1410 and 1840, considering a confidence interval of 95%). Interestingly, the comparison between the ancestral haplotype and the haplotypes observed in each parental population that contributed to the current admixed Brazilian population indicated that the ancestral haplotype on which the 30delG mutation coincides has the greatest genetic similarity with several haplotypes present in individuals from Portugal. It is historically known that, as the Portuguese and Spanish crowns disputed sovereignty over this region in the beginning of the 18th century, the King of Portugal encouraged the immigration of approximately 6000 settlers from the Azores Islands to establish Portuguese domination

over Southern Brazil. Thus, it is hypothesized that the *GLA* 30delG mutation occurred on an X chromosome from Portuguese immigrants during the initial colonization period of Southern Brazil [299].

Although interesting and plausible from an historical point of view, no genealogical data was provided to support these historical hypotheses generated by genetic research. Our study, on the other hand, is original and innovative, as the demonstration of the founder effect is based on genealogical evidence and integrated in the historical and socioeconomic context of the real individuals carrying this disease throughout generations.

Interestingly, FD prevalence in HCM in the region of Guimarães, after adjusting for the founder effect, is still higher (3.3%) than the prevalence found in our multicentre study (0.9%) or in a recent systematic review of the literature and meta-analysis (0.9%).

Assuming that the FD prevalence in HCM in the region of Guimarães, adjusted for the founder effect, is the same as in other regions, this result could be explained by an efficient diagnosis rate of FD in this region due to an increased awareness of health professionals on FD red-flags, motivated by the knowledge of a founder effect of the disease in this region, and the systematic referral of patients with higher risk of FD, based on the coexistence of HCM with other FD red-flags, to the Reference Centre for screening and diagnosis.

The possibility that FD prevalence in HCM, adjusted for the founder effect, is higher in the region of Guimarães than in other regions is less likely, as the frequency of "*de novo*" mutation has been reported to be low in FD and similar among different ethnic groups. In Japan, the frequency of "*de novo*" mutations has been reported as 6.8% (five patients in 74 unrelated families), although this frequency might be overestimated because the confirmation that the parents were the biological fathers or mothers was not performed [300]. In Spain, this frequency was estimated as 4.5% (one male patient in 22 unrelated families, whose mother was negative for FD) [301]. The size and structure of the gene and its position within the genome may contribute to the frequency of *"de novo"* mutations. In other X-linked disorders, such as Duchenne muscular dystrophy and hemophilia A, one-third of the mutations are expected to arise *"de novo"* [300,302], which might be explained by the unusually large size (2400 kb) of *DMD* gene and the rich CpG dinucleotide content in the *F8* gene, respectively. In FD, the smaller size of *GLA* gene (12 kb) as well as its lower content of CpG dinucleotides may contribute to the lower frequency of *"de novo"* mutations [300].

Therefore, a possible referral bias may explain the higher prevalence of FD in HCM, adjusted for the founder effect, in the region of Guimarães; indeed, some of the non-F113L Fabry families presented other FD red-flags besides HCM. Nevertheless, the same bias is not likely to explain the prevalence of the

p.F113L mutation in HCM (21/150; 14.0%), as Fabry patients with the p.F113L mutation are essentially referred for unexplained HCM and not following the recognition of other FD red-flags by health professionals.

The demonstration of a high prevalence of a rare genetic disease that carries high healthcare related-costs, in a geographical region, due to a founder effect, justifies the implementation of specific health policies and measures targeting equity and excellence in healthcare, early diagnosis and treatment, genetic counselling, as well as disease awareness, education of health professionals and top-level scientific research in this field. The achievement of these goals should lie in the joint action of the governmental health authorities, the Reference Centre on Lysosomal Storage Disorders of Hospital Senhora da Oliveira – Guimarães, the Primary Care and affilliated hospital centres, the patient organizations and the research institutes and universities.

5.2 Natural history of late-onset phenotypes of Fabry disease with predominant cardiac involvement

Our research provides a detailed description of the phenotype and an accurate overview of the natural history of the disease caused by the *GLA* mutation p.F113L, thereby improving the knowledge on the natural history of late-onset phenotypes of FD with predominant cardiac involvement.

This investigation was based on one of the largest cohorts of adult patients with late-onset FD caused by the same *GLA* mutation, which offered the unique opportunity not only to perform a gender and age-stratified analysis and obtain the chronology of the clinical manifestations in both genders, decade by decade, but also to appreciate the clinical heterogeneity of the disease in the overall cohort as well as within the same gender and age group. Furthermore, most patients were diagnosed through family screening, which allowed the diagnosis of patients from both genders, all age categories and disease status, capturing the full spectrum of the disease. The female to male ratio in this cohort was 1.6, closer to the expected ratio in X-linked diseases, thereby considerably reducing the bias related to the female underrepresentation of previous studies, in which this ratio ranged from 0.3 to 1.3 [181,182,184,186-189,209,210,212]. Patients were systematically submitted to a predefined multidisciplinary diagnostic protocol, which assured a high level of completeness and detail of the data on all organ system manifestations, overcoming common bias of retrospective studies and registries. The studies evaluated only baseline data obtained at the time of FD diagnosis, so the disease status of the patients reflected the natural course of the disease until that moment, without the influence of FD specific therapy. Finally, the studies accounted for the influence of comorbidities and medications on the disease phenotype.

However, the natural history provided by this investigation does not represent a longitudinal follow-up of these patients, rather it was built based on the baseline data presented by untreated adult patients distributed through the full range of age categories. The longitudinal follow-up of untreated patients in a time where therapies are available raises ethical issues and, given the attenuated and slow course of the disease, a long time of follow-up would be needed to capture its natural history. Nevertheless, considering the large size of our cohort, the fact that it includes all the patients diagnosed with FD within 34 families and the fact that most of them were diagnosed through family screening and not because of medical attention driven by symptoms, it is reasonable to expect that the data within each age category is a reliable representation of the clinical heterogeneity of the disease at each timeframe of its natural course.

The results of this investigation supported the concept that the mutation p.F113L is associated to a late-onset phenotype of FD with predominant cardiac involvement. To better understand the natural history of late-onset FD due to the mutation p.F113L, this thesis provides a comparative analysis with the natural history of late-onset FD associated to the mutations p.N215S and IVS4+919G>A, which are so far the most common and studied *GLA* mutations associated with late-onset phenotypes with predominant cardiac involvement [181-190,208-211] (Tables 1 and 2).

The mutation p.N215S results in the substitution of an asparagine by a serine at a highly conserved N-glycosylation consensus site [194], thereby disrupting glycosylation at this site and impairing enzyme activity, solubility, and transport from the endoplasmic reticulum to the lysosome [303]. Consequently, only 5% and 25% of normal α-galactosidase A enzymatic activity are expressed in peripheral leukocytes and lymphoblasts, respectively [194]. The p.N215S variant is a common cause of FD, apparently the most common mutation in Western countries [187], occurring in 4.8% of people in a large cohort of primarily North American and European descent [304]. A cluster of patients with the p.N215S mutation in the United Kingdom suggests a founder effect of FD in this region, although haplotype analysis would be useful to clarify this hypothesis [181].

The mutation IVS4+919G>A results in an alternative splicing event that inserts an additional 57-nucleotide sequence (pseudoexon) in the *GLA* messenger ribonucleic acid (mRNA) between exons 4 and 5. This misspliced mRNA could be a target for nonsense-mediated decay. Moreover, this in-frame insertion generates a premature stop codon at the 27th nucleotide downstream from exon 4, resulting in a premature truncated protein of 222 amino acids residues with no enzyme activity and that is rapidly degraded. Low levels of correctly spliced mRNA account for the residual enzyme activity [195,305,306]. In healthy individuals, the aberrant spliced form occurred in <1% of the *GLA* mRNA transcripts in heart and small intestine, <10% in kidney, spleen and liver and more frequently in muscle and lung. In contrast, in patients

with the IVS4+919G>A mutation, the aberrant spliced form occurred in >70% of the *GLA* mRNA transcripts [195]. There is a differential tissue-specific expression of the aberrant splicing variant [207,306], i.e., it is more expressed in some tissues (skeletal muscle, lungs, blood) than others (brain, kidneys, pancreas) [207]. Consequently, the IVS4+919G>A mutation is associated to a reduction of the enzymatic activity of α-galactosidase A (typically 8-15%) [195]. Due to a documented founder effect [298], this mutation is highly prevalent in Taiwan. In an enzymatic screening of 90288 Taiwanese male newborns, FD was found in ~1/1250 males and 86.1% of them had the IVS4+919G>A mutation (~1/1460 males) [34]. In a genetic screening of 20063 Taiwanese newborns, FD due to the IVS4+919G>A mutation was found in 1/875 males and 1/399 females [206].

As this research has shown, the mutation p.F113L is another common *GLA* mutation due to a founder effect in the Portuguese region of Guimarães and a cross-border spread of the disease due to migration. Therefore, the results of this investigation on the natural history of the late-onset FD due to this mutation will have an impact on the clinical decisions of many patients in Portugal and around the world.

Table 1: Summary of previous studies on the natural history of the late-onset phenotype of FD with predominant cardiac involvement caused by the *GLA* mutation p.N215S

N215S Mutation	Oder et al., 2017		Alharbi et al., 2018		Lavalle et al., 2018		Germain et al. 2018	
Population	FD patients with LVH (n=26)		FD patients (n=34)		FD patients (n=84)		FD patients from Fabry Registry (n=125)	
Gender	M (n=13)	F (n=13)	M (n=20)	F (n=14)	M (n=37)	F (n=47)	M (n=59)	F (n=66)
Age at diagnosis								
Mean	49±17 (14-74)		N/A	N/A	51.1±19.2	38.3±16.1	49.1 (8.7-72)	41 (0.5-69.3)
Median	N/A		58.6 (18-86)	41.6 (25-85)	58 (13-74)	36 (11-78)	N/A	N/A
α-GAL A enzyme activity (median)								
Plasma	N/A		N/A	N/A	0.2 (0.0, 0.6)	4 (1.9, 7.4)	N/A	N/A
Leukocytes	N/A		N/A	N/A	7.0 (2.1, 14)	49 (7.0, 73)	N/A	N/A
Plasma lyso-Gb3								
Mean	N/A		9.7 ± 1.0 nM	5.4 ± 0.8 nM	6.7±2.1nmol/L	1.5±0.9 nmol/L	N/A	N/A
Median	4.9 ng/mL (IQR 1.3–9.1)		N/A	N/A	6.6 (3.0, 9.8) nmol/L	1.2 (0.4, 4.1) nmol/L	N/A	N/A
Urinary lyso-GB3 (pmol/mmol creatinine)	N/A		14.5±5.7	20.3±9.0	N/A	N/A	N/A	N/A
Age at symptom onset								
Mean	N/A		N/A	N/A	51.7±14.5	36.8±21.6	N/A	N/A
Median	N/A		N/A	N/A	57 (7-73)	28.5 (13-66)	N/A	N/A
First signs and symptoms								
Cardiac	N/A		N/A	N/A	18 (81.8%)	1 (50.0%)	N/A	N/A
Renal	N/A		N/A	N/A	2 (9.1%)	1 (50.0%)	N/A	N/A
GI	N/A		N/A	N/A	2 (9.1%)	-	N/A	N/A
NYHA Class I-II	92%		N/A	N/A	N/A	N/A	N/A	N/A
LVH	N/A		14/20 (70%)	2/14 (14%)	17 (46%)	4 (8.5%)	88% had abnormal IVS	49% had abnormal IVS
Echocardiogram								
LV mass index (mean) ($g/m^{2.7}$)	N/A		N/A	N/A	58.2±22.7	33±16.3	N/A	N/A
LV mass index (median) ($g/m^{2.7}$)	N/A		N/A	N/A	50.6 (27.6,105.3)	29.3 (9.8, 94.4)	N/A	N/A
IVS thickness (mm)	12±4 (7-23)		N/A	N/A	N/A	N/A	N/A	N/A
PW thickness (mm)	11±4 (7-21)		N/A	N/A	N/A	N/A	N/A	N/A
LV end-diastolic diameter (mm)	46±6 (36–58)		N/A	N/A	N/A	N/A	N/A	N/A
LV end-systolic diameter (mm)	31±7 (20–48)		N/A	N/A	N/A	N/A	N/A	N/A
LV Ejection fraction (%)	61±7 (47-77)		N/A	N/A	N/A	N/A	N/A	N/A
LV Global Longitudinal Strain (%)	−15.0±5.6		N/A	N/A	N/A	N/A	N/A	N/A
LV Global Longitudinal Strain Rate (s^{-1})	−0.8±0.3		N/A	N/A	N/A	N/A	N/A	N/A
Cardiac MRI								
LV mass (g/m^2)	86±41 (46–195)		N/A	N/A	N/A	N/A	N/A	N/A
End-systolic volumes (mL/m^2)	30±13 (12–56)		N/A	N/A	N/A	N/A	N/A	N/A
End-diastolic volumes (mL/m^2)	74±18 (53–114)		N/A	N/A	N/A	N/A	N/A	N/A
Stroke volume (mL/m^2)	44±11 (25–65)		N/A	N/A	N/A	N/A	N/A	N/A
Cardiac index ($L/min/m^2$)	2.8±0.6 (1.9–3.9)		N/A	N/A	N/A	N/A	N/A	N/A
LV Ejection fraction (%)	60±10 (42–79)		N/A	N/A	N/A	N/A	N/A	N/A
LGE	69%		5/8 (62.5%)	3/7 (42.9%)	N/A	N/A	N/A	N/A
Sinus bradycardia	N/A		N/A	N/A	N/A	N/A	7%	
Bradyarrythmias (2nd degree or complete heart block)	N/A		1/12 (8%)	0/7 (0%)	N/A	N/A	N/A	N/A
Conduction abnormalities	N/A		N/A	N/A	11 (29.7)	8 (17)	N/A	N/A
Arrhythmias	N/A		N/A	N/A	11 (29.7)	0 (0)	N/A	N/A
Tachyarrhythmias (VT, AF)	N/A		3/12 (25%)	2/7 (29%)	N/A	N/A	N/A	N/A
Atrial fibrillation	N/A		N/A	N/A	N/A	N/A	4%	
VT on Holter	2/26		N/A	N/A	N/A	N/A	N/A	N/A
Cardiac devices								
Pacemaker	12%		N/A	N/A	7 (18.9%)	1 (2.1%)	N/A	N/A
ICD	4/26 (15.4%)		N/A	N/A	6 (16.2%)	0 (0%)	N/A	N/A
Pacemaker/ICD	N/A		2/12 (17%)	0/7 (0%)	N/A	N/A	N/A	N/A
Median age at pacemaker/ICD implantation (years)	N/A		N/A	N/A	69	63	N/A	N/A

Age-predicted heart rate on stress testing	24%	N/A	N/A	N/A	N/A	N/A	N/A
NT-proBNP (median) (pg/mL)	207 (IQR 69, 955)	N/A	N/A	N/A	N/A	N/A	N/A
Hs-TnT (pg/mL)	<5 (<5-162)	N/A	N/A	N/A	N/A	N/A	N/A
Endomyocardial biopsy	Lysosomal inclusions on cardiomyocytes and fibrosis	N/A	N/A	N/A	N/A	N/A	N/A
GFR (mL/min)	Mean: 102±23 Median: 106 (IQR 84–113)	N/A	N/A	N/A	N/A	N/A	N/A
Serum creatinine (mg/dL)	Mean: 0.87±0.20 Median: 0.80 (IQR 0.70-1.01)	N/A	N/A	N/A	N/A	N/A	N/A
CKD stages							
1 (> 90 mL/min/1.73m^2)	N/A	N/A	N/A	16/37 (43.2%)	27/41 (65.9%)	N/A	N/A
2 (60±89 mL/min/1.73m^2)	N/A	N/A	N/A	13/37 (35.1%)	13/41 (31.7%)	N/A	N/A
3A (45±59 mL/min/1.73m^2)	N/A	N/A	N/A	4/37 (10.8%)	0/41 (0%)	N/A	N/A
3B (30±44 mL/min/1.73m^2)	N/A	N/A	N/A	1/37 (2.7%)	1/41 (2.4%)	N/A	N/A
4 (>15±29 mL/min/1.73m^2)	N/A	N/A	N/A	1/37 (2.7%)	0/41 (0%)	N/A	N/A
5 (<15 mL/min/1.73m^2 or dialysis)	N/A	N/A	N/A	2/37 (5.4%)	0/41 (0%)	N/A	N/A
CKD stage ≥3	N/A	2/20 (10.0%)	0 (0%)	N/A	N/A	17%	3%
Renal replacement therapy	0 (0%)	0 (0%)	0 (0%)	N/A	N/A	2/59 (3.4%)	3/66 (4.5%)
Pathological albuminuria	2/26 (7.7%)	N/A	N/A	N/A	N/A	N/A	N/A
Microalbuminuria	N/A	11/20 (55.0%)	6/14 (42.9%)	N/A	N/A	N/A	N/A
Proteinuria	N/A	N/A	N/A	12 (32.4%)	3 (6.4%)	N/A	N/A
Kidney biopsy	Lysosomal inclusions on podocytes	Lysosomal inclusions on podocytes		N/A		N/A	
Cerebrovascular events	N/A	N/A	N/A	3 (8.1%)	1 (2.1%)	N/A	N/A
Stroke	0	1/12 (8.3%)	0 (0%)	N/A	N/A	N/A	N/A
TIA	0	N/A	N/A	N/A	N/A	N/A	N/A
Brain WML	3/14 (21.4%)	5/12 (41.7%)	1/7 (14.3%)	19 (51.4%)	13 (27.7%)	N/A	N/A
Other neurological manifestations							
Pain/Acroparesthesias	0 (0%)	4/12 (33%)	2/7 (28.6%)	8 (21.6%)	7 (14.9%)	N/A	N/A
Hypohidrosis	0 (0%)	0 (0%)	0 (0%)	N/A	N/A	N/A	N/A
ORL manifestations							
Hearing impairment	N/A	3/12 (25.0%)	1/7 (14.3%)	N/A	N/A	N/A	N/A
Vertigo	7/26 (26.9%)	N/A	N/A	N/A	N/A	N/A	N/A
Tinnittus	5/26 (19.2%)	N/A	N/A	N/A	N/A	N/A	N/A
GI symptoms	N/A	1/12 (8.3%)	1/7 (14.3%)	N/A	N/A	N/A	N/A
Diarrhoea	4/26 (15.4%)	N/A	N/A	N/A	N/A	N/A	N/A
Cornea *verticillata*	1/26 (3.8%)	2/12 (16.7%)	0 (0%)	N/A	N/A	N/A	N/A
Angiokeratoma	1/26 (3.8%)	0 (0%)	0 (0%	9 (24.3%)	5 (10.6%)	N/A	N/A
MSSI score							
Mild	N/A	N/A	N/A	19 (51.4%)	42 (91.3%)	N/A	N/A
Moderate	N/A	N/A	N/A	17 (46.0%)	4 (8.7%)	N/A	N/A
Severe	N/A	N/A	N/A	1 (2.7%)	-	N/A	N/A
Comorbidities							
Hypertension	6/26 (23.1%)	N/A	N/A	13 (35.1%)	7 (14.9%)	N/A	N/A
Diabetes	0 (0%)	N/A	N/A	5 (13.5%)	3 (6.4%)	N/A	N/A
Obesity	0 (0%)	N/A	N/A	3 (8.1%)	1 (2.1%)	N/A	N/A
Smoking history	N/A	N/A	N/A	15 (40.5%)	8 (17%)	N/A	N/A
Medications							
ARB/ACEI at any time	N/A	N/A	N/A	17 (46%)	6 (12.8%)	N/A	N/A
Beta-blocker at any time	N/A	N/A	N/A	12 (32.4%)	3 (6.4%)	N/A	N/A
Antiplatelet at any time	N/A	N/A	N/A	19 (51.4%)	9 (19.2%)	N/A	N/A
Anticoagulant at any time	N/A	N/A	N/A	4 (10.8%)	0 (0%)	N/A	N/A
Statins at any time	N/A	N/A	N/A	20 (54.1%)	8 (17%)	N/A	N/A

Table 2: Summary of previous studies on the natural history of the late-onset phenotype of FD with predominant cardiac involvement caused by the *GLA* mutation IVS4+919G>A

IVS4+919G>A Mutation	Lin et al., 2010		Lin et al., 2013		Liu et al., 2014		Hsu et al., 2014		Chien et al., 2016		Lee et al., 2016		Lee et al., 2017		Hsu et al., 2017		Auray-Blais et al. 2018		Wang et al, 2020	
Population	FD patients (n=94)		FD patients on ERT (n=21)		FD patients with cardiac symptoms (n=23)		FD patients with LVH who underwent endomyocardial biopsy (n=22)		FD patients with LVH (n=25)		FD patients (n=26)		FD patients (n=25)		FD patient from FOS registry who underwent endomyocardial biopsy (n=30)		FD patients (n=112)		FD patients (n=22)	
Gender	M (n=22)	F (n=72)	M (n=15)	F (n=6)	M (n=16)	F (n=7)	M (n=17)	F (n=5)	M (n=24)	F (n=1)	M (n=20)	F (n=6)	M (n=19)	F (n=6)	M (n=24)	F (n=6)	M (n=25)	F (n=87)	M (n=16)	F (n=6)
Age at diagnosis Mean Median	57.8±6.0 (42-68) N/A	39.1±14.1 (19-82) N/A	52.7 N/A	57.3 N/A	57.8 (18.2-81.2) N/A		N/A 61		58.4±5.8 N/A		59.7±7.5 N/A	57.8±6.5 N/A	57.6±6.9 59 (42-67)		N/A 59.0 (53.5-63.0)	N/A 59.5 (57.0-62.0)	N/A 57.8 (26.3-74.3)	N/A 36.8 (19.6-85.5)	N/A 49.5 (33.8-57.0)	
α-GAL A enzyme activity (mean) Plasma (nmol/h/mL) Leukocytes	1.29±1.39 N/A	6.03±2.42 N/A	1.16 N/A	6.24 N/A	N/A	N/A	N/A	N/A	N/A		N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	
Plasma lyso-Gb3 Mean (nM) Median	N/A N/A	N/A N/A	7.66±3.53 N/A	3.03±0.82 N/A	8.5±3.3 N/A	2.6±1.0 N/A	N/A N/A	N/A N/A	3.5±0.4 N/A		N/A N/A	N/A N/A	N/A N/A	N/A N/A	N/A N/A	N/A N/A	N/A 8.6 (3.5-20.2)	N/A 1.5 (0.2-5.5)	N/A N/A	
Urinary lyso-GB3 Median (pmol/mmol creatinine)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A		N/A	N/A	N/A	N/A	N/A	N/A	Nd (Nd-Nd)	Nd (Nd-67)	N/A	
Age at symptom onset Mean Median	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A		N/A N/A	N/A N/A	50.3±7.8 48 (38-65)		N/A 49.0 (47.0-54.0)	N/A 48.0 (47.0-48.0)	N/A	N/A	N/A	
First signs and symptoms Cardiac Renal GI	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A		N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	
NYHA Class I-II	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A		N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	7/22 (31.8%)	
LVH	14/21 (66.7%)	5/69 (7.2%)	N/A	N/A	N/A	N/A	N/A	N/A	25 (100%)		N/A	N/A	N/A	N/A	N/A	N/A	18/25 (72.0%)	11/87 (12.6%)	19/22 (86.4%)	
Echocardiogram LV mass index (mean) (g/m$^{2.7}$) LV mass index (median) (g/m$^{2.7}$) LV mass index (median) (g/m^2) IVS thickness (mm) PW thickness (mm) LV end-diastolic diameter (mm) LV end-systolic diameter (mm) LV Ejection fraction (%) LV Global Longitudinal Strain (%) LV GLSR (s^{-1})	N/A	N/A	72.8±25.3 N/A N/A 16.1±4.9 12.3±5.7 N/A N/A N/A N/A N/A	57.2±4.8 N/A N/A 12.1±0.9 10.9±2.1 N/A N/A N/A N/A N/A	81.1±32.2 N/A N/A N/A N/A N/A N/A N/A N/A N/A	59.4±12.8 N/A N/A N/A N/A N/A N/A N/A N/A N/A	N/A	N/A	71.5±3.5 N/A N/A N/A N/A N/A N/A 69% N/A N/A		N/A	N/A	N/A	N/A	N/A 65.3 (52.7-93.1) N/A N/A N/A N/A N/A N/A N/A N/A	N/A 53.2 (42.0-55.0) N/A N/A N/A N/A N/A N/A N/A N/A	N/A	N/A	N/A N/A 90.1 (66.9-133.9) 10.0 (8.0-13.25) 10.5 (8.0-13.75) 45.5 (41.25-50.25) 27.5 (25.0-32.0) 68.0 (60.5-73.8) N/A N/A	
Cardiac MRI LV mass (g/m^2) End-systolic volumes (mL/m^2) End-diastolic volumes (mL/m^2) Stroke volume (mL/m^2) Cardiac index (L/min/m^2) LV Ejection fraction (%) LGE	N/A	N/A	N/A	N/A	N/A	N/A	N/A N/A N/A N/A N/A N/A 8/15 (53.3%)	N/A N/A N/A N/A N/A N/A 2/5 (40.0%)	N/A		N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	
Sinus bradycardia	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A		N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	
Bradyarrythmias (2nd degree or complete heart block)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A		N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	
Conduction abnormalities	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A		N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	
Arrhythmias	N/A	N/A	N/A	N/A	N/A	N/A	3/17 (17.6%)	0 (0%)	N/A		N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	
Tachyarrhythmias (VT, AF)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A		N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	
Atrial fibrillation	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A		N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	
VT on Holter	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A		N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	
Cardiac devices Pacemaker ICD Pacemaker/ICD	N/A N/A N/A	N/A N/A N/A	2/15 (13.3%) N/A N/A	0/6 (0%) N/A N/A	N/A N/A N/A	N/A N/A N/A	N/A N/A N/A	N/A N/A N/A	N/A N/A N/A		N/A N/A	N/A N/A	N/A N/A N/A	N/A N/A N/A	N/A N/A N/A	N/A N/A N/A	0/25 (0%) N/A N/A	1/87 (1.1%) N/A N/A	1 (4.5%) N/A N/A	
Median age at pacemaker/ ICD implantation (years)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A		N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	
Age-predicted HR on stress testing	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A		N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	
NT-proBNP (pg/mL)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	703±12.5		N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	42.7 (19.2-156.4)	
Hs-TnT (pg/mL)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A		N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	
Endomyocardial biopsy	N/A		N/A		Myelin bodies in cardiomyocytes		GB3 deposits in cardiomyocytes		N/A		N/A		N/A		GB3 accumulation in cardiomyocytes		N/A		N/A	
GFR (mL/min/1.73m^2)	N/A	N/A	86.3±13.7	79.4±31.7	N/A	N/A	N/A	N/A	N/A		N/A	N/A	N/A	N/A	79.8 (66.1-92.1)	73.2 (55.0-86.5)	N/A	N/A	86.1 (66.4-102.3)	
Serum creatinine (mg/dL)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A		N/A	N/A	N/A	N/A	1.0 (0.9-1.2)	0.8 (0.7-1.1)	N/A	N/A	N/A	
CKD stages 1 (> 90 mL/min/1.73m^2) 2 (60±89 mL/min/1.73m^2) 3A (45±59 mL/min/1.73m^2) 3B (30±44 mL/min/1.73m^2) 4 (>15±29 mL/min/1.73m^2) 5 (<15 mL/min/1.73m^2 or dialysis)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A		N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	
CKD stage ≥3	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	25 (100%)		N/A	N/A	N/A	N/A	N/A	N/A	0	0	N/A	
Renal replacement therapy	N/A	N/A	0/15 (0%)	0/6 (0%)	N/A	N/A	1/17 (5.9%)	0 (0%)	N/A		N/A	N/A	N/A	N/A	N/A	N/A	0	0	N/A	

Pathological albuminuria	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Microalbuminuria	5/20 (25.0%)	12/66 (18.2%)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Proteinuria	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	7/25 (28.0%)	8/87 (9.2%)	3/22 (13.6%)
Kidney biopsy	N/A		N/A		N/A		N/A		N/A	N/A		N/A		N/A		N/A		N/A
Cerebrovascular events	N/A	N/A	1/15 (6.7%)	0/6 (0%)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A		N/A	N/A	N/A	N/A	2/22 (9.1%)
Stroke	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	1/25 (4.0%)		N/A	N/A	0 (0%)	1/87 (1.1%)	N/A
TIA	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A		N/A	N/A	N/A	N/A	N/A
Brain WML	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	7/20 (35.0%)	4/6 (66.6%)	10/25 (40.0%)		N/A	N/A	N/A	N/A	N/A
Other neurological manifestations																		
Pain/Acroparesthesias	N/A	N/A	0/15 (0%)	0/6 (0%)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	3/25 (12.0%)	3/87 (3.4%)	3/22 (13.6%)
Hypohidrosis	N/A	N/A	0/15 (0%)	0/6 (0%)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	1/25 (4.0%)	1/87 (1.1%)	0/22 (0%)
Carpal tunnel syndrome	N/A	N/A	N/A	N/A	N/A	N/A	1/17 (5.9%)	0 (0%)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Depression	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	2/26 (7.7%)		1/25 (4.0%)		N/A	N/A	N/A	N/A	0/22 (0%)
ORL manifestations																		
Hearing impairment	N/A	N/A	0/15 (0%)	0/6 (0%)	N/A	N/A	N/A	N/A	N/A	N/A		N/A		N/A	N/A	N/A	N/A	N/A
Vertigo	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	3/26 (11.5%)		5/25 (20.0%)		N/A	N/A	2/25 (8.0%)	1/87 (1.1%)	1/22 (4.5%)
Tinnitus	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	4/26 (15.4%)		3/25 (12.0%)		N/A	N/A	4/25 (16.0%)	1/87 (1.1%)	1/22 (4.5%)
GI symptoms	N/A	N/A	3/15 (20.0%)	0/6 (0%)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Abdominal pain	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	7/25 (28.0%)	4/87 (4.6%)	0/22 (0%)
Diarrhoea/constipation	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	5/25 (20.0%)	1/87 (1.1%)	0/22 (0%)
Cornea *verticillata*	1/7 (14.3%)	14/45 (31.1%)	1/15 (6.7%)	1/6 (16.7%)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0/25 (0%)	1/87 (1.1%)	0/22 (0%)
Cataract	0/7 (0%)	10/45 (22.2%)	3/15 (20.0%)	2/6 (33.3%)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Retinal vessel tortuosity	2/7 (28.6%)	32/45 (71.1%)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Conjunctival vessel tortuosity	1/7 (14.3%)	7/45 (15.6%)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Angiokeratoma	N/A	N/A	0/15 (0%)	0/6 (0%)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0/25 (0%)	1/87 (1.1%)	0 /22 (0%)
MSSI score			21.3±5.4	18.8±3.6					12.1±2.2									14.5 (9.75-18.0)
Mild	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Moderate			N/A	N/A					N/A									N/A
Severe			N/A	N/A					N/A									N/A
Comorbidities																		
Hypertension							9/17 (52.9%)	4/5 (80.0%)	12 (48.0%)									10/22 (45.5%)
Diabetes	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	2/22 (9.1%)
Obesity							N/A	N/A	N/A									N/A
Smoking history							N/A	N/A	N/A									4/22 (18.2%)
Dyslipidemia							7/17	0 (0%)	N/A									1/22 (4.5%)
Coronary heart disease							3/17	0 (0%)	N/A									N/A
Medications																		
ARB/ACEI at any time									20 (80.0%)									
Beta-blocker at any time	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Antiplatelet at any time									N/A									
Anticoagulant at any time									N/A									
Statins at any time									N/A									

5.2.1 Cardiac involvement

Our research has shown that, in the late-onset phenotype due to the p.F113L mutation, cardiac manifestations carry the highest prognostic impact. The first cardiac manifestations are LVH and LGE, which arise in males over 30 years and are followed by heart failure, non-sustained VT and cardiac conduction disorders, which arise in males over 40 years, culminating with the development of bifascicular block and complete atrioventricular block in males beyond the age of 50 years. Cardiac manifestations are more common and arise one to two decades earlier in males and their frequency increases with age in both genders. Likewise, the severity of LVH and cardiac blocks also increases with age, as demonstrated by the significant increase of LV mass, IVS and PW thickness and QRS interval with age.

5.2.1.1 Left ventricular hypertrophy

This investigation has demonstrated that LVH remains the main cardiac manifestation in the late-onset phenotype of FD due to the p.F113L mutation (40.2%), occurring more commonly in males (73.1%; mean age: 49±15 years) than in females (19.0%; mean age: 44±19 years). LVH arises in males over 30 years and females over 50 years, and its frequency increases with age in both genders, affecting all males beyond 60 years and all females beyond 80 years of age. LVH severity also increases with age, as demonstrated by the significant correlations of LV mass, IVS and PW thickness with age in both genders. Mean age at diagnosis of LVH was 57±10 years in males and 73±8 years in females, reflecting both a late occurrence and a late diagnosis of LVH in this phenotype. In patients with LVH, the frequency and severity of cardiac manifestations is similar in both genders except for a higher prevalence of heart failure in females (75.0% vs. 36.4%).

In our research, LVH has been defined as a maximal LV wall thickness >12mm, as this criterion carries a therapeutic impact, being widely used to establish the presence of LVH and mandate the initiation of specific therapy in Fabry patients. However, the frequency of LVH in other cardiac predominant phenotypes has ranged from 21.1-25.9% in the IVS4+919G>A phenotype [183,185,307] to 29-67% in the p.N215S phenotype [181,182,187], which may be explained by the *GLA* mutation, characteristics of the studied patients, but also different definitions of LVH.

In the phenotype associated to the p.N215S mutation, Lavalle et al. reported LVH (≥50 g/m$^{2.7}$ in males and ≥48 g/m$^{2.7}$ in females) in 46% of the males [median age: 58 (13-74)] and 8.5% of the females [median age: 36 (11-78)] [181]; while, in a registry-based cohort, Germain et al. reported that 88% of the males [mean age: 49.1 (8.7-72.0)] and 49% of the females [mean age: 41.0 (0.5-69.3)] had abnormal IVS thickness (≥11 mm in males and ≥10 mm in females) [187]. Like in the p.F113L phenotype, LVH has

been described to arise as early as the third decade of life in males [187,188] and two decades later in females [187]. In males, mean thickness of IVS and PW were found first mildly abnormal in the 25–34-year age group; and these values also gradually increased with advancing age reaching severely abnormal mean values in the 65–74-year age group [187]. In females, mean thickness of IVS was found first mildly abnormal in the 45–54-year age group, while mean thickness of PW was only mildly abnormal in the 55–64-year age group; however, they remained only mildly abnormal with advancing age [187], contrasting with the p.F113L phenotype, in which LVH severity also increases with age leading to severe LVH in females. Median survival free from LVH was found to be 64 years in males and 72 years in females [181], also reflecting a late occurrence and/or diagnosis of LVH in this phenotype. Nevertheless, all males and females come to develop LVH with advancing age [181].

In the phenotype associated to the IVS4+919G>A mutation, LVH (>259 g in men and >166 g in women) was found in 21.1% of the patients, namely in 66.7% of the males [mean age: 57.8±6.0 (42-68)] and 7.2% of the females [mean age: 39.1±14.1 (19-82)] [183]. In another study, LVH (LVMI >51 g/m$^{2.7}$ in men and LVMI >48 g/m$^{2.7}$ in women) was found in 72.0% of the males [median age: 57.8 (26.3-63.0)] and 12.6% of the females [median age: 36.8 (19.6-85.5)] [185]. Like in the p.F113L phenotype, LVH was reported after 30 years of age [189] and its frequency has also been observed to increase with age, occurring in 4% of males and 2% of females under 40 years of age; 67% of males and 32% of females over 40 years of age; 82% of males and 48% of females over 60 years of age and 100% of males and 63% of females over 70 years of age [307]. However, it is noteworthy that, in the IVS4+919G>A phenotype, the complete penetrance of LVH in males is only achieved one decade later than in the p.F113L phenotype, while in females it is not achieved at all, unlike in the p.F113L and the p.N215S phenotypes [181].

5.2.1.2 Late gadolinium enhancement

Cardiac MRI is a valuable tool for the assessment of LVH and cardiac fibrosis [308]. However, most studies on cardiac predominant phenotypes do not provide cardiac MRI data [181,183-187,190,209-212].

Our study has provided cardiac MRI data on a large cohort of 166 patients with the cardiac predominant phenotype due to the p.F113L mutation. LVH [309] has been detected in 36.1% of the patients, in agreement with echocardiography findings. LGE has been demonstrated in 21.4% of the patients, mostly in males (39.1% vs. 10.6%). LGE was found to arise in males over 30 years and females over 40 years and its frequency to increase with age in both genders, although its penetrance is not complete at least in females and is later than the penetrance of LVH. LV mass on MRI has been identified as a predictor of

LGE. Accordingly, LGE has been detected in 52.5% of the patients with LVH, but in only 4.8% of the patients without LVH, both without gender difference. LGE has been associated with ST depression and negative T waves on the ECG, but still 17.8% of the patients without ST depression and 13.4% without negative T waves presented LGE, arguing against the hypothesis raised by other authors that their absence almost excludes LGE [120].

In the p.N215S phenotype, only two small studies have reported cardiac MRI data, both reporting an apparently higher frequency of LGE compared to the p.F113L phenotype. LGE has been found in 53% of the patients (8/15), namely in 62.5% of males and 42.9% of females [182]; and in 69% of the patients with LVH (18/26) [188]. However, this apparently higher frequency of LGE might be explained by the small size of the samples, low female to male ratio (0.9 in one study and 1.0 in the other) [182,188] and also an apparently higher median age of males (58.6 years) in one of the studies [182]. Nevertheless, as in p.F113L phenotype, LGE in the absence of LVH remained a rare finding (6.7%; 1/15) [182], and LGE was described as early as the third decade of life [188].

In a larger cohort of 100 patients over 35 years of age with the IVS4+919G>A mutation, who were submitted to cardiac MRI, LVH (LVMI >39 g/m$^{2.7}$ in women and >48 g/m$^{2.7}$ in men) was found in only 28% (34.4% of males and 16.7% of females), whereas LGE was found in 38% (51.6% of males and 13.9% of females). This apparently higher frequency of LGE, compared to the p.F113L phenotype, may be explained by the *GLA* mutation and the characteristics of the studied patients, such as the female to male ratio of 0.6 and the age>35 years in all patients (mean age not reported), although it is not possible to ascertain, as the baseline characteristics of the majority of the patients were not reported and an age and gender-stratified analysis was not performed for LGE in this study. Interestingly, in patients without LVH, LGE was apparently more frequent than in the phenotypes associated to the p.F113L and p.N215S mutations, occurring in 38.1% of males (16/42) and 16.7% of females (5/30). Most remarkably, among the 16 males without LVH, there were 11 males aged 60-66 years, who did not present LVH (LV mass 35.2-47.7g/m$^{2.7}$; IVS thickness 8.2-12mm) and in whom only four had cardiac conduction blocks and none had VT, despite their age and the demonstration of lamellar myelin bodies in cardiomyocytes [307]. Moreover, the same study also reported echocardiography results from 524 patients that showed LVH in 78% of males aged 60-69 years [307] and in 100% of males aged 70-79 years [307]. These results led the authors to suggest that cardiac fibrosis progresses in silence before the development of LVH. However, LGE quantification was not performed in the study to understand its magnitude in these patients, T1 and T2 mapping was not performed to understand the potential contribution of fibrosis and inflammation to the documented LGE and the study did not report the presence of fibrosis in the endomyocardial biopsies of these patients.

Moreover, the clinical impact of LGE in the absence of LVH in these patients seemed limited [307]. On the other hand, in patients with LVH, LGE was found in 61%, exclusively in males (77.3%), being surprisingly absent in females, although the characteristics of those six females were also not reported [307].

Taken together, these results suggest a later development of LVH in this phenotype compared to the p.F113L phenotype. It would be interesting to determine the percentage of the aberrant splicing variant in the *GLA* mRNA transcripts to understand if patients with later development of LVH had lower percentage of aberrant transcripts. Moreover, many patients with the IVS4+919G>A mutation do not exhibit clinical manifestations and there are even reports of asymptomatic males at the age of 92 years [310]. Juang *et al.* performed exome sequencing in IVS4+919G>A patients presenting cardiac manifestations and found several GVUS in genes commonly involved in heart diseases. These observations raise the hypothesis that other factors may be determinant for the development of the phenotype, such as other genetic modifiers, comorbidities and environmental factors [310].

On the other hand, further studies are necessary to determine if there is a lower frequency of LGE in the p.F113L phenotype compared to other cardiac predominant phenotypes. Finally, cardiac MRI studies including T1 and T2 mapping are needed to better characterize the natural history of lipid deposition, inflammation and fibrosis in cardiac predominant phenotypes.

5.2.1.3 Heart failure

Studies on the cardiac predominant phenotypes have reported the prevalence of LVH and LGE, but data on the specific prevalence or natural course of heart failure, which is a clinical event associated with LVH and cardiac fibrosis, were very scarce. Therefore, our research came to fill the evidence gap regarding the natural history of heart failure on cardiac predominant phenotypes.

Our research has demonstrated that, in late-onset FD due to the p.F113L mutation, heart failure develops in 21.9% of the patients, more commonly in males; and in 55.0% of the patients with LVH, more commonly in females. Heart failure arises in males over 40 years and females over 50 years of age. By the age of 41-50 years, 28.6% of the males already present heart failure and its frequency increases with age, so that 80% of males over 60 years and nearly 70% of females over 70 years suffer from heart failure. Accordingly, pro-BNP levels also increase with age in both genders. Mean survival free from heart failure is lower in males (64 ± 1 vs. 76 ± 2 years). Most patients are in NYHA class I or II.

5.2.1.4 Cardiac rhythm and conduction disorders

Despite carrying the highest impact on survival, limited data have been reported on dysrhythmias and cardiac conduction disturbances in cardiac predominant phenotypes.

In the p.N215S phenotype, knowledge was mostly limited to generic prevalences of tachydysrhythmias and bradyarrhythmias, but the specific frequency and chronology of each rhythm and conduction disorder has not been reported yet. In a large cohort, arrhythmias (Wolf-Parkinson-White syndrome, supraventricular tachycardia, atrial fibrillation, ventricular fibrillation (VF), non-sustained VT, VT and paroxysmal atrial fibrillation) have been described in 29.7% of males, but not in females [181]. A smaller study reported tachyarrhythmias (sustained and non-sustained VT and atrial fibrillation) in 25.0% of males and 28.6% of females [182]. Atrial fibrillation has been reported in 4% of patients [187]. A small study reported VT in 7.7% of the patients with LVH [188]. On the other hand, conduction abnormalities (long QT, short PR, and conduction blocks) were reported in 29.7% of males and 17% of females [181]. A smaller study reported bradyarrhythmias (second-degree or complete heart block) in 8.3% of males, but not in females [182].

In the phenotype associated to the IVS4+919G>A mutation, knowledge was limited to the frequency of arrhythmias in patients with LVH who underwent endomyocardial biopsy (17.6% of males and 0% in females) [189].

Our investigation provided for the first time the frequency and natural history of each cardiac rhythm and conduction disorder in a cardiac predominant phenotype.

Our study has shown that non-sustained VT occurs in 8.9% of the patients, mainly in males (14.1% vs. 5.6%), arising in males over 40 years and females over 50 years. By the age of 41–50 years, 16.7% of the males already present non-sustained VT and its frequency increases with age, so that one third of the patients over 70 years present non-sustained VT. Atrial fibrillation occurs in 4.4% with a mean age at diagnosis of 70 ± 10 years.

Left anterior fascicular block occurs in 23.0% of the patients, right bundle branch block in 19.6%, bifascicular block in 13.1% and complete atrioventricular block in 5.9%, all more commonly in males. Left anterior fascicular block develops in both genders since the age of 40 years and right bundle branch block in females over 40 years and males over 50 years. Bifascicular block occurs in males over 50 years, but only in females over 70 years. Likewise, complete atrioventricular block occurs in males since the age of 50 years, but only in females over 80 years. The frequency of all cardiac conduction disturbances increases with age in both genders, as well as the duration of the QRS interval. By the age of 51-60 years, 59.1% of the males already present left anterior fascicular block, 57.1% have right bundle branch block,

36.4% have bifascicular block and 14.8% already suffered complete AV block and therefore implanted pacemaker.

Bradydysrhythmias and tachydysrhythmias are more common as disease progresses. Implantable loop recorders revealed clinically relevant dysrhythmias, including four episodes of asystole, seven of bradycardia, five of paroxysmal atrial fibrillation, and five of VT (three sustained and two non-sustained) in 16 patients with advanced cardiomyopathy, with no abnormalities on Holter, followed for a median of 1.2 years [311]. In our studies, patients did not receive implantable loop recorders, so the real prevalence of dysrhythmias may be underestimated.

Our research also provided data on the frequency and chronology of cardiac devices implantation in the cardiac predominant phenotype caused by the p.F113L mutation. Our study has shown that pacemaker implantation occurs in 6.4% of the patients, most commonly in males (12.7% vs. 2.4%), nearly two decades earlier in males [60 ± 7 (49–71) vs. 81 ± 5 (75–84), years] and mainly in the emergency setting of a complete AV block. Median survival free of pacemaker was estimated as 71 years in males and 87 years in females. On the other hand, the frequency of implantation of ICD was lower (1.3% of males and 0.8% of females), all in the setting of primary prevention of SCD due to severe LVH with diffuse LGE and frequent non-sustained VT. In patients with LVH, cardiac device implantation rate is higher, both for pacemaker (14.3%) and ICD (4.1%), without significant differences between genders.

In the phenotype associated to the p.N215S mutation, pacemaker implantation has been reported in a similar frequency as in the p.F113L phenotype (18.9% of males and 2.1% of females) [181]. ICD implantation has been reported in 0% of females, but in an apparently higher frequency of males (16.2%) [181]. Nevertheless, despite a probably more conservative strategy of primary prevention of SCD, no cases of sustained VT, VF or SCD due to VF or VT have been observed in our p.F113L cohort. The same study reported a median age of pacemaker/ICD implantation of 69 years in p.N215S males and the only p.N215S female with a cardiac device in this study had implanted a pacemaker at a younger age of 63 years [181].

In the phenotype associated to the IVS4+919G>A mutation, knowledge was limited to the frequency of pacemaker implantation, which needs, however, further clarification. In a large study, pacemaker implantation has been reported in 1.1% of females, but surprisingly in 0% of males [185]. A smaller study has reported pacemaker implantation in 4.5% of patients [186]. In patients under ERT, pacemaker implantation has been described in 13.3% of males, but not in females [184].

5.2.1.5 Myocardial ischemic events

This investigation has shown that myocardial ischemic events secondary to late-onset FD are rare, as most of them are indeed acute coronary syndromes secondary to atherosclerotic coronary heart disease, which is a common comorbidity with advancing age. Notably, the typical absence of endothelial GB3 deposits in late-onset FD is also consistent with this finding [202].

Some previous studies on late-onset FD reported myocardial ischemic events as Fabry cardiac events [15,187], but no information was given on coronary angiography findings or atherosclerotic risk of the patients. Moreover, percutaneous coronary intervention and coronary artery bypass graft have been considered as cardiac events [15], despite the virtually null probability of representing manifestations of late-onset FD. Our investigation has shown that one should cautiously attribute myocardial ischemic events to late-onset FD and this is a relevant finding that should be accounted in future studies evaluating FD natural history or efficacy of its therapies.

5.2.2 Extracardiac involvement

Our research has shown that, although cardiac involvement carries the highest prognostic impact, extracardiac involvement is common in the late-onset phenotype due to the p.F113L mutation; therefore, the designation of "cardiac variant" underestimates the systemic involvement by FD. Moreover, the earlier manifestations were extracardiac, with increased albuminuria, brain WML and sensorineural deafness arising before 30 years of age in both genders. Nevertheless, their prognostic impact seems low, as the frequency of renal insufficiency is similar to the one in the general population, stroke is rare and hearing aids are seldom needed.

5.2.2.1 Renal manifestations

This investigation has reported the frequency of increased albuminuria in the p.F113L phenotype, taking into account the influence of concomitant medications and comorbidities. Albuminuria A2/A3 was found in 33.7%, mainly in males (52.6% vs. 21.7%). Nevertheless, it should be noted that 23.1% of the patients with normoalbuminuria were under angiotensin-converting-enzyme inhibitors or angiotensin II receptor blockers. Conversely, 51.5% of the patients with albuminuria A2/A3 had concomitant hypertension and 22.7% diabetes, although medically controlled. More importantly, albuminuria A2/A3 was found in 23.8% of the non-hypertensive nondiabetic patients, with a male predominance (39.1% vs. 15.5%), supporting the notion that albuminuria A2/A3 in these patients is secondary to FD. Moreover, our research has shown that albuminuria A2/A3 is an early manifestation, affecting 22.2% of males and 12.9% of females before

the age of 30 years. In males, its frequency increases progressively with age, reaching a peak of 71.4% in the 7th decade, while, in females, its frequency slowly increases until it affects nearly one third of them by the age of 40 years.

In other cardiac predominant phenotypes, different results have been reported, which may be explained by the *GLA* mutation, the sample size of the studies and the definition of pathological albuminuria/proteinuria.

In the p.N215S phenotype, conflicting results regarding the frequency of increased albuminuria have been reported in two small studies. Microalbuminuria (used definition not reported) has been detected in 50% of the patients (55.0% of males and 42.9% of females) in one study [182], whereas pathological albuminuria (urinary albumin-to-creatinine ratio ≥30 mg/g creatinine) has been reported in 7.7% of the patients with LVH in another study [188]. In a larger study, proteinuria (>150mg/24h) has been found in 32.4% of males and 6.4% of females [181]. Like in the p.F113L phenotype, proteinuria has been detected as early as the second decade of life and its frequency has been found to increase with age in both genders and to stabilize in middle-aged women [181].

In the IVS4+919G>A phenotype, microalbuminuria (urinary albumin-to-creatinine ratio ≥2.0 mg/mmol for men and ≥2.8 mg/mmol for women on at least two occasions) was found in 20% of the patients (25% of males and 18% of females). Like in the p.F113L phenotype, microalbuminuria occurred before 40 years of age and there was no significant difference on the frequency of microalbuminuria in females under and over the age of 40 years [183]. Proteinuria (albumin/creatinine ratio >30 mg/g creatinine) was described in 28.0% of males and 9.2% of females in a larger study [185].

This research has also demonstrated lysosomal inclusions in renal podocytes of young patients presenting with increased albuminuria, without any known morbidities besides late-onset FD due to the p.F113L mutation, therefore supporting the hypothesis that increased albuminuria in these patients is indeed caused by FD. Likewise, lysosomal inclusions have also been found in renal podocytes of p.N215S patients [182,188].

However, despite the common pathological albuminuria/proteinuria, our investigation has shown that the frequency of renal insufficiency in patients with the p.F113L mutation is similar to the one in the general population. In p.F113L patients, chronic kidney disease (CKD) stage ≥G3 (eGFR <60 mL/min/1.73m^2) was rarely observed (6.6%), without gender dominance (9.3% in males vs. 4.9% in females); CKD G5 was only observed in 0.5%; and there were no cases of renal replacement therapy. Similarly, CKD stage ≥G3 has been described in 6.1% of the Portuguese adults aged 20-79 years [312]; and, in a more recent study,

CKD stage ≥G3 has been reported in 10.6% of the Portuguese adults aged 18-89 years and CKD stage G5 in 0.08% of them [313].

In the p.N215S phenotype, although CKD is also not frequent, it seems to be more common and carry a higher impact than in the p.F113L phenotype. Although mean eGFR has been reported to remain >60 ml/min/1.73 m^2 in males and females of all age groups, except in males aged 65–74 years [mean eGFR 54.1 (21.5) ml/min/1.73 m^2] [187], CKD stage ≥G3 has been reported to affect 10-22% of males and 0-3% of females [181,182,187]. End-stage renal failure has also been reported as rare, but ranging from 0% in smaller series [182,194] to 2.4% of patients (5.4% of males and 0% of females) [181] and 4.0% of patients (3.4% of males and 4.5% of females) in larger series [187]. Moreover, unlike the p.F113L phenotype, severe CKD has been reported at young age [181,187,314-316]. In a large cohort of 125 patients with the p.N215S mutation, one female had a renal event (dialysis, age 45 years) as a first severe event; and four additional patients experienced a renal event during their natural history follow-up before the age of 65 years (i.e., kidney transplant in one 54-year-old male; dialysis in one 39-year-old male, followed by kidney transplant at the age of 43 years; and dialysis in two females aged 51 and 63 years) [187]. In another large cohort of 84 patients, end-stage renal failure has been described in two young p.N215S males (25 and 38 years old) (2.4%) with marked persistent proteinuria [181].

In the IVS4+919G>A phenotype, although evidence on CKD is more limited, it suggests a lower impact than in the p.N215S phenotype. In a small study of 22 patients, median eGFR was reported to be 86.1 (66.4-102.3) mL/min/1.73m^2 [186]. In 21 patients under ERT, mean eGFR at baseline was 86.3±13.7 and 79.4±31.7mL/min/1.73m^2 in males and females, respectively, and no patients were under renal replacement therapy [184].

5.2.2.2 Neurologic manifestations

Our research has shown that brain WML are common manifestations (50.3%) in the p.F113L phenotype, both in males (53.8%) and females (48.0%). Brain WML arise early, affecting 11.1% of males and 26.9% of females under 30 years of age, and increase progressively thereafter, becoming a universal finding in males and females above 70 years of age. They were found in patients with cardiovascular risk factors, despite medically controlled, but also in 38.4% of the patients without these risk factors. Moreover, our investigation demonstrated the presence of brain WML in young patients without comorbidities, which supports the notion that they are indeed caused by FD and not by ageing or other conditions.

Brain WML have also been frequently found in other cardiac predominant phenotypes. In a small cohort of IVS4+919G>A patients, brain WML were found in 35.0% of males and 66.6% of females [211]. In a

large cohort of p.N215S patients, brain WML were found in 51.4% of males and 27.7% of females, and, like in the p.F113L phenotype, they appeared before 40 years of age, increased progressively thereafter and affected most elderly patients [181].

In the general population, brain WML have been associated with cerebrovascular risk factors and ageing [317,318]. A previous study reported the following age-related prevalences of brain WML in the general population: 3.5% (<40 years), 22% (41–50 years), 47% (51–60 years), 64% (61–70 years), 84% (71–80 years) and 95% (>81 years) [317]. Although our studies did not include a comparison between Fabry patients and age-matched controls to account for the effect of age on the development of brain WML, there seems to be a higher prevalence of brain WML in young patients with the p.F113L phenotype compared to the general population, which further supports the hypothesis that brain WML are caused by FD and not by ageing.

Nevertheless, the clinical significance of brain WML, namely in terms of cognitive function and neuropsychiatric manifestations, remains to be elucidated [319]. Although neuropsychiatric manifestations, such as depression and anxiety disorder, were also common in the p.F113L phenotype, it remains to be established to what extent these are due to FD pathogenesis or just a reaction to the disease burden [319].

Despite the high frequency of brain WML, our investigation has shown that stroke was rare (3.0%) in both males (3.8%) and females (2.4%) with the p.F113L mutation. Moreover, all the stroke events occurred in the setting of cardiovascular risk factors and one of them in the setting of atrial fibrillation.

Likewise, a low frequency of cerebrovascular events has been reported in other cardiac predominant phenotypes. In the p.N215S phenotype, cerebrovascular events have been described in 8.1% of males and 2.1% of females [181]. In the IVS4+919G>A phenotype, a small study reported cerebrovascular events in 9.1% of the patients [186]. In a small cohort, stroke was reported in 4.0% of the IVS4+919G>A patients, but haemorrhages were not reported [210]. In a larger study, stroke occurred in 1.1% of females, but not in males [185].

The pathophysiology of central nervous system manifestations is poorly understood in FD. GB3 deposits within the endothelial and smooth muscle cells of the small and medium-size brain arteries may decrease vascular compliance and impair autoregulation of cerebral perfusion and lead to endothelial dysfunction, increase of pro-thrombotic/pro-inflammatory cytokines, and upregulation of the renin–angiotensin system, which may result in ischemic events [320]. In this regard, however, it is noteworthy that late-onset phenotypes of FD are typically characterized by the absence of endothelial GB3 deposits [202]. On the other hand, GB3 deposits were also described in brain neurons and glia [134,321] and glial dysfunction

and neuroinflammation may also contribute to the development of brain WML [320]. Finally, stroke and transient ischemic attack may also result from cardioembolism [320].

Our research has shown that acroparesthesias occur in 23.3% of the patients with the p.F113L mutation, mostly in females (34.2% vs. 6.4%), and are predominantly mild. Moreover, our investigation was the first to report the prevalence of carpal tunnel syndrome in a late-onset phenotype (22.3%) and to show that acroparesthesias in a cardiac predominant phenotype were attributed to carpal tunnel syndrome in nearly one third of the cases (34.6%). As endothelial GB3 storage is characteristically absent in late-onset phenotypes of FD [202], carpal tunnel syndrome may be caused by GB3 storage on fibroblasts and subsequent increase of extracellular matrix within the carpal tunnel [322]. Repetitive wrist movement related to the high prevalence of factory professionals in our cohort may also have caused or aggravated a FD-related carpal tunnel syndrome. The acroparesthesias of the patients without carpal tunnel syndrome were predominantly mild and non-disabling, unlike the ones typically seen in the small-fiber neuropathy of classical phenotypes, and, given the unspecific nature of acroparesthesias and the high prevalence of factory professionals in our cohort, are probably not related to FD.

In contrast to the p.F113L phenotype, the frequency of acroparesthesias in other cardiac predominant phenotypes is higher in males instead of females, which may be explained by the high frequency of factory workers in our female cohort. In the p.N215S phenotype, acroparesthesias were reported in 21.6% of males and 14.9% of females [181], while, in the IVS4+919G>A phenotype, acroparesthesias were described in 12.0% of males and 3.4% of females [185]. However, no data was reported on the prevalence of carpal tunnel syndrome or the characteristics of these acroparesthesias [181,185].

5.2.2.3 Otorhinolaryngological manifestations

Our investigation has shown that sensorineural deafness is common in p.F113L patients (44.7%), particularly in males (62.2% vs.32.4%). It arises early, affecting 20.0% of males and 3.6% of females under 30 years, and its frequency increases with age in both genders, affecting all elderly individuals. However, its impact seems low, as hearing aids are seldom indicated.

To our knowledge, our study was the first to describe the natural history of sensorineural deafness in a cardiac predominant phenotype. In other cardiac predominant phenotypes, data on hearing impairment was limited to its frequency in small size studies. In the p.N215S phenotype, a study including 19 patients reported hearing impairment in 25.0% of males and 14.3% of females [182], while, in the IVS4+919G>A phenotype, hearing impairment was not reported at baseline in a study including 21 patients who underwent ERT [184]. However, the low frequency of hearing impairment in these studies may be biased

by their small sample size. Conversely, the high frequency of sensorineural deafness in the p.F113L phenotype highlights the importance of a systematic otorhinolaryngological evaluation in cardiac predominant phenotypes. However, it should be noted that our research did not include a comparison between Fabry patients and age-matched controls to account for the effect of age on the development of sensorineural deafness.

5.2.2.4 Other clinical manifestations

This investigation has shown that cornea *verticillata* is uncommon in the p.F113L phenotype. Still, it is detected in 10.3% of the patients, both in males and females (15.5% vs. 7.1%). Our study identifies its first detection in patients over 40 years (mean age 60±8 years), much later than in classic patients [323,324], and its frequency is observed to increase with age in both genders. Nevertheless, this is a milder form of cornea *verticillata* compared to the one that is typically observed in the classic patients.

In the p.N215S phenotype, data is limited to small size studies. In a cohort of 19 patients, cornea *verticillata* has been detected in a similar frequency in males (16.7%), but not in females [182], which may be explained by the small sample size. In the IVS4+919G>A phenotype, large series have reported conflicting results regarding the frequency of cornea *verticillata*. In a study of 112 adult patients, cornea *verticillata* has been reported in 0% of males and 1.1% of females (median age: 57.8 years in males and 36.8 years in females) [185], while in another cohort of 94 adult patients it has been detected in 14.3% of males and 31.1% of females (mean age: 57.8±6.0 vs. 39.1±14.1) [183].

Our investigation has also shown that angiokeratomas are extremely rare (2.1%) and solitary in patients with the p.F113L mutation, being uncertain if they are related to FD. Likewise, in a large cohort of patients with the IVS4+919G>A mutation, angiokeratomas have been found to be extremely rare (0% of males and 1.1% of females) [185]. However, in the p.N215S phenotype, the frequency of angiokeratomas has widely ranged from 0% in small series [182] to 24.3% of males and 10.6% of females in large series [181].

5.2.3 Disease biomarkers

Only 25% of the plasma glycosphingolipids pool is newly synthesized each day in the liver and a portion is derived from the turnover of senescent erythrocytes. Moreover, the rate of exchange of plasma glycosphingolipids with those found in plasma membranes or stored in disease cells has not been determined. Conversely, nearly 75% of the urinary cells shed by an affected individual are derived from desquamated lipid-laden distal tubular epithelial cells, which suggests that changes in urinary GB3 may

be a more sensitive and specific measure of tissue and body GB3 burden [179]. Nevertheless, plasma and urinary GB3 may be normal in females and non-classic males [42,43] and changes in plasma and urine GB3 levels do not predict progression of eGFR and LV mass index over 1 year of therapy with agalsidase alfa [325], suggesting that they are not suitable markers for monitoring the clinical response to FD therapies.

While lyso-GB3 accumulates to a lesser extent compared to GB3, its usefulness has been demonstrated as a biomarker for disease screening [326,327], determination of pathogenicity of a mutation [328], prediction of clinically relevant disease or events [328,329] and monitoring of therapeutic efficacy [328,330]. Furthermore, lysoGB3 values relate to FD phenotype. All men and women with a classical phenotype and men with a non-classical phenotype had higher plasma lysoGB3 values than controls (ie, there was no overlap), while lysoGB3 values of non-classical females showed some overlap with control values [44].

This research has demonstrated that plasma lyso-GB3 values in the p.F113L phenotype are in agreement with the values reported in the late-onset phenotypes [44], including the cardiac predominant phenotypes due to the p.N215S [181] or the IVS4 mutation [185]. Plasma lyso-GB3 is always increased in males and below the lower limit of quantification in females, independently of the development of clinical manifestations, being therefore a poor biomarker for predicting the risk of complications in females with the p.F113L mutation.

Similarly, in other cardiac predominant phenotypes, normal plasma lyso-GB3 values have been reported in females. Although plasma lyso-GB3 is higher in p.N215S males and females than in healthy males and females, respectively, an overlap has been observed between the values in the p.N215S females and the healthy females [182]. In the IVS4+919G>A phenotype, increased plasma lyso-GB3 has been reported in all adult males but in only 18% of females [185]. However, unlike in the p.F113L phenotype, another study has suggested that plasma lyso-GB3 values may be different according to symptomatic status. In this study, plasma lyso-GB3 was higher in symptomatic than in asymptomatic patients, both in males and females, although there was a considerable overlap between the groups. Moreover, plasma lyso-GB3 was increased in all adult males and symptomatic females, being clearly distinguishable from male and female controls. In asymptomatic females, although it was reported to be higher than in controls, it was within normal range in 19% of them [331].

In summary, our research on the natural history of the p.F113L phenotype found many similarities to the natural history of other predominant cardiac phenotypes, suggesting a general common natural course to these FD variants, despite the different *GLA* gene mutations. Nevertheless, this research came to fill many evidence gaps on the natural history of these FD phenotypes, namely on heart failure, cardiac rhythm and conduction disturbances, ischemic myocardial events, sensorineural deafness and carpal tunnel syndrome. In addition, it also pointed out some differences between the phenotypes associated to these mutations, such as a later development of LVH in the IVS4+919G>A phenotype and a higher frequency and prognostic impact of CKD in the p.N215S phenotype.

Therefore, this investigation improved the knowledge on the natural history of late-onset FD, which is essential to make evidence-based clinical decisions and guidelines and to accurately evaluate the clinical efficacy of FD therapies.

5.3 Fabry disease prevalence and predictors in Hypertrophic cardiomyopathy

5.3.1 Fabry disease prevalence in Hypertrophic cardiomyopathy

Our research has revealed a FD prevalence of 0.9% in patients with HCM, based on a multicentre screening study, which had the advantages of including a large cohort of 780 patients, both males and females, with well-defined diagnostic criteria of HCM according to the ESC guidelines and using a combined enzymatic and genetic testing that ensures accurate FD diagnosis in both genders. Furthermore, solid clinical, biochemical and histological evidence supported the pathogenicity of the *GLA* variants that accounted for FD prevalence calculation in this study.

In previous studies, inclusion criteria widely ranged (i) from extremely restrictive (patients submitted to myectomy [162] or endomyocardial biopsy [163]); (ii) to extremely broad (LVH ≥12 mm [174], ≥13 mm [161,164,170,171], >13 mm [165] or ≥13 mm without exclusion of hypertension and valve disease [160,172]); and (iii) from more elaborated, combining LV thickness with several factors (no obstruction [173]), negative genetic test for sarcomeric HCM [164,167,176] or exclusion of family history of sudden death in >2 relatives, inheritance pattern inconsistent with X-linked pattern and histology compatible with sarcomeric HCM [176]; (iv) to more straightforward (ESC definition of HCM [166,168,169,175].

Our study inclusion criteria consisted on the ESC definition of HCM and therefore the characteristics of our HCM cohort were similar to the literature data on HCM [280,285]. Accordingly, patients were mainly males, HCM was mainly asymmetrical, diastolic dysfunction was common, LV ejection fraction was

preserved, LGE occurred in nearly 60% of the cases, heart failure was common, syncope occurred in 12.6% and a pacemaker was implanted in nearly 8% and an ICD in 9% of the cases. Of note, the mean age of our cohort was 66±14 years, which might be explained by the inclusion of patients followed for a long time since the diagnosis of HCM in whom FD had never been excluded and/or patients diagnosed with HCM at an older age, due to either late penetrance, late diagnosis or underdiagnosed phenocopies (e.g., wild-type transthyretin amyloidosis). Accordingly, arterial hypertension, although not considered to be the cause of LVH, was a common comorbidity, reflecting its high prevalence in the general population, particularly in the older age groups. The patients diagnosed with HCM at an older age have been increasingly recognized as a subset of HCM patients with higher probability of negative genetic testing/family history of HCM and lower risk of adverse events [332,333], which might explain the apparently lower frequencies of family history of HCM (22.1% vs. 33%) and positive genetic testing (24.9% vs. 40%) compared to the Portuguese Registry of HCM [285], and LVOT obstruction at rest (23.8% vs. 33%), atrial fibrillation (11.7% vs. 22.5%) and VT (15% vs. 25%) compared to the literature [280]. Still, it is noteworthy that a significant number of patients in our cohort was genetically tested for HCM (63.7%) and the genes more frequently mutated were the same that have been reported in the literature (*MYBPC3*, *MYH7*, *TNNT2*, *TPM1*) [285,334].

Screening methods also widely ranged in previous studies on FD prevalence in HCM. Some included only males and used enzymatic assay as the screening method [160,161,170,171,175]. One study included only females and used electron microscopy of endomyocardial biopsies as the screening method [163]. Other studies included both genders and used urinary GB3 [174], electron microscopy of myectomy specimens [162], the enzymatic assay alone [168], the genetic testing [164,165,167,169] or a combined enzymatic and genetic approach [166,172,173,176].

Our study used a combined enzymatic and genetic testing, which allows the most accurate FD diagnosis in both genders [335], since it helps to eliminate GVUS that are found to be associated with normal enzymatic activity in males and it avoids missed FD diagnoses in females by applying genetic testing to all females including the ones with normal enzymatic activity.

Screening of high-risk groups, such as LVH or HCM, frequently leads to the finding of GVUS, which, if not appropriately integrated in the clinical context, may result in an erroneous diagnosis of FD and overestimation of FD prevalence. In patients with LVH and GVUS, a definite diagnosis of FD has been consensually defined as ≤5% of the mean reference value of enzymatic activity of α-galactosidase A in leucocytes (males only) with at least one characteristic FD symptom/sign (neuropathic pain, cornea *verticillata*, angiokeratoma) or increased plasma lyso-GB3 (classical male range) or family members with

definite FD. Subjects with LVH failing these criteria were considered to have a GVUS and an uncertain diagnosis. The gold standard for FD diagnosis has been defined as characteristic storage in an endomyocardial biopsy on electron microscopy. Abnormally low voltages on ECG and severe LVH (wall thickness >15 mm) before the age of 20 years were considered to exclude FD [278].

Based on this consensus, in addition to the patients with the *GLA* variants previously identified in the FD screening in the Portuguese region of Guimarães (p.C94S, p.F113L, p.G183V, p.E203X, p.M290I), the patients with the *GLA* variants p.M96V and p.R356Q/p.G360R were also classified as Fabry patients and included for FD prevalence calculation.

The novel mutation c.286A>G (p.M96V) was found during the course of this investigation and its pathogenicity and association to a classical phenotype was based on the biochemical and clinical findings of the proband and her son. Indeed, our 56-year-old female patient presented reduced enzymatic activity of α-galactosidase A on plasma and leukocytes, increased plasma lyso-GB3 in the expected range for a classic female and clinical findings specific of a classical phenotype, such as cornea *verticillata*, besides other manifestations compatible with FD, such as severe HCM, short PR interval, atrial fibrillation, microalbuminuria and deafness. Her 19-year-old son presented increased FD biomarkers and several manifestations specific of a classical phenotype, such as cornea *verticillata*, angiokeratomas, acroparesthesias, hypohidrosis, abdominal pain and diarrhoea, besides proteinuria and renal failure. Moreover, the pathogenicity of this mutation was also supported by the demonstration of lysosomal inclusions on skin biopsy. Of note, further study has also revealed near-null enzymatic activity (0.68pmol/h; normal range: 8.75-15.6) in this male patient as well as increased plasma lyso-GB3 in the classic male range (38.5ng/mL before ERT and 10.82ng/mL after starting ERT) and lysosomal inclusions in kidney biopsy, further supporting the pathogenicity of this mutation and its association to the classical phenotype.

The patient carrying the *GLA* variants p.R356Q/p.G360R has been classified as a Fabry patient based on the histological evidence of lysosomal inclusions in skin biopsy and the biochemical and clinical findings compatible with a classical phenotype of FD [336]. Indeed, our 63-year-old male patient presented near-null/null enzymatic activity on plasma and leukocytes, increased plasma lyso-GB3 in the classic male range and several manifestations specific of FD, such as cornea *verticillata*, angiokeratomas, acroparesthesias, hypohidrosis, abdominal pain and diarrhoea, along with other clinical manifestations compatible with FD, such as severe HCM, myocardial infarct, stroke, brain WML and deafness. Moreover, his sister, who carried the same *GLA* variants, also presented clinical manifestations compatible with FD

(HCM and microalbuminuria), whereas the absence of manifestations in his daughter with the same variants may occur in young Fabry females.

The variant c.1078G>C (p.G360R) had been previously described as a GVUS [337]. However, in-silico methods consistently predict a damaging effect on protein function (MutationTaster, Polyphen-2, SIFT, Provean) [290,291,338,339]. Moreover, mutations in the same amino acid of α-galactosidase A have already been reported in association with a classical phenotype of FD (p.G360C [340,341,342] and p.G360S [343]) or clinical manifestations compatible with the disease, such as end-stage renal disease (p.G360D) [344]. Indeed, this amino acid has been reported to be in the dimmer interface of α-galactosidase A, which supports the pathological nature of these mutations [343]. Therefore, this variant was classified as pathogenic and associated to the classical phenotype exhibited by the patient.

Conversely, the variant c.1067G>A (p.R356Q) was considered as GVUS possibly benign. This variant occurs at the end of a beta strand of the protein [288], where the arginine residue is weakly conserved, existing a small physicochemical difference between arginine and glutamine [345]. This variant has been found in 1.1e-05 in 183292 control chromosomes (gnomAD) [346]. It was first described in a newborn screening in Taiwan in two males with α-galactosidase A activities between 5 and 30% of mean normal leukocyte activity [34,342]. The mutant enzyme has also been reported to exhibit a residual activity in the COS-1 cells (15% of expressed mean wild-type activity) [34]. In addition, the "in vitro" enzymatic activity of the mutant α-galactosidase A in HEK-293 cells has been reported as 36.1±1.5% of the enzymatic activity of the wild-type enzyme [289]. Moreover, Liao et al. found that, although this variant was not detected in > 1000 X-chromosomes from normal Taiwanese individuals, it was associated with an enzyme activity on a dried blood spot test > 10% of the normal mean enzyme activity (12.9 ± 1.5%) in a male patient, an "in vitro" enzyme activity of 82.3 ± 4.4% compared to wild-type enzyme, normal plasma lyso-GB3 levels (< 2.6 nM) in eight males (1.5 ± 0.9; 0.1–2.4) and seven females (1.2 ± 0.6; 0.1–1.9), and no obvious clinical manifestations of FD in those individuals [336,340]. Although it has been recently reported in association with a classic phenotype of FD, data on enzyme activity, biomarker levels, and clinical manifestations were not specified in this report and the concomitant presence of a large deletion/insertion or an intronic variant could not be completely excluded, as *GLA* gene analysis only included the seven exons, the regulatory sequences flanking them, and the cryptic exon [347]. Finally, most in-silico models predict this variant to be benign or tolerated (Polyphen2: benign; Provean: neutral; SIFT: tolerated; and Mutation-Taster: disease causing) [290,291,338,339]. Taken together, this *GLA* variant was considered a GVUS, probably benign, and the classic phenotype of FD exhibited by our patient was considered to be most probably due to the p.G360R mutation.

On the other hand, the screening study has also found other *GLA* variants, whose pathogenicity came to be excluded or remain uncertain and which have not been accounted for the FD prevalence calculation and have been excluded from the statistical analysis related to the identification of FD predictors in HCM (p.V22A/p.A73S, p.R118C, p.A143T, p.D175E, p.D313Y).

To the best of our knowledge, the variants p.V22A and p.A73S had never been reported before. The variant p.V22A is predicted to be benign by in-silico models (Polyphen-2, Provean) [291,339]. The variant p.A73S occurs in a position of a helix in the protein and in-silico models predict conflicting results regarding its impact on the protein function, with Polyphen-2 predicting it as probably damaging and Provean predicting it as neutral (Polyphen-2, Provean) [291,339]. Although other variants have been reported in the same amino acid (p.A73V, p.A73E) [27,348], the finding of a normal enzymatic activity of α-galactosidase A on plasma and leukocytes in a male relative of our patient (brother) carrying the p.A73S variant excluded this variant as pathogenic. Furthermore, our patient carrying the p.V22A/p.A73S variants was a 61-year-old female with normal enzymatic activity of α-galactosidase A on plasma and only slightly decreased on leukocytes, no other manifestations compatible with FD, besides cardiac manifestations related to HCM and acroparesthesias, and no available biomarkers, as the patient refused further diagnostic testing. Finally, a pathogenic sarcomeric mutation in the *MYBPC3* gene (p.R502W) [349] has been found in our patient and her mother and hence considered the cause of HCM in these patients.

The variant c.352C>T (p.R118C) occurs at a CpG dinucleotide, a mutational hotspot, and results in the replacement of the weakly conserved basic amino acid arginine by the neutral sulphydryl-containing cysteine residue [281,350] in a position of a helix in the protein [288]. This variant was first described in a newborn screening and considered pathogenic based on the fact that it can be accommodated within the crystal structure of the enzyme, it affects a similar number of atoms in their main and side chains as known late-onset mutations and it is associated to an “in vitro” enzymatic activity in COS-7 cells of 29% of the activity of the wild-type enzyme [27]. Moreover, it was hypothesized that the additional thiol could interfere with the correct formation of the five disulfide bonds critical to the enzyme structure [350]. However, in-silico models predict conflicting results regarding its impact on the protein function (Polyphen-2: damaging; MutationTaster: polymorphism) [290,291]. Furthermore, enzymatic activity on plasma has been reported as 58% of the normal mean in males and 67% in females and on leukocytes as 32% in males and 58% in females [351]; and in some males it has been reported as normal both in plasma and leukocytes [350,352,353]. On the other hand, this variant has been found in a male newborn with <5% of residual enzymatic activity on plasma, although genetic analysis was limited to PCR sequencing of the exons and flanking intronic regions and large deletions/duplications have not been formally excluded [27].

Homozygous females have also been described and found to present an enzymatic activity of 25-33% of the normal average on dried blood spot test [350]. Plasma lyso-GB3 has also been reported as normal both in males and females [287,350,354]. Moreover, this variant has been found in screenings of high-risk groups, such as cardiac disease [353], HCM [169], haemodialysis [281,355], stroke [351,354,356], small fibre and mixed neuropathy [352] and newborn screenings [27,357]. However, it does not seem to segregate with FD clinical phenotypes in a highly Mendelian fashion [350]. In addition, a 69-year-old male with this variant has been described as presenting normal enzymatic activity and plasma lyso-GB3 and no LVH and no other clinical manifestations, besides mild neuropathy [352]. Another 56-year-old male has been described with normal enzymatic activity on plasma and leukocytes, normal plasma lyso-GB3 and only mild LVH and right bundle branch block. Endomyocardial biopsy revealed no hypertrophy or vacuolization of cardiomyocytes and no interstitial fibrosis and only small myelin figures were found within rare cardiomyocytes. Moreover, no progression of cardiac or renal disease was documented over 10 years of follow-up, although brain WML have been detected at the age of 61 years [350]. Finally, this variant has been found in 2.3e-04 among 205229 X-chromosomes (gnomAD) [358]. Despite apparently more common on Iberian cohorts, haplotype analysis did not support the hypothesis of a founder effect; instead, it seems to be a relatively frequent mutational event, in accordance with its location at a CpG dinucleotide [350]. Taken together, this variant seems to be non-pathogenic, or mildly pathogenic at most. The higher allelic frequency in stroke patients than in the general population (0.0087) [351] and the predominant association of the variant with stroke in the posterior circulation [351] suggest that it could be a modulator of the multifactorial risk of cerebrovascular disease [350].

Our study found three patients with the variant p.R118C (3/780; 0.0038). However, alternative causes of LVH could not be fully excluded. In our 73-year-old male with asymmetrical obstructive HCM with LGE in the septum, it is reasonable to hypothesize that it could be a sarcomeric HCM, given the pattern of LGE and LVH which is more typical of a sarcomeric cause than FD, the presence of obstruction which is rare in FD and the fact that the genetic testing, besides revealing a GVUS in the *MYBPC3* gene, included only 11 HCM genes. In our 69-year-old female with apical HCM, although genetic testing was not performed, a sarcomeric cause is also possible, as apical HCM is rare in FD. In the 75-year-old female with moderate symmetrical non-obstructive HCM with no LGE, besides the GVUS on the *MYH7* gene, we should also consider the possibility of isolated basal septal hypertrophy of the elderly. Therefore, based on the unclear role of this *GLA* variant and the possibility of alternative causes of HCM, these patients were excluded from the FD prevalence calculation.

The variant c.427G>A (p.A143T) also occurs at a CpG dinucleotide, a known mutational hotspot [293], and its pathogenicity has also been questioned. It consists of a conservative mutation of alanine to threonine, which results in no change in charge of the residues, but in a slightly bulkier side chain [359]. This variant occurs on the surface of protein's functional domain and is close to Cys142 that forms a disulfide bridge with Cys172. As a result of this variant, formation of Cys142-Cys172 disulfide bond results in local misconformation of the protein. Moreover, the loss of Cys142-Cys172 disulfide bond may also affect the accessibility of nucleophilic residue, Asp170, in the active site. In addition, difference in physicochemical properties between wild-type and mutated residue may cause loss of interactions with the ligand, alpha-D-galactose, thereby affecting the function of the enzyme [360]. In-silico methods also predict a damaging effect on protein function (MutationTaster, Polyphen-2) [290,291]. Nevertheless, the variant p.A143T was shown to be localized in lysosomes [293] and to express ~35% of the enzymatic activity of the wild-type enzyme in COS-7 cells [27,293].

This variant was first described in a 2-month-old male infant with low enzymatic activity and no family history of FD [192]. Now evidence shows that the original Fabry patient described by Anderson in 1898 with a classical phenotype had the p.A143T variant, but his relatives showed a substantial variation in phenotypic expression of the disease throughout multiple generations [360].

This variant is a common finding in screenings of high-risk populations, such as kidney disease [361-364], LVH or HCM [166,169,172,365], stroke [366-368], and neonatal screenings [27,31,205,357]. It has been found in 5.1e-04 in 205433 X-chromosomes and its frequency in European population is estimated as 0.09% (gnomAD) [369] and its frequency in high-risk populations as 0.42% [370]. Microsatellite markers analysis revealed that different patients found with this variant in the same screening were not related, supporting the hypothesis that this was a common mutational event, in accordance to the location at a CpG dinucleotide [27].

Classical signs and symptoms of FD, such as angiokeratomas, hypohidrosis, acroparestesias and cornea *verticillata*, are frequently absent [370]. Still, angiokeratomas [169,360,365,371] and cornea *verticillata* [362,365] have been reported in some patients.

Patients with this variant commonly present high residual enzymatic activity; and in males the enzymatic activity has been reported to widely range between 25 and 72% [372]. However, enzymatic activity below 25% has also been reported [27,172,366,368,373,374,375]. Conversely, a male has been described with an enzymatic activity that was normal in plasma and mildly low in leukocytes [376].

According to the threshold theory [377], only the decrease of enzyme activity below the critical threshold value causes storage, and in sphingolipidosis a decrease of enzyme activity down to values of 20 % of

normal has been considered as having no impact on the turnover rate. Accordingly, plasma lyso-GB3 has been found normal in 77.8% of males and 85.7% of females [372]; however, it has also been found to be increased in some patients [360,372].

Some authors argue that this variant is non-pathogenic and its association to clinical manifestations may be coincidental, as patients are frequently found in screenings of high-risk groups [370,372,375] and frequently do not present other FD clinical manifestations [370,372]. Plasma lyso-GB3 is normal in most males and females [287,368,372,375,376]. Moreover, in some cases, tissue biopsies have not shown lysosomal inclusions [370,375] and alternative diagnoses have been found, such as sarcomeric HCM [375], AL amyloidosis [370] and vascular kidney disease [363]. Recently, Valtola et al. described one female and two male relatives with LVH, who carried this variant and in whom other pathogenic/likely pathogenic variants have been excluded in 59 cardiomyopathy-related genes. All the patients presented characteristic features of Fabry cardiomyopathy. The female had positive FDG-signal in Positron Emission Tomography-Computed Tomography (PET/CT) in the basal inferolateral wall; one male had LGE, high T1 value and positive FDG-signal in PET/CT in the basal inferolateral wall; and one male had low T1 value in the basal inferolateral wall. Endomyocardial biopsy showed lysosomal inclusions in cardiomyocytes in both males and positive staining for anti-GB3 antibody was available in one of the males. Taken together with the current evidence, the authors concluded that this is a late-onset disease-causing variant with incomplete penetrance [360]. The –10C>T polymorphism in the 5' untranslated region of exon 1 of *GLA* gene is present in 8% of Caucasians and has been proposed as a possible modifier of the clinical phenotype of patients with *GLA* variants associated to high residual enzymatic activity, such as p.A143T, resulting in earlier and more severe manifestations [378,379].

Nevertheless, our p.A143T patient presented severe HCM at the age of 28 years, which is too young for an HCM due to late-onset FD, even for a male, which means that even if we considered p.A143T variant as pathogenic, this variant alone could not explain HCM at this age neither its severity. Moreover, he had a documented pathogenic mutation in the *MYBPC3* gene (p.R943X) [380] that could fully explain the HCM phenotype. Therefore, we did not classify this HCM case as caused by FD, although we cannot exclude a contributing role of this *GLA* variant to the progression of HCM with advancing age.

The variant c.525C>G (p.D175E) was considered a GVUS most probably benign. This variant leads to a replacement of aspartic acid by glutamic acid, existing a small physicochemical difference between them. In-silico methods also predict this variant to be benign (Polyphen-2) or a polymorphism (MutationTaster) [290,291]. This variant has been found in 3.3e-05 in 183473 X-chromosomes (gnomAD) [381]. The "in vitro" enzymatic activity of mutant α-galactosidase A in HEK-293 cells has been reported as 44.3±2.9%

of the enzymatic activity of the wild-type enzyme [289]. Moreover, although our 83-year-old female patient presented asymmetrical severe HCM, alternative causes of LVH, such as sarcomeric HCM, isolated basal septal hypertrophy or wild-type transthyretin amyloidosis, could not be fully excluded. Likewise, proteinuria, renal failure and stroke could also be explained by concomitant cardiovascular risk factors (arterial hypertension, diabetes *mellitus* and dyslipidemia). There was no family history suggestive of FD and further clinical or biochemical data regarding this variant were not available, as the patient and family refused additional diagnostic study. Taken together, this variant was classified as GVUS most probably benign.

The variant c.937G>T (p.D313Y) was first reported in a male with a classical phenotype of FD [194]; however, further study came to reveal a second *GLA* mutation (p.C172G) in this patient, which was pathogenic [382]. Although Polyphen-2 predicts p.D313Y variant as probably damaging [291], the expression of this variant in COS-7 cells eventually showed an enzymatic activity of 60-75% of the enzymatic activity of the wild-type enzyme [382,383] and demonstrated that the mutant enzyme does reach the lysosomes [382]. Furthermore, the expressed mutant enzyme was shown to be stable at the lysosomal pH (pH 4.6), but unstable at the neutral pH (pH 7.4), thereby explaining a nearly normal enzymatic activity in leukocytes and a lower enzymatic activity in plasma of patients carrying this variant [382]. In addition, this is a non-conserved residue [383] and this variant has been found in 3,04e-03 in 205260 X-chromosomes (gnomAD) [384]. A recent systematic review of 35 studies on the p.D313Y variant [385] has shown that (i) most males and females carrying this variant present normal enzymatic activity of α-galactosidase A on leukocytes; (ii) males and females with decreased enzymatic activity still exhibit a high residual enzymatic activity, above the cut-off of 35% of the enzymatic activity of the wild-type enzyme, i.e., above the FD diagnostic range; (iii) males and females with this variant present normal plasma lyso-GB3; (iv) clinical manifestations specific for FD, such as angiokeratomas and cornea *verticillata*, are rarely reported in subjects with this *GLA* variant; (v) no GB3 accumulation has been demonstrated so far in tissue biopsies of individuals carrying the p.D313Y variant; (vi) the prevalence of this variant in high-risk populations, such as kidney (0.20%), cardiac (0.42%), neurologic (0.59%) and small-fibre neuropathy (0.80%) cohorts, is similar to the prevalence of the variant in the general population (0.3-0.5%) [382,384], with the possible exception of a higher frequency in patients with cerebrovascular disease [351,386,387]. Taken together, current evidence suggests that the variant p.D313Y is non-pathogenic, or mildly pathogenic at most with a possible role in the development of brain WML and stroke.

In accordance to the current evidence, our study found four patients with the variant p.D313Y (4/780; 0.5%), which resulted in a variant prevalence in HCM that is similar to the variant prevalence in the general

population (0.5%) [382]. Furthermore, although enzymatic activity of α-galactosidase A on plasma and leukocytes was not available, plasma lyso-GB3 was also normal both in our male and female patients. Finally, alternative (and probable) causes of HCM could not be excluded in these four patients. HCM genetic testing would be appropriate to assess a sarcomeric cause in the (i) 36-year-old male with severe asymmetrical non-obstructive HCM and antero-septal LGE; the (ii) 70-year-old female with severe asymmetrical non-obstructive HCM and no LGE; and the (iii) 70-year-old female with symmetrical obstructive HCM, short PR interval, left bundle branch block, family history of HCM and no history of hypertension. The possibility of isolated basal septal hypertrophy of the elderly should be considered in the (iv) 76-year-old female with non-obstructive LVH and maximal LV wall thickness of 15mm at the septum, no LGE and no family history of HCM. Amyloidosis by wild-type transthyretin should also be excluded in these elderly females with HCM. Therefore, based on current evidence on the variant p.D313Y and these study data, the pathogenicity of this variant could not be ascertained and therefore this variant was not included in the FD prevalence calculation.

Prevalence results have to be constantly recalculated as knowledge on Genetics evolves on the pathogenicity of *GLA* variants. The variants p.R118C, p.A143T and p.D313Y are common findings in FD screenings and therefore their inclusion/exclusion from FD prevalence calculation has a major impact on prevalence results. An in-depth study is mandatory in case GVUS are found in order to better understand their role in disease pathogenesis and not to overdiagnose FD.

Based on these considerations on the pathogenicity of the identified *GLA* variants, this study classified 37 patients as FD cases, including 31 with the founder p.F113L mutation, four with the pathogenic *GLA* mutations previously identified in the FD screening in the region of Guimarães (p.C94S, p.G183V, p.E203X and p.M290I) and two with the *GLA* variants p.M96V and p.R356Q/p.G360R (37/780, 0.9%).

In line with our results, a recent literature review, which included 17 FD screening studies encompassing a total of 5491 patients with HCM or LVH, re-evaluated the pathogenicity of the identified *GLA* variants and reported a FD prevalence of 0.94% in males (38/4054) and 0.9% in females (13/1437) with HCM or LVH. Of note, this review also excluded variants p.R118C, p.A143T and p.D313Y from the FD prevalence calculation [388].

Our research determined FD prevalence in patients with HCM as 0.9%, after adjusting for the founder effect. Other FD screenings in HCM have also found different patients with the same *GLA* variant [161,166,168,169,172,173,174,176,389], but most of them did not provide any genetic or genealogical analysis to exclude a family relation between the identified cases [161,166,168,169,172,173,176,389].

Considering that mutations in FD are frequently private, the absence of these analyses can lead to erroneous prevalence results, particularly in regions with documented founder effects.

Hagege et al. screened 392 patients with HCM and found four Fabry cases (4/392, 1.0%), two of them with the *GLA* mutation p.F113L; interestingly, the authors noted that both patients had Portuguese ancestry, suggesting a family connection between them, which would impact prevalence result (3/392, 0.8%) [168]. Sadasivan et al. screened 266 patients with unexplained LVH and found five Fabry patients, all from Hong Kong, with the c. IVS4 + 919G>A mutation (5/266, 1.9%) [389]; however, although a family connection was not recognized, these individuals most probably descend from a common ancestor given the documented founder effect of this mutation in mainland China [298], which significantly decreases the FD prevalence found in the study (1/266, 0.4%). Monserrat et al. screened 508 patients with HCM and found five Fabry cases (5/508, 1.0%), two of them with the mutation p.S238N, both from the Spanish region of Alicante [166], where this mutation is especially prevalent, strongly suggesting a founder effect (4/508, 0.8%). Moreover, the mutation p.N215S, which is particularly common in UK, possibly also due to a founder effect, and one of the most common *GLA* mutations associated with late-onset FD with predominant cardiac involvement, has also been commonly found in more than one case during FD screenings in patients with HCM [161,169,173,176]. The possibility of a family connection between the identified cases would also decrease estimates of FD prevalence. Sachdev et al. screened 79 males with HCM diagnosed at ≥40 years of age and found five FD cases (5/79, 6.3%), three of them with this mutation (3/79, 3.8%) [161]. Favalli et al. screened 473 patients with HCM, concentric mild LVH and no LVOT obstruction and found eight Fabry cases (8/473, 1.69%), five of them with this mutation (4/473, 0.8%) [173].

Conversely, mutations at CpG dinucleotides, known mutational hot spots, occur more often in unrelated families. This is the case of the variant p.A143T, which was found by Terryn et al. in four out of five Fabry cases among 540 patients with LVH (5/540, 0.9%). Although no further study was performed to exclude a family connection between them, this hypothesis seems less likely, given the location of the variant at a CpG dinucleotide. Accordingly, in a newborn screening of 37104 males, Spada et al. found three cases with the p.A143T variant and two with the p.F113L variant. Analysis of microsatellite markers surrounding *GLA* gene revealed that markers differed among patients with the p.A143T variant, but not in the cases with the p.F113L variant, suggesting that p.F113L patients could be related, unlike p.A143T patients [27]. Therefore, this is another issue that should be accounted in studies aiming to determine FD prevalence.

Finally, our study is one of the largest studies on FD prevalence in HCM. From the studies published subsequently on FD prevalence in HCM, a recent study stands out for being the largest so far [390] (Table

3). Yoshida et al. screened 4057 patients (2853 males, 1176 females, 28 with unspecified gender) with LVH detected by electrocardiography or echocardiography. FD screening was performed by measurement of α-galactosidase A enzymatic activity on DBS samples followed by genetic analysis (PCR sequencing of all *GLA* exons and flanking intronic sequences and search for the c.639+919G > A mutation) in patients with enzymatic activity below a predefined cut-off. This study presented some limitations: genetic analysis was not performed in females with enzymatic activity above the predefined cut-off; LVH definition was not stated; and the study registered a drop-out rate. Nevertheless, it is noteworthy that this study reached a similar result with a FD prevalence adjusted to the drop-out rate of 0.94% [390], further supporting our finding of a FD prevalence in HCM of 0.9%.

Table 3: Summary of studies on Fabry disease prevalence in patients with HCM or LVH, published after our study

Authors	Year	Screened population	Screening methods	*GLA* gene variants found	Fabry disease reported prevalence	Comments
Kim *et al.*	2019	988 males with LVH (LV wall thickness ≥13mm)	Plasma α-galactosidase A activity was performed in all males and repeated in males with low enzymatic activity	p.H46R p.R301Q p.G328R	0.3% (3/988)	Study only in males Two males were found with the variant p.E66Q associated to polymorphism
			Genetic analysis (PCR sequencing of exons and intronic flanking regions) in males with two results of low enzymatic activity			No *GLA* variant was found in two males, despite low enzymatic activity
Barman *et al.*	2019	80 patients (53 males) with non-obstructive HCM (unexplained LVH with LV wall thickness ≥15mm)	Genetic analysis in all patients (PCR sequencing of exons and exon-intron connections)	p.R112C p.R301Q	2.5% (2/80)	Small sample
			Plasma α-galactosidase A activity in patients with *GLA* mutations			
Barman *et al.*	2019	190 patients (119 males) with unexplained LVH (LV wall thickness ≥13mm)	Genetic analysis in all patients (PCR sequencing of exons and exon-intron connections)	p.A143T p.D313Y	1.05% (2/190)	One patient had the variant p. A143T, whose pathogenicity has been questioned
			Plasma α-galactosidase A activity and lyso-GB3 in patients with *GLA* mutations			One patient with the variant p.D313Y currently described as an enzymatic pseudodeficiency
Tran Vu *et al.*	2019	104 patients (66 males) with HCM (unexplained LVH with LV wall thickness ≥15mm)	HCM gene panel including *GLA* gene by NGS	p.N215S	1.0% (1/104)	Small sample
Yamashita *et al.*	2019	277 patients (215 males) with unexplained LVH (LV wall thickness >12mm)	Plasma α-galactosidase A activity and lyso-GB3 in all patients	p.R112H Unspecified *GLA* variant	0.7% (2/277)	Genetic analysis was not performed in females with normal enzymatic activity and lyso-GB3

			Genetic analysis in patients with low enzymatic activity or high lyso-GB3			5 patients were positive (low enzymatic activity or high lyso-GB3), but only 2 were Fabry cases; 2 patients had the variant p.E66Q associated to polymorphism; 1 patient had no *GLA* variant One of the Fabry cases had an unspecified *GLA* variant. Despite being classified by the authors as GVUS, lyso-GB3 was high and endomyocardial biopsy showed lysosomal inclusions, so this patient was considered a Fabry case.
Fuller *et al.*	2020	511 patients (332 males) aged 18-75 years with unexplained mild-to-moderate LVH (LV wall thickness 12-15mm)	α-galactosidase A activity in DBS Plasma lyso-GB3 *GLA* gene testing by NGS	p.A143T p.N215S	0.4% (2/511)	One patient had the variant p.A143T, whose pathogenicity has been questioned
Sadasivan *et al.*	2020	266 patients (167 males) with unexplained LVH (LV mass >115 g/m² in men and 95 g/m² in women, in the absence of increased LV end-diastolic dimension (<58.4 mm and <52.2 mm for men and women, respectively))	α-galactosidase A activity in DBS Genetic analysis (PCR sequencing of all *GLA* exons and up to 20 base pairs upstream and downstream of the intron-exon boundaries at both sites + search for IVS4 + 919G >A mutation) in DBS - in males and females with low enzymatic activity (IDUA/α-GAL A activity ratio ≥10) of the Hong Kong cohort - in all patients of the Edmonton cohort	c.IVS4 + 919G>A	1.9% (5/266)	Genetic analysis was not performed in the majority of females with IDUA/α-GAL A enzymatic activity ratio <10 The possibility of a founder effect is very likely and should be accounted for the calculation of FD prevalence

Yoshida *et al.*	2020	4057 patients (2853 males; 28 with unspecified gender) with cardiac manifestations, such as LVH detected using electrocardiography or echocardiography	α-galactosidase A activity in DBS Genetic analysis (PCR sequencing of all *GLA* exons and the flanking intronic sequences + search for c.639 + 919G > A mutation) by Sanger and NGS - in patients with enzymatic activity below cut-off in two samples (Method I: < 12 [AgalU] for males and < 20 [AgalU] for women; and Method II: < 15 [AgalU] for males and < 20 [AgalU] for women)	p.G43D p.N53Y p.F69L p.M76T p.R112C p.R112H c.369+806_c.640-416del p.V137Lfs28 p.S148N p.L166V p.G183S p.V199M p.C202Y c.639+919G>A p.N215S p.D231Y p.W236X p.Q279E p.R301X p.R301P p.I303T p.I317T p.G328R p.Y329X p.R342X p.G375E p.W24R/p.N419D	0.94% (29/4057) – prevalence adjusted to the drop-out rate	Genetic analysis was not performed in females with enzymatic activity above the cut-off LVH definition was not stated Drop-out rate

5.3.2 Fabry disease predictors in Hypertrophic cardiomyopathy

Our research has identified the symmetric pattern of HCM, ST-segment depression, bifascicular block and basal inferolateral LGE as independent predictors of FD in HCM. Moreover, it has revealed bifascicular block and basal inferolateral LGE as the most powerful predictors of FD in HCM.

Although many diagnostic red-flags of FD have been described in the literature [219], increasing awareness on these red-flags have not translated into an earlier diagnosis of FD in the clinical practice [35], either because (i) they are not perceived by cardiologists as strong red-flags due to limited specificity or sensitivity (symmetric pattern of LVH) [55,71,220]; (ii) they are not routinely evaluated, quantified or mentioned in the exams reports (prominence of papillary muscles, low T1 native time); (iii) they are rare findings in FD (short PR interval) [114]; (iv) they are extracardiac and frequently escape the attention or detection by cardiologists, or demand clinical expertise or evaluation by other medical specialties to allow their detection (angiokeratomas, cornea *verticillata*, deafness, acroparesthesias); or (v) they are frequently absent in heterozygous females and late-onset phenotypes, particularly with single-organ involvement (angiokeratomas, cornea *verticillata*, acroparesthesias, renal failure, stroke) [15,181].

Therefore, our study addressed the medical need to find predictors that could be useful for the cardiologists in the clinical practice in the differentiation of FD from other causes of HCM and could translate into an earlier diagnosis and treatment of the disease. Accordingly, we included in the multivariate binary logistic regression analysis cardiac categorical variables that were readily available from ECG, echocardiogram and cardiac MRI, in order to ensure their easy application in the clinical practice.

The LVH pattern is readily known as soon as the diagnosis of HCM is established and, therefore, is one of the first characteristics that will guide the etiological study of HCM. In FD, HCM is typically concentric and symmetric [25,71]. However, this LVH pattern is also common in other phenocopies, such as amyloidosis, mitochondrial diseases and Danon disease [219]. Moreover, in advanced stages of FD, there is thinning and akinesia of the basal PW, due to fibrosis [55], which may result in asymmetric LVH [71]. Finally, other LVH patterns have been described in FD, such as apical LVH [220]. Accordingly, the symmetric pattern of HCM has been demonstrated in our study as an independent but not the strongest predictor of FD in HCM.

Our study, on the other hand, emphasized the importance of ECG, a widely and readily accessible exam, in the differential diagnosis of FD from other causes of HCM. Only a few studies had explored in the past the role of ECG in this differential diagnosis. These studies were only focused on PR interval and showed that a PQ interval minus P-wave duration in lead II <40ms in combination with a corrected QT duration

<440ms was 100% sensitive and 99% specific for the diagnosis of FD [391]. However, short PR interval has been shown to be uncommon in FD [114].

Our study was pioneer in pointing out the importance of other ECG findings, particularly ST-segment depression and conduction blocks, in the differential diagnosis of FD from other causes of HCM. After the publication of our study, two other studies have been published on the role of ECG in the differential diagnosis of FD from other HCM causes, also identifying ST-segment depression and conduction blocks as independent predictors of FD [392,393].

ST-segment depression has been significantly associated with LGE in FD [120]; nevertheless, it emerged as an independent predictor of FD in HCM, alongside with basal inferolateral LGE. In fact, although it was suggested that ST-segment depression and LGE could signal the same patients and that the absence of ST-segment or T wave alterations on ECG could almost exclude LGE in FD [120], LGE has been found in up to 17.8% of the patients without ST-segment depression and 13.4% of the patients without negative T waves in our large cohort of patients with late-onset FD with predominant cardiac involvement due to the p.F113L mutation.

However, it should be noted that, in our study, ST-segment depression has been found in only 27.6% of non-Fabry cases. In a large cohort of 1004 patients (mean age of 50±16 years) with HCM and a mean maximal LV wall thickness of 20±5mm, ST-segment depression was reported in 56% of the cases [394], and this frequency reached 76% in a cohort of 148 adolescents (mean age 16.1±1.8 years) with HCM and a mean IVS thickness of 24±10mm [395]. ST-segment depression at rest has been identified as an independent predictor of exercise-induced subendocardial perfusion abnormalities as detected by myocardial scintigraphy [396] and as an independent predictor of sudden death or appropriate ICD therapy [397] in patients with HCM. Our apparently lower frequency of ST-segment depression might be related to the previously mentioned characteristics of our HCM cohort, which also includes patients diagnosed at an older age, a subset of HCM patients that has been increasingly recognized as a lower-risk subset of HCM.

Accordingly, ST-segment depression has been demonstrated in our study as an independent but not the strongest predictor of FD in HCM and its clinical usefulness for the differential diagnosis of FD is probably dependent on the age of patients, being more useful in higher age groups, which correspond to the age groups, in which Fabry patients at the HCM stage (≥15mm) are still currently diagnosed. Indeed, in our study, the median age of Fabry and non-Fabry group is high but similar [64.0 (57.5, 75.0) vs. 68.0 (57.0, 77.0)], and probably it is in this age group in which ST-segment depression may be more useful in the

differential diagnosis of FD. More recently, Vitale et al. also found that inferior ST-segment depression was an independent predictor of FD in patients with HCM [392].

Basal inferolateral LGE is a very specific red-flag that allows to differentiate FD from other causes of HCM, such as sarcomeric HCM, which usually results in LGE at the RV junction points [398]; and amyloidosis, which usually results in a global subendocardial pattern of LGE [399]. Although it may be found in other medical conditions, such as myocarditis, Chagas disease, sarcoidosis, PRKAG2 cardiomyopathy and mitochondrial myopathy [228-230], most of these conditions are also rare. In addition, although LGE may come to involve other LV segments as disease progresses [75], the involvement of the basal inferolateral segment is always constant as soon LGE arises. Accordingly, our study identified basal inferolateral LGE as a powerful predictor of FD in HCM. However, many Fabry patients at HCM stage (≥15mm) present disease complications that constitute contraindications to cardiac MRI, such as renal failure or nonconditional pacemakers. Indeed, nine of our 37 Fabry cases (24.3%) did not perform cardiac MRI due to medical contraindications.

Therefore, bifascicular block emerges as a powerful and useful predictor of FD in the clinical practice, as it is readily detectable on the ECG, a basic exam performed in all patients with cardiac diseases. To our knowledge, our study was the first to demonstrate that bifascicular block is an independent and powerful predictor of FD in HCM.

Cardiac conduction blocks are known manifestations of FD. As FD progresses, GB3 accumulation and fibrosis lead to the development of bundle-branch and atrioventricular blocks [55,115,116]. A QRS interval duration >120ms has been reported in 9% of the cases and a PR interval duration >200ms in 3%. The durations of these intervals have been shown to increase with age [117], and the duration of QRS interval has been shown to positively correlate with LV mass on cardiac MRI [120]. Our research on the late-onset phenotype with predominant cardiac involvement due to the p.F113L mutation has found bifascicular block in 25.3% of males and 5.7% of females with a mean age at diagnosis of 62 ± 6 years in males and 78 ± 6 years in females. The frequency of bundle branch blocks and complete atrioventricular block has also been found to increase with age as well as the duration of QRS interval.

In the HCM stage (≥15mm), our Fabry patients, who presented a similar median age (64 vs. 68 years) compared to non-Fabry patients, also presented similar IVS thickness (18 vs. 17mm); and, at this advanced stage of disease, more than half (54.2%) of Fabry patients, against only 17.7% of non-Fabry patients, already presented bundle branch blocks, the most striking difference being noted at this stage of the disease on the frequency of bifascicular block (37.1% vs. 3.5%). A cohort of milder Fabry cases with HCM (with maximal LV wall thickness closer to 15mm) could have found the most striking difference on

right bundle branch block, which commonly precedes bifascicular block. Accordingly, a more recent study compared LVH secondary to FD with LVH secondary to sarcomeric cause; however, mean age of these Fabry patients was 55.6±11.5 years and their maximal LV wall thickness widely ranged from 13 to 26mm, meaning that some patients were on pre-HCM stage (<15mm), which was reflected in a mean maximal LV wall thickness of 16.2±3.5mm. Therefore, this cohort of patients was apparently younger and presented a less advanced stage of disease, which might explain why the statistical difference between the groups was noted on the frequency of right bundle branch block (54% vs. 22%), a common step before the development of bifascicular block. In this study, right bundle branch block was identified as a strong predictor of FD [393]. More recently, another study also found that right bundle branch block was an independent predictor of FD in patients with HCM [392].

The identification of these independent FD predictors in HCM enabled the creation of a score that aimed to help clinicians to identify FD cases among patients with HCM: ID FABRY-HCM = −0.729 + (2.781 x Bifascicular block on ECG) + (0.590 x ST depression on ECG) + (0.831 x Symmetric LVH) + (2.130 x basal inferolateral LGE). The cut-off value of 1.0 showed a sensitivity of 63.4%, a specificity of 97.2%, a positive predictive value of 68.3% and a negative predictive value of 95.8% for FD.

From the analysis of this score, it was possible to conclude that neither the presence of symmetrical HCM or ST-segment depression alone, nor the combination of the two, was enough to obtain a score in favor of FD. Conversely, the presence of either bifascicular block or basal inferolateral LGE alone achieved a favorable score for FD. More importantly, in the absence of both bifascicular block and basal inferolateral LGE, the negative predictive value for FD was 95.8%. Therefore, it became clear that the score application was not needed in the clinical practice and that the algorithm to define the most appropriate diagnostic strategy in the etiological study of HCM could be simplified into a very pragmatical rule: (i) in the presence of either bifascicular block or basal inferolateral LGE, targeted FD screening is the most appropriate next step in the etiological study of HCM; and (ii) in the absence of both bifascicular block and basal inferolateral LGE, FD is a less probable cause of HCM and a wide HCM gene panel is the most adequate strategy to its etiological study.

The score sensitivity of 63.4% may be explained by the fact that patients with milder forms of HCM (LV wall thickness closer to 15mm) may not present bifascicular block or basal inferolateral LGE, as they represent later stages of the disease. The positive predictive value of 68.3% is explained by the low prevalence of the disease and the fact that bifascicular block or basal inferolateral LGE are not exclusive of FD and may appear in other HCM causes. It should also be recognized that the high negative predictive

value (95.8%) is less meaningful in the field of rare diseases, because negative predictive value increases as disease prevalence decreases.

FD is a treatable condition and should be systematically screened in all patients with unexplained LVH, either by targeted FD screening or a wider HCM gene panel, which currently also includes the *GLA* gene. Therefore, this algorithm does not preclude FD screening in any patient with HCM, rather it guides clinicians on the most probable cause of HCM and the most appropriate next diagnostic step in the etiological study of HCM. Considering the significantly lower cost of targeted FD screening compared to a wide HCM gene panel, the application of this diagnostic algorithm may prove useful in the clinical practice.

This study presented some limitations that should be acquainted. The diagnosis of FD was considered according to the current knowledge on genetics that is constantly evolving and may reveal in the future different pathophysiological roles for the *GLA* variants that were found in this study. Most Fabry cases in this study carried the same *GLA* mutation, so this score needs to be validated in other *GLA* mutations. This score only applies to adult patients with the ESC definition of HCM, and therefore it is not applicable to paediatric patients or patients with milder LVH (LV wall thickness <15mm), in whom FD diagnosis could carry a higher prognostic impact, as earlier diagnosis and treatment result in better outcomes. Nevertheless, it is relevant to note that the diagnosis of Fabry cases at the HCM stage will enable the diagnosis of several relatives at earlier stages of the disease. Finally, this score needs external validation in other large cohorts of patients with HCM.

Innovative features of our research include (i) the identification of independent predictors of FD in HCM, and particularly from a clinically useful perspective; (ii) the highlight on the importance of basic ECG findings such as cardiac conduction blocks to the differential diagnosis of FD from other HCM causes, and specifically bifascicular block; and (iii) the definition of a simple pragmatic algorithm for FD screening in patients with HCM.

5.4 Left ventricular noncompaction and Fabry disease

The multicentre FD screening of 78 consecutive patients with LVNC did not reveal any additional Fabry patient besides the single case that had been previously reported by our centre, thus arguing against the hypothesis that LVNC is a clinical manifestation of FD.

The most likely explanation for the finding of LVNC in this Fabry patient is an overdiagnosis of LVNC. Studies have increasingly exposed the lack of specificity of the current diagnostic criteria of LVNC,

potentially leading to its overdiagnosis, either by echocardiography or cardiac MRI [235,236,263,264,400,401].

Kohli et al., in a study including 199 patients referred to a heart failure clinic and 60 healthy controls, found that 23.6% of the patients and 8.3% of the controls (3.3% of the Caucasian controls and 13.3% of the African controls) fulfilled one or more echocardiographic criteria for LVNC. Among the heart failure patients with LVNC criteria, 29.8% fulfilled the three echocardiographic criteria and 36.2% fulfilled only a single criterion with the frequency of each criterion ranging from 12.1 to 18.6% [235]. In a large study of 1146 athletes aged 14–35 years, Gati et al. also showed that 9.5% fulfilled the Jenni criteria, 10.1% the Chin criteria and 8.1% both criteria [236].

In a large population-based cohort of 2742 subjects from the Multi-Ethnic Study of Atherosclerosis (MESA) registry who underwent cardiac MRI, the Petersen criteria were fulfilled in 25.7% of the individuals [400]. In 700 patients referred to cardiac MRI, Ivanov et al. have found high prevalences of LVNC by any of the cardiac MRI criteria (Petersen 39%, Stacey 23%, Jacquier 25%, and Captur 3%) [401]. De la Chica et al., in a study of 705 healthy subjects submitted to cardiac MRI, have also shown high overall prevalences of LVNC criteria (Petersen 17%, Jacquier 16%, Grothoff 8%, Stacey 4%), which were even higher in subjects with vigorous physical activity (Petersen 31%, Jacquier 27%, Grothoff 16%, Stacey 6%) [264]. Finally, a recent systematic review and meta-analysis also confirmed high overall prevalences of LVNC imaging criteria in individuals aged ≥12 years, although higher in cardiac MRI (14.79%) than in echocardiography (1.28%); and even higher prevalences in athletes, both by MRI (27.29%) and echocardiography (3.16%) [263].

Moreover, growing evidence suggests that LVNC has no independent prognostic impact compared to conventional risk factors, such as LV dilation, dysfunction or fibrosis, and that patients with LVNC without LV dilation, dysfunction or fibrosis have an excellent prognosis [250,400-404].

The large population-based study including 2742 subjects from the MESA registry submitted to cardiac MRI showed that a greater extent of LV trabeculations in asymptomatic individuals appears benign and is not associated with deterioration of LV volumes or function over an almost 10 year-period [400]. In 700 patients referred to cardiac MRI, there were no statistically significant differences in the assessed outcomes (death, ischemic stroke, ventricular tachycardia/fibrillation, and heart failure hospitalization) during a median follow-up of 7 years, between patients with and without LVNC, irrespective of the applied criteria (Petersen, Stacey, Jacquier or Captur) [401].

In 105 adult patients with echocardiographic criteria of LVNC from a French registry, the factors associated with death or heart transplantation were high LV filling pressures, LV ejection fraction, NYHA class III/IV

and hospitalization for heart failure, but not the number or percentage of noncompacted LV segments [402]. In 98 patients with Petersen criteria for LVNC, LV dysfunction and LGE were predictors of major adverse cardiovascular and cerebrovascular events (cardiac death, cardiac transplantation, sustained VT/VF and ischemic stroke), unlike the degree of trabeculation as measured by the noncompacted mass/total mass ratio [405]. Andreini et al., in a study including 113 patients with LVNC according to the Jenni and Petersen criteria, found that the degree of hypertrabeculation had no prognostic impact over and above LV dilation, dysfunction or LGE. Indeed, patients without LV dilation, dysfunction or LGE had an excellent prognosis at the 4-year follow-up [403]. Vaidya et al. have shown that patients with LVNC with preserved LV ejection fraction and isolated apical noncompaction had similar survival to the general population [404]. Finally, a recent systematic review and meta-analysis of 28 studies enrolling 2501 patients with LVNC has shown that LV ejection fraction, but not the degree of hypertrabeculation as measured by the noncompacted/compacted layer ratio, was associated to cardiovascular death [250].

Additionally, LGE has been demonstrated as an independent predictor of LV ejection fraction [406], VT [407] and cardiac events (thromboembolic events, hospitalization for heart failure, ventricular arrhythmias, and cardiac death due to ventricular arrhythmias or refractory heart failure) [403]. A recent meta-analysis showed that LGE was associated with the (i) combined endpoint of cardiac death, SCD, appropriate ICD shock, resuscitated cardiac arrest, cardiac transplantation, LV assist device implantation, heart failure hospitalization, ischemic stroke and other thromboembolic events; (ii) hard cardiac events (cardiac death, SCD, appropriate ICD shock, resuscitated cardiac arrest, cardiac transplantation, and LV assist device implantation); and (iii) cardiac death. Notably, no hard cardiac events were recorded in patients with LVNC, preserved ejection fraction and negative LGE [408].

Other imaging criteria have shown to be useful in the differentiation of LVNC from healthy controls, such as the reduction of LV GLS, particularly in the apical segments [409,410], and the impairment of LV apical rotation and twist, being particularly typical the pattern of rigid body rotation in which the LV base and apex rotate in the same direction [410,411]. Cardiac MRI may also show high T1 native values both in patients with and without LGE [412,413].

Moreover, other factors have been demonstrated to carry a prognostic impact in patients with LVNC. Long QTc interval has been associated to lower LV ejection fraction, higher T1 values and higher frequency of adverse events (sudden death, deteriorating heart failure requiring hospital admission, cardiogenic shock, syncope, and severe arrhythmia) [414]. Fragmented QRS has been identified as an independent predictor of arrhythmic events and cardiovascular mortality [415].

Genetic testing has also emerged as an important factor in prognostic stratification of LVNC, as patients with pathogenic mutations were found to have an earlier onset [258], worse LV function [256,258,259], higher risk of major adverse cardiovascular events (cardiac death, implantation of a LV assistance device, heart transplantation, (aborted) SCD, appropriate ICD shock, or ischemic stroke) [256] and worse survival [258]. A positive genotype was identified as an independent predictor for the composite endpoint of death or heart transplantation [259] as well as the combined endpoint of death, heart transplantation or ICD insertion [258]. Multiple mutations have been associated to higher risk of LV systolic dysfunction [256] and even worse survival [258]. Patients with a mutation in the sarcomere genes had lower risk of major adverse cardiovascular events (stroke, heart failure requiring hospital admission, LV assist device, heart transplantation, sustained VT/VF or appropriate shock, and cardiac death) than patients with other genetic causes [254,258]. Patients with a mutation in *MYBPC3* and *TTN* had a much higher risk of major adverse cardiovascular events than patients with a *MYH7* mutation [254,256]. Pathogenic variants in the *LMNA* and *RBM20* genes were associated with unfavourable outcome [257] and mutations in *DSP* gene were described in patients with ventricular dysrhythmias [259].

Finally, cardiovascular complications at presentation [416], NYHA class III-IV [416,417], sustained ventricular arrhythmias [417] have been also identified as independent predictors of cardiovascular death or heart transplantation. In patients with LVNC and a dilated phenotype, the coexistence of a neuromuscular disorder was also associated with higher mortality [418].

Based on this evidence, in 2019, Negri et al. proposed a diagnostic algorithm for LVNC [248]. According to this algorithm, in the presence of echocardiographic (Jenni) and cardiac MRI (Jacquier, Grothoff) criteria of LVNC, LVNC diagnosis should only be considered when other cardiac diseases have been excluded and at least one of the following factors is present: (i) Family history of LVNC; (ii) Positive genotype in children; (iii) Abnormal 12-lead ECG; (iv) Long QTc interval; (v) Fragmented QRS; (vi) Medical history of heart failure, thromboembolic events, arrhythmias (VT) or unexplained syncope; (vii) Neuromuscular disease; (viii) LV ejection fraction <50% or 50-54% and GLS <15% on echocardiography; (ix) Diastolic dysfunction grade II or III; (x) LGE, high T1 or global longitudinal/radial/circumferential strain impairment on cardiac MRI [248].

In light of this evidence, we must consider the hypothesis of an overdiagnosis of LVNC, without any associated prognostic impact, in these particular three cases of FD published in the literature. In fact, all three case reports of LVNC occurred in young females with no family history of LVNC on Fabry or non-Fabry relatives, no LV hypertrophy, dilation or dysfunction, no LGE suggestive of FD involvement, no or

few clinical manifestations of FD, no clinical complications of LVNC (heart failure, thromboembolic events, ventricular arrhythmias or unexplained syncope) and no history of neuromuscular disorder [270-275].

Moreover, no history of syncope and no ECG abnormalities were found in the first two cases, besides a sinus bradycardia with a heart rate of 50bpm in the second case. Although the third patient presented a history of syncope and short PR interval and delta waves on ECG, the electrophysiological study detected an accessory pathway in the para-Hisian region and induced an atrioventricular reentrant tachycardia. A complete ECG normalization occurred after successful ablation and no recurrences of syncope were reported during the 2-year follow-up. Although there are reports of FD cases with short PR interval and accessory pathways [419], enhanced AV conduction rather than ventricular pre-excitation is the most likely cause of the short PR interval. Short PR interval has been documented in FD patients in whom pre-excitation due to accessory pathways has been formally excluded [420]. Moreover, a normalization of PR interval has been documented with ERT [421]. Therefore, it is possible that ventricular pre-excitation is an unrelated condition and the cause of syncope in this patient.

Strain imaging data were not originally reported in these three cases; yet it is noteworthy that GLS by speckle tracking was normal in our patient both at baseline and follow-up. T1 mapping results were also not reported, as this technique was not part of the routine study in cardiac MRI at the time of the original publications of these three cases. Finally, genetic data are only available in our patient; however, the genetic study of LVNC was performed more than 10 years ago and was limited to *TAZ* and *LDB3* genes, revealing no pathogenic mutations. Nevertheless, a wider genetic approach as well as T1 mapping could provide further data at this point to this investigation.

Notably, during the eight-year follow-up of our patient, no significant change has been documented on the location or extension of LVNC or the LV chamber size, mass or function, despite the absence of FD therapy. This patient has remained asymptomatic and without any known clinical manifestation of FD or complication of LVNC, which further supports the hypothesis of an overdiagnosis of LVNC in this Fabry patient.

Overdiagnosis of LVNC is more likely to occur in physiological conditions of cardiac overload, such as pregnancy [261], sport practice [236,262,263] or vigorous exercise [264]. Nevertheless, history of recent pregnancies or vigorous exercise practice was not reported in these three cases. Our patient had been pregnant once, more than 10 years before the diagnosis of LVNC; however, as LVNC has been reported to revert after pregnancy [261], it is not likely that LVNC in our case was due to previous pregnancy.

On the other hand, the hypothesis that LVNC and FD may coexist in these patients as coincidental and unrelated conditions is less plausible. Although FD prevalence in the general population has been largely

underestimated as 1: 117,000 and later shown in newborn screening studies to be much higher, ranging from 1: 2913 to 1: 11854 [27,29-33], it remains a rare condition. Moreover, although LVNC imaging criteria are commonly fulfilled due to their low specificity [235,236,263,264,400,401], the addition of other factors in the recently proposed diagnostic algorithm [248] increases specificity to the diagnosis of LVNC, reducing its probability in patients presenting with imaging criteria of LVNC.

Although current evidence supports an overdiagnosis of LVNC as the most likely explanation for these three cases, given the low specificity of the current imaging criteria of LVNC [235,236,263,264,400,401], it is plausible that more cases fulfilling the LVNC imaging criteria may appear among Fabry patients, which raises concerns on its therapeutic implications. This matter is particularly important in Fabry females, who, in part due to the inactivation pattern of the X chromosome [22], present a highly variable prognosis, some of them exhibiting a benign disease course and a lifelong prognosis without significant clinical manifestations in the absence of specific therapy.

So far, there is no evidence that FD causes LVNC neither by a compensatory or a genetic mechanism. Indeed, despite FD, none of these patients had concomitant LVH, dilation or dysfunction that could lead to a cardiac overload condition and, consequently, to LVNC. Furthermore, there is no evidence that the *GLA* mutation, the mutant α-galactosidase A or the intramyocardial deposition of glycosphingolipids due to FD are the cause or are anyway related to the development of LVNC and any assumption on that matter remains speculative. Lysosomal accumulation of GB3 begins in utero and has been demonstrated, as early as the 17th week of gestation, in various foetal cell types, such as cardiomyocytes [8], kidney podocytes [8-10], hepatocytes [9,11], cornea epithelial cells [12] and myenteric plexus cells [10]. Although GB3 storage begins in utero, clinical manifestations only develop several years later. Therefore, GB3 deposits are expected findings in biopsies, including endomyocardial biopsies, of all Fabry patients, independently of the presence of clinical manifestations and its demonstration cannot constitute by itself evidence that LVNC is due to FD.

The fact that our clinical case remained stable and did not develop any complications during an eight-year follow-up period without specific therapy for FD also argues against the hypothesis that LVNC is a clinical manifestation or a risk marker for the development of other cardiac manifestations of FD.

Furthermore, the only screening study of FD in patients with LVNC did not reveal any positive case. However, this was a small cohort of 26 male patients and the only criteria for the LVNC diagnosis were the Stöllberger echocardiographic criteria. Yet, these patients presented a high frequency of ECG abnormalities (25/26), LVH (20/26), LV dilation (21/26), LV systolic dysfunction (19/26) and heart failure (21/26) [276].

Our study, on the other hand, included a larger cohort of patients and the inclusion criteria allowed LVNC diagnosis based on one or more criteria, either by echocardiography or cardiac MRI. Although a significant proportion of cases were incidental findings, there was a high agreement between echocardiogram and cardiac MRI regarding LVNC diagnosis. Of note, the absence of patients fulfilling the Stacey and Captur cardiac MRI criteria for LVNC may be explained by the lower use of these criteria in the clinical practice.

Moreover, our cohort of patients with LVNC seems to be representative of LVNC and its wide clinical spectrum. Nearly half of the patients presented LV systolic dysfunction and in the follow-up most patients developed complications (heart failure, dysrhythmias, thromboembolism). In a recent systematic review and meta-analysis including 2271 patients with LVNC from 35 studies, the demographic characteristics and the estimated mean frequencies of the clinical manifestations were comparable to the respective frequencies in our study: male gender (62% vs. 62%), mean age (45.7 vs. 47 years), VT (20% vs. 21.6%), supraventricular tachycardia (17% vs. 17.6%), thromboembolism (9% vs. 11.5%) [260]. Although the frequency of heart failure at clinical presentation seems lower in our study (42.7% vs. 34.6%) [260], 60.3% of the patients in our study came to develop heart failure in the follow-up. A recent literature review reported LV dysfunction on MRI in 40-73% and LGE in 10-55% [248]; in our study, LV systolic dysfunction by echocardiography and LGE were also within the reported ranges of frequencies (48.7% and 15.4%, respectively). Moreover, the same review reported 50-69% of asymptomatic cases [248], while in our study the frequency of incidental finding of LVNC was apparently lower (44.9%). Nevertheless, only 35.9% of our patients underwent genetic testing; the gene panel that was used included a variable number of genes and it is noteworthy that *TTN*, one of the most commonly involved genes in LVNC [254,256,257,259,260], was only tested in 2.6% of the patients, which is probably explained by the fact that some of these genetic studies had been performed many years before FD screening at a time *TTN* was not part of the gene panels and genetic study was not routinely used in the clinical practice given its lower diagnostic yield in LVNC. Moreover, only 6.4% of the patients had family history of LVNC, which might also explain the low frequency of pathogenic mutations (1/28, 3.6%). Neuromuscular diseases were also not systematically investigated in these patients.

Our study was also limited by a small sample size, which may have prevented the detection of FD. Although LVNC imaging criteria are commonly fulfilled in several patients' cohorts as well as in healthy controls and the general population [235,236,263,264,400,401], the frequency of patients with a clinical diagnosis of LVNC is lower, which limited the sample size, despite the inclusion of all patients diagnosed with LVNC in the study centres.

Nevertheless, no Fabry patients were diagnosed besides the single case already reported in the literature. Therefore, our study did not find any evidence to support the hypothesis that LVNC is a clinical manifestation of FD. Taken together with the available evidence, our results suggest that we should not start specific therapy for FD in young asymptomatic females presenting only imaging criteria for LVNC, in the absence of other cardiac abnormalities or known manifestations of FD that would constitute clinical indication to start therapy according to the current guidelines.

5.5 Novel pathogenic *GLA* variants

In the course of this investigation, the novel *GLA* variants c.607G>T (p.E203X) and c.286A>G (p.M96V) were detected and their pathogenicity was demonstrated as well as their association to the classical phenotype of FD, on the basis of the premises enumerated on the sections 5.1 and 5.3 of this doctoral thesis.

CHAPTER 6 – CONCLUSIONS

In the Portuguese region of Guimarães, the prevalence of FD in patients with HCM was unexpectedly high (16.6%), a finding that has been explained by the discovery of a founder effect of FD due to one of the most common *GLA* gene mutations, the p.F113L mutation.

Probands with the p.F113L mutation were originated from a particular region of Guimarães and genealogy research revealed a 12-generation family tree with a common ancestor to these patients, who lived in the 17th century in this region. Haplotype analysis of the p.F113L individuals was in line with the historical family structure. This founder effect occurred as a result of high endogamy and low migration rates, which were explained by the occurrence of marriages within the same socioeconomic stratum, the low socioeconomic status and the progressive impoverishment of these families and the fact that distant migration was not affordable to poor individuals until the 19th century.

More recently, with the generalization of migration, this founder mutation has been spread to many countries around the world (Spain, France, Monaco, United Kingdom, Luxembourg, Switzerland, Germany, Angola, Colombia). Therefore, the characterization of this late-onset phenotype has also a cross-border impact in the therapeutic decisions of many patients.

The mutation p.F113L was associated to a late-onset phenotype of FD with predominant cardiac involvement (LVH 40.2%, LGE 21.4%, heart failure 21.9%, atrial fibrillation 4.4%, non-sustained VT 8.9%, bifascicular block 13.1%, atrioventricular block 14.8%), in which bradydysrhythmias carried a higher prognostic impact than tachydysrhythmias, potentially leading to pacemaker implantation (6.4%), while no cases of sustained VT or SCD due to ventricular dysrhythmias were found.

The first cardiac manifestations were LVH and LGE, which emerged in males over 30 years and were followed by heart failure, non-sustained VT and cardiac conduction disorders, which emerged in males over 40 years, culminating with the development of bifascicular block and complete atrioventricular block in males beyond the age of 50 years. Cardiac manifestations were more common and arose one to two decades earlier in males and their frequency increased with age in both genders. Likewise, the severity of LVH and cardiac blocks also increased with age.

Myocardial ischemic events were rare manifestations of late-onset FD; most of them were acute coronary syndromes secondary to atherosclerotic coronary heart disease, which is a common comorbidity with ageing.

The designation of this phenotype as “cardiac variant” clearly underestimates its systemic involvement. Extracardiac manifestations included albuminuria A2/A3 (33.7%), brain WML (50.3%), sensorineural deafness (44.7%), cornea *verticillata* (10.3%) and carpal tunnel syndrome (22.3%). Moreover, the earlier manifestations were extracardiac, with pathological albuminuria, brain WML and sensorineural deafness arising before 30 years of age in both genders.

The demonstration of lysosomal inclusions on renal podocytes of young, otherwise healthy, p.F113L patients with proteinuria, as well as the finding of pathological albuminuria and brain WML in young patients without comorbidities, support the conviction that proteinuria and brain WML in p.F113L patients are secondary to FD.

However, the prognostic impact of extracardiac manifestations seems to be lower, as no cases of renal dialysis or transplant were found, the frequency of CKD stage ≥3 was similar to the one observed in the general population, cerebrovascular events were rare and hearing aids were seldom indicated. Moreover, the clinical significance of brain WML remains to be elucidated.

Plasma lyso-GB3 levels were within the range reported in late-onset phenotypes, i.e. modestly high in males and always below the lower limit of quantification in females regardless of clinical manifestations, being therefore a poor biomarker for predicting the risk of complications in females.

The natural history of the p.F113L phenotype presented many similarities to the natural history of other predominant cardiac phenotypes, suggesting a general common natural course to these FD variants, despite the different *GLA* gene mutations. Nevertheless, our research came to fill many evidence gaps on the natural history of these FD phenotypes, namely on heart failure, cardiac rhythm and conduction disturbances, ischemic myocardial events, sensorineural deafness and carpal tunnel syndrome.

Our research improved the knowledge on the natural history of late-onset FD, which is essential to make evidence-based clinical decisions and guidelines and to accurately evaluate the clinical efficacy of FD therapies.

In our large Portuguese multicentre study, FD prevalence in HCM was 0.9%. Bifascicular block and basal inferolateral LGE were the most powerful predictors of FD in patients with HCM. In the absence of both bifascicular block and basal inferolateral LGE, FD is a less probable cause of HCM, being more appropriate to perform an HCM gene panel as the next step of the etiological study of HCM.

Screening of FD in patients with LVNC found no Fabry patients besides the single case that we had previously reported, which argues against the hypothesis that LVNC is a cardiac manifestation of FD.

Taken together with the available evidence, our results do not support the use of the current diagnostic criteria of LVNC for the decision to start specific therapy for FD, either enzyme replacement or chaperone therapy, in young asymptomatic females presenting LVNC criteria in the absence of other cardiac abnormalities or known manifestations of FD that would constitute a clinical indication to start therapy according to the current guidelines.

Our FD screenings led to the identification of novel pathogenic *GLA* variants, namely the c.286A>G (p.M96V) and the c.607G>T (p.E203X), both causing a classical phenotype of FD.

Finally, our investigation has contributed to the diagnosis of a large number of FD patients in our centre in the past decade, which currently makes of our centre the largest in Portugal and one of the largest in Europe on FD. Consequently, a great experience and knowledge has been accumulated and an intense investigation has been promoted on this field in the past decade in our centre, which has culminated in the designation of Hospital Senhora da Oliveira – Guimarães as National Reference Centre on Lysosomal Storage Disorders (Despacho n.º 5911-A/2016) and European Reference Centre on Inherited Metabolic Disorders, which officially integrates since 2016 the European Reference Network on Hereditary Metabolic Disorders (MetabERN).

CHAPTER 7 – FUTURE DIRECTIONS

The discovery of a founder effect of FD in the Portuguese region of Guimarães and the subsequent diagnosis of a large cohort of patients with late-onset FD, who carry the same *GLA* gene mutation and share common environmental factors, offered the unique opportunity to better understand and define the natural history of the late-onset phenotypes of FD with predominant cardiac involvement.

Now that the natural history of FD associated with the p.F113L mutation has been documented, it is possible to proceed with the next steps of the investigation: (i) To find the genetic modifiers and determinants of FD phenotype; (ii) To unravel early markers of cardiac damage and predictors of progression of cardiac disease; (iii) To evaluate the efficacy of current FD specific therapies and particularly its impact on prognosis of patients with late-onset phenotypes with predominant cardiac involvement; (iv) To contribute to clarify some unresolved issues related to FD therapy, such as the optimal timing to start therapy, the optimal dose of ERT and the clinical impact of anti-drug antibodies; (v) To evaluate the efficacy and safety of emerging new FD therapies in late-onset phenotypes with predominant cardiac involvement.

7.1 Genetic determinants of Fabry disease phenotype

The study on the natural history of this late-onset phenotype showed that the patients presented a high clinical heterogeneity, despite sharing the same *GLA* mutation as well as some other genetic background and common environmental factors, as a result of the founder effect.

Indeed, some patients seem to develop an earlier and more severe phenotype, while others seem to progress more slowly. Cardiac involvement occurred in all males, but the age of onset of LVH widely varied from their thirties to their fifties. The age of development of LGE, an imaging correlate of cardiac fibrosis, also varied in males from their thirties to beyond 60 years of age, occurring in some patients before or simultaneously with the development of LVH, but in others only later when severe LVH was already established. This clinical heterogeneity on the onset and progression of cardiac manifestations was also encountered in females. Furthermore, some patients presented renal involvement, while others did not, and our research has already shown that the renal manifestations were indeed caused by FD, as lysosomal inclusions were demonstrated on renal podocytes of young Fabry patients, without known comorbidities, who presented with proteinuria. Moreover, some males and females exhibited brain WML before 30 years of age and in the absence of other comorbidities, while others only developed them at advanced age.

Random X-chromosome inactivation occurs in heterozygote females and prediction of their ultimate disease course is challenging. In females with non-skewed X-chromosome inactivation, a cross-correction of the cells with enzymatic deficiency by the cells with normal enzymatic activity does not seem to occur, so most of these females will develop symptoms with increasing age. Skewed X-chromosome inactivation occurs in 29% of the females, either preferentially expressing or suppressing the pathogenic mutation, and the direction of skewing has been correlated with the disease severity and progression [22].

However, this high clinical heterogeneity [277], within the same family and in both genders, raises the hypothesis that other genetic factors, beyond the *GLA* mutation and the X-chromosome inactivation in females, could determine the resultant FD phenotype. Some studies have also suggested that phenotype may be influenced by other genetic modifiers beyond the *GLA* mutation [310,422-429], as well as epigenetics [430] and other comorbidities and environmental factors, such as cardiovascular risk factors which are common in the general population and carry a major impact in Fabry vasculopathy [178,431].

The study of our large cohort of Fabry patients, who share the same *GLA* mutation and other genetic and environmental factors, provides the perfect opportunity to find the genetic determinants of FD phenotype, using broad genetic approaches, such as whole exome sequencing or whole genome sequencing, as well as X-chromosome inactivation studies.

To address this question, a study, based on whole exome sequencing and X-chromosome inactivation analyses, is already underway in our centre.

7.2 Early markers of cardiac damage and predictors of faster progression of cardiac damage in Fabry disease

7.2.1 Analytical biomarkers

Biomarkers of glomerular (transferrin and type IV collagen) and tubular (α1-microglobulin, N-acetyl-β-glucosaminidase, and alanine aminopeptidase) dysfunction have been shown to be elevated in FD patients, even in patients without pathological albuminuria, emerging as candidate early markers of Fabry nephropathy. Moreover, significant inverse correlations have been found between eGFR and type IV collagen or N-acetyl-β-glucosaminidase, which were stronger than with albuminuria [432].

A biomarker of collagen synthesis, procollagen III N-terminal propeptide (PIIINP), has been found elevated in FD patients with mild symptoms, being a possible early marker of organ damage in FD [433].

Early markers of Fabry cardiomyopathy have also been described. Collagen biomarkers have been shown to be elevated in Fabry patients, both with and without LGE, compared to healthy controls [123]. Additionally, carboxyterminal propeptide of procollagen type I (PICP), a biomarker of collagen type I synthesis, has been reported to be increased in Fabry cardiomyopathy, even in the earlier stages of the disease, when echocardiogram is still normal, thus being the best predictor of LV mass and a good predictor of LGE [434]. Angiostatin and matrix metalloproteinase 9 (MMP-9) have also been found to be elevated in FD patients compared to controls independently of the presence of Fabry cardiomyopathy, further supporting an increased extracellular matrix turnover in FD patients [435]. Galectin-3, a marker of cardiac fibrosis, has also shown to be increased in unaffected FD patients compared to healthy controls, and a correlation has been reported between this marker and LV mass index, LVH, NYHA class, NT-proBNP and high sensitivity troponin T levels [436].

Proteomics based-studies have already identified some potential candidates for early biomarkers of Fabry nephropathy in urine, blood and renal cells/tissue. While some of them hold promise, such as uromodulin, none has yet been adopted into the clinical practice [437,438]. Moreover, the detection of specific up or downregulated proteins may enable the definition of proteomic signatures specific to FD and to its classic/late-onset phenotypes as well as gender-related fingerprints of the disease, which may be helpful in the interpretation of the significance of a GVUS [437,438]. In the SOPHIA study, urinary proteomics has revealed increased levels of collagen alpha-1 (I), (III), (VII), and collagen alpha-3 (V) in FD patients with mild symptoms, which may be potential early biomarkers of organ damage in FD. Moreover, a significant correlation has been found between these markers and LGE volume in these patients [433]. However, proteomics based-studies specifically on Fabry cardiomyopathy are lacking.

Metabolomics studies have revealed several altered analogs/isoforms structurally related to GB3 and lyso-GB3 in urine and in plasma of FD patients [438]. Plasma lyso-Gb3 and sphingosine-1-phosphate have been shown to be increased in FD patients with mild symptoms; and plasma lyso-GB3 has been shown to be correlated with LGE volume [433]. However, there are no metabolomics based-studies specifically on Fabry nephropathy or cardiomyopathy [438].

Transcriptomics approach has also revealed that fibrosis suppressors miR-29 and miR-200 are decreased in the urine of FD patients compared to healthy subjects, while the fibrosis activators miR-21, miR-192, and miR-433 are similarly expressed. This transcriptomic profile has been detected in an early stage, before pathological albuminuria has occurred, supporting its usefulness as an early biomarker of renal fibrosis [439]. However, transcriptomics based-studies on Fabry cardiomyopathy are lacking.

7.2.2 Cardiac imaging biomarkers

Several cardiac imaging biomarkers have been proposed as early markers of cardiac damage in FD, based on TDI and strain parameters on echocardiography, T1 and T2 mapping on cardiac MRI and ^{18}F-FDG uptake on PET [7].

On echocardiography, LV subclinical dysfunction can be detected before the development of LVH both by TDI [104,440] and two-dimensional speckle-tracking [96,97]. Indeed, E' and S' velocities [104,440] and LV longitudinal [96,97], circumferential and radial strain [97] have been found to be reduced in the pre-hypertrophic stage.

On cardiac MRI, a low native T1 time has been documented in 40% of Fabry patients without LVH [84], being associated with ECG abnormalities and worse LV wall thickness and mass [85,86], LA size [86,87], E/E' ratio, LV GLS and inferolateral longitudinal strain [87], LV GLS by feature tracking [88], LGE [85] and worse MSSI and clinical worsening (Fabry Stabilization Index >20%) [86]. In addition, LGE has been associated with increased troponin and high basal inferolateral wall T2 [85], which in turn was associated with increased troponin and NT-proBNP, GLS impairment and electrocardiographic abnormalities (long PR, complete bundle branch block, LVH voltage criteria, long QTc, and T-wave inversion) and predicted clinical worsening after 1 year (Fabry stabilization index >20%) [80].

Accordingly, it has been proposed that normal values of high sensitivity troponin, in combination with normal ECG and echocardiogram, indicate that FD cardiomyopathy is unlikely; elevated values of high sensitivity troponin indicate advanced FD cardiomyopathy; while borderline values should lead to a thorough investigation including cardiac MRI [441].

Finally, on PET, focal ^{18}F-FDG uptake has been shown in FD females before LGE [442], but also before LVH, being associated with lower LV GLS [82].

The mutation p.F113L is associated to a late-onset phenotype of FD with a predominant cardiac involvement [27,192]. Our studies on this late-onset phenotype have already described its cardiac manifestations and their natural history, demonstrating a considerable heterogeneity among male and female patients regarding their age of onset and progression.

Now the next step of our investigation should be to perform an in-depth characterization of the cardiac phenotype throughout the course of the disease, considering all the cellular and subcellular phenomena that take place in the heart of Fabry patients, including sphingolipid deposition, hypertrophy, fibrosis and inflammation. For that purpose, it would be useful to evaluate (i) analytical markers of collagen turnover and inflammation; (ii) proteomics, metabolomics and transcriptomics profile; (iii) and advanced cardiac

imaging findings (two and three-dimensional echocardiography with strain analysis and cardiac MRI including T1 and T2 mapping) in patients throughout the different stages of the disease.

This study might enable to find early markers of cardiac damage as well as predictors of cardiac disease, its earlier onset or faster progression.

The best timing to start therapy in FD is not clear, particularly in late-onset phenotypes [46]. While current guidelines recommend to start therapy in these patients as soon as there are signs/symptoms of FD [41], some studies have already shown that early treatment is better than late treatment [443,149,217,218,444,445]. However, how early should the treatment be started is not yet defined, although from a theoretical point view it would make sense to start treatment before target organ damage occurs.

If future studies come to demonstrate clinical benefit from early treatment in the subset of patients presenting with these high-risk biomarkers, the treatment paradigm may change in FD and biomarkers may become providential in the clinical practice to guide decisions on therapy in individual patients at an early stage, before target organ damage occurs (Precision Medicine).

Moreover, studies specifically addressing the efficacy of therapies on late-onset phenotypes are also needed to ascertain if all patients benefit from therapy. If that would not be the case, the holy grail would be, within the high clinical heterogeneity of late-onset phenotypes, to correctly select the patients who could benefit or benefit the most from therapy. In this setting, these biomarkers could also be useful to identify the patients already with target organ damage, who present higher risk of faster disease course and potentially derive benefit or more benefit from therapy (Precision Medicine).

In order to find these high-risk biomarkers, a study, based on advanced cardiac imaging, markers of collagen turnover and transcriptomics, is already underway in our centre.

7.3 Efficacy of current Fabry disease specific therapies

A large body of literature has demonstrated the efficacy and safety of ERT on the treatment of FD [46].

In males, agalsidase alfa has been demonstrated to decrease plasma GB3 [142,446,447] and lyso-GB3 [448] and urinary GB3 [142,447] levels; to decrease GB3 deposits in kidney endothelial cells [446]; to slow the decline of the eGFR [446,447]; to reduce/stabilize LV mass [142-144] and wall thickness [144]; to improve/stabilize vestibular/auditory symptoms [449,450]; to improve nerve sensitivity [451], gastrointestinal symptoms [452], sweat function [452], pain [446], and pain-related quality of life [446].

In females, agalsidase alfa has been demonstrated to decrease plasma GB3 [145,146], plasma lyso-GB3 [448,453] and urinary GB3 [145,146] (when pre-treatment values were elevated); to stabilize/decrease the decline of eGFR [144,145,146,454]; to decrease/stabilize LV mass [143-146] and wall thickness [144]; to improve exercise capacity [146]; to stabilize hearing loss and vestibular function [450]; and to improve quality of life [145].

In males, agalsidase beta has been shown to decrease plasma GB3 [147,453,455,456] and lyso-GB3 [453] and urinary GB3 [456] levels; to decrease GB3 deposits in different kidney cell types [455-457] and endothelial cells in skin [455,456]; to slow the decline of eGFR [148,456,458]; to reduce/stabilize LV mass [147-150] and wall thickness [147,150]; to improve nerve sensitivity [459], gastrointestinal symptoms [460], sweat function [459,461], pain [461], and quality of life [455,461].

In females, agalsidase beta has been shown to stabilize plasma GB3 (when pre-treatment values were normal) [453]; to decrease plasma lyso-GB3 [453] (when pre-treatment values were elevated); to stabilize eGFR [148]; to decrease/stabilize LV mass [148,150] and wall thickness [150]; and to improve quality of life [462].

More recently, evidence has been growing on the efficacy of the pharmacological chaperone migalastat [46].

In the FACETS trial, in the modified-intention to treat population (i.e., ERT-naïve patients with migalastat-amenable *GLA* mutations), migalastat, compared to placebo, significantly reduced the plasma lyso-GB3 and the mean number of GB3 inclusions/kidney interstitial capillary at 6 months. There were no significant differences in baseline levels or changes from baseline to month 6 between the groups regarding eGFR, measured glomerular filtration rate (GFR), 24 h-urine protein excretion, and 24 h urinary GB3. At 24 months of migalastat therapy (i.e., after 18 months of migalastat in patients who switched from placebo or 24 months of continuous migalastat), there was a significant reduction of the mean LV mass index compared to the baseline [152]. An improvement in diarrhea was also found after 6 months of migalastat compared to placebo, and this benefit was sustained at 24 months [152,463]. Those results were also confirmed in the subgroup of classic males [464]. Migalastat treatment was also demonstrated to decrease the mean total GB3 inclusion volume per podocyte in renal biopsies from baseline to 6 months; and this reduction correlated with the reduction in mean podocyte volume [465].

In the ATTRACT trial, in ERT-experienced patients with amenable *GLA* mutations who were randomized to switch to migalastat or continue ERT, renal function and plasma lyso-GB3 levels were maintained during 18 months of migalastat or ERT. Migalastat significantly reduced the mean LV mass index at 18 months, and changes on LV mass index correlated with changes in the thickness of the IVS and not the PW. No

significant change of the mean LV mass index was found in the ERT group; however, the randomization ratio of migalastat to ERT of 1.5 to 1, the existence of non-amenable mutations in the initial cohort, and the higher discontinuation rate on the ERT group resulted in a small number of evaluable patients under ERT at the end of the study (16 patients), which may have influenced the results regarding ERT [153]. In the open-label extension study, a significant decrease of LV mass index was found after 30 months of migalastat in patients with LVH at baseline [154].

In real world conditions, Muntze et al. reported one patient with an improvement of LV mass, LGE, troponin, and NT-proBNP after 12 months of treatment with migalastat [155]. Later, the same authors showed that, in 14 patients treated with migalastat for 1 year, LV mass index significantly decreased, while plasma lyso-GB3 significantly decreased in naïve patients and remained stable in patients switched from ERT. However, eGFR significantly worsened, a finding that contrasted to the results in the pivotal clinical trials. The authors hypothesized that these results could have been partly explained by the simultaneous initiation of angiotensin converting enzyme inhibitors and underlined the need for further studies with longer follow-up of the renal function [156].

In a larger study including 59 patients, treatment of previously ERT-treated and untreated FD patients with migalastat for 12 months was generally safe and also resulted in a significant decrease of LV mass index. However, plasma lyso-GB3 levels were stable both in males and females, irrespective of previous treatment regimen; and increasing plasma lyso-GB3 was noted in a few males and females, some of whom carried *GLA* mutations whose amenability to migalastat has been questioned. Moreover, eGFR continued to decline under migalastat in both males and females. The loss of renal function did not seem to be explained by a more severe renal impairment and disease load at baseline. Instead, higher decline of eGFR was more common in patients with a systolic blood pressure below 120 mmHg. Additionally, females with *GLA* mutations, classified as non-amenable by the in-house assay based on *GLA*-knockout HEK cells, had higher decline of eGFR. This study recommended to avoid systolic blood pressure values below 120 mmHg and alerted that “in vitro” amenability may not always reflect “in vivo” amenability to migalastat, emphasizing the importance of monitoring clinical response to therapy [158].

In a smaller study of seven FD males, who switched from ERT to migalastat, a significant decrease of LV mass index was also observed after 1 year of treatment with migalastat, while plasma lyso-GB3 remained stable. Unlike previous real-life studies, eGFR was stable and proteinuria significantly decreased [157]. Therefore, further studies are needed to understand the discrepant results of migalastat on renal function.

7.3.1 Effect of Fabry disease therapies on central nervous system

Knowledge on the pathophysiology of central nervous system manifestations is limited in FD [320], particularly in the late-onset phenotypes, which are typically characterized by the absence of endothelial GB3 deposits [202]. Our studies have shown that cerebrovascular events are rare, but brain WML are common in the late-onset phenotype due to the p.F113L mutation and the same has been reported for other *GLA* mutations associated to late-onset phenotypes with predominant cardiac involvement, such as the IVS4+919G>A mutation [184,185,186,210,211] and the p.N215S mutation [181,182].

However, none of the recombinant enzyme preparations can pass the blood–brain barrier and the effect of ERT on the incidence of cerebrovascular events or the development or progression of brain WML remains to be elucidated [46].

Although a recent meta-analysis has suggested that ERT may have a protective role against stroke occurrence [466], there are no studies so far evaluating the effect of agalsidase alfa or agalsidase beta specifically on cerebrovascular events. Moreover, stroke has been reported to occur despite ERT, either with agalsidase alfa [146,447,467] or beta [468,469].

During a 2-year follow-up of six patients under agalsidase alfa, brain WML remained stable in three patients, worsened in one, fluctuated in one, and improved in one patient; during this time, some brain WML disappeared and others appeared on brain MRI [470]. During a mean follow-up of 27 months, agalsidase beta seemed to stabilize brain WML on MRI compared to placebo, as a statistically significant greater proportion of younger patients (≤50 years) under agalsidase beta had stable WML compared with younger patients under placebo (44% vs. 31%) [471]. However, new brain WML have been detected in 48% of males over a median period of 3.1 years and in 28% of females over a median period of 4.0 years of ERT with either agalsidase alfa or agalsidase beta [472]. Finally, ERT has not been identified as an independent predictor of the annual progression rate of brain WML [473].

On the other hand, migalastat, as a small molecule, is likely to have enhanced cellular and tissue distribution and potential to cross the blood-brain barrier [46,474], as suggested by the finding of increased α-galactosidase A activity and reduced GB3 levels in the brain of Fabry transgenic mice [475]. However, so far there is no evidence on the clinical efficacy of migalastat at the central nervous system manifestations [46].

Therefore, studies will be needed to understand the effect of ERT and migalastat on central nervous system manifestations, including in late-onset phenotypes. Efficacy data of these therapies on our cohort of FD patients will be valuable to better understand this matter.

7.3.2 Impact of Fabry disease therapies on mortality and major clinical events

Evidence is specifically lacking on the effect of these therapies on hard-endpoints, such as mortality and major clinical events and data on this subject are restricted to very few studies on ERT that present some important limitations.

In a randomized clinical trial, agalsidase beta, compared to placebo, significantly reduced the time to first clinical event in protocol-adherent patients, when adjusted for baseline proteinuria (considering clinical events defined as renal: 33% increase in serum creatinine level from baseline (2 consecutive values) or end-stage kidney disease requiring long-term dialysis or transplantation; cardiac: myocardial infarction; new symptomatic arrhythmia requiring antiarrhythmic medication, pacemaker, direct current cardioversion, or defibrillator implantation; unstable angina defined by national practice guidelines and accompanied by electrocardiographic changes resulting in hospitalization; or worsening congestive heart failure requiring hospitalization; cerebrovascular: stroke or transient ischemic attack; or death). However, most clinical events were renal and these were limited to the increase in serum creatinine, while there was a low rate of cerebrovascular events and death [445]. Moreover, agalsidase beta has reduced the incidence rate of clinicals events after 6 months of treatment, maintaining, from 6 months to up to 5 years of treatment, a stable incidence rate of clinical events (renal: chronic dialysis (>40 days) or renal transplantation; cardiac: myocardial infarction, first-time congestive heart failure, atrial fibrillation, VT, evidence of progressive heart disease sufficiently severe to require a pacemaker, heart bypass surgery, coronary artery dilatation or implantation of a cardioverter-defibrillator; cerebrovascular: haemorrhagic or ischaemic stroke; or death due to any cause) [476].

Likewise, in a study comparing patients treated with agalsidase alfa from the Fabry Outcome Survey registry with historical cohorts of untreated patients, the median age at first event seemed to be higher in patients under agalsidase alfa than in untreated patients, both in males (48 vs. 41 years) and females (57 vs. 53 years). The median survival time also seemed to be higher in males under agalsidase alfa than in untreated males (77.5 vs. 60 years). However, these findings have to be interpreted with caution because this study was a retrospective analysis that used a historical cohort as a comparator group [477].

On the other hand, there are no data so far regarding the effect of migalastat on mortality or composite endpoints of major clinical events.

It is particularly relevant to determine the effect of FD therapies on these hard-endpoints in late-onset phenotypes, given the later onset, slower progression and more attenuated clinical course of these phenotypes.

To fill in these evidence gaps, large cohorts of patients are needed and our large cohort of patients with a well-characterized late-onset phenotype may contribute to increase the knowledge on this subject.

7.3.3 Impact of Fabry disease therapies on late-onset phenotypes

Early studies on the natural history of FD included mainly classic patients with severe phenotypes. The lower awareness of the disease and the absence of specific therapies contributed in the past to a low diagnostic rate, a late diagnosis and a preferential diagnosis of the most severe cases. Therefore, those historical cohorts of FD in early studies were mainly composed by classic patients with severe phenotypes and long-standing disease.

Since FD therapy became available, studies evaluating therapeutic efficacy have included patients with a high genetic heterogeneity and a wide range of phenotypes, from the most severe classic phenotypes to the attenuated late-onset phenotypes with variable organ involvement. However, studies aiming to evaluate the effect of FD therapies specifically on late-onset phenotypes with predominant cardiac involvement are scarce and, given their slower and attenuated disease course, a longer follow-up is probably necessary to assess the prognostic impact of these therapies.

The evaluation of ERT efficacy in the late-onset phenotypes with predominant cardiac involvement has been limited to a few small studies on the IVS4+919G>A mutation [7]. In these patients, a significant negative correlation has been described between ERT duration and GB3 accumulation in cardiomyocytes and cardiomyocytes size [209]. In patients under ERT for more than 3 years, GB3 deposits were not found in cardiomyocytes [307]. In a study including 23 patients with the IVS4+919G>A mutation, plasma lyso-GB3 decreased under ERT both in males and females, reaching the lowest value at 11.1 months and increasing gradually thereafter, even when LV mass index was still improving or remaining stable. Indeed, no correlation was found between plasma lyso-GB3 change and LV mass index change under ERT, making it an unreliable marker for the long-term monitoring of the therapeutic response [208]. Finally, in another study, including 26 patients with the IVS4+919G>A mutation under ERT for 6-39 months, a significant reduction of mean plasma lyso-GB3 (on average by 28%) has been reported in males and females, with 89% of patients experiencing a reduction of plasma lysoGB3. A stabilization or reduction of LV mass index, IVS and PW thickness has been observed in 83%, 83% and 67% of patients, respectively, with an average decrease of 12% in LV mass index, 14% in IVS and 13% in PW thickness. Mean LV mass index, IVS and PW thickness have been found to decrease both in males and females, although only reaching statistical significance for LV mass index and PW thickness in females. Mean eGFR has been reported to be stable both in males and females. A reduction of microalbuminuria has been observed in 15 of 16 patients, with

an average decrease of mean urinary albumin/creatinine ratio of 66%; however, the respective values at baseline and after ERT were not statistically different neither in males nor in females. MSSI has also been found to decrease in males and females, although statistical significance has only been achieved in females [184].

No data has been published on the efficacy of migalastat specifically on the late-onset phenotypes.

Hence, more and larger studies with longer follow-up periods and focused on other *GLA* mutations are needed to understand the effect of FD therapies on late-onset phenotypes with predominant cardiac involvement.

Now that the natural history of the phenotype due to the p.F113L mutation has been established and now that a significant number of these patients are under ERT and migalastat for a sufficient period of time, it will be possible, during the coming years, to accurately determine the effect of these therapies on the disease course and prognosis of patients with this late-onset cardiac predominant phenotype.

It would be particularly relevant to determine if all patients with late-onset phenotypes benefit from therapy and what is the therapy (or combination of therapies) that results in the best clinical outcome.

7.4 Other unresolved issues on Fabry disease therapy

7.4.1 Optimal timing to start Fabry disease therapy

The optimal timing to start FD therapy is not clear, particularly on late-onset phenotypes [46]. According to the current recommendations, ERT should be initiated (i) in classic males at the age of 16 years regardless of symptomatic status, although it should be considered earlier, on an individual basis, since the age of 8–10 years old; and (ii) in late-onset males and in classic/late-onset females with signs/symptoms of FD (LVH, cardiac fibrosis, cardiac rhythm or conduction abnormality, microalbuminuria/proteinuria non-attributed to other causes, CKD (eGFR <90 mL/min/1.73 m2), stroke or transient ischemic attack, neuropathic pain, gastrointestinal symptoms such as abdominal pain or diarrhoea, anhidrosis/hypohidrosis). In asymptomatic late-onset males and classic/late-onset females, ERT could be considered in the presence of moderate or severe GB3 deposits, podocyte foot process effacement, or glomerulosclerosis on kidney biopsy [41].

Nevertheless, early treatment has been shown to achieve better outcomes. Classic males who started ERT before the age of 25 years achieved greater reduction of plasma lyso-GB3 than the ones who started after that age [443]. Patients who started agalsidase beta at the age <30 years experienced a statistically

significant decline in LV mass, while those who started it at the age ≥50 years suffered an increase in LV mass [149]. Likewise, patients who started agalsidase beta at the age <40 years had stable thickness of the IVS and PW over a period of 10 years, whereas patients who started it at the age ≥40 years experienced a significant progression of those parameters over time [217]. Moreover, in patients without fibrosis, agalsidase beta resulted in a statistically significant decline of LV mass and improvement of exercise capacity and LV radial strain rate, while no effect was observed in patients with mild or severe fibrosis at the time of treatment initiation [218]. Additionally, the slope of eGFR decline was lower in patients who started agalsidase beta earlier since symptom onset and before the development of significant glomerulosclerosis and proteinuria [217,458].

Finally, the risk of clinical events was lower in patients who started agalsidase beta at younger age and before severe organ damage [444]. Accordingly, agalsidase beta, compared to placebo, significantly reduced the time to first clinical event in the subgroup of patients with eGFR >55mL/min/1.73m^2, but not in the subgroup of patients with eGFR≤55mL/min/1.73m^2 [445]. Likewise, in patients treated with agalsidase alfa, LVH or low eGFR at the time of treatment initiation were associated with a higher risk of a cardiovascular event; and low eGFR at the time of treatment initiation was associated with a higher risk of a renal event [478].

However, how early should the treatment be started is not yet clear and further studies will be needed to ascertain the best timing to start FD therapy, particularly in patients with late-onset phenotypes, and if that timing is the same for all these patients.

7.4.2 Optimal dose of enzyme replacement therapy

The optimal dose of ERT remains unclear both in classic and late-onset phenotypes [46].

In classic males, the reduction in plasma lyso-GB3 was significantly greater in patients treated for 1 year with agalsidase beta at the dose of 1.0 mg/kg than in patients treated with agalsidase alfa or beta at the dose of 0.2 mg/kg [453]. In another study, the reduction of plasma lyso-GB3 in classic males treated for 1 year with agalsidase beta 1 mg/Kg was greater than with agalsidase alfa 0.2 mg/Kg, and the same was observed in females and non-classic males. Additionally, a higher proportion of patients had a decrease in LV mass index when treated for 1 year with agalsidase beta than with agalsidase alfa (79% vs. 62%). Nevertheless, no difference between agalsidase alfa and beta was found regarding eGFR or clinical events [479]. Another study showed that 19.4% of patients on agalsidase alfa 0.2 mg/Kg and 13.3% of patients on agalsidase beta 1 mg/Kg progressed, during the 59-month study duration, to a composite clinical endpoint consisting of renal events (development of end-stage renal disease or sustained decline in GFR

of 50% or greater for more than 30 days and for which other causes besides FD had been excluded), cardiovascular events (pacemaker or other intracardiac device, coronary artery bypass grafting, valve replacement surgery, coronary angioplasty or stent, cardioversion, hospitalization or emergency room visit for unstable angina/acute coronary syndrome, myocardial infarction, congestive heart failure, tachy or brady-arrhythmia, heart block, cardiac arrest), cerebrovascular events (transient ischemic attack or stroke documented by a physician or acute hearing loss), or death. However, these differences were not significant due to limited power [480].

The dose reduction of agalsidase beta from 1 mg/Kg eow to 0.3 mg/Kg eow for 18 months allowed to maintain the clearance of GB3 deposits in the kidney interstitial capillary endothelium in 90% of the patients; and the clearance/reduction of GB3 deposits in other renal cells and superficial dermal capillary endothelium in only 70% of the patients. Additionally, urinary GB3 increased significantly after dose reduction [456]. In another study, the dose reduction of agalsidase beta from 1 mg/Kg eow to 0.5 mg/Kg per month for 1 year led to a significant increase of plasma lyso-GB3 in males, but no differences were seen on clinical events, MSSI, pain, eGFR, or LV mass [481]. In the Fabry registry, the dose reduction of agalsidase beta resulted in lower self-reported energy levels in males, but no difference in MSSI, FD Severity Scoring System (DS3), Brief Pain Inventory (BPI), or Short Form 36 Health Survey Questionnaire (SF-36) scores [482].

Patients under agalsidase beta 1 mg/kg eow, who switched directly to agalsidase alfa 0.2 mg/Kg eow or suffered a dose reduction to agalsidase beta 0.3–0.5 mg/kg eow and then switched to agalsidase alfa 0.2 mg/Kg eow, showed a greater eGFR decline and an increase of MSSI, gastrointestinal symptoms [483,484], and pain [483], although clinical events remained stable for 2 years [484]. In three young classic males under agalsidase beta 1 mg/kg eow for 5 years, there was a complete clearance of GB3 from mesangial and endothelial cells and a partial clearance from podocytes. Three years after the switch to agalsidase alfa 0.2 mg/Kg eow, there was reaccumulation of GB3 in podocytes. One patient switched back to agalsidase beta 1 mg/Kg eow and two years later again showed reduction of GB3 in podocytes. Additionally, pain and gastrointestinal symptoms worsened in all three patients following the switch from agalsidase beta to alfa [485]. However, in another study, patients under agalsidase beta 1 mg/kg eow who switched to agalsidase alfa 0.2 mg/Kg eow did not show any differences on plasma lyso-GB3 or GB3, eGFR, proteinuria, or LV mass index for 2 years [448].

A study comparing patients under agalsidase alfa 0.2 mg/Kg eow and 0.2 mg/kg weekly for one year did not find any significant differences in plasma GB3, LV mass, eGFR, albuminuria, 6-min walk test, or quality of life. Exploratory analyses of patients under 0.4 mg/kg weekly did not find any differences either [486].

Additionally, a study comparing patients under agalsidase alfa 0.1 mg/kg weekly, and 0.2 mg/Kg eow and 0.2 mg/Kg weekly for 4 weeks, did not find any significant differences on plasma GB3, quality of life, or pain, although there was a trend for a higher sweat volume and a lower urinary GB3 with the weekly dose of 0.2 mg/Kg [487]. Conversely, in patients with progressive decline of renal function despite agalsidase alfa 0.2 mg/kg eow for 2–4 years, the switch to 0.2 mg/Kg weekly significantly decreased the annual slope of eGFR during a 10 year-study, significantly delaying the time to end-stage renal disease [488,489].

Further studies are needed to clarify what is the optimal dose of ERT both in patients with classic and late-onset phenotypes and our unique cohort of Fabry patients with a late-onset phenotype can contribute to answer this question.

7.4.3 Clinical impact of anti-drug antibodies

The clinical impact of anti-drug antibodies in Fabry patients treated with ERT remains to be clarified [46]. Anti-drug antibodies develop in the first 3–6 months of ERT [490,491], mostly in classic males [492]. Anti-drug antibodies have been reported in 91% of males treated with agalsidase beta [493] and 20% of males treated with agalsidase alfa [142], although no significant difference in their formation has been reported when the same dosage (0.2 mg/kg eow) was used for both drugs at ERT initiation [494]. Antibodies have shown in vitro cross-reactivity to both agalsidase alfa and beta [491].

IgG antibodies measured by enzyme-linked immunoassay (ELISA) mediate a neutralizing activity [491]. Neutralizing anti-drug antibodies develop in about 40% of all ERT-treated males [490,495] and mostly in patients treated with agalsidase beta [479]. Their formation seems to be irreversible, and the majority of patients positive for neutralizing anti-drug antibodies remain as so over 10 years [479,494,495]. These anti-drug antibodies bind and neutralize ERT in plasma and lead to activation of macrophages that internalize the ERT-antibody complexes, decreasing the cellular uptake of ERT [491].

Neutralizing anti-drug antibodies may attenuate ERT efficacy. Anti-drug antibodies have been associated to higher frequency of GB3 deposits in endothelial cells of the skin [496] and higher plasma lyso-GB3 [479,494,495,497] and urinary GB3 [491,494]. Although previous reports have suggested that anti-drug antibodies had no effect on the time to first clinical event or eGFR slope [496], other studies have shown that they were associated to worse renal function [495,497] and higher LV mass, disease severity scores, and frequency of symptoms [495]. Increasing the ERT dose in patients with established anti-drug antibodies may saturate them and allow for the excess enzyme to perform its catalytic function, thereby

attenuating the negative effect of anti-drug antibodies in plasma lyso-GB3, eGFR, and LV wall thickness [498].

Although anti-drug antibodies develop mostly in in classic males [492], little is known about its clinical impact specifically on late-onset phenotypes. To clarify this question, a study is underway in our centre.

7.5 Emerging new therapies for Fabry disease

Current therapies have not shown to be able to fully revert FD pathology and clinical manifestations. Therefore, several new therapies are under research, including new forms of ERT, such as pegunigalsidase alfa and moss-derived α-galactosidase A; substrate reduction therapy, such as lucerastat and venglustat; mRNA therapy; and genetic therapy [46,499,500].

Pegunigalsidase alfa, a chemically modified α-galactosidase A enzyme produced in a tobacco plant cell based ProCellEX system, is constituted by two subunits of α-galactosidase A covalently bound by a chain of polyethylene glycol (PEG), which increases its stability and reduces its clearance, thereby extending its plasma half-life [501-503] and allowing a monthly infusion [503]. Being plant-derived, this enzyme does not display mannose 6-phosphate (M6P) on their surface glycans [501], which suggests an alternative mechanism of cell uptake and may result in a different biodistribution profile from agalsidase alfa and agalsidase beta. This enzyme has already been demonstrated (i) to decrease plasma lyso-GB3 levels and peritubular capillary GB3 deposits in the kidney; (ii) to improve the BPI score, gastrointestinal symptoms, and MSSI; (iii) and to maintain eGFR, proteinuria and LV mass on MRI with no “de novo” cardiac fibrosis [502]. Further studies on its efficacy are ongoing [503,504]. Anti-drug antibodies occurred in 19% of cases and became negative after 1 year, suggesting induction of immune tolerance [502], but its effect on the immune system is not yet completely clear [500]. Immune tolerance may occur as a result of the extended half-life and higher exposure of the immune system to the enzyme; or because pegylation may mask the enzyme to the immune system. However, concerns about immunogenicity have also been raised, because of the different glycosylation pattern of a plant-derived protein and the PEG component. Of note, the extended half-life may also interfere with the laboratory assays for anti-drug antibodies detection, as the circulating enzyme at the sampling time may bind antibodies and prevent their detection [500].

Moss-derived α-galactosidase A is produced in the moss *Phycomitrella patens* and is a glycoengineered variant devoid of α-1,3-fucose and β-1,2-xylose residues on its N-glycans, which are plant-specific and may elicit immunogenic response in mammals [505]. Being plant-derived, this enzyme does not display M6P on their surface glycans; instead, it carries >90% mannose-terminated glycans [505]. This enzyme

has a shorter half-life, which might be due to a higher cellular uptake via the mannose receptors [505,506], and has shown to reduce urinary GB3 in a higher proportion than previously reported for α-galactosidase A produced in mammalian cells [506].

The glucosylceramide synthetase (GCS) inhibitors, such as venglustat and lucerastat, block the enzyme catalyzing the first step of glycosphingolipid biosynthesis, reducing glucosylceramide and GB3 [500]. In patients with residual enzyme activity, GCS inhibitors might be sufficient to reduce the synthesis of GB3 to a level manageable by the residual enzyme activity, while in classic patients, with minimal to no residual enzyme activity, they may not be sufficient in monotherapy, but can still be useful in combination with ERT [500]. Venglustat, also known as ibiglustat, has shown to reduce GB3 deposits in tissues (including in brain) and plasma GB3 and lyso-GB3 in mice, this effect being more pronounced when added to ERT, except in the brain, where ERT had no effect [507]. Preliminary data also showed the reduction of GB3 from superficial skin capillary endothelium and plasma lyso-GB3 in treatment-naïve Fabry patients [500]. Lucerastat added to ERT resulted in a reduction of plasma GB3, while no reduction was seen in patients under ERT alone; and in stabilization of renal and cardiac parameters at 12 weeks [508]. Despite the clear advantages of oral administration, absence of anti-drug antibodies, and the possibility of passing the blood-brain barrier, caution in dosing should be taken, because the complete block of a single enzymatic reaction could potentially disrupt the cell homeostasis [500].

mRNA therapy, encapsulated in lipid nanoparticles, primarily targets hepatocytes in which the enzyme is produced and secreted into the circulation [500]. In mice and non-human primates, mRNA therapy has been shown to reduce GB3 and lyso-GB3 in heart and kidney [509,510] and plasma [510], and this effect was maintained for up to six weeks after infusion, suggesting that it could have the advantage of a larger interval between infusions [509,510]. Unlike ERT, mRNA therapy carries the advantage of using the endogenous protein translation system to ensure proper folding, glycosylation, and intracellular trafficking of α-galactosidase A; and, unlike DNA-based therapy, it does not carry a risk for insertional mutagenesis [500].

Gene therapy, using viral or non-viral vectors, may introduce a correct version of *GLA* gene, through "in vivo" or "ex vivo" technology [499]. The first FD patients have been treated in phase I and II clinical trials using an "ex vivo" approach, in which hematopoietic stem cells of the patient were recruited, transfected using lentiviruses, and re-administered to the patient. The main challenge is to target all affected cell types and tissues. Additionally, it is unclear whether classic males will develop antibodies against the expressed enzyme [500].

More evidence is needed to support the clinical use of these new therapies in FD patients. Our cohort, with its large size and unique characteristics, provides an excellent opportunity to test the effect of new emerging therapies in late-onset phenotypes of FD with predominant cardiac involvement.

In summary, it is essential to find the determinants of phenotype, the predictors of organ damage and faster disease progression and to evaluate the effect of specific therapies of FD in male and female patients with late-onset phenotypes, so we could practice Precision Medicine for each individual patient, based on factors much beyond gender, age or *GLA* gene mutation, and accurately determine, for each individual, if he/she is a good candidate for therapy, the best therapy and the best timing to start it in order to avoid target organ damage.

CHAPTER 8 – REFERENCES

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CHAPTER 9 – OTHER ACHIEVEMENTS

This doctoral thesis also led to the publication of two review articles, one on FD and the heart and the other on FD therapy (Appendices 1 and 2):

- Azevedo O, Cordeiro F, Gago MF, Miltenberger-Miltenyi G, Ferreira C, Sousa N, Cunha D. Fabry Disease and the Heart: A Comprehensive Review. *Int J Mol Sci*. 2021 Apr 23;22(9):4434. doi: 10.3390/ijms22094434. (JIF 5.923, JCR Q1 77.61%)
- Azevedo O, Gago MF, Miltenberger-Miltenyi G, Sousa N, Cunha D. Fabry Disease Therapy: State-of-the-Art and Current Challenges. *Int J Mol Sci*. 2020 Dec 28;22(1):206. doi: 10.3390/ijms22010206. (JIF 4.556, JCR Q1 75.25%)

The investigation carried out for this doctoral thesis was instrumental for the implementation of a Reference Centre on Lysosomal Storage Disorders in the Hospital Senhora da Oliveira – Guimarães, the official recognition of this hospital as a National and European Reference Centre on this field and the integration of this Reference Centre into the European Reference Network on Hereditary Metabolic Disorders (MetabERN).

This Reference Centre on Lysosomal Storage Disorders has been pivotal not only in providing highly specialized and multidisciplinary healthcare to patients suffering from these diseases, but also in promoting and securing funds for innovative research in this field, in collaboration with Universidade do Minho and other research partners.

CHAPTER 10 – APPENDICES

Chapter 10.1

Azevedo O, Cordeiro F, Gago MF, Miltenberger-Miltenyi G, Ferreira C, Sousa N, Cunha D.

Fabry Disease and the Heart: A Comprehensive Review.

Int J Mol Sci. 2021 Apr 23;22(9):4434. doi: 10.3390/ijms22094434.

International Journal of
Molecular Sciences

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Review

Fabry Disease and the Heart: A Comprehensive Review

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Abstract: Fabry disease (FD) is an X-linked lysosomal storage disorder caused by mutations of the *GLA* gene that result in a deficiency of the enzymatic activity of α-galactosidase A and consequent accumulation of glycosphingolipids in body fluids and lysosomes of the cells throughout the body. GB3 accumulation occurs in virtually all cardiac cells (cardiomyocytes, conduction system cells, fibroblasts, and endothelial and smooth muscle vascular cells), ultimately leading to ventricular hypertrophy and fibrosis, heart failure, valve disease, angina, dysrhythmias, cardiac conduction abnormalities, and sudden death. Despite available therapies and supportive treatment, cardiac involvement carries a major prognostic impact, representing the main cause of death in FD. In the last years, knowledge has substantially evolved on the pathophysiological mechanisms leading to cardiac damage, the natural history of cardiac manifestations, the late-onset phenotypes with predominant cardiac involvement, the early markers of cardiac damage, the role of multimodality cardiac imaging on the diagnosis, management and follow-up of Fabry patients, and the cardiac efficacy of available therapies. Herein, we provide a comprehensive and integrated review on the cardiac involvement of FD, at the pathophysiological, anatomopathological, laboratory, imaging, and clinical levels, as well as on the diagnosis and management of cardiac manifestations, their supportive treatment, and the cardiac efficacy of specific therapies, such as enzyme replacement therapy and migalastat.

Keywords: Fabry disease; heart; cardiomyopathy; enzyme replacement therapy; migalastat

check for updates

Citation: Azevedo, O.; Cordeiro, F.; Gago, M.F.; Miltenberger-Miltenyi, G.; Ferreira, C.; Sousa, N.; Cunha, D. Fabry Disease and the Heart: A Comprehensive Review. *Int. J. Mol. Sci.* **2021**, 22, 4434. https://doi.org/10.3390/ijms22094434

Academic Editor: Grzegorz Wegrzyn

Received: 19 March 2021
Accepted: 13 April 2021
Published: 23 April 2021

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1. Fabry Disease Overview

Fabry disease (FD) (OMIM 301500) is a rare lysosomal storage disorder caused by mutations in the *GLA* gene, leading to deficiency of the enzymatic activity of α-galactosidase A. Globotriaosylceramide (GB3) and other neutral glycosphingolipids consequently accumulate in body fluids and lysosomes of cells throughout the body, including in those that are particularly relevant for the disease, such as in the heart (cardiomyocytes, conduction system cells, vascular endothelial and smooth muscle cells, and fibroblasts), kidney (podocytes, tubular, glomerular, mesangial, and interstitial cells), nervous system (neu-

rons in autonomic and posterior root ganglia) and vascular endothelium and smooth muscle [1,2].

GLA mutations causing a virtually null enzymatic activity (<5% of the normal mean) are associated with severe and early onset classical phenotypes, which are characterized by the development of clinical manifestations in childhood or adolescence, such as acroparesthesias, neuropathic pain, hypohidrosis, heat, cold and exercise intolerance, cornea *verticillata*, angiokeratomas, gastrointestinal symptoms, and proteinuria. In adulthood, patients may also suffer from cardiomyopathy, heart failure, dysrhythmias, cardiac conduction blocks, renal failure, brain white matter lesions, cerebrovascular events, and sensorineural deafness. In contrast, *GLA* mutations leading to a residual enzymatic activity are associated to attenuated and late-onset phenotypes, which are characterized by the development of cardiac, renal and/or cerebrovascular manifestations in adulthood [2–5]. In this X-linked disorder, heterozygote females are not merely carriers and their clinical spectrum widely ranges from asymptomatic to full-blown disease as severe as in affected males [6,7].

2. Cardiac Involvement in FD

2.1. Pathophysiology

FD leads to GB3 accumulation in virtually all cardiac cells, but the mechanisms by which substrate accumulation leads to cellular dysfunction or organ damage remain less defined [8]. GB3 accumulation may affect mitochondrial function, either directly through accumulation within the mitochondrial membrane or indirectly by preventing mitophagy [9], likely contributing to the reduction in the activities of the respiratory chain enzymes that has been shown in fibroblasts [10]. On the other hand, substrate accumulation and organelle damage have also been shown to induce oxidative stress [9,11]. GB3 has also been demonstrated to promote a higher proinflammatory cytokine production and expression [12], to mediate apoptosis [13,14], and to induce endothelial dysfunction [15]. Lyso-GB3, a deacylation product of GB3 [16], has also been shown to inhibit α-galactosidase A activity and to promote the proliferation of smooth muscle cells [17], likely contributing to the increased intima-media thickness.

2.2. Pathology

GB3 deposits are found in cardiomyocytes, valve fibroblasts, endothelial and smooth muscle vascular cells, and cardiac conduction system cells [18–21], representing 1–2% of the cardiac mass. Nevertheless, in cardiac variants, lysosomal inclusions are only found in cardiomyocytes [22]. In women, a mosaic pattern of normal and vacuolated cells caused by random X-chromosome inactivation is observed [23].

GB3 accumulation activates common signaling pathways leading to hypertrophy, inflammation, apoptosis, necrosis, and fibrosis. Accordingly, anatomopathological analysis of hearts of FD patients have shown hypertrophy of the cardiomyocytes, myocyte apoptosis and necrosis, inflammatory infiltrate, replacement and interstitial fibrosis, valve thickening and vascular intima and media thickening with vascular narrowing [18,21]. Myocardial disarray may be observed, although it is less pronounced than in sarcomeric HCM [21].

The cardiac involvement by FD is progressive. Progressive cardiomyocyte hypertrophy ultimately ends in cell death of enlarged substrate-engorged cardiomyocytes, either by necrosis or apoptosis, which presumably leads to fibrosis. Accordingly, the cardiomyocyte diameter, the lysosomal glycosphingolipid area and the extent of necrosis, apoptosis and fibrosis are all positively correlated with disease severity and age. In the "pre-hypertrophy" stage, the cardiomyocytes are already mildly hypertrophied and contain numerous glycosphingolipid-engorged vacuoles, mostly in the perinuclear zone, while the intramural vessels and interstitium are essentially unaffected, and myocardial fibrosis is not detectable. In moderate hypertrophy, vacuolar areas occupy > 30% of myocytes, and there is increased cell apoptosis and necrosis, moderate fibrosis, and thickening and some luminal narrowing of intramural vessels. In severe hypertrophy, lysosomal inclusions occupy

~60% of the myocardial cells, and there is extensive myocardial fibrosis and severe narrowing of arteriole lumens. Cell apoptosis seem to prevail in patients with moderate cardiac hypertrophy, while myocyte necrosis is more pronounced in severe hypertrophy [23].

Endomyocardial biopsies have shown myocarditis in 56% of FD patients. Myocarditis was detected even before left ventricular hypertrophy (LVH) or late gadolinium enhancement (LGE) and its frequency correlated with disease severity. Myocarditis is immune-mediated and positive antiheart and antimyosin antibodies were found in all FD patients with myocarditis [24].

3. Cardiac Manifestations of FD

Cardiac manifestations of FD include ventricular hypertrophy and fibrosis, valve thickening or regurgitation, heart failure, angina, dysrhythmias, cardiac conduction abnormalities, and sudden death [25]. Cardiac signs or symptoms have been reported in 60% of males and 50% of females, with a mean age of onset of 29.2 ± 14.4 and 34.5 ± 17.6 years, respectively [26]. Cardiac symptoms were the presenting symptoms of FD in 13% of males and 10% of females [27] (Table 1). Despite available therapies and supportive treatment, cardiac involvement carries a major prognostic impact, representing the main cause of death in FD [26,28].

Table 1. Frequency of cardiac symptoms in FD.

Cardiac Manifestations	Frequencies	References
Cardiac signs or symptoms	• 60% in males Mean age of onset 29.2 ± 14.4 years • 50% in females Mean age of onset 34.5 ± 17.6 years	• Mehta et al. [26]
Cardiac symptoms as presenting symptoms	• 13% in males • 10% in females	• Eng et al. [27]

3.1. Hypertrophic Cardiomyopathy

Hypertrophic cardiomyopathy (HCM) is the main cardiac manifestation of FD [25]. FD has been diagnosed in 0.9% of the patients with HCM [29,30] and, being a treatable disease, it should be systematically ruled out in all patients with HCM, either by targeted FD screening or by a wider HCM gene panel including the *GLA* gene [29]. Basal inferolateral LGE and bifascicular block were identified as the most powerful predictors of FD in patients with HCM; therefore, in their presence, a targeted FD screening should be performed, while in their absence, an HCM gene panel would be the most appropriate next step in the etiological study of HCM [29].

3.1.1. Left Ventricular Hypertrophy

LVH was found in 43% of males and 26% of females, arising earlier and progressing more rapidly in males than in females (mean age of onset: 39 ± 10 vs. 50 ± 11 years) [31,32]. The prevalence of LVH has been reported to increase with age, occurring in 76.9% of patients aged ≥ 75 years [33]. Left ventricular (LV) mass index was also found to increase with age [34,35] and to correlate inversely with estimated glomerular filtration rate (eGFR) [34]. In a late-onset phenotype with predominant cardiac involvement, LVH was detected in 73.1% of males and 19.0% of females, with a mean age at diagnosis of 57 ± 10 and 73 ± 8 years, respectively. The frequency and severity of LVH also increased with age [4,5].

Although electrocardiographic changes of LVH may predate imaging evidence of LVH in Fabry patients [35], multimodality cardiac imaging is useful to suggest the diagnosis of FD, to detect LVH, as well as to monitor the progression of cardiac involvement and response to therapy [36,37]. Cardiac magnetic resonance imaging (MRI) can detect cardiac involvement even when the LVH severity is mild, allowing to reclassify 21% of FD patients

as having cardiac involvement that was previously unrecognized [38]. Of note, recent studies have suggested that machine learning applied to 3D myocardial architecture and deformation obtained by cardiac MRI may present increased ability to perform differential diagnosis of the cause of HCM [39].

LVH secondary to FD is most commonly concentric and symmetric [25,32] (Figure 1); however, other patterns can also occur, including asymmetric septal hypertrophy, eccentric hypertrophy, and apical hypertrophy [25,40,41].



Figure 1. Left ventricular hypertrophy (LVH) secondary to Fabry disease (FD). Transthoracic echocardiogram of a 70-year-old male Fabry patient showing a severe symmetrical LVH with prominent papillary muscles in parasternal long-axis (**A**), four-chambers (**B**) and short-axis (**C**) views.

A binary appearance of the LV endocardial border, known as the "binary sign," was correlated with a characteristic pattern of glycosphingolipid compartmentalization on histological examination and once thought to represent a hallmark feature of Fabry cardiomyopathy [42]. However, it was later found to be an unreliable marker of Fabry cardiomyopathy, with an estimated sensitivity of 28.0% and a specificity of 80.0%, occurring more commonly in patients with LVH and in a more advanced stage of the disease [43].

Prominent papillary muscles are a characteristic feature of FD [25,44] (Figure 1), while papillary muscles anomalies, such as anterior displacement of the anterolateral papillary muscles or direct insertion of the papillary muscle into the mitral valve, are very specific for HCM [45]. Indeed, the papillary muscle contribution to LV mass was found to be significantly increased in Fabry patients, both with and without LVH, compared to controls [38]. Hence, the inclusion of papillary muscle mass in LV mass calculation is recommended for the earlier detection of LVH in Fabry patients [46], although exclusion of papillary muscles seems to be a better predictor of adverse cardiac events (composite endpoint of ventricular tachycardia (VT), bradycardia requiring device implantation, severe heart failure, and cardiac death) [47].

3.1.2. Left Ventricular Storage, Inflammation, and Fibrosis

Intramyocardial LGE on the basal inferolateral LV segments is typically seen on cardiac MRI in approximately 50% of Fabry patients [48] (Figure 2), which correlates histologically with focal replacement fibrosis [49]. This LGE pattern was hypothesized to be due to increased stress at the interface of the fibrous skeleton of the mitral annulus and the LV midwall [50]. It allows to differentiate FD from sarcomeric HCM, which usually results in LGE at the right ventricular (RV) junction points, and amyloidosis, which usually results in a global subendocardial pattern of LGE [51]. However, this LGE pattern is not exclusive of FD and may also be found in myocarditis, Chagas disease, and sarcoidosis [52].

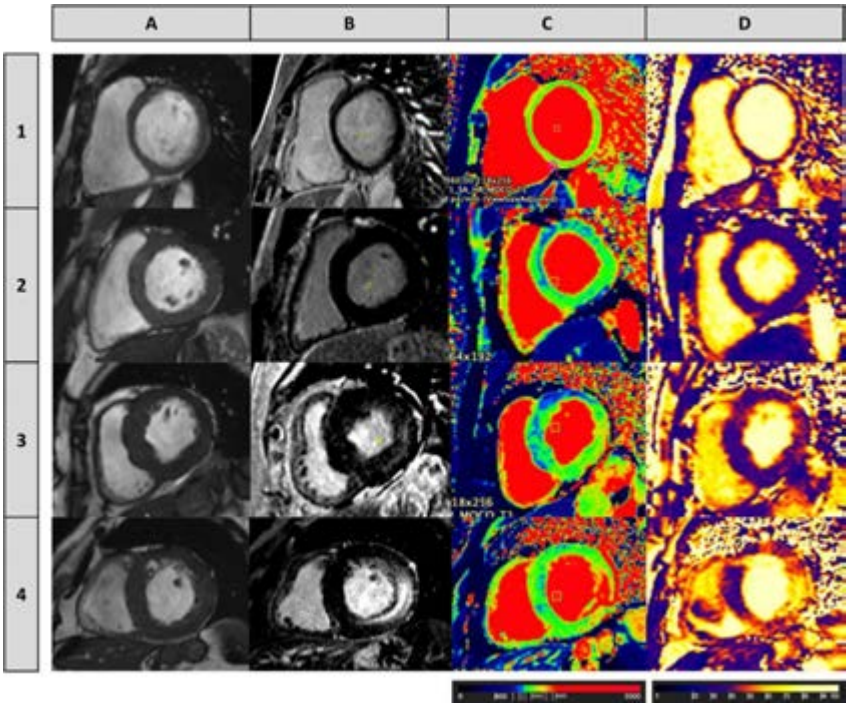


Figure 2. Cardiac magnetic resonance imaging (MRI) (3.0 Tesla) in four Fabry patients, illustrating different stages of myocardial involvement by the disease. (**A**) Cine (balanced steady-state free precession sequence) image at the basal left ventricular (LV) short-axis slice; (**B**) Late gadolinium enhancement (LGE) at the basal LV short-axis slice; (**C**) Native T1 mapping (precontrast) performed using a modified Look-Locker inversion (MOLLI) recovery sequence at the basal LV short-axis slice (the resulting pixel-by-pixel T1 color maps were displayed using a customized lookup table, in which normal myocardium was green, increasing T1 was yellow and red, and decreasing T1 was blue); and (**D**) T2 mapping (fast low angle shot (FLASH)) at basal LV short-axis slice (the resulting pixel-by-pixel T2 color maps were displayed using a customized lookup table, in which normal myocardium was purple and increasing T2 was red and yellow). **Patient 1:** A 26-year-old female without LVH or LGE, presenting normal values of T1 (1265 ± 68 ms at the basal septum) and T2 (41.76 ± 5.40 ms at the basal septum); **Patient 2:** A 41-year-old male without LVH or LGE, presenting low T1 (1118 ± 40 ms) and normal T2 (40.93 ± 5.80 ms) values at the basal septum; **Patient 3:** A 76-year-old female with LVH (LV mass 83 g/m^2, maximum wall thickness 19 mm at the basal septum) and diffuse LGE in the basal segment of the inferolateral wall, who presents low T1 (1093 ± 36 ms) at the basal septum and T1 pseudonormalization particularly at the inferolateral wall (1276 ± 59 ms), where a mild increase in T2 values (49.10 ± 2.30 ms) was also observed; **Patient 4:** A 69-year-old male patient with LVH (LV mass 123 g/m^2, maximum wall thickness 18 mm at the septum) and diffuse and extensive LGE in the inferolateral wall, who presents areas of T1 pseudonormalization but also areas of T1 increase, such as in the inferolateral wall (1425 ± 144 ms), where T2 values (64.44 ± 8.56 ms) are also increased.

LGE may develop before LVH, and a study has reported that 50% of females would not have been identified with FD cardiomyopathy without the aid of LGE imaging [53]. The prevalence of FD in patients with unexplained LGE has been reported as 2.5% [54].

The "double-peak sign" on strain rate by Tissue Doppler Imaging (TDI), depicted as a sharp first peak in early systole followed by a rapid fall of strain rate approaching zero and finally a second peak during isovolumetric relaxation, identifies segments of LGE with 99% of sensitivity and 93% of specificity in patients with HCM, aortic stenosis, and FD [55]. Two-dimensional speckle-tracking can also detect LGE (Figure 3). LV global longitudinal strain (GLS) is lower in patients with LGE and reduced longitudinal systolic strain (worse than −12.5%) in the posterolateral segment identifies LGE with 90% of sensitivity and 97%

of specificity [56]. It was suggested that the absence of ST segment or T wave alterations on electrocardiogram could almost exclude LGE in FD [57]; however, although LGE has been significantly associated with ST segment depression and negative T waves, it has been described in up to 17.8% of the patients without ST depression and 13.4% of the patients without negative T waves in a large cohort of patients with late-onset FD with predominant cardiac involvement [5].

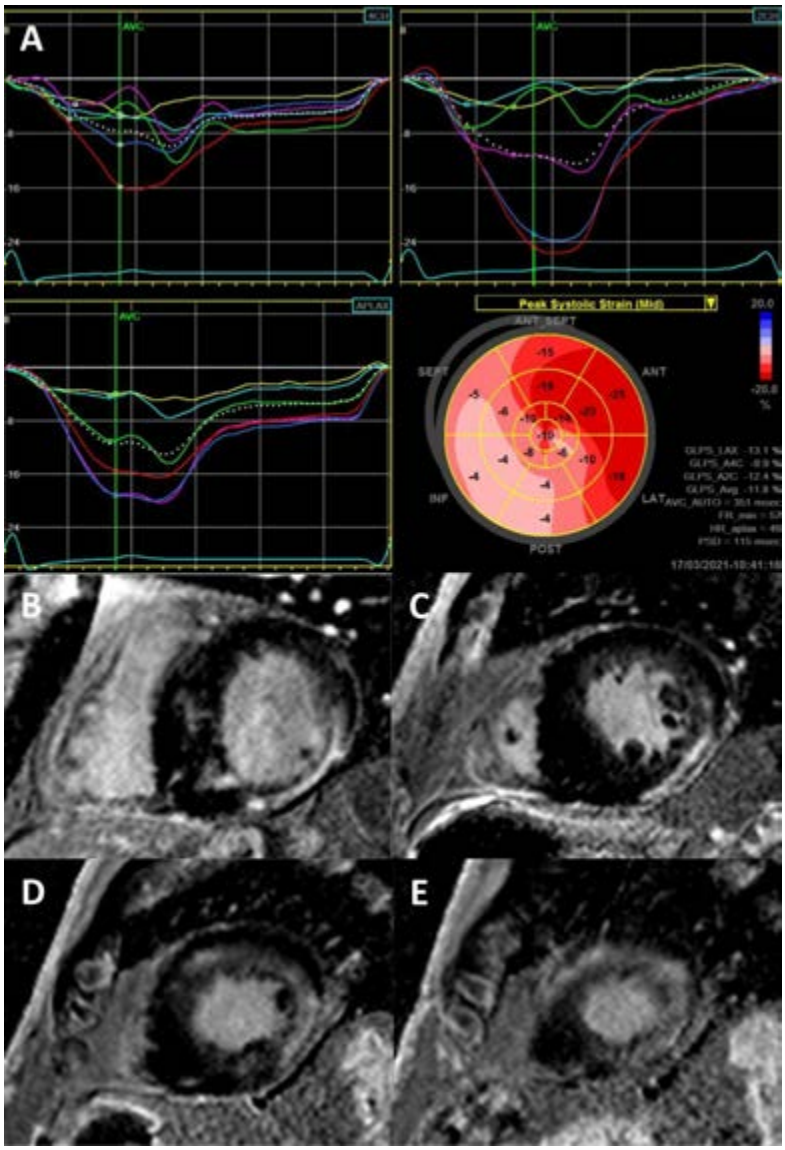


Figure 3. Strain echocardiography for the detection of LGE. (**A**) Transthoracic echocardiogram with 2D-strain analysis by speckle tracking showing reduction of longitudinal strain, particularly in the inferolateral, inferior and inferoseptal walls and apex, in a 73-year-old male Fabry patient; (**B–E**) Cardiac MRI (3.0 Tesla) with LGE in short-axis slices, from the LV base to the apex, showing fibrosis in the same regions where the reduction in longitudinal strain was more pronounced.

In FD patients with basal inferolateral LGE, troponin levels and T2 values are increased, suggesting that LGE may also represent inflammation [58] (Figure 2).

LGE was shown to correlate with increased high-sensitivity troponin, and it has been proposed that normal values of high-sensitivity troponin, in combination with normal ECG and echocardiogram, indicate that FD cardiomyopathy is unlikely; elevated values of high-sensitivity troponin indicate advanced FD cardiomyopathy, while borderline values should lead to a thorough investigation including cardiac MRI. In addition, patients with increased high-sensitivity troponin presented decreased LV wall thickness and ejection fraction, suggesting progression of cardiomyopathy [59]. Increased inflammatory markers, such as interleukin-6 and tumor necrosis factor, were also associated to increased disease burden (LVH and fibrosis) [60].

In another study, LGE in the basal inferolateral wall was associated to high global and basal inferolateral wall T2. High basal inferolateral wall T2 was, in turn, associated with increased troponin and N-terminal prohormone of brain natriuretic peptide (NT-proBNP), GLS impairment, and electrocardiographic abnormalities (long PR, complete bundle branch block, LVH voltage criteria, long QTc, and T-wave inversion) and predicted clinical worsening after 1 year (Fabry stabilization index > 20%) [61].

Finally, LGE regions also showed focal 18F-FDG uptake, further supporting the notion that LGE represents inflammation [62]. Focal 18F-FDG uptake was also shown before the development of LGE in FD females, in association to a pseudonormalization of T1 time, suggesting an intermediate stage of Fabry cardiomyopathy [63]. Focal 18F-FDG uptake was also shown in 50% of the patients before LVH, being associated with lower LV GLS [64].

LGE at the time of initiation of enzyme replacement therapy (ERT) was associated to no improvement in LV mass, LV strain, and exercise capacity [65]. Moreover, LGE was associated with a higher risk of ventricular arrhythmias and sudden cardiac death (SCD) [66,67] and the annual increase in fibrosis was the only independent predictor of ventricular arrhythmias [66].

FD has also been associated to low native T1 time on cardiac MRI [68] (Figure 2), which has been attributed to intracellular glycosphingolipid accumulation [68]. In FD patients without LVH, low T1 was found in 40% of the cases [68,69] (Figure 2), being associated with ECG abnormalities and worse LV wall thickness and mass [69,70], LA size [70,71], E/E' ratio, LV GLS and inferolateral longitudinal strain [71], LV GLS by feature tracking [72], LGE [69], and worse Mainz Severity Score Index (MSSI) and clinical worsening (Fabry Stabilization Index > 20%) [70]. In FD with LVH, T1 correlated inversely with LV mass [68]. In patients with LVH, low native T1 time accurately differentiates FD from other causes of LVH [68], as low T1 times are only otherwise noted in iron overload [73]. Pseudonormalization or elevation of T1 in LV inferolateral wall was correlated with the presence of LGE [68] (Figure 2). In FD patients with RV hypertrophy, low T1 was also found in the RV [74].

Nordin et al. proposed three stages of cardiac involvement in FD: (1) storage stage with normal or low native T1 times without LVH; (2) inflammation and myocyte hypertrophy stage with low native T1 times, inflammation, LGE, chronic troponin elevation, with LVH in males and without LVH in females; and (3) fibrosis and impairment stage with pseudonormalization of native T1 times, extensive LGE, LVH, troponin and NT-proBNP elevation, LV dysfunction, and heart failure [75].

In advanced stages of FD, there is thinning and akinesia of the basal posterior wall, which may result in asymmetrical LVH [25] (Figure 4) and correlates histologically with fibrosis [22]. The thinning of the basal posterior wall was found to significantly precede severe heart failure [New York Heart Association (NYHA) class III] and cardiac death—LV septum/posterior wall thickness ratio >1.3, 1.5, or 1.7 significantly preceded NYHA class III heart failure and cardiac death by 4.0, 3.8, or 3.4 and 4.7, 4.5, or 4.1 years, respectively [76].

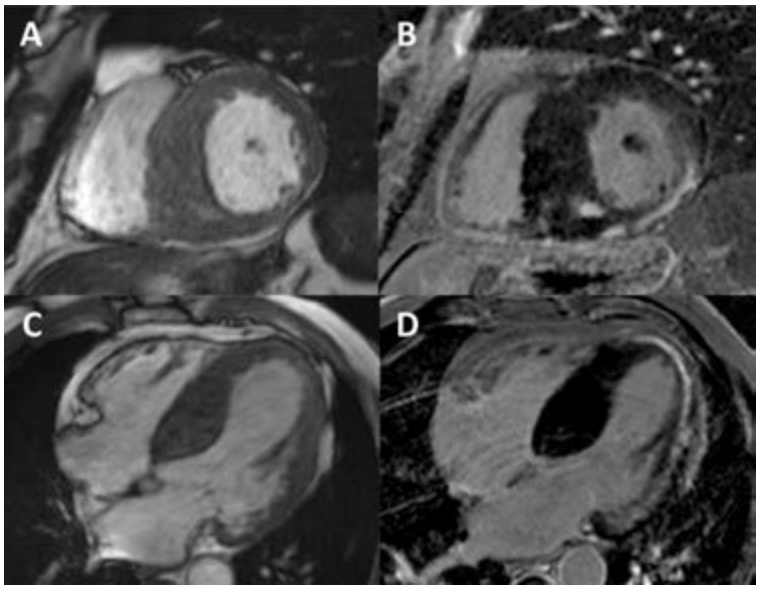


Figure 4. Cardiac MRI (3.0 Tesla) in a Fabry patient with advanced cardiomyopathy. Cine (balanced steady-state free precession sequence) images at the basal LV short-axis slice (**A**) and four-chamber view (**C**) showing massive and asymmetrical LVH (maximal thickness 30. mm at the septum) with thinning of the posterior wall (2 mm). LGE at the basal LV short-axis slice (**B**) and four-chamber view (**D**) showing fibrosis of the inferior and inferolateral walls and apex and focal fibrosis in the septum, where the LVH is more pronounced.

3.1.3. Left Ventricular Function

Diastolic function is abnormal in 69.4% of those with LVH [4] and 63% of those with LGE [77]. Diastolic dysfunction occurs more commonly as an abnormal relaxation or a pseudonormal pattern [25], and it has been associated with the presence of LGE [78] and to correlate with NT-proBNP [79]. LV systolic dysfunction with reduction in ejection fraction is rare (6.7%) [80], occurring in late stages of advanced Fabry cardiomyopathy [22,25].

LV systolic and diastolic dysfunction can, however, be detected before the development of LVH, not only by TDI [81] but also by speckle-tracking [82,83]. LV longitudinal [82], circumferential and radial strain [83] were found to be reduced in the pre-hypertrophic stage; basal segmental longitudinal strain is also impaired even when LV wall thickness is normal [84], and the strain rate during isovolumic relaxation and the ratio of transmitral E-wave velocity to the strain rate during isovolumic relaxation have shown to differentiate FD patients from controls regardless of LVH [85].

Strain echocardiography has also shown that mechanical dispersion is higher in FD patients with LVH than in FD patients without LVH or healthy controls [86]. An apical sparing pattern on longitudinal strain has also been described in FD, similarly to amyloidosis [84,87] (Figure 5). Additionally, the loss of the normal base-to-apex circumferential strain gradient was suggested to be a specific LV deformation pattern of Fabry cardiomyopathy, as opposed to non-obstructive HCM that has been associated to a higher global circumferential strain and a normal base-to-apex gradient [88].

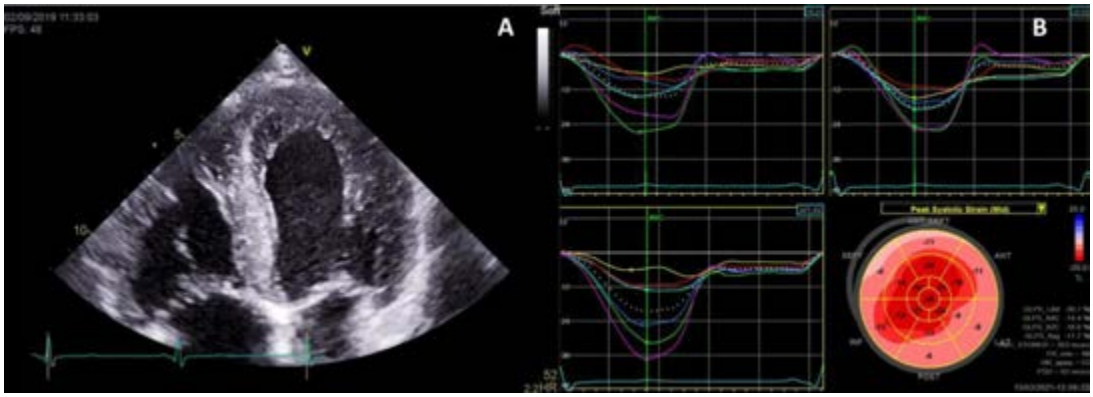


Figure 5. Apical sparing pattern of global longitudinal strain (GLS) in a Fabry patient. Transthoracic echocardiogram of a 48-year-old male Fabry patient showing symmetrical LVH in a four-chamber view (**A**) and 2 D-strain analysis by speckle tracking revealing an apical sparing pattern of GLS (**B**).

3.1.4. Left Ventricular Obstruction

Obstruction at the LV outflow tract (LVOT) may occur, but massive LVH involving the papillary muscles has also been reported to cause mid-ventricular obstruction [89]. Obstruction at rest is rare, but it may be elicited by exercise in 43% of the patients [90], also contributing to heart failure. During exercise, FD patients also present lower augmentation of stroke volume than healthy controls, being E/E' ratio the independent predictor of exercise-induced change in stroke volume [91].

3.1.5. Right Ventricular Involvement

RV hypertrophy occurs in 31–71% of the patients [92,93]. RV systolic function in patients with Fabry cardiomyopathy tends to be preserved [93]. Nevertheless, RV global and free wall systolic strain may be reduced despite normal function on conventional echocardiography, and RV systolic dysfunction has been associated to RV wall thickness and fibrosis [94]. Although RV hypertrophy and RV systolic function indexes (TAPSE and S') have shown significant association to clinical events, they were not identified as independent predictors of their occurrence [95].

3.1.6. Atrial Involvement

Glycolipid deposition in the atria [19] may ultimately cause atrial dilation, which occurs more commonly in patients with LVH [81] and fibrosis [56]. Left atrial (LA) systolic and early diastolic strain rate were found preferentially reduced in patients with LVH, but LA systolic strain was decreased even before LVH [96]. Peak atrial longitudinal strain was inversely associated to Fazekas score of brain white matter lesions, even after adjusting for LV mass index [97]. Atrial dilation is associated to the occurrence of atrial fibrillation [98], which also contributes to heart failure.

3.1.7. Heart Failure

Ventricular hypertrophy and fibrosis result in diastolic and systolic dysfunction, which together with dysrhythmias and conduction disorders, valve disease, and myocardial ischemia, contribute to heart failure [25]. Dyspnea or heart failure has been reported in 19.7% of untreated females and 19.4% of untreated males [34]. The prevalence of heart failure increases with age, occurring in 34.6% of the patients aged $\geq$ 75 years [33]. In a late-onset phenotype with predominant cardiac involvement, heart failure was found in 32.9% of males and 14.8% of females and mean survival free from heart failure was 64 $\pm$ 1 and 76 $\pm$ 2 years, respectively. The frequency of heart failure also increased with age [4,5].

NT-proBNP levels were correlated to symptom class, LV mass, E/E' ratio, and LA size, reaching higher values in patients with LVH, diastolic dysfunction, and LGE [75,99].

Severe heart failure (NYHA class $\geq$ III) was reported in 10% of FD patients. The annual incidence of severe heart failure was 1.62 per 100 person-years, and age and MSSI were independent predictors of its development [100] (Table 2).

Table 2. Frequency of manifestations related to ventricular hypertrophy, fibrosis and dysfunction in FD.

Cardiac Manifestations	Frequencies	References
LVH	• 43% in males Mean age of onset 39 ± 10 years • 26% in females Mean age of onset 50 ± 11 years	• Mehta et al. [31]
	• 76.9% in patients ≥ 75 years	• Lidove et al. [33]
	In late-onset phenotype with predominant cardiac involvement • 73.1% of males Mean age at diagnosis of 57 ± 10 years • 19.0% of females Mean age at diagnosis of 73 ± 8 years	• Azevedo et al. [5]
LGE	• 50% of patients	• Moon et al. [48]
	In late-onset phenotype with predominant cardiac involvement • 21.4% of patients	• Azevedo et al. [5]
LV diastolic dysfunction	• 63% of patients with LGE	• Niemann et al. [77]
	In late-onset phenotype with predominant cardiac involvement • 29.2% of patients • 69.4% of patients with LVH	• Azevedo et al. [4]
LV systolic dysfunction (reduced EF)	• 6.7% of patients	• Wu et al. [80]
Latent LVOT obstruction	• 43% of patients	• Calcagnino et al. [90]
RV hypertrophy	• 31–71% of patients	• Niemann et al. [92] • Graziani et al. [93]
Heart Failure (or Dyspnoea)	• 19.4% in untreated males • 19.7% in untreated females	• Linhart et al. [34]
	• 34.6% in patients ≥ 75 years	• Lidove et al. [33]
	• Severe heart Failure (NYHA class ≥III) in 10% • Annual incidence of severe heart failure: 1.62 per 100 person-years	• Patel et al. [100]
	In late-onset phenotype with predominant cardiac involvement • 32.9% of males Mean survival free from heart failure: 64 ± 1 years • 14.8% of females Mean survival free from heart failure: 76 ± 2 years	• Azevedo et al. [5]

EF, ejection fraction; LGE, late gadolinium enhancement; LV, left ventricular; LVH, left ventricular hypertrophy; LVOT, left ventricular outflow tract; NYHA, New York Heart Association; RV, right ventricular.

3.2. Dysrhythmias and Cardiac Conduction Disorders

As a consequence of dysrhythmias and cardiac conduction disorders, Fabry patients may experience symptoms, such as palpitations and syncope. Palpitations have been reported in 15.3% and 21.3% of untreated Fabry male and female patients, while syncope has been, respectively, reported in 5.6% and 2.4% [34].

3.2.1. Bradycardia, Chronotropic Incompetence, and Cardiac Conduction Disorders

FD involvement of the autonomous nervous system may result in a significantly reduced heart rate variability in pediatric male patients, reflecting a reduction in parasympathetic stimulation of the heart [101].

GB3 accumulation in the cardiac conduction system cells [20] is constant in men and variable in women due to X-chromosome inactivation [102]. In an earlier stage, it may lead to accelerated atrioventricular (AV) conduction, which manifests as a short PR interval [102,103] (Figure 6). Although there are reports of FD cases with short PR interval and accessory pathways [104], enhanced AV conduction rather than ventricular pre-excitation is the most likely cause of the short PR interval. Short PR interval has been documented in FD patients in whom pre-excitation due to accessory pathways has been formally excluded [105]. Moreover, a normalization of PR interval has been documented with ERT [106]. Likewise, in PRKAG2 glycogen-storage cardiomyopathy, the short PR interval was shown to be directly caused by glycogen storage in and around the AV node, being reverted by glycogen depletion in the heart [107]. Still, although characteristic, short PR interval is not common; it has been found in only 14% of FD patients [108].

As FD progresses, GB3 accumulation and fibrosis lead to the development of AV and bundle-branch blocks [22,109,110] (Figure 6) and sinus node dysfunction [111], which may require a pacemaker. Bradycardia at rest is common (72%) [112]. Chronotropic incompetence with exercise due to autonomic nervous dysfunction may also occur and contribute to heart failure; hence, exercise stress testing or cardiopulmonary exercise testing could be useful in the differential diagnosis of dyspnea [112,113]. Bradyarrhythmic events were reported in 23% of the patients and were associated with age, LV mass, ejection fraction, and LA strain [114]. There was also a positive correlation between LV mass on cardiac MRI and QRS duration [57]. PR interval duration > 200 ms was reported in 3% and QRS interval duration > 120 ms in 9%. The durations of PR and QRS intervals were shown to increase with age and were identified as independent predictors of need of a pacemaker [111]. In a late-onset phenotype with predominant cardiac involvement, bifascicular block was reported in 25.3% of males and 5.7% of females with a mean age at diagnosis of 62 $\pm$ 6 years in males and 78 $\pm$ 6 years in females, and complete AV block was reported in 12.7% of males and 1.6% of females with a mean age at diagnosis of 60 $\pm$ 7 years in males and 79 $\pm$ 6 years in females. The frequency of bundle branch blocks and complete AV block increased with age as well as the duration of QRS interval [4,5].

3.2.2. Tachydysrhythmias

GB3 deposition in the atria [115] and subsequent fibrosis, together with LVH and diastolic dysfunction and atrial dilation, are the proposed mechanisms of development of atrial fibrillation [98], which was reported in 3% of FD patients [111]. Another study reported that 3.9% of the patients had persistent atrial fibrillation and 13.3% had paroxysmal atrial fibrillation, and age was the only independent predictor of this dysrhythmia [98]. Its annual incidence was reported as 1 per 100 person-years and age and LV mass were independent predictors of its development [100]. In a late-onset phenotype with predominant cardiac involvement, atrial fibrillation was found in 7.6% of males and 2.4% of females, while atrial flutter in 2.5% and 0.8%, respectively. Mean age at diagnosis of atrial fibrillation was 67 $\pm$ 11 years in males and 77 $\pm$ 3 years in females [4,5].

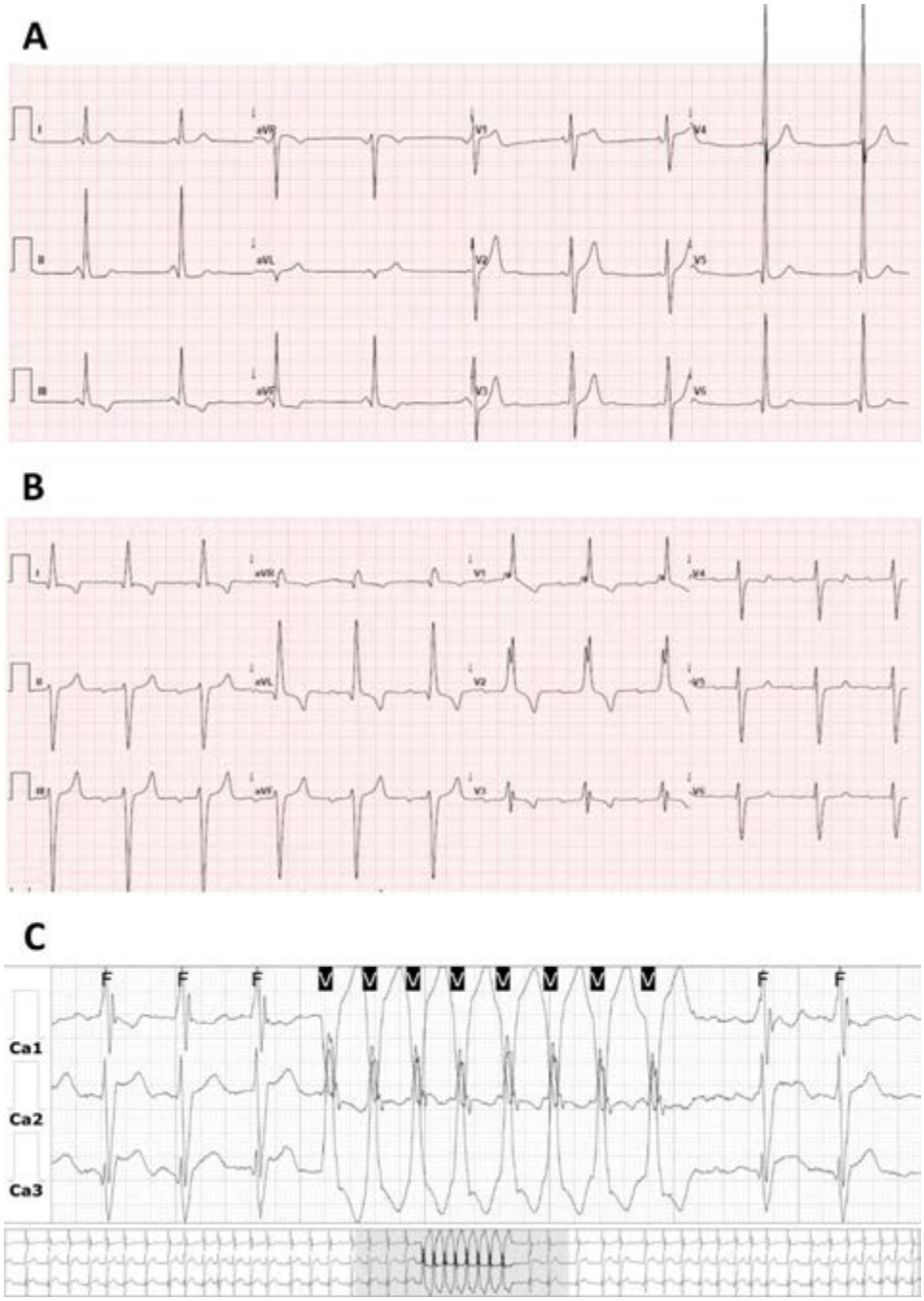


Figure 6. Electrocardiographic findings in FD. (**A**) Short PR interval in a 34-year-old male, (**B**) bifascicular and first-degree AV blocks and electrocardiographic criteria of LVH in a 72-year-old male, and (**C**) non-sustained VT in 24 h-Holter monitoring of a 76-year-old male.

A study of 1448 untreated Fabry patients showed ventricular arrhythmias in 13% of men and 20% of women [116]. In a further study, non-sustained VT was reported in 21%, while sustained VT in only 1% and bradycardia requiring device in 6% of Fabry patients during a median follow-up of 3.6 years, suggesting a higher impact of bradyarrhythmic clinical events [117]. A systematic review of the literature estimated the occurrence of VT in 15.3% of the patients and reported that 75% of the deaths were due to cardiac disease and 62% due to SCD, ranging the incidence of SCD events from 0.34% to 1.4% per annum [67]. In a late-onset phenotype with predominant cardiac involvement, non-sustained VT (Figure 6) was found in 14.1% of males and 5.6% of females, with a mean age at diagnosis of 57 ± 8 and 70 ± 6 years, respectively [4,5].

GB3 accumulation in cardiac conduction system has been reported in FD patients presenting with VT in the absence of LVH, suggesting that GB3 deposits may precipitate VT [118]. However, the major mechanism of sustained VT in FD appears to be re-entry related to myocardial fibrosis [119].

Indeed, the annual increase in fibrosis during follow-up was identified in a study as the only independent predictor of malignant ventricular arrhythmias [66]. Other study showed that SCD only occurred in patients with documented VT and LGE [120]. Patients with elevated LV mass index also had more overall arrhythmia, ventricular arrhythmia, and sustained VT [50]. Finally, a systematic review of the literature identified age, male gender, LVH, LGE, and non-sustained VT as risk factors associated with SCD events [67].

Sympathetic nerve damage, demonstrated by MIBG defects in the inferolateral wall, occurs in FD patients without LGE and, in patients with LGE, MIBG defects are larger than the LGE areas, suggesting that sympathetic nerve damage precedes myocardial fibrosis [121]. Therefore, MIBG may have a unique role in assessing the risk of ventricular arrhythmia and SCD [36].

Dysrhythmias occur especially in late stages of the disease [22]. Implantable loop recorders (ILR) revealed clinically relevant dysrhythmias, including four episodes of asystole, seven of bradycardia, five of paroxysmal atrial fibrillation, and five of VT (three sustained and two non-sustained) in 16 patients with advanced cardiomyopathy, with no abnormalities on Holter, followed for a median of 1.2 years. These findings led to new management decisions (pacemaker or ICD implantation, anticoagulation, and termination of beta-blockers) in more than half of them (n = 9) [122].

3.2.3. Cardiac Devices

In a study, the annual rate of cardiac device implantation was estimated at 1.90 per 100 person-years. A pacemaker was needed in 12.5% of the patients due to AV blocks or sinus node dysfunction, and an implantable cardioverter-defibrillator (ICD) was placed in 4.2% due to non-sustained VT. Age at diagnosis of FD and age at the last follow-up visit were independently associated with an increased risk of rhythm/conduction abnormalities requiring cardiac device [123]. The annual incidence of cardiac device implantation for the treatment of bradycardia was 1.07 per 100 person-years and age and QRS duration were independent predictors of device implantation [100]. Another study reported a 5-year cumulative incidence of pacemaker implantation of 8%, which is more than 25 times greater than in general population [111]. In a late-onset phenotype with a predominant cardiac involvement, a pacemaker was implanted in 12.7% of males and 2.4% of females and an ICD in 1.3% and 0.8%, respectively. Mean survival free from pacemaker was 71 ± 2 and 86 ± 1 years, respectively [4,5] (Table 3).

Patients with devices were older, had greater LV mass, more scar tissue and larger atrial size. A class I indication for device implantation was found in 92% of the patients with permanent pacemakers, but in only 28% of the patients with ICD. Moreover, further 44% of patients had ICD inserted for primary prevention outside of current guidance [124].

Table 3. Frequency of dysrhythmias and cardiac conduction disorders in FD.

Cardiac Manifestations	Frequencies	References
Palpitations	• 15.3% in untreated males • 21.3% in untreated females	• Linhart et al. [34]
Syncope	• 5.6% in untreated males • 2.4% in untreated females	• Linhart et al. [34]
Short PR interval	• 14% of patients	• Namdar et al. [108]
Bradycardia	• 72% of patients	• Lobo et al. [112]
Bradyarrhythmic events	• 23% of patients	• Di et al. [114]
PR interval > 200 ms	• 3% of patients	• O'Mahony et al. [111]
QRS duration > 120 ms	• 9% of patients	• O'Mahony et al. [111]
Right bundle branch block	In late-onset phenotype with predominant cardiac involvement • 38.8% of males Mean age at diagnosis of 61 ± 6 years • 8.5% of females Mean age at diagnosis of 76 ± 9 years	• Azevedo et al. [5]
Left anterior fascicular block	In late-onset phenotype with predominant cardiac involvement • 45.5% of males Mean age at diagnosis of 60 ± 7 years • 10.3% of females Mean age at diagnosis of 72 ± 14 years	• Azevedo et al. [5]
Bifascicular block	In late-onset phenotype with predominant cardiac involvement • 25.3% of males Mean age at diagnosis of 62 ± 6 years • 5.7% of females Mean age at diagnosis of 78 ± 6 years	• Azevedo et al. [5]
Complete AV block	In late-onset phenotype with predominant cardiac involvement • 12.7% of males Mean age at diagnosis of 60 ± 7 years • 1.6% of females Mean age at diagnosis of 79 ± 6 years	• Azevedo et al. [5]
Atrial Fibrillation	• 3% of patients	• O'Mahony et al. [111]
	• 3.9% of patients had persistent atrial fibrillation • 13.3% of patients had paroxysmal atrial fibrillation	• Shah et al. [98]
	• Annual incidence: 1 per 100 person-years	• Patel et al. [100]
	In late-onset phenotype with predominant cardiac involvement • 7.6% of males Mean age at diagnosis of 67 ± 11 years • 2.4% of females Mean age at diagnosis of 77 ± 3 years	• Azevedo et al. [5]

Table 3. *Cont.*

Cardiac Manifestations	Frequencies	References
Atrial Flutter	In late-onset phenotype with predominant cardiac involvement • 2.5% of males • 0.8% of females	• Azevedo et al. [5]
Ventricular Arrhythmias	• 13% in males • 20% in females	• Pinderski et al. [116]
	• Non-sustained VT in 8.3% of patients during 1.9 years of follow-up (n = 78)	• Shah et al. [98]
	• Non-sustained VT in 21% of patients • Sustained VT in 1% of patients (Median follow-up of 36 months)	• Hanneman et al. [117]
	Systematic review of the literature • VT in 15.3% of the patients	• Baig et al. [67]
	In late-onset phenotype with predominant cardiac involvement • Non-sustained VT in 14.1% of males Mean age at diagnosis of 57 ± 8 years • Non-sustained VT in 5.6% of females Mean age at diagnosis of 70 ± 6 years	• Azevedo et al. [5]
SCD	• SCD in 1 patient during 1.9 years of follow-up (n = 78)	• Shah et al. [98]
	• VF in 1 patient in the setting of multiorgan failure during mean follow-up of 53 months (n = 19)	• Acharya et al. [125]
	Systematic review of the literature • 62% of cardiac deaths are sudden • Incidence of SCD events: 0.34–1.4% per annum	• Baig et al. [67]
Cardiac device	• Annual incidence: 1.9 per person-years	• Sené et al. [123]
	• Annual incidence of devices for the treatment of bradycardia (pacemaker/ICD): 1.07 per person-years	• Patel et al. [100]
Pacemaker	• 12.5% of patients	• Sené et al. [123]
	• 5-year incidence: 8%	• O'Mahony [111]
	In late-onset phenotype with predominant cardiac involvement • 12.7% in males Mean survival free from pacemaker: 71 ± 2 • 2.4% in females Mean survival free from pacemaker: 86 ± 1	• Azevedo et al. [5]
ICD	• 4.2% of patients	• Sené et al. [123]
	In late-onset phenotype with predominant cardiac involvement • 1.3% in males • 0.8% in females	• Azevedo et al. [5]

AV, atrioventricular; ICD, implantable cardioverter-defibrillator; SCD, sudden cardiac death; VF, ventricular fibrillation; VT, ventricular tachycardia.

3.3. Coronary Manifestations

GB3 accumulation results in hypertrophy and proliferation of smooth muscle and endothelial cells and widespread narrowing of intramural coronary arteries [21,126].

On angiography, epicardial coronary arteries have been described as structurally normal, but presenting slow flow, and small vessel disease correlated with coronary slow flow and extent of fibrosis [126].

Hence, FD patients with angina present myocardial ischemia on exercise stress test, myocardial perfusion defects on single-photon emission computed tomography [126], and reduced myocardial blood flow and coronary flow reserve on positron emission tomography (PET) [127–129]. Myocardial blood flow is reduced in patients without LVH, both males and females, although it is more reduced in patients with LVH [129]. Perfusion defects showed marked regional heterogeneity, with prevalent hypoperfusion of the apical region [129]. In cardiac MRI including vasodilator stress perfusion mapping, the stress myocardial blood flow is also lower in all FD patients regardless of LVH status, although the reduction is more pronounced in patients with LVH. The reduction in stress myocardial blood flow is more pronounced in the subendocardium than in the subepicardium. LGE and low T1 were identified as independent predictors of stress global myocardial blood flow, while LV wall thickness, LGE, and T2 value as predictors of stress segmental myocardial blood flow [130].

Besides coronary microvascular dysfunction, coronary vasospasm may also contribute to angina [131,132] and may precipitate dysrhythmias and sudden death [133].

Oxygen supply to myocardium may also be impaired by LVOT obstruction and increased LV diastolic filling pressure, which reduce blood perfusion of the subendocardial layer of myocardium. Taken together with an increased demand of oxygen by the hypertrophied myocardium, the result is myocardial ischemia, which may be asymptomatic or manifest as angina and myocardial infarct or contribute to dysrhythmias and heart failure [134,135].

Moreover, FD patients commonly present cardiovascular risk factors and may develop coronary atherosclerotic disease. It is not clear if FD may increase the risk of accelerated atherosclerosis [136].

Angina has been reported in 22% of males and 23% of females with FD [134]. Mean age of onset was reported to be 42 ± 5 and 49 ± 13 years in males and females, respectively [31]. It may be the first manifestation of FD cardiomyopathy, preceding the development of LVH [137]. However, myocardial infarct is rare (<2%) [134], occurring in 2.7% of males and 1.5% of females [138] (Table 4). Hence, FD remains a rare cause of chest pain in patients without obstructive coronary artery disease (0.15%) [139].

3.4. Valvular Disease

GB3 accumulation in valve fibroblasts may ultimately lead to valve thickening and fibrosis [18,19] and regurgitation [20,35], which are usually mild/moderate and rarely require intervention [25,35]. Left-sided valves are most affected, probably due to greater hemodynamic stress [134]. Aortic valve was reported to be affected in 47% of the cases and mitral valve in 57% [35] (Table 4). Mitral valve prolapse has also been described [140], although now known to be less prevalent than originally reported [35]. Aortic root dilation may also occur in late stages of the disease, thereby contributing to aortic regurgitation [35]. The development of valve dysfunction also contributes to heart failure [25].

3.5. Aortic Dilation

Degenerative changes in the aortic media from glycolipid deposition [19] can lead to aortic dilatation at the sinuses of Valsalva and ascending aorta in 32.7% and 29.6% of males and 5.6% and 21.1% of females, respectively [141]. An aortic aneurysm, defined as an aortic diameter exceeding 1.5 times the upper limit of normal at the sinuses of Valsalva, was found in 9.6% of male and 1.9% of female patients [141] (Table 4).

Table 4. Frequency of other cardiac manifestations in FD.

Cardiac Manifestations	Frequencies	References
Angina	• 22% in males • 23% in females	• Linhart et al. [134]
	• Mean age of onset in males: 42 ± 5years • Mean age of onset in females: 49 ± 13 years	• Mehta et al. [31]
Myocardial infarct	• <2%	• Linhart et al. [134]
	• 2.7% in males • 1.5% in females	• Patel et al. [138]
Aortic valve dysfunction	• 47% of patients	• Linhart et al. [35]
Mitral valve dysfunction	• 57% of patients	• Linhart et al. [35]
Aortic dilation	At the sinuses of Valsalva • 32.7% in males • 5.6% in females At the ascending aorta • 29.6% in males • 21.1% in females	• Barbey et al. [141]
Aortic aneurysm	• 9.6% in males • 1.9% in females	• Barbey et al. [141]

3.6. Cardiac Events

The incidence of adverse cardiac events (composite endpoint of VT, bradycardia requiring device implantation, severe heart failure, or cardiac death) was 7.6% per year; LVH and LGE were predictors of adverse cardiac events and patients with extensive LGE ($\geq$15% of LV mass) were at highest risk [117]. In another study, the incidence of the primary endpoint (a composite of new onset atrial fibrillation, NYHA $\geq$ III symptoms, device insertion for bradycardia, or cardiac death) was 2.64 per 100 person-years. Age, MSSI, and QRS duration were independent predictors of the primary endpoint [100]. Age, eGFR, high-sensitivity troponin I, NT-proBNP, LV mass index, E/E' ratio, and GLS have shown incremental value in the prediction of adverse cardiac events (defined as composite of cardiac death, malignant VT, atrial fibrillation, or severe heart failure) [142] (Table 5).

Cardiac events (defined as myocardial infarction, arrhythmia, angina pectoris, congestive heart failure, or significant cardiac procedures, such as pacemaker placement, coronary bypass, stent placement, and valve replacement) were reported in 69.9% of males and 81.6% of females. Cardiac events were the first clinical events in 21.4% of males and 16.9% of females. Age at first cardiac event was 41.7 (5.3–80.4) in males and 49.8 (17.3–78.2) in females [28].

Table 5. Predictors/Factors associated to cardiac events in FD.

Cardiac Endpoints	Predictors/Factors Associated to Outcome	References
Composite endpoint of VT, bradycardia requiring device implantation, severe heart failure or cardiac death	Predictors • LVH • LGE Extensive LGE (≥15% of LV mass) were at highest risk	• Hanneman et al. [117]
Composite endpoint of new onset atrial fibrillation, NYHA ≥ III symptoms, device insertion for bradycardia or cardiac death	Predictors • Age • MSSI • QRS duration	• Patel et al. [100]
Composite endpoint of myocardial infarction, heart failure, or cardiac-related death	Predictor • LVH	• Patel et al. [138]
Composite endpoint of death, myocardial infarction, cerebral vascular accident, exacerbation of heart failure, arrythmia, or implantation of permanent pacemaker or cardioverter-defibrillator	Associated factor • Impaired basal segmental longitudinal strain	• Zada et al. [84]
Composite endpoint of sudden death, arrhythmia or pacing device insertion	Predictor • End-stage renal disease	• Talbot et al. [143]
Composite of cardiac death, malignant VT, atrial fibrillation or severe heart failure	Predictors • Age • eGFR • High-sensitivity troponin I • NT-proBNP • LV mass index • E/E' ratio • GLS	• Spinelli et al. [142]

eGFR, estimated glomerular filtration rate; GLS, global longitudinal strain; LGE, late gadolinium enhancement; LV, left ventricular; LVH, left ventricular hypertrophy; MSSI, Mainz severity score index; NT-proBNP, N-terminal prohormone of brain natriuretic peptide; NYHA, New York Heart Association; VT, ventricular tachycardia.

Ultimately, heart disease is the main cause of death of Fabry patients (40% in males and 41.7% in females) [28]. Cardiac death occurred in 3.4% over a mean follow-up period of 7.1 years (2.4% due to SCD and 1.0% due to heart failure). The annual incidence of cardiac death was 0.52 per 100 person years and the only independent predictor was LV mass [100]. Cardiac death was reported to occur at a median age of 55.5 years in males and 66.0 years in females [28]. All-cause mortality and heart failure-related mortality is higher in patients with FD than with sarcomeric HCM [144].

Table 6 summarizes the main recommendations for the diagnosis and monitoring of cardiac manifestations in FD.

Table 6. Summary of the main recommendations for the diagnosis and monitoring of cardiac manifestations in FD [145].

Recommendations for the Diagnosis and Monitoring of Cardiac Manifestations in FD
ECG
A standard 12-lead ECG is recommended in all adult patients at first clinical evaluation, every 6–12 months and when there is development of new symptoms.
Echocardiogram
Echocardiogram is recommended in all patients at baseline, every 12–24 months and with the development of new symptoms.
Exercise echocardiography
Exercise echocardiography should be performed in all symptomatic patients to exclude latent obstruction and exercise-induced mitral regurgitation.
Cardiac MRI
Cardiac MRI should be considered in all adult patients at baseline to assess cardiac morphology and function and myocardial fibrosis; and may be considered, every 2–5 years in patients without cardiac abnormalities and every 2–3 years in patients with progressive disease, in order to assess progression of fibrosis and cardiac function. T1 mapping may also be considered to detect early cardiac involvement or to help in the differential diagnosis of LVH.
Holter monitoring
A 24 h-Holter monitoring should be considered in all adult patients at first clinical evaluation, every 6–12 months and when there is development of new symptoms.
ILR
A prolonged Holter monitoring or preferably an ILR should be considered in patients with recurrent episodes of unexplained syncope. An ILR may also be considered in patients with palpitations or recent stroke and negative Holter monitoring.
Cardiopulmonary exercise testing
Cardiopulmonary exercise testing should be considered in patients with exercise intolerance.
Coronary angiography
Coronary angiography (or CT coronary angiography) is recommended in all patients with angina CCS class $\geq$ II. Invasive coronary angiography is recommended in adult survivors of cardiac arrest, in patients with sustained VT and in patients with severe stable angina (CCS class III) or unstable angina.
BNP/NT-proBNP
Measurement of plasma BNP/NT-proBNP is recommended in symptomatic patients with suspected heart failure.
High-sensitivity troponin
High-sensitivity troponin may be considered to assess disease severity.
Renal function
Regular assessment of renal function and albuminuria/proteinuria is recommended in all patients.
Endomyocardial biopsy
When a genetic variant of uncertain significance is found in the *GLA* gene, an endomyocardial biopsy with electron microscopy should be considered, particularly in females or in patients with high residual enzyme activity (>10%) and low lyso-GB3 levels, in order to exclude FD as the cause of LVH.

BNP, brain natriuretic peptide; CCS, Canadian Cardiovascular Society; CT, computed tomography; ECG, electrocardiogram; FD, Fabry disease; ILR, implantable loop recorder; LVH, left ventricular hypertrophy; MRI, magnetic resonance imaging; NT-proBNP, N-terminal prohormone of brain natriuretic peptide; VT, ventricular tachycardia.

4. Late-Onset Phenotypes with Predominant Cardiac Involvement ("Cardiac Variants")

Cardiac involvement may be the predominant feature in some late-onset phenotypes. These phenotypes, commonly designated by some authors as "cardiac variants" of FD, have been described in association to the *GLA* mutations p.F113L [4,5,146], p.N215S [147–150], IVS4+919G>A [151], p.A20P [152], p.I91T [146], p.N139S [153], p.I232T [154], p.I239M [155], p.Q279E [156,157], p.M296I [152,158], p.M296V [159], p.R301Q [156,157,160], and p.G328R [161].

Some of these mutations are very common and, despite their widespread distribution around the world, they occur in large clusters in specific geographical areas, such as the IVS4+919G>A mutation in Taiwan, the p.N215S mutation in the United Kingdom, and the p.F113L mutation in the Portuguese region of Guimarães. Indeed, a founder effect has already been documented for the IVS4+919G>A mutation in Southern China [162] and the p.F113L mutation in the Portuguese region of Guimarães [4,29].

These *GLA* mutations are associated to residual enzymatic activity of α-galactosidase A [4,5,150,163]. Plasma lyso-GB3 levels are lower than in patients with classic phenotypes in both genders, and normal or near-normal values are usually found in these females [4,5,150,164–169]. Cardiac deposits of GB3 are only found in myocardial cells [22,164,167,170,171].

Cardiac manifestations are common and carry the highest prognostic impact [4,5]. Despite the attenuated course of late-onset phenotypes, the severity of cardiac involvement is the same or greater than in classic phenotypes [164,172]. LVH remains the most common cardiac manifestation, occurring in 40.2% of the patients (21–67%) [5]. The first cardiac manifestations are LVH and LGE, which arise in males over 30 years and are followed by heart failure, non-sustained VT, and cardiac conduction disorders, which arise in males over 40 years, culminating with the development of bifascicular block and complete AV block in males beyond the age of 50 years. Cardiac manifestations are more common and arise one to two decades earlier in males, and their frequency and severity increase with age in both genders [5].

In these late-onset phenotypes, acroparesthesias, neuropathic pain, hypohidrosis, heat, cold and exercise intolerance, gastrointestinal symptoms, angiokeratomas, and cornea *verticillata* are characteristically absent or rare [4,5,150,163,165,166,169,172]. However, other extracardiac manifestations, such as proteinuria, brain white matter lesions, and deafness, are common and arise early, before 30 years of age. Their prognostic impact, however, is lower, as renal insufficiency, stroke, and need for a hearing device is uncommon [4,5,150,165,166,169,172–174].

5. Cardiac Treatment in FD

5.1. ERT

ERT with recombinant α-galactosidase A has been approved for clinical use since 2001. There are two commercially available preparations, agalsidase alfa and agalsidase beta, both administered intravenously every other week at the doses of 0.2 and 1 mg/kg of body weight, respectively [175].

According to the current recommendations, ERT should be initiated in classic males at the age of 16 years regardless of symptomatic status, although it should be considered earlier, on an individual basis, since the age of 8–10 years old. In late-onset males and in classic/late-onset females, LVH, cardiac fibrosis or cardiac rhythm, or conduction abnormalities constitute indications to start ERT [176].

Agalsidase alfa has been demonstrated to decrease/stabilize LV mass [177–179] and wall thickness [179] in males, as well as to decrease/stabilize LV mass [178–181] and wall thickness [179] and to improve exercise capacity [181] in females. However, myocardial GB3 content did not significantly decrease on endomyocardial biopsies taken at 6 months of treatment with agalsidase alfa [177]. In a study comparing patients treated with agalsidase alfa from the Fabry Outcome Survey registry with historical cohorts of untreated patients, the median age at first event seemed to be higher in patients under agalsidase alfa than in untreated patients, both in males (48 vs. 41 years) and females (57 vs. 53 years). The median survival time also seemed to be higher in males under agalsidase alfa than in untreated males (77.5 vs. 60 years). However, these findings have to be interpreted with caution because this study was a retrospective analysis that used a historical cohort as a comparator group [182].

Agalsidase beta has been shown to reduce/stabilize LV mass [128,183–185] and wall thickness [128,185] in males, as well as to decrease/stabilize LV mass [183,185] and wall thickness [185] in females. Complete clearance of GB3 deposits was also achieved in interstitial capillary endothelial cells from 72% of the patients treated with agalsidase beta for

5 months, and this benefit seemed to be sustained up to 60 months. Nevertheless, no clearance of GB3 was observed in the cardiomyocytes [186]. Conversely, in another study, there was no improvement of GB3 deposits in endothelial and smooth muscle cells, myocardial blood flow, perfusion defects, or electrocardiographic changes on exercise stress test with agalsidase beta for ≥12 months [126]. In a randomized clinical trial, agalsidase beta, compared to placebo, significantly increased the time to first clinical event in protocol-adherent patients, when adjusted for baseline proteinuria (considering clinical events as cardiac: myocardial infarction; new symptomatic arrhythmia requiring antiarrhythmic medication, pacemaker, direct current cardioversion, or defibrillator implantation; unstable angina defined by national practice guidelines and accompanied by electrocardiographic changes resulting in hospitalization; or worsening congestive heart failure requiring hospitalization; renal: 33% increase in serum creatinine level from baseline (2 consecutive values) or end-stage kidney disease requiring long-term dialysis or transplantation; cerebrovascular: stroke or transient ischemic attack; or death). However, most clinical events were renal and these were limited to the increase in serum creatinine, while there was a low rate of cerebrovascular events and death [187]. Moreover, agalsidase beta has reduced the incidence rate of clinicals events after 6 months of treatment, maintaining, from 6 months to up to 5 years of treatment, a stable incidence rate of clinical events (cardiac: myocardial infarction, first-time congestive heart failure, atrial fibrillation, VT, evidence of progressive heart disease sufficiently severe to require a pacemaker, heart bypass surgery, coronary artery dilatation or implantation of an ICD; renal: chronic dialysis (>40 days) or renal transplantation; cerebrovascular: hemorrhagic or ischemic stroke; or death due to any cause) [188].

The evaluation of ERT efficacy in the late-onset phenotypes with predominant cardiac involvement has been limited to a few small studies in the IVS4+919G>A mutation. In these patients, a significant negative correlation has been described between ERT duration and GB3 accumulation in cardiomyocytes and cardiomyocyte size [189]. In patients under ERT for more than 3 years, GB3 deposits were not found in cardiomyocytes [170]. In a study including 23 patients with the IVS4+919G>A mutation, plasma lyso-Gb3 decreased under ERT both in males and females, reaching the lowest value at 11.1 months and increasing gradually thereafter, even when LV mass index was still improving or remaining stable [167]. Finally, in another study, including 26 patients with the IVS4+919G>A mutation under ERT for 6–39 months, a significant reduction in mean plasma lyso-GB3 (on average by 28%) has been reported in males and females, with 89% of patients experiencing a reduction in plasma lysoGB3. A stabilization or reduction in LV mass index, interventricular septum and posterior wall thickness has been observed in 83%, 83%, and 67% of patients, respectively, with an average decrease in 12% in LV mass index, 14% in interventricular septum, and 13% in posterior wall thickness. Mean LV mass index, interventricular septum, and posterior wall thickness have been found to decrease both in males and females, although only reaching statistical significance for LV mass index and posterior wall thickness in females [166].

Better cardiac outcomes are achieved with early treatment. Agalsidase beta was associated to a statistically significant decline in LV mass when started at the age < 30 years, but an increase in LV mass was seen when it was started at the age ≥ 50 years [184]. Likewise, agalsidase beta started at the age < 40 years was associated to a stable thickness of the interventricular septum and posterior wall over a period of 10 years, whereas a significant worsening of these parameters was observed over time when it was started at the age ≥ 40 years [190]. Moreover, in patients without fibrosis, agalsidase beta resulted in a statistically significant decline of LV mass and improvement of exercise capacity and LV radial strain rate, while no effect was observed in patients with mild or severe fibrosis at the time of treatment initiation [65]. Similarly, in patients treated with agalsidase alfa, LVH or low eGFR at the time of treatment initiation were associated with a higher risk of cardiovascular events [191].

A study comparing agalsidase alfa and agalsidase beta found that a higher proportion of patients had a decrease in LV mass index when treated for 1 year with agalsidase beta than with agalsidase alfa at licensed doses (79% vs. 62%). Nevertheless, no difference between agalsidase alfa and beta was found regarding clinical events [192].

Neutralizing anti-drug antibodies may attenuate ERT efficacy. Although previous reports have suggested that anti-drug antibodies had no effect on the time to first clinical event [193], other studies have shown that they were associated to higher LV mass, disease severity scores, and frequency of symptoms [194] and worse renal function [194,195].

5.2. Migalastat

Migalastat is a first-in-class pharmacological chaperone therapy for FD, administered orally at the dosage of 123 mg once every other day, which has been approved by the European Medicines Agency for the treatment of FD patients aged $\geq$ 16 years, with eGFR $\geq$ 30 mL/min/1.73 m^2 and amenable *GLA* mutations [175,196].

Migalastat has consistently shown to decrease LV mass [197–203]. In the FACETS trial, in the modified-intention to treat population (i.e., ERT-naïve patients with migalastat-amenable *GLA* mutations), there was a significant reduction in the mean LV mass index compared to the baseline after 24 months of migalastat therapy (i.e., after 18 months of migalastat in patients who switched from placebo or 24 months of continuous migalastat) [197]. In the ATTRACT trial, in ERT-experienced patients with amenable *GLA* mutations who were randomized to switch to migalastat or continue ERT, migalastat significantly reduced the mean LV mass index at 18 months, and changes on LV mass index correlated with changes in the thickness of the interventricular septum and not the posterior wall [198]. In the open-label extension study, a significant decrease in LV mass index was found after 30 months of migalastat in patients with LVH at baseline [199]. Muntze et al. reported one patient who showed improvement of LV mass, LGE, troponin, and NT-proBNP under treatment with migalastat for 12 months [200]. Later, the same authors reported a decrease in LV mass index in 14 patients treated with migalastat for 1 year [201]. Riccio et al. also reported a significant decrease in LV mass index after 1 year of treatment with migalastat in seven FD males previously treated with ERT [202]. In the larger FAMOUS study, including 59 previously ERT-treated and untreated FD patients, treatment with migalastat for 12 months was also associated to a significant decrease in LV mass index [203].

No data have been published on the efficacy of migalastat specifically on the late-onset phenotypes.

5.3. Supportive Treatment

Control of cardiovascular risk factors, including arterial hypertension and dyslipidaemia is indicated [134]. Systolic blood pressure was higher in patients with LGE and highest in patients with a faster progression of LGE [204]. In fact, hypertension increased the odds of cardiovascular events (myocardial infarction, heart failure, or cardiac-related death) by 7.8 in men and 4.5 in women [138].

Angiotensin converting enzyme inhibitors or angiotensin II receptor blockers should be used in patients with LVH, LV systolic dysfunction, and heart failure, but also in the presence of proteinuria [25,51,145,205,206]. Mineralocorticoid receptor antagonists should also be considered in patients with heart failure and LV systolic dysfunction [51,145]. However, these drugs must be used with caution in patients with nephropathy due to the possible development of hyperkalemia or worsening of renal function [145]. Evidence is lacking on the use of sacubitril/valsartan in Fabry patients.

Beta-blockers are recommended to relieve LVOT obstruction symptoms or control the rate of atrial fibrillation/flutter and should be considered in patients with angina or heart failure and LV systolic dysfunction [25,51,145,207,208]. Verapamil should also be considered for the treatment of angina and is recommended for the treatment of LVOT obstruction symptoms. Diltiazem should also be considered in patients with LVOT obstruction symp-

toms or angina. Ivabradine should be considered for the treatment of heart failure or angina, according to the European Society of Cardiology (ESC) guidelines [51,145,207,208]. However, these drugs must be handled with care, due to the propensity of FD patients to develop chronotropic incompetence and bradydysrhythmias. Dihydropyridine calcium channel blockers may be safer alternatives and should be considered for angina treatment [134,208].

Loop diuretics should be considered to treat symptoms of congestion in patients with heart failure [134,207].

Cardiac resynchronization therapy (CRT) has also been applied in a few FD cases at burn-out stage with LV systolic dysfunction and heart failure [209], but its efficacy in this cohort of patients may be hampered by extensive scar burden. Nevertheless, it should be considered in patients with LV dysfunction (ejection fraction $\leq$ 35%), according to the current ESC guidelines [207]. CRT-P should also be considered in symptomatic patients with a pacing indication, LV ejection fraction < 50%, and QRS duration > 120 ms [145].

In patients with resting or latent LVOT obstruction, digoxin is not recommended and vasodilators, such as angiotensin converting enzyme inhibitors, angiotensin II receptor blockers, dihydropyridines, and nitrates, should be avoided, if possible [51,145]. Myectomy should be performed in the rare cases of LVOT obstruction associated with severe symptoms of heart failure or syncope, resulting in excellent operative outcome and relief of LVOT obstruction and symptoms [210]. Septal alcohol ablation may be a safe alternative treatment for alleviation of LVOT obstruction and improvement of related heart failure in Fabry patients [211].

Antiplatelet aggregation therapy should be started in patients who suffered a stroke or myocardial infarction [134]. Anticoagulation should be immediately started once atrial fibrillation or flutter is detected [134], based on the evidence extrapolated from HCM, and no risk score should be used for this purpose as none has been validated in FD [145]. Direct oral anticoagulants (DOACs) should be considered as the first-line choice in Fabry patients without contraindications, namely, related to renal failure, because, despite the lack of systematic data on their use on FD, they are associated to lower risk of intracranial bleeding and may avoid the risk of warfarin-induced nephropathy [145].

Prior stroke/transient ischemic attack (TIA), angiokeratoma, LV posterior wall thickness >14 mm, creatinine $\geq$1.0 mg/dL, and GLS > $-$13.5% were identified as independent risk factors for new or recurrent stroke/TIA in FD patients without atrial fibrillation. A new predicting score based on these risk factors was proposed to predict stroke/TIA in FD patients without atrial fibrillation. It remains to clarify if high-risk patients, according to this score, might benefit from antithrombotic therapy [212].

Amiodarone should be avoided in FD patients, as it induces lysosomal dysfunction and may precipitate clinical worsening [213]. It induces phospholipidosis by inhibiting lysosomal degradation of phospholipids [214]. Additionally, dronedarone is contraindicated in patients with heart failure (NYHA class III–IV) and renal failure (eGFR < 30 mL/min). Sotalol, flecainide, and propafenone are also contraindicated in patients with heart failure [145].

Isolation of pulmonary veins has been performed [215], but it may require longer and repeated procedures [216].

Pacemaker may be required to treat symptomatic bradycardia or symptomatic/advanced cardiac blocks, according to ESC guidelines [134,145].

ICD implantation is recommended in patients who suffered sudden cardiac arrest due to VT/ventricular fibrillation or sustained VT causing syncope or hemodynamic compromise and have a life expectancy of >1 year [145]. ICD implantation should be considered in patients with advanced hypertrophy and fibrosis, who require pacemaker implantation and have a life expectancy of >1 year [145]. Evidence is lacking to guide ICD implantation in primary prevention in FD patients and the HCM RISK-SCD score should not be used for this purpose [145]. Nevertheless, it is generally accepted that FD patients should receive ICD in the presence of heart failure (NYHA class II–III) and LV ejection

fraction of ≤35% [207]. Patients who have significant fibrosis on MRI and those who have non-sustained VT on Holter monitoring are at higher risk for arrhythmic complications and may be considered for ICD [66,123,145]. VT ablation has also been performed with success in some cases of recurrent VT or ICD storms [119]. However, radiofrequency ablation by endocardial and/or epicardial approaches is challenging, as the target tissue is frequently localized at the midwall [216]. Asymptomatic runs of non-sustained VT do not usually require anti-arrhythmic therapy [145].

Heart transplantation should be considered in patients with advanced heart failure with severe LV systolic and diastolic dysfunction and NYHA class III–IV despite optimal medical therapy, or intractable ventricular arrhythmia, depending on the extension of the extracardiac involvement by the disease and considering that the disease does not affect the transplanted organ [134,217] (Table 7).

Table 7. Summary of the main recommendations for the supportive treatment of cardiac manifestations in FD.

Recommendations for the Supportive Treatment of Cardiac Manifestations in FD
Angiotensin Converting Enzyme Inhibitors or Angiotensin II Receptor Blockers [1,2]
Angiotensin converting enzyme inhibitors (or angiotensin II receptor blockers, if not tolerated) should be used in patients with LV systolic dysfunction and heart failure [25,51,145].
Beta-blockers [3]
Beta-blockers should be considered in patients with heart failure and LV systolic dysfunction; or in patients with angina [25,51,145,207,208]. Beta-blockers are recommended to relieve LVOT obstruction symptoms and to control the rate of atrial fibrillation/flutter [25,51,145].
Mineralocorticoid receptor antagonists [1]
Mineralocorticoid receptor antagonists should be considered in patients with heart failure and LV systolic dysfunction [51,145].
Loop diuretics
Loop diuretics should be considered to treat symptoms of congestion in patients with heart failure [134,207].
Calcium channel blockers
Dihydropyridines[2] should be considered for the treatment of angina [134,208]. Verapamil[3] is recommended in patients with LVOT obstruction symptoms and should be considered in patients with angina [51,145,208]. Diltiazem[3] should be considered in patients with LVOT obstruction symptoms or angina [51,145,208].
Ivabradine [3]
Ivabradine should be considered for the treatment of heart failure or angina, according to ESC guidelines [145,207,208].
Antiplatelet therapy
Antiplatelet therapy should be started in patients who suffered a stroke or myocardial infarction [134].
Anticoagulation
Anticoagulation should be immediately started once atrial fibrillation or flutter is detected [145]. Direct oral anticoagulants (DOACs) should be considered as the first-line choice in patients without contra-indications [145].
Anti-arrhythmic drugs
Amiodarone should be avoided in FD [213,214]. Dronedarone is contra-indicated in patients with heart failure (NYHA class III–IV) and renal failure (eGFR < 30mL/min) [145]. Sotalol, flecainide and propafenone are contra-indicated in patients with heart failure [145].
Management of cardiovascular risk factors
Control of cardiovascular risk factors, including arterial hypertension, diabetes and dyslipidaemia, is indicated [134].

Table 7. *Cont.*

Recommendations for the Supportive Treatment of Cardiac Manifestations in FD
Pacemaker
Pacemaker may be required to treat symptomatic bradycardia or symptomatic/advanced cardiac blocks, according to ESC guidelines [134,145]. Dual chamber pacemakers should be implanted unless patients are in permanent atrial fibrillation [145].
ICD
ICD implantation is recommended in patients who suffered sudden cardiac arrest due to VT/fibrillation or sustained VT causing syncope/haemodynamic compromise and have a life expectancy >1 year [209]. ICD implantation should be considered in patients with advanced hypertrophy and fibrosis, who require pacemaker implantation and have a life expectancy >1 year [145]. ICD implantation may be considered in patients with severe LVH and advanced fibrosis or non-sustained VT, who have a life expectancy >1 year [145]. ICD implantation is recommended in patients with heart failure (NYHA class II-III) and LV ejection fraction ≤35%, despite ≥3 months of optimal treatment, who have a life expectancy >1 year [207].
CRT
CRT should be considered in patients with LV ejection fraction ≤35%, according to ESC guidelines [207]. CRT-P should be considered in symptomatic patients with a pacing indication, LV ejection fraction <50% and QRS duration >120ms [145].
Septal reduction therapy (myectomy/alcohol ablation therapy)
Septal reduction therapy is recommended in patients with a resting or provoked LVOT gradient ≥50 mm Hg, who are in NYHA class III–IV, despite maximum tolerated medical therapy [51,210,211]. Septal reduction therapy should be considered in patients with a resting or provoked LVOT gradient ≥50 mm Hg, who suffer recurrent exertional syncope, despite maximum tolerated medical therapy [51,210,211].
Heart transplantation
Heart transplantation should be considered in patients with advanced heart failure with severe LV dysfunction and NYHA class III–IV despite optimal medical therapy, or intractable ventricular arrhythmia, depending on the extension of the extracardiac involvement by the disease [134,217].

CRT, Cardiac resynchronization therapy; eGFR, estimated glomerular filtration rate; ESC, European Society of Cardiology; FD, Fabry disease; ICD, Implantable cardioverter-defibrillator; LV, left ventricular; LVH, left ventricular hypertrophy; LVOT, left ventricular outflow tract; NYHA, New York Heart Association; VT, ventricular tachycardia [1] Caution should be taken in Fabry patients with nephropathy due to the risk of hyperkalaemia or worsening of renal function; [2] Should be avoided, if possible, in patients with resting/latent LVOT obstruction; [3] Caution should be taken due to the increased risk of bradycardia in Fabry patients.

6. Conclusions

Cardiac involvement remains the leading cause of death in Fabry patients. GB3 deposits are found in virtually all cardiac cells, but the mechanisms leading to organ damage remain less defined. Fabry hearts exhibit myocardial hypertrophy, inflammation, apoptosis, necrosis and fibrosis, valve thickening, and narrowing of intramural coronary arteries. Cardiac manifestations therefore include LVH, heart failure, angina, valve disease, dysrhythmias, cardiac conduction blocks, and sudden cardiac death, and the severity of cardiac involvement is the same either in classic or late-onset phenotypes.

ERT has demonstrated to reduce or stabilize LV mass and wall thickness and to reduce the incidence and delay the occurrence of clinical events, whereas migalastat has consistently shown to reduce LV mass. Early treatment has shown to achieve better outcomes, while cardiac fibrosis is deemed to be irreversible. Still, evidence is lacking on the efficacy of available therapies on hard-endpoints, such as total mortality, cardiovascular mortality, heart failure, dysrhythmias, or need for a cardiac device, as well as in late-onset phenotypes with predominant cardiac involvement. Furthermore, despite several therapies targeting, the enzymatic defect or substrate accumulation are under research, it remains to be clarified if other therapeutic strategies will be needed to avoid or revert cardiac damage in FD.

Author Contributions: Conceptualization, O.A.; methodology, O.A., F.C. and M.F.G.; validation, O.A., M.F.G. and G.M.-M.; formal analysis, O.A., F.C., M.F.G. and C.F.; investigation, O.A., F.C., M.F.G., G.M.-M. and C.F.; resources, O.A.; writing—original draft preparation, O.A.; writing—review and editing, O.A., F.C., M.F.G., G.M.-M., C.F., N.S. and D.C.; supervision, O.A., N.S. and D.C. All authors have read and agreed to the published version of the manuscript.

Funding: There were no funding sources.

Acknowledgments: The authors of this publication O.A., F.C., M.F.G. and G.M.-M. are members of the European Reference Network for Rare Hereditary Metabolic Disorders (MetabERN).

Conflicts of Interest: O.A. and G.M.-M. have received educational/research grants from Shire Human Genetic Therapies/Takeda and travel/accommodation support for conferences from Shire Human Genetic Therapies/Takeda, Amicus and Sanofi Genzyme. M.F.G. has received travel/accommodation support for conferences from Shire Human Genetic Therapies, Amicus, and Sanofi Genzyme. C.F. has received travel/accommodation support for conferences from Shire Human Genetic Therapies/Takeda, Amicus, and Sanofi Genzyme. The remaining authors declare no conflicts of interest.

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Chapter 10.2

Azevedo O, Gago MF, Miltenberger-Miltenyi G, Sousa N, Cunha D.

Fabry Disease Therapy: State-of-the-Art and Current Challenges.

Int J Mol Sci. 2020 Dec 28;22(1):206. doi: 10.3390/ijms22010206.

International Journal of
Molecular Sciences

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Review

Fabry Disease Therapy: State-of-the-Art and Current Challenges

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Abstract: Fabry disease (FD) is a lysosomal storage disorder caused by mutations of the *GLA* gene that lead to a deficiency of the enzymatic activity of α-galactosidase A. Available therapies for FD include enzyme replacement therapy (ERT) (agalsidase alfa and agalsidase beta) and the chaperone migalastat. Despite the large body of literature published about ERT over the years, many issues remain unresolved, such as the optimal dose, the best timing to start therapy, and the clinical impact of anti-drug antibodies. Migalastat was recently approved for FD patients with amenable *GLA* mutations; however, recent studies have raised concerns that "in vitro" amenability may not always reflect "in vivo" amenability, and some findings on real-life studies have contrasted with the results of the pivotal clinical trials. Moreover, both FD specific therapies present limitations, and the attempt to correct the enzymatic deficiency, either by enzyme exogenous administration or enzyme stabilization with a chaperone, has not shown to be able to fully revert FD pathology and clinical manifestations. Therefore, several new therapies are under research, including new forms of ERT, substrate reduction therapy, mRNA therapy, and gene therapy. In this review, we provide an overview of the state-of-the-art on the currently approved and emerging new therapies for adult patients with FD.

Keywords: fabry disease; enzyme replacement therapy; agalsidase alfa; agalsidase beta; migalastat; pegunigalsidase alfa; moss-derived alfa galactosidase A; substrate reduction; mRNA; gene therapy

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Citation: Azevedo, O.; Gago, M.F.; Miltenberger-Miltenyi, G.; Sousa, N.; Cunha, D. Fabry Disease Therapy: State-of-the-Art and Current Challenges. *Int. J. Mol. Sci.* **2021**, 22, 206. https://dx.doi.org/10.3390/ijms22010206

Received: 2 December 2020
Accepted: 21 December 2020
Published: 28 December 2020

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1. Fabry Disease Overview

Fabry disease (FD) (OMIM 301500) is a rare X-linked lysosomal storage disorder caused by mutations in the *GLA* gene, leading to deficiency of the enzymatic activity of α-galactosidase A. Subsequent accumulation of globotriaosylceramide (GB3) and other neutral glycosphingolipids occurs in body fluids and lysosomes of cells throughout the body, including in those that are particularly relevant to disease pathology, such as in the heart (cardiomyocytes, conduction system cells, vascular endothelial, and smooth muscle cells and fibroblasts), kidney (podocytes, tubular, glomerular, mesangial, and interstitial cells), nervous system (neurons in autonomic and posterior root ganglia) and vascular endothelium and smooth muscle [1,2].

GLA mutations causing a virtually null enzymatic activity (<5% of the normal mean) are associated to severe and early onset classical phenotypes, while mutations leading to a residual enzymatic activity are associated to attenuated and late-onset phenotypes [1–3]. Classical phenotypes are characterized by early development, in childhood or adolescence, of acroparesthesias, neuropathic pain, hypohydrosis, heat, cold and exercise intolerance, cornea *verticillata*, angiokeratomas, gastrointestinal symptoms, and proteinuria. In adulthood, patients also suffer from sensorineural deafness and cardiac, renal, and cerebrovascular manifestations. In contrast, late-onset phenotypes are characterized by the development of cardiac, renal, and/or cerebrovascular manifestations in adulthood, and the phenotype may be dominated by the involvement of an organ, such as the heart or the kidneys [2–5]. Cardiac manifestations include left ventricular hypertrophy (LVH), heart failure, angina, dysrhythmias, cardiac conduction abnormalities, and sudden death. Renal involvement may lead to end-stage renal failure, and brain involvement is characterized by the development of brain white matter lesions (WML) and the occurrence of strokes or transient ischemic attacks (TIA) [2–5]. In this X-linked disorder, heterozygote females are not merely carriers, and their clinical spectrum ranges from asymptomatic to full-blown disease as severe as in affected males [6,7]. Ultimately, FD leads to a reduction of quality of life [8] and survival, with death being mainly driven by heart disease [9,10].

Available therapies for FD include enzyme replacement therapy (ERT) (agalsidase alfa and agalsidase beta) and the chaperone migalastat [11]. However, several new therapies are under research, including new forms of ERT, substrate reduction therapy, mRNA therapy, and gene therapy [12,13].

2. ERT

2.1. Efficacy and Safety of ERT

ERT with recombinant α-galactosidase A has been approved for clinical use since 2001. There are two commercially available preparations: agalsidase alfa, produced from human fibroblasts; and agalsidase beta, produced in Chinese hamster ovary (CHO) cells. Both are administered intravenously every other week (eow) at doses of 0.2 mg/kg and 1 mg/kg of body weight, respectively [11].

In males, agalsidase alfa has been demonstrated to decrease plasma GB3 [14–16] and lyso-GB3 [17] and urinary GB3 [14,16] levels; to decrease GB3 deposits in kidney endothelial cells [15]; to slow the decline of the estimated glomerular filtration rate (eGFR) [15,16]; to reduce/stabilize left ventricular (LV) mass [14,18,19] and wall thickness [19]; to improve/stabilize vestibular/auditory symptoms [20,21]; to improve nerve sensitivity [22], gastrointestinal symptoms [23], sweat function [23], pain [15], and pain-related quality of life [15].

In males, agalsidase beta has been shown to decrease plasma GB3 [24–27] and lyso-GB3 [26] and urinary GB3 [25] levels; to decrease GB3 deposits in different kidney cell types [24,25,28] and endothelial cells in skin [24,25]; to slow the decline of eGFR [25,29,30]; to reduce/stabilize LV mass [27,29,31,32] and wall thickness [27,32]; to improve nerve sensitivity [33], gastrointestinal symptoms [34], sweat function [33,35], pain [35], and quality of life [24,35].

In females, agalsidase alfa has been demonstrated to decrease plasma GB3 [36,37], plasma lyso-GB3 [17,26] and urine GB3 [36,37] (when pre-treatment values were elevated); to stabilize/decrease the decline of eGFR [19,36–38]; to decrease/stabilize LV mass [18,19,36,37] and wall thickness [19]; to improve exercise capacity [37]; to stabilize hearing loss and vestibular function [21]; and to improve quality of life [36].

In females, agalsidase beta has been shown to stabilize plasma GB3 (when pre-treatment values were normal) [26]; to decrease plasma lyso-GB3 [26] (when pre-treatment values were elevated); to stabilize eGFR [29]; to decrease/stabilize LV mass [29,32] and wall thickness [32]; and to improve quality of life [39].

Agalsidase alfa and beta are generally safe. The most common adverse events are infusion associated reactions (IAR), which are mild or moderate and tolerable in most cases [14,15,40,41] (Table 1).

Table 1. Currently approved therapies for adult patients with FD.

	Agalsidase Alfa	Agalsidase Beta	Migalastat
Definition	Recombinant α-galactosidase A produced from human fibroblasts [11]	Recombinant α-galactosidase A produced from Chinese hamster ovary (CHO) cells [11]	Low molecular weight iminosugar analogue of the terminal galactose residue of GB3 [42–44]
Mechanism of action	Enzyme replacement therapy [11]	Enzyme replacement therapy [11]	Pharmacological chaperone that selectively and reversibly binds to the active site of amenable mutant forms of α-galactosidase A, stabilizing it, preventing its retention in the ER, and enabling its trafficking to the lysosomes [42–44]
Administration route	Intravenous [45]	Intravenous [46]	Oral [47]
Dose and frequency of administration	0.2 mg/Kg every other week [45]	1 mg/Kg every other week [46]	123 mg once every other day [47]
Efficacy	*Males* ■ Decreases plasma GB3 [14–16] and lyso-GB3 [17] and urinary GB3 [14,16] levels ■ Decreases GB3 deposits in kidney endothelial cells [15] ■ Slows the decline of eGFR [15,16] ■ Reduces/stabilizes LV mass [14,18,19] and wall thickness [19] ■ Improves/stabilizes vestibular/auditory symptoms [20,21] ■ Improves nerve sensitivity [22], gastrointestinal symptoms [23], sweat function [23], pain [15] and pain-related quality of life [15] *Females* ■ Decreases plasma GB3 [36,37], plasma lyso-GB3 [17,26], and urine GB3 [36,37] (when pre-treatment values were elevated) ■ Stabilizes/decreases the decline of eGFR [19,36–38] ■ Decreases/stabilizes LV mass [18,19,36,37] and wall thickness [19] ■ Improves exercise capacity [37] ■ Stabilizes hearing loss and vestibular function [21] ■ Improves quality of life [36]	*Males* ■ Decreases plasma GB3 [24–27] and lyso-GB3 [26] and urinary GB3 [25] levels ■ Decreases GB3 deposits in different kidney cell types [24,25,28] and endothelial cells in skin [24,25] ■ Slows the decline of eGFR [25,29,30] ■ Reduces/stabilizes LV mass [27,29,31,32] and wall thickness [27,32] ■ Improves nerve sensitivity [33], gastrointestinal symptoms [34], sweat function [33,35], pain [35], and quality of life [24,35] *Females* ■ Stabilizes plasma GB3 (when pre-treatment values were normal) [26] ■ Decreases plasma lyso-GB3 [26] (when pre-treatment values were elevated) ■ Stabilizes eGFR [29] ■ Decreases/stabilizes LV mass [29,32] and wall thickness [32] ■ Improves quality of life [39]	■ Decreases plasma lyso-GB3 [48,49] ■ Decreases mean number of GB3 inclusions/kidney interstitial capillary [48] and mean total GB3 inclusion volume per podocyte [50] ■ Reduces mean LV mass index [48,49,51–55] ■ Improves diarrhoea [48,56] ■ No change in eGFR, mGFR, 24 h-urine protein excretion and 24 h urinary GB3 in pivotal clinical trials [48,51]; however, decline of eGFR was reported in some real-life studies [49,54]
Safety	■ IAR are the most common side effects (mainly mild, such as fever and chills) [14,15] ■ Hypersensitivity reactions are rare [45] ■ Anti-drug antibodies in 20% of treated males [14]	■ IAR are the most common side effects (mainly mild, such as fever and chills) [40,41] ■ Hypersensitivity reactions are rare [46] ■ Anti-drug antibodies in 91% of treated males [57]	■ Headache and nasopharyngitis are the most common side effects [48,51] ■ No immunogenicity issues [58]

eGFR, estimated glomerular filtration rate; ER, endoplasmic reticulum; IAR, infusion associated reactions; LV, left ventricular; mGFR, measured glomerular filtration rate.

2.2. Best Timing to Start ERT

According to the current recommendations, ERT should be initiated (i) in classic males at the age of 16 years regardless of symptomatic status, although it should be considered earlier, on an individual basis, since the age of 8–10 years old; and (ii) in late-onset males and in classic/late-onset females with signs/symptoms of FD (LVH, cardiac fibrosis, cardiac rhythm or conduction abnormality, microalbuminuria/proteinuria non-attributed to other causes, chronic kidney disease (eGFR <90 mL/min/1.73 m^2), stroke or transient ischemic attack, neuropathic pain, gastrointestinal symptoms such as abdominal pain or diarrhoea, anhidrosis/hypohidrosis). In asymptomatic late-onset males and classic/late-onset females, ERT could be considered in the presence of moderate or severe GB3 deposits, podocyte foot process effacement, or glomerulosclerosis on kidney biopsy [11].

Early treatment has clearly been shown to achieve better outcomes.

Classic males who started ERT before the age of 25 years achieved greater reduction of plasma lyso-GB3 than the ones who started after that age [59]. Patients who started agalsidase beta at the age <30 years experienced a statistically significant decline in LV mass, while those who started it at the age ≥50 years suffered an increase in LV mass [31]. Likewise, patients who started agalsidase beta at the age <40 years had stable thickness of the interventricular septum and posterior wall over a period of 10 years, whereas patients who started it at the age ≥40 years significantly progressed over time [60]. Moreover, in patients without fibrosis, agalsidase beta resulted in a statistically significant decline of LV mass and improvement of exercise capacity and LV radial strain rate, while no effect was observed in patients with mild or severe fibrosis at the time of treatment initiation [61].

Additionally, the slope of eGFR decline was lower in patients who started agalsidase beta earlier since symptom onset and before the development of significant glomerulosclerosis and proteinuria [30,60].

Finally, the risk of clinical events was lower in patients who started agalsidase beta at younger age [62] and before severe organ damage [62,63]. Likewise, the risk of clinical events seemed to be lower in classic males who started ERT before the age of 25 years compared to the ones who started ERT later [59].

2.3. Optimal Dose of ERT

2.3.1. Agalsidase Alfa vs. Agalsidase Beta at Licensed Dose

In classic males, the reduction in plasma lyso-Gb3 was significantly larger in patients treated for 1 year with agalsidase beta at the dose of 1.0 mg/kg than in patients treated with agalsidase alfa or beta at the dose of 0.2 mg/kg [26].

In another study, the reduction of plasma lyso-GB3 in classic males treated for 1 year with agalsidase beta 1 mg/Kg was greater than with agalsidase alfa 0.2 mg/Kg, and the same was observed in females and non-classic males. Additionally, a higher proportion of patients had a decrease in LV mass index when treated for 1 year with agalsidase beta than with agasidase alfa (79% vs. 62%). Nevertheless, no difference between agalsidase alfa and beta was found regarding eGFR or clinical events [64].

Another study showed that 19.4% of patients on agalsidase alfa 0.2 mg/Kg and 13.3% of patients on agalsidase beta 1 mg/Kg progressed, during the 59-month study duration, to a composite clinical endpoint consisting of renal events (development of end-stage renal disease or sustained decline in GFR of 50% or greater for more than 30 days and for which other causes besides FD had been excluded), cardiovascular events (pacemaker or other intracardiac device, coronary artery bypass grafting, valve replacement surgery, coronary angioplasty or stent, cardioversion, hospitalization or emergency room visit for unstable angina/acute coronary syndrome, myocardial infarction, congestive heart failure, tachy- or brady-arrhythmia, heart block, cardiac arrest), cerebrovascular events (TIA or stroke documented by a physician or acute hearing loss), or death. However, these differences were not significant due to limited power [65].

2.3.2. Agalsidase Beta at Reduced Dose

The dose reduction of agalsidase beta from 1 mg/Kg eow to 0.3 mg/Kg eow for 18 months allowed for maintaining the clearance of GB3 deposits in the kidney interstitial capillary endothelium in 90% of the patients; and the clearance/reduction of GB3 deposits in other renal cells and superficial dermal capillary endothelium in only 70% of the patients. Additionally, urinary GB3 increased significantly after dose reduction [25].

In another study, the dose reduction of agalsidase beta from 1 mg/Kg eow to 0.5 mg/Kg per month for 1 year led to a significant increase of plasma lyso-GB3 in males, but no differences were seen on clinical events, Mainz Severity Score Index (MSSI), pain, eGFR, or LV mass [66].

In the Fabry registry, the dose reduction of agalsidase beta resulted in lower self-reported energy levels in males, but no difference in MSSI, DS3, Brief Pain Inventory (BPI), or SF-36 scores [67].

2.3.3. Switch between Therapies

Patients under agalsidase beta 1 mg/kg eow, who switched directly to agalsidase alfa 0.2 mg/Kg eow or suffered a dose reduction to agalsidase beta 0.3–0.5 mg/kg eow and then switched to agalsidase alfa 0.2 mg/Kg eow, showed a greater eGFR decline and an increase of MSSI, GI symptoms [68,69], and pain [68], although clinical events remained stable for 2 years [69].

In three young classic males under agalsidase beta 1 mg/kg eow for 5 years, there was complete clearance of GB3 from mesangial and endothelial cells and partial clearance from podocytes. Three years after the switch to agalsidase alfa 0.2 mg/Kg eow, there was reaccumulation of GB3 in podocytes. One patient switched back to agalsidase beta 1 mg/Kg eow and two years later again showed reduction of GB3 in podocytes. Additionally, pain and GI symptoms worsened in all three patients following the switch from agalsidase beta to alfa [70].

However, in another study, patients under agalsidase beta 1 mg/kg eow who switched to agalsidase alfa 0.2 mg/Kg eow did not show any differences on plasma lyso-GB3 or GB3, eGFR, proteinuria, or LV mass index for 2 years [17].

2.3.4. Agalsidase Alfa at Increased Dose

A study comparing patients under agalsidase alfa 0.2 mg/Kg eow and 0.2 mg/kg weekly for one year did not find any significant differences in plasma GB3, LV mass, eGFR, albuminuria, 6-min walk test, or quality of life. Exploratory analyses of patients under 0.4 mg/kg weekly did not find any differences either [71].

Additionally, a study comparing patients under agalsidase alfa 0.1 mg/kg weekly, and 0.2 mg/Kg eow and 0.2 mg/Kg weekly for 4 weeks, did not find any significant differences on plasma GB3, quality of life, or pain, although there was a trend for a higher sweat volume and a lower urinary GB3 with the weekly dose of 0.2 mg/Kg [72].

Conversely, in patients with progressive decline of renal function despite agalsidase alfa 0.2 mg/kg eow for 2–4 years, the switch to 0.2 mg/Kg weekly significantly decreased the annual slope of eGFR during a 10 year-study, significantly delaying the time to end-stage renal disease [73,74].

2.4. Limitations of ERT

ERT presents several potential limitations: (i) It has limited tissue penetration; (ii) it does not pass the blood-brain barrier; (iii) it may induce infusion adverse reactions; (iv) it may induce the production of anti-drug antibodies with neutralizing effect, reducing the efficacy of the therapy; (v) it is a lifelong therapy requiring intravenous administration every 2 weeks; and (vi) it is associated to a high cost (Table 2).

Table 2. Advantages and limitations of current therapies of FD.

	Advantages	Limitations
ERT	▪ Large body of evidence supporting its efficacy and safety ▪ Long clinical experience, being commercially available since 2001	▪ Incomplete reversion of FD pathology and clinical manifestations ▪ Limited tissue penetration ▪ No crossing of the blood–brain barrier ▪ Infusion adverse reactions ▪ Anti-drug antibodies with neutralizing effect may reduce the efficacy of ERT ▪ Lifelong therapy requiring intravenous administration every 2 weeks ▪ High cost
Migalastat	▪ Oral administration ▪ Small molecule, likely to have enhanced cellular and tissue distribution ▪ Sustained and stable enzyme levels ▪ Non-immunogenic molecule ▪ Favorable safety profile	▪ Therapeutic option only for patients with amenable *GLA* mutations ▪ "In Vitro" amenability may not always reflect "in vivo" amenability ▪ Not recommended in patients aged ≥75 or <16 years, pregnant or breastfeeding, or with severe renal impairment (eGFR <30 mL/min/1.73 m^2) due to lack of data ▪ Incomplete reversion of FD pathology and clinical manifestations ▪ High cost

eGFR, estimated glomerular filtration rate; ERT, enzyme replacement therapy; FD, Fabry disease.

2.4.1. Limited Tissue Penetration

The inefficient biodistribution of the exogenous recombinant enzyme may contribute to the limited efficacy of ERT. While most of the administered recombinant enzyme is taken up by the liver, cardiomyocytes and podocytes, despite being severely affected by FD, only take up few amounts of recombinant enzyme, which might contribute to the limited clearance of GB3 deposits in these cells [75,76].

Mannose 6-phosphate (M6P) mediated endocytosis has been considered the main mechanism of uptake of recombinant α-galactosidase A. However, the rate of cellular uptake among different tissues depends on the pattern of glycosylation and phosphorylation of the enzyme, which is different between agalsidase alfa and agalsidase beta, because post-translational protein modifications are specific to the species [77].

Nevertheless, blocking the M6P receptor inhibited the uptake of recombinant α-galactosidase A in fibroblasts, but not in endothelial cells [78]. Moreover, in podocytes, enzyme uptake is known to be mediated by M6P, megalin, and sortilin receptors, but blocking all three receptors only inhibited the uptake of recombinant enzyme by 39% [79]. These results suggest the existence of additional uptake mechanisms, which might explain the existence of different biodistribution profiles among tissues.

2.4.2. No Crossing of the Blood-Brain Barrier

Stroke and TIA in Fabry patients have been attributed to cardioembolism or vascular involvement by the disease; however, the pathophysiology of brain WML is probably more complex. GB3 deposits within the endothelial and smooth muscle cells of the small and medium-size brain arteries may decrease vascular compliance and impair autoregulation of cerebral perfusion and lead to endothelial dysfunction, increase of pro-thrombotic/pro-inflammatory cytokines, and upregulation of the renin–angiotensin system, which may result in ischemic events [80]. On the other hand, GB3 deposits were also found in brain neurons and glia [81,82], although a clinical correlate remains to be established [83]. Glial dysfunction and neuroinflammation may also contribute to the development of brain WML [80]. Yet, their clinical impact is not fully understood.

None of the recombinant enzyme preparations can pass the blood–brain barrier. The effect of ERT on the development or progression of brain WML remains to be elucidated. During a 2-year follow-up of six patients under agalsidase alfa, brain WML remained stable in three patients, worsened in one, fluctuated in one, and improved in one patient; during this time, some brain WML disappeared and others appeared on brain MRI [84]. During a mean follow-up of 27 months, agalsidase beta seemed to stabilize brain WML on MRI compared to placebo, as a statistically significant greater proportion of younger patients (≤50 years) under agalsidase beta had stable WML compared with younger patients under placebo (44% vs. 31%) [85].

2.4.3. Infusion-Associated Reactions

ERT leads to IAR, which occur mostly in the first 13 infusions and are mostly limited to fever and chills [86], although life-threatening reactions have also been reported [87]. IAR occur in 14% of patients under agalsidase alfa [45] and 67% under agalsidase beta [46]; and the risk of IAR seems higher in males with nonsense or null mutations (CRIM negative) [86] and in patients with an anti-agalsidase IgG antibody–positive status [86,88].

2.4.4. Anti-Drug Antibodies

Anti-drug antibodies develop in the first 3–6 months of ERT [86,89], mostly in classic males [90]. Anti-drug antibodies have been reported in 91% of males treated with agalsidase beta [57] and 20% of males treated with agalsidase alfa [14], although no significant difference in their formation has been reported when the same dosage (0.2 mg/kg every 2 weeks) was used for both drugs at ERT initiation [91]. Antibodies have shown in vitro cross-reactivity to both agalsidase alfa and beta [89].

IgG antibodies measured by ELISA mediate a neutralizing activity [89]. Neutralizing anti-drug antibodies develop in about 40% of all ERT-treated males [86,88] and mostly in patients treated with agalsidase beta [64]. Their formation seems to be irreversible, and the majority of patients positive for neutralizing anti-drug antibodies remain as so over 10 years [64,88,91].

These anti-drug antibodies bind and neutralize ERT in plasma and lead to activation of macrophages that internalize the ERT-antibody complexes, decreasing the cellular uptake of ERT [89].

Neutralizing anti-drug antibodies may attenuate ERT efficacy. Anti-drug antibodies have been associated to higher frequency of GB3 deposits in endothelial cells of the skin [92] and higher plasma lyso-GB3 [64,88,91,93] and urinary GB3 [89,91]. Although previous reports have suggested that anti-drug antibodies had no effect on the time to first clinical event or eGFR slope [92], other studies have shown that they were associated to worse renal function [88,93] and higher LV mass, disease severity scores, and frequency of symptoms [88].

Increasing the ERT dose in patients with established anti-drug antibodies may saturate them and allow for the excess enzyme to perform its catalytic function, thereby attenuating the negative effect of anti-drug antibodies in plasma lyso-Gb3, eGFR, and LV wall thickness [94].

3. Migalastat

3.1. Efficacy and Safety of Migalastat

Migalastat is a first-in-class pharmacological chaperone therapy for FD. It is a low molecular weight iminosugar analogue of the terminal galactose residue of GB3 that selectively and reversibly binds to the active site of amenable mutant forms of α-galactosidase A, thereby stabilizing the enzyme, preventing its retention and degradation in the endoplasmic reticulum, and facilitating its trafficking to lysosomes. Once in lysosomes, migalastat dissociates from α-galactosidase A, due to the more acidic pH and higher concentration of substrates, allowing the enzyme to exert its activity on GB3 [42–44].

Migalastat is an oral drug administered at the dosage of 123 mg once every other day, which has been approved in 2016 by the European Medicines Agency for the treatment of FD patients aged ≥16 years, with eGFR ≥30 mL/min/1.73 m^2 and amenable *GLA* mutations [47].

In the FACETS trial, in the modified-intention to treat population (i.e., ERT-naïve patients with migalastat-amenable *GLA* mutations), migalastat, compared to placebo, significantly reduced the plasma lyso-GB3 and the mean number of GB3 inclusions/kidney interstitial capillary at 6 months. There were no significant differences in baseline levels or changes from baseline to month 6 between the groups regarding eGFR, mGFR, 24 h-urine protein excretion, and 24 h urinary GB3. At 24 months of migalastat therapy (i.e., after 18 months of migalastat in patients who switched from placebo or 24 months of continuous migalastat), there was a significant reduction of the mean LV mass index compared to the baseline [48]. An improvement in diarrhea was also found after 6 months of migalastat compared to placebo, and this benefit was sustained at 24 months [48,56]. Those results were also confirmed in the subgroup of classic males [95]. Migalastat treatment was also demonstrated to decrease the mean total GB3 inclusion volume per podocyte in renal biopsies from baseline to 6 months; and this reduction correlated with the reduction in mean podocyte volume [50].

In the ATTRACT trial, in ERT-experienced patients with amenable *GLA* mutations who were randomized to switch to migalastat or continue ERT, renal function and plasma lyso-GB3 levels were maintained during 18 months of migalastat or ERT. Migalastat significantly reduced the mean LV mass index at 18 months, and changes on LV mass index correlated with changes in the thickness of the interventricular septum and not the posterior wall. No significant change of the mean LV mass index was found in the ERT group; however, the randomization ratio of migalastat to ERT of 1.5 to 1, the existence of non-amenable mutations in the initial cohort, and the higher discontinuation rate on the ERT group resulted in a small number of evaluable patients under ERT at the end of the study (16 patients), which may have influenced the results regarding ERT [51]. In the open-label extension study, a significant decrease of LV mass index was found after 30 months of migalastat in patients with LVH at baseline [52].

In real world conditions, Muntze et al. reported one patient with an improvement of LV mass, LGE, troponin, and NT-proBNP after 12 months of treatment with migalastat [53]. Later, the same authors showed that, in 14 patients treated with migalastat for 1 year, LV mass index significantly decreased, while plasma lyso-GB3 significantly decreased in naïve patients and remained stable in patients switched from ERT. However, eGFR significantly worsened, a finding that contrasted to the results in the pivotal clinical trials. The authors hypothesized that these results could have been partly explained by the simultaneous initiation of angiotensin converting enzyme inhibitors and underlined the need for further studies with longer follow-up of the renal function [49].

In a larger study including 59 patients, treatment of previously ERT-treated and untreated FD patients with migalastat for 12 months was generally safe and also resulted in a significant decrease of LV mass index. However, plasma lyso-GB3 levels were stable both in males and females, irrespective of previous treatment regimen; and increasing plasma lyso-GB3 was noted in a few males and females, some of whom carried *GLA* mutations whose amenability to migalastat has been questioned. Moreover, eGFR continued to decline under migalastat in both males and females. The loss of renal function did not seem to be explained by a more severe renal impairment and disease load at baseline. Instead, higher decline of eGFR was more common in patients with a systolic blood pressure below 120 mmHg. Additionally, females with *GLA* mutations, classified as non-amenable by the in-house assay based on *GLA*-knockout HEK cells, had higher decline of eGFR. This study recommended to avoid systolic blood pressure values below 120 mmHg and alerted that "in vitro" amenability may not always reflect "in vivo" amenability to migalastat, emphasizing the importance of monitoring clinical response to therapy [54].

In a smaller study of seven FD males, who switched from ERT to migalastat, a significant decrease of LV mass index was also observed after 1 year of treatment with migalastat, while plasma lyso-GB3 remained stable. Unlike previous real-life studies, eGFR was stable and proteinuria significantly decreased [55]. Therefore, further studies are needed to understand the discrepant results of migalastat on renal function.

Migalastat is generally well tolerated, with headache and nasopharyngitis being the most common side effects [48,51]. Transient and fully reversible infertility was also found in male rats, but its occurrence in humans remains to be established [47] (Table 1).

3.2. "In Vitro" and "In Vivo" Amenability to Migalastat

The amenability of *GLA* mutations to migalastat is determined by a good laboratory practice (GLP) "in vitro" pharmacogenetics assay, which has been clinically validated. This assay uses human embryonic kidney (HEK) 293 cells that have been transfected with individual *GLA*-containing DNA plasmids to measure increases in α-galactosidase A activity in response to migalastat. According to Benjamin et al., *GLA* mutations that do not qualify for testing include large deletions, insertions, truncations, frameshift mutations, and splicing mutations; these mutations are classified as non-amenable. *GLA* mutations that qualify for testing include missense mutations, nonsense mutations near the carboxyl terminus, small insertions and deletions that maintain reading frame, and complex mutations comprising two or more of these types of mutations on a single *GLA* allele. Migalastat-amenable mutations are defined as *GLA* mutations that translate to mutant forms of α-galactosidase A that, in the presence of 10 μmol/L migalastat, display an increase of the enzymatic activity ≥1.2-fold over baseline and an absolute increase ≥3% of the enzymatic activity quantified as a percentage of the enzymatic activity of the wild-type α-galactosidase A [96]. It is estimated that 35–50% of FD patients have mutations that are amenable to migalastat [51].

However, recent studies have reported that migalastat was associated to an insufficient increase of the enzymatic activity of α-galactosidase A and increasing values of plasma lyso-GB3 in patients with certain *GLA* gene mutations classified as amenable based on the "in vitro" GLP-HEK assay, thereby raising concerns that "in vitro" amenability may not always reflect "in vivo" amenability [54,97,98]. Hence, serial measurement of the enzymatic activity of α-galactosidase A in leukocytes and monitoring of the clinical response are mandatory in order to assess "in vivo" amenability to migalastat.

3.3. Limitations and Potential Advantages of Migalastat

Migalastat is a therapeutic option only for patients with amenable *GLA* mutations [47,48,51,96]. Due to the lack of data, migalastat is not recommended in patients aged ≥75 or <16 years, pregnant or breastfeeding, or with severe renal impairment (eGFR <30 mL/min/1.73 m^2) [47,48,51].

Migalastat presents several potential advantages: (i) It is an oral therapy, thereby eliminating the requirement for lifelong intravenous infusions; (ii) it is a non-immunogenic molecule, avoiding antibody-related tolerability issues of ERT; (iii) it allows for sustained and stable enzyme levels that more closely mimic those of endogenous wild-type enzymes, whereas ERT leads to fluctuating and intermittent enzymatic activity; (iv) as a small molecule, it is likely to have enhanced cellular and tissue distribution; (v) and potential to cross the blood-brain barrier [58], as suggested by the finding of increased α-galactosidase A activity and reduced GB3 levels in the brain of Fabry transgenic mice [99] (Table 2).

3.4. Emerging New FD Therapies

Current therapies have not shown to be able to fully revert FD pathology and clinical manifestations. Therefore, several new therapies are under research, including new forms of ERT (pegunigalsidase alfa, moss-derived α-galactosidase A), substrate reduction therapy (lucerastat, venglustat), mRNA therapy, and genetic therapy [12,13] (Table 3).

Pegunigalsidase alfa, a chemically modified α-galactosidase A enzyme produced in a tobacco plant cell based ProCellEX system, is constituted by two subunits of α-galactosidase A covalently bound by a chain of polyethylene glycol (PEG), which increases its stability and reduces its clearance, thereby extending its plasma half-life [100–102] and allowing a monthly infusion [102]. Being plant-derived, this enzyme does not display M6P on their surface glycans [100], which suggests an alternative mechanism of cell uptake and may result in a different biodistribution profile from agalsidase alfa and agalsidase beta. This enzyme has already been demonstrated (i) to decrease plasma lyso-Gb3 levels and peritubular capillary Gb3 deposits in the kidney; (ii) to improve the BPI score, gastrointestinal symptoms, and MSSI; (iii) and to maintain eGFR, proteinuria and LV mass on MRI with no "de novo" cardiac fibrosis [101]. Studies to further test its efficacy are ongoing [102,103]. Anti-drug antibodies occurred in 19% of cases and became negative after 1 year, suggesting induction of immune tolerance [101], but its effect on the immune system is not yet completely clear [13]. Immune tolerance may occur as a result of the extended half-life and higher exposure of the immune system to the enzyme; or because pegylation may mask the enzyme to the immune system. However, concerns about immunogenicity have also been raised, because of the different glycosylation pattern of a plant-derived protein and the PEG component. Of note, the extended half-life may also interfere with the laboratory assays for anti-drug antibodies detection, as the circulating enzyme at the sampling time may bind antibodies and prevent their detection [13].

Table 3. Potential advantages and limitations of emerging therapies of FD.

	Potential Advantages	Potential Limitations
Pegunigalsidase alfa	■ Higher plasma half-life [100–102], allowing a monthly infusion [102]	■ Anti-drug antibodies in 19% of cases [101] ■ Plant-derived protein with a different glycosylation pattern—possible immunogenicity issues? [100] ■ Unclear effect on the immune system [13] ■ No crossing of the blood–brain barrier [13] ■ Lifelong therapy requiring intravenous administration [13]
Moss-derived α-galactosidase A	■ Higher cellular uptake via the mannose receptors [104,105]	■ Plant-derived protein with a different glycosylation pattern—possible immunogenicity issues? [104] ■ Unclear effect on the immune system [104] ■ No crossing of the blood–brain barrier [13] ■ Lifelong therapy requiring intravenous administration [13]
Substrate reduction therapy	■ Oral administration [13] ■ Non-immunogenic [13] ■ Possible crossing of the blood-brain barrier [106]	■ Complete block of a single enzymatic reaction could potentially disrupt the cell homeostasis [13] ■ May not be sufficient as monotherapy for patients with minimal/no residual enzymatic activity [13]
mRNA therapy	■ Potential for a larger interval between infusions [107,108] ■ Uses endogenous protein translation system to ensure proper folding, glycosylation, and intracellular trafficking of α-galactosidase A [13] ■ No risk of insertional mutagenesis [13]	■ Primarily targets hepatocytes [13] ■ Unclear effect on immune system in classic males [13]
Gene therapy	■ Introduces a correct version of the *GLA* gene [12] ■ Uses endogenous protein translation system to ensure proper folding, glycosylation, and intracellular trafficking of α-galactosidase A [13]	■ Targeting all affected cell types and tissues is a challenge [13] ■ Risk of insertional mutagenesis [13] ■ Unclear effect on immune system in classic males [13]

Moss-derived α-galactosidase A is produced in the moss *Physcomitrella patens* and is a glycoengineered variant devoid of α-1,3-fucose and β-1,2-xylose residues on its N-glycans that are plant-specific and may elicit immunogenic response in mammals [104]. Being plant-derived, this enzyme does not display M6P on their surface glycans; instead, it carries >90% mannose-terminated glycans [104]. This enzyme has a shorter half-life, which might be due to a higher cellular uptake via the mannose receptors [104,105], and has shown to

reduce urinary Gb3 in a higher proportion than previously reported for α-galactosidase A produced in mammalian cells [105].

The glucosylceramide synthetase (GCS) inhibitors, such as venglustat and lucerastat, block the enzyme catalyzing the first step of glycosphingolipid biosynthesis, reducing glucosylceramide and Gb3 [13]. In patients with residual enzyme activity, GCS inhibitors might be sufficient to reduce the synthesis of GB3 to a level manageable by the residual enzyme activity, while in classic patients, with minimal to no residual enzyme activity, they may not be sufficient in monotherapy, but can still be useful in combination with ERT [13]. Venglustat, also known as ibiglustat, has shown to reduce GB3 deposits in tissues (including in brain) and plasma GB3 and lyso-GB3 in mice, this effect being more pronounced when added to ERT, except in the brain, where ERT had no effect [106]. Preliminary data also showed the reduction of Gb3 from superficial skin capillary endothelium and plasma lyso-Gb3 in treatment-naïve Fabry patients [13]. Lucerastat added to ERT resulted in a reduction of plasma GB3, while no reduction was seen in patients under ERT alone; and in stabilization of renal and cardiac parameters at 12 weeks [109]. Despite the clear advantages of oral administration, absence of anti-drug antibodies, and the possibility of passing the blood-brain barrier, caution in dosing should be taken, because the complete block of a single enzymatic reaction could potentially disrupt the cell homeostasis [13].

mRNA therapy, encapsulated in lipid nanoparticles, primarily targets hepatocytes in which the enzyme is produced and secreted into the circulation [13]. In mice and non-human primates, mRNA therapy has been shown to reduce GB3 and lyso-GB3 in heart and kidney [107,108] and plasma [108], and this effect was maintained for up to six weeks after infusion, suggesting that it could have the advantage of a larger interval between infusions [107,108]. Unlike ERT, mRNA therapy carries the advantage of using the endogenous protein translation system to ensure proper folding, glycosylation, and intracellular trafficking of α-galactosidase A; and, unlike DNA-based therapy, it does not carry a risk for insertional mutagenesis [13].

Gene therapy, using viral or non-viral vectors, may introduce a correct version of *GLA* gene, through "in vivo" or "ex vivo" technology [12]. The first FD patients have been treated in phase I and II clinical trials using an "ex vivo" approach, in which hematopoietic stem cells of the patient were recruited, transfected using lentiviruses, and re-administered to the patient. The main challenge is to target all affected cell types and tissues. Additionally, it is unclear whether classic males will develop antibodies against the expressed enzyme [13].

4. Conclusions

Despite ERT has demonstrated efficacy and safety on the treatment of FD, there are still pending questions about the best regimen and timing to start therapy as well as limitations, such as the limited tissue penetration, the immunogenicity issues, and the inconvenience of lifelong biweekly intravenous administrations.

Migalastat, the first-in-class pharmacological chaperone therapy for FD, has proven to be safe and efficacious in patients with amenable *GLA* mutations. Being an oral non-immunogenic drug, migalastat has overcome some of the limitations of ERT; however, clinical evidence is still growing, amenability issues have been reported, and its clinical use is limited to about 35–50% of FD patients.

As both therapies have been unable to fully revert FD pathology and clinical manifestations, this remains an enthusiastic field of investigation, with several new emerging therapies under research and development, such as new forms of ERT, substrate reduction therapy, and mRNA and gene therapies. So far, it remains unclear if optimal treatment lies on a single therapy or combination of therapies; and whether other therapeutic strategies, beyond the correction of the enzymatic defect alone, will be needed to avoid or revert organ damage in FD.

Author Contributions: Conceptualization, O.A.; methodology, O.A. and M.F.G.; validation, O.A., M.F.G., and G.M.-M.; formal analysis, O.A. and M.F.G.; investigation, O.A., M.F.G., and G.M.-M.; resources, O.A.; writing—original draft preparation, O.A.; writing—review and editing, O.A., M.F.G., G.M.-M., N.S., and D.C.; supervision, O.A., N.S., and D.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Acknowledgments: The authors of this publication O.A., M.F.G., and G.M.-M. are members of the European Reference Network for Rare Hereditary Metabolic Disorders (MetabERN).

Conflicts of Interest: O.A. and G.M.-M. have received educational/research grants from Shire Human Genetic Therapies/Takeda and travel/accommodation support for conferences from Shire Human Genetic Therapies/Takeda, Amicus and Sanofi Genzyme. M.F.G. has received travel/accommodation support for conferences from Shire Human Genetic Therapies and Sanofi Genzyme. The remaining authors declare no conflict of interest.

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