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Faculdade de Medicina

Early markers of heart and kidney damage in Fabry disease

Patrício Ricardo da Terra Aguiar

Orientadores: Professor Doutor José Luís Bliebernicht Ducla Soares

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Tese especialmente elaborada para obtenção do grau de Doutor em Medicina, especialidade de Medicina Interna

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2018

“Põe quanto És no Mínimo que Fazes

Para ser grande, sê inteiro: nada
Teu exagera ou exclui.

Sê todo em cada coisa. Põe quanto és
No mínimo que fazes.

Assim em cada lago a lua toda
Brilha, porque alta vive.”

Ricardo Reis, in "Odes"

Declaration of work and integrity

I confirm that the work presented in this thesis is my own and I did not resort to the practice of plagiarism or any form of falsification of results. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Moreover, I state that I was the main responsible for the research idea and design, as well as the data acquisition, the data analysis and interpretation and the statistical analysis. I also participated in laboratorial tasks, but under the supervision and with help of other members of the research team.

I also declare that I acted according to the code of ethical conduct of the University of Lisbon and of the University College London.

Lisbon, 17th of December, 2018

Patrício Ricardo da Terra Aguiar

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Introductory note

This thesis focuses in the identification of new biomarkers of heart and kidney involvement in Fabry disease, not only of the very early pre-clinical stages, but also their prognostic value.

In the introduction section I present a brief overview of Fabry disease epidemiology, pathophysiology, clinical manifestations and treatment, with a special attention to the rational underlying the urgent need for better biomarkers in Fabry disease. Furthermore, I also review the biomarker concept and its usefulness in this disorder, as well as the current state of the art in terms of the available biomarkers in Fabry disease. Moreover, an overview about searching new biomarkers and the available evidence supporting the studied biomarkers related to myocardial fibrosis and glomerular and tubular injury is also presented.

The global goals of this thesis are enumerated at the end of the introduction in the aims section and the subsequent sections are the result of the original work performed during the PhD:

- The "cardiomyopathy study" was designed to understand the role of the biomarkers related to collagen metabolism, not only in the identification of the pre-clinical stage of Fabry disease cardiomyopathy, but also their prognostic value in terms of the identification of patients at greater risk of a progressive disorder (increase in left ventricular mass). Part of the results have been accepted for publication in the *Journal of American Heart Association* and another paper is being prepared with the remaining results.

- In the "nephropathy study" I have studied several biomarkers of glomerular and tubular injury in terms of the identification of patients with incipient Fabry disease nephropathy and patients prone to a progressive decline in glomerular filtarion rate. Some results have been published in the *Molecular, Genetics and Metabolism* and the remaining are being prepared for publication.

Finally, I present the ongoing projects of biomarkers research and the future perspectives.

All the publications are included as appendices.

Table of Contents

Abbreviations

A: atrial contraction transmitral peak velocity

A1MG: α1-microglobulin

AAP: alanine aminopeptidase

ACEi: angiotensin converting enzyme inhibitors

ACR: urinary albumin to creatinine ratio

ADAMST: a disintegrin-like and metalloproteinase domain with thrombospodin type motif

A dur: duration of transmitral forward wave

ARB: angiotensin receptor blockers

Ar dur: duration of the pulmonary venous retrograde wave

Ar vel: velocity of the pulmonary venous retrograde wave

ATP: adenosine triphosphate

AUC: area under the curve

AV: atrioventricular

B-AP: bone-specific alkaline phosphatase

BP: blood pressure

C: carboxy

CITP: collagen type I carboxy-terminal telopeptide

CI: confidence interval

CKD: chronic kidney disease

CKD-EPI: Chronic Kidney Disease Epidemiology Collaboration

ColIV: collagen type IV

DC: dilated cardiomyopathy

DMT: divalent metal transporter

DNA: deoxyribonucleic acid

DOR: diagnostic odds ratio

E: rapid filling transmitral peak velocity

E': early diastolic mitral annular velocity

ECLIA: electro-chemiluminescence immunoassay

EDTA: ethylenediamine tetra-acetic acid

ELISA enzyme-linked immunosorbent assay

EOW: every other week

ERT: enzyme replacement therapy

ESRD: end-stage renal disease

FD: Anderson-Fabry disease

FIPI: Fabry International Prognostic Index

FLC: free light chains

Gb3: globotriaosylceramide

Gb4: globoside

GBM: glomerular basement membrane

GFR: glomerular filtration rate

GHL: tripeptide glycyl-histidyl-lysine

HbA1C: glycated haemoglobin

HBP: arterial hypertension

HCM: hypertrophic cardiomyopathy due sarcomere protein gene mutations

IL-6: interleukin 6

IQR: interquartile range

IVRT: isovolumic relaxation time

IVS: interventricular septum

KDIGO: Kidney Disease Improving Global Outcomes

LA: left atrial

LGE: late gadolinium-contrast enhancement

LR-: likelihood ratio for a negative test result

LSD: lysosomal storage disorder

LV: left ventricular

LVEDP: left ventricular end-diastolic pressure

LVPW: left ventricular posterior wall

MDRD: modification of diet in renal disease

MMP: matrix metalloproteinase

MRI: magnetic resonance imaging

MSSI: Mainz severity score index

mTOR: mammalian target of rapamycin

N: amino

NAG: N-acetyl-β-D-glucosaminidase

NO: nitric oxide

NT-proBNP: amino-terminal fragment of the pro-hormone of brain natriuretic peptide

NYHA: New York Heart Association

OR: odds ratio

PICP: procollagen type I carboxy-terminal propeptide

PINP: procollagen type I amino-terminal propeptide

PIIINP: procollagen type III amino-terminal propeptide

PCr: creatine phosphate

PPV: positive predictive value

PW: posterior wall

RBP: retinol-binding protein

RNA: ribonucleic acid

ROC: receiver operating characteristic

ROS: reactive oxygen species

RV: right ventricle

S: sensitivity

S': systolic mitral annular velocity

SD: standard deviation

Sp: specificity

SPARC: secreted protein acidic and rich in cysteine

SR: strain-rate

sVCAM-1: soluble vascular adhesion molecule-1

T1DM: type 1 diabetes mellitus

T2DM: type 2 diabetes mellitus

TDI: tissue Doppler imaging

TGF-β1: transforming growth factor-β1

TIMP: tissue inhibitor of metalloproteinases

TM: thrombomodulin

UTE: urinary transferrin excretion

VEGF: vascular endothelial growth factor

List of figures

List of tables

Abstract

Anderson-Fabry disease is an X-linked lysosomal storage disorder, causing significant morbidity and premature death; cardiac and renal involvements are the major determinants of overall disease prognosis. Fabry cardiomyopathy is characterized by a hypertrophic phenotype, in the setting of histological cardiomyocyte hypertrophy and myocardial fibrosis; myocardial fibrosis is an irreversible event and affects the prognosis. A progressive chronic kidney disease characterizes Fabry disease nephropathy; however it is clinically silent for a long period, because heavy storage may occur in renal cells with minimal or no changes on standard renal tests. Furthermore, accumulating evidence suggests that early enzyme replacement therapy is effective in preventing progression of both cardiomyopathy and nephropathy. Therefore, in this thesis I have studied two linked research questions in a multicenter, prospective, longitudinal (evaluation at baseline, 12 months and 24 months) and diagnostic test study:

1) Role of biomarkers related to collagen type I metabolism in the diagnosis of incipient and prognosis of Fabry cardiomyopathy, in a cohort of 60 patients with Fabry disease and 20 healthy controls, according to subgroups of increasing disease severity. I found that collagen type I synthesis is increased in Fabry disease cardiomyopathy, even in the earlier stages of the disease, and this profibrotic state has good prognostic value for and is likely to be critical to the development of overt left ventricular hypertrophy. Moreover, inhibition of enzymes involved in collagen type I cleavage also seems crucial to myocardial collagen deposition and is related to risk of progressive diastolic dysfunction.

2) Identification of early and prognostic biomarkers of Fabry nephropathy in a cohort of 78 patients with Fabry disease and 25 healthy controls, according to subgroups of increasing disease severity. I have shown that two biomarkers of glomerular damage (urinary transferrin and collagen type IV excretion) and three biomarkers of tubular injury (urinary α1-microglobulin, N-acetyl-β-D-glucosaminidase and alanine aminopeptidase excretion) may overcome the limitations of albuminuria as a sensitive marker of early renal dysfunction; furthermore N-acetyl-β-D-glucosaminidase presented the better prognostic value in the identification of patients at risk for chronic kidney disease progression. These biomarkers may also define novel early stages of nephropathy characterized by mesangial expansion and/or tubular damage.

Resumo

A Doença de Fabry é uma doença lisossomal de sobrecarga com um padrão de hereditariedade ligado ao cromossoma X, que condiciona morbilidade significativa e mortalidade precoce; o envolvimento cardíaco e renal são os principais determinantes do prognóstico global. A cardiomiopatia da Doença de Fabry é caracterizada por um fenótipo hipertrófico, condicionado pela hipertrofia dos cardiomiócitos e fibrose miocárdica; esta última é irreversível e afecta o prognóstico. A doença renal crónica progressiva caracteriza a nefropatia da Doença de Fabry; contudo, esta é clinicamente silenciosa por um longo período, na medida em que pode ocorrer extensiva acumulação de substractos nas células renais na ausência de alterações nos testes *standard* de avaliação da função renal. Mais ainda, evidência crescente sugere que a instituição precoce de terapêutica de substituição enzimática é efectiva na prevenção da progressão quer da cardiomiopatia quer da nefropatia. Por conseguinte, na presente tese eu abordei duas questões interrelacionadas, num estudo de testes de diagnóstico, multicêntrico, prospectivo e longitudinal (avaliação basal e aos 12 e 24 meses):

1) A função dos biomarcadores relacionados com o metabolismo do colagénio tipo I no diagnóstico precoce e na determinação do prognóstico na cardiomiopatia da Doença de Fabry, num coorte de 60 doentes com Doença de Fabry e 20 controlos saudáveis, de acordo com subgrupos de gravidade crescente. Demonstrei que a síntese de colagénio tipo I encontra-se aumentada na cardiomiopatia da Doença de Fabry, mesmo nos estádios mais precoces da doença e que este estado profibrótico apresenta bom valor prognóstico para e parece ser essencial ao desenvolvimento de hipertrofia ventricular esquerda. Constei ainda que a inibição das enzimas envolvidas na degradação do colagénio tipo I também afigura-se como crucial à deposição de colagénio no miocárdio e encontra-se relacionada com o risco de disfunção diastólica progressiva.

2) Identificação de biomarcadores precoces e de prognóstico na nefropatia da Doença de Fabry num coorte de 78 doentes com Doença de Fabry e 25 controlos saudáveis, de acordo com subgrupos de gravidade crescente. Evidenciei que dois biomarcadores urinários de lesão glomerular (transferrina e colagénio tipo IV) e três biomarcadores urinários de dano tubular (α1-microglobulina, N-acetil-β-D-glucosaminidase e alanina aminopeptidase) podem ultrapassar as limitações da

albuminuria como marcador sensível de disfunção renal precoce; mais ainda, a excreção de N-acetil-β-D-glucosaminidase apresentou melhor valor prognóstico na identificação de doentes em risco de doença renal crónica progressiva. Estes biomarcadores também poderão definir novos estádios precoces da nefropatia da Doença de Fabry caracterizados por expansão do mesângio e/ou lesão tubular.

Introduction

1. Anderson-Fabry disease

1.1 Aetiology, genotype-phenotype correlations and epidemiology

Anderson-Fabry disease (FD) is an X-linked lysosomal storage disorder (LSD), independently first described by Johannes Fabry and William Anderson in 1898,[1] who reported patients with "angiokeratoma corporis diffusum", the red-purple maculopapular skin lesions that are characteristic of the disease. It was only in 1967 that FD was found to be caused by mutations in the GLA gene (that encodes the enzyme α-galactosidase A).[2] The deficiency of this enzyme leads to the lysosomal accumulation of neutral glycosphingolipids (mainly globotriaosylceramide [Gb3]) in several cells, causing organ failure. Involvement of the heart, kidney and brain in FD causes significant morbidity and premature death.[3, 4]

The GLA gene was mapped to the q22.1 region of the X chromosome. It contains seven exons and the coding part of the gene consists of 1290 base pairs, encoding a polypeptide of 429 amino acids.[5] A total of 672 mutations / variants have been described, most of them (455) missense / nonsense.[6] The great majority of the GLA mutations are private (each family has its own mutation) and theoretical considerations point to 3-10% of patients with *de novo* mutations.[7]

GLA gene encodes the lysosomal enzyme α-galactosidase A (a homodimeric molecule), responsible for the degradation of neutral glycosphingolipids with terminal α-galactosyl moieties in cells throughout the body. Its main substrate is Gb3, but there are also other minor substrates, including digalactosylceramide, P1 antigen and blood group B glycolipids. Gb3 originate from metabolic turnover of membrane glycosphingolipids (like globoside [Gb4]) present in large amounts in the kidneys, liver, vascular endothelium, lungs and erythrocytes.[5]

Despite its "monogenic" origin, FD is clinically heterogeneous, with some patients presenting a classical phenotype (full-blown clinical picture with "acroparesthesias", angiokeratomas, hypohydrosis, corneal opacity and renal, cardiac and cerebrovascular involvement) and others a late-onset / attenuated form (mainly affecting the heart).[8] Genetic factors certainly contribute to this heterogeneity, with patients exhibiting out-of-frame short length rearrangements and splice-site and nonsense mutations, leading to a

premature stop codon and a truncated and most likely non-functional enzyme, presenting a classical phenotype.[5] In patients with missense mutations, enzymatic activity may be variably affected: mutations in functionally important regions or leading to large structural changes show the classical phenotype, whereas late-onset forms are associated with mutations located apart from the active site, conditioning small structural changes.[9] However, in patients within the same family or with the same mutation, the clinical picture may vary widely, thus other genetic, epigenetic and environmental factors also contribute to clinical heterogeneity.

Furthermore, unlike many other X-chromosomal conditions, female FD patients may show signs and symptoms commonly seen in male patients. However, disease onset occurs later and the phenotype tends to be milder (though clinically heterogeneous with several patients with classic FD).[10, 11] Thus, with this state of intermediate disease penetrance in female FD patients, the terms "X-linked recessive" or "X-linked dominant" may not capture the wide spectrum of variable expression in heterozygotes and FD may be referred to as "X-linked".[12]

Phenotype and penetrance in FD female patients may be explained according to the principles of random X chromosome inactivation (lionization), resulting in a mosaic with some cells expressing "wild type" and others "mutant" GLA gene.[13] The celular and tissue levels of α-galactosidase A activity will depend on the balance between "wild type" and "mutant" X-inactivation, varying from organ to organ and with female FD patients exhibiting skewed X-inactivation favouring "mutant" GLA gene, presenting a more severe phenotype.[14] However, there is no correlation between plasma or leucocyte α-galactosidase A activity and disease severity, and cross correction of the metabolic defect by uptake of the "wild type" enzyme by the cells with the "mutant" GLA gene would prevent females from developing clinically significant disease.[15, 16] Thus, these findings suggest that cross complementation is impaired or insufficient in FD, possibly due to defective uptake, for reasons as yet unclear, of "wild type" enzyme by the "mutant" cells.[16] Moreover, "cross-induction" of the defect may be another pathophysiological link explaining the clinical manifestations in female FD patients. Lyso-Gb3, a deacylated form of Gb3, is increased in the great majority of female FD patients; it is widely diffusible and has the capacity to inhibit α-galactosidase A activity in "wild type" cells.[17] Moreover, lyso-Gb3 may have a direct "toxic" effect on the disease pathophysiology, because it induces

smooth muscle cells proliferation (directly contributing to FD vasculopathy) and production of extracellular matrix proteins by podocytes (leading to glomerular damage).[17, 18]

FD is pan-ethnic, but due to its rarity, it is difficult to determine its true prevalence. Moreover, in most countries, there are various diagnostic centres, making it difficult to collect the diagnoses. Three main studies addressing the birth prevalence of FD reported incidences ranging from 1:117,000 in Australia, to 1:833,000 in Northern Portugal.[19-21] In the Australian epidemiological study, no data was obtained on heterozygote females, but the incidence determined in hemizygote males could be extrapolated to give a combined incidence of 1 in 58,000. However, the true prevalence of FD is likely to be much higher, due to the existence of late-onset forms / attenuated phenotypes of the disease as identified by the screening of high-risk populations and newborns. Several newborn screening initiatives of FD raised the incidence of FD up to 1:3,100 male births in Italy or even 1:1,250 male births in Taiwan, with most of the identified mutations associated with attenuated phenotypes with predominant cardiac involvement, giving an estimated ratio as high as 11:1 of attenuated to classic phenotypes.[22, 23] A summary of prevalence and newborn screening studies is shown in table 1.

1.2 Pathology and pathophysiology

1.2.1 *Cardiomyopathy*

In FD cardiomyopathy, glycosphingolipid storage (mainly Gb3) occurs in all cardiac cell types, namely cardiomyocytes, conduction system cells, valvular fibroblasts, endothelial cells, vascular smooth muscle cells and cardiac nerves (including autonomic nervous system).[24-26] In female FD patients there is a mosaic pattern of storage, due to the random X-inactivation.[27] However, autopsy studies in extremely hypertrophied hearts showed that Gb3 infiltration of the myocardium accounts only for 1-3% of the wet weight of the hypertrophic heart and cannot explain its degree, which indicates that activation of several pathological pathways and other histological features are also important.[28]

Hence, Gb3 storage is probably a primer for lysosomal and cellular malfunctioning, triggering intracellular common signalling pathways leading, ultimately, to

cardiomyocytes hypertrophy, apoptosis and necrosis, followed by replacement fibrosis. These events are responsible for the diastolic dysfunction and contractility impairment seen in the clinical setting.[28, 29]

Table 1: Studies on prevalence of Fabry disease. LSD: lysosomal storage disorder; FD: Fabry disease; UK: United Kingdom; NL: Netherlands; USA: United States of America (adapted from Germain DP[30]).

Methods	Source	Period	Prevalence	Country reference
Birth prevalence (pre and postnatal enzymatic diagnosis divided by number of births)	Two centres conducting all enzyme assays in Australia	1980-1996	1:117.000	Australia[19]
Birth prevalence (pre and postnatal enzymatic diagnosis divided by number of births)	One centre providing all pre and postnatal diagnosis in Portugal	1982-2001	1:833.000	Northern Portugal[20]
Birth prevalence (cases born in a certain period divided by total number of live births in the same period)	All the laboratories making pre and postnatal diagnosis in Netherlands	1970-1996	1:476.000	NL[21]
Birth prevalence (cases born in a certain period divided by total number of live births in the same period)	Two main reference centres providing enzyme assay for patients under 5 years old suspected of LSD	1997-2002	1:6.666.000	Turkey[31]
Prevalence	Records from regional genetic units, enzyme reference laboratories and individual doctors	1980-1995	1:370.000	UK (♂)[3]
Prevalence of obligate carriers	By family history, from the UK FD register	1980-1995	1:345.000	UK (♀)[4]
Neonatal screening	Northern Italy (males only)	2003-2005	1:3,100	Italy[22]
Neonatal screening	Taiwan (males only)	2006-2008	1:1,250	Taiwan[23]
Neonatal screening	Austria	2010	1:3,900	Austria[32]
Neonatal screening	Hungary	2010	1:5,000	Hungary[33]
Neonatal screening	Washington state (males only)		1:7,800	USA[34]
Neonatal screening	Missouri state	2013	1:2,900	USA[35]

The link between lysosomal storage and cellular dysfunction is not well understood, but energy depletion (related to respiratory chain enzymes dysfunction), abnormal production of reactive oxygen species (ROS), ischaemia and circulating growth-promoting factors are certainly involved.

Cellular energy depletion, due to inefficient cardiomyocytic energy utilization, is one of the most accepted hypotheses. It has been proposed as a common mechanism for several types of metabolic and even sarcomeric hypertrophic cardiomyopathy.[36] A study using cultured skin fibroblasts from four FD patients and ten controls has shown, in FD patients, a significant reduction of the activity of respiratory chain complexes I+III, IV and V, as well as a significant decrease of cellular content of high-energy phosphate compounds (creatine phosphate [PCr] and adenosine diphosphate) and adenosine monophosphate.[37] Moreover, studies with cardiac phosphorus-31 magnetic resonance spectroscopy (a non-invasive technique enabling *in vivo* determination of PCr and

adenosine triphosphate [ATP]) in FD have shown a significant decrease in PCr, ATP and the PCr to ATP ratio, as well as a significant inverse correlation between the PCr to ATP ratio and the annual increase in left ventricular (LV) mass, indicating mitochondrial energy depletion.[38, 39] This mitochondrial dysfunction will lead to inefficient sarcomeric ATP utilization and impaired contractility, resulting in hypertrophy. The reason for the reduction of respiratory chain enzyme activities in FD remains unclear. However, *in vitro*, lysosphingolipids may have direct deleterious effects on mitochondrial function, by direct binding to mitochondrial membranes and inhibition of oxidative phosphorylation.[40]

Several studies suggest that oxidative stress is also implicated in the link between lysosomal storage and cellular dysfunction in FD cardiomyopathy and vasculopathy. In cultured FD endothelial cells, incubation with increasing doses of Gb3 increased intracellular ROS generation in a dose-dependent manner, which was significantly lowered (even in the cells loaded with Gb3) by addition of an antioxidant (vitamin C). In the same study, incubation of the cell line with plasma from FD patients also led to increased ROS production, even if the endothelial cells were pre-treated with a glycosphingolipid synthase inhibitor or recombinant enzyme replacement therapy (ERT) to decrease Gb3 loading, suggesting that other factors in FD patient plasma may also contribute to oxidative stress in vascular endothelial cells.[41] This data was confirmed *in vivo* by demonstration, in FD patients under ERT, of reduced glutathione and glutathione peroxidase activity and increased superoxide dismutase / catalase ratio in erythrocytes (leading to decreased levels of antioxidant defences), as well as oxidative damage of lipid and proteins (increased levels of malondialdehyde [a product of lipid peroxidation] and carbronyl group and dityrosine [both resulting from oxidative damage to proteins]).[42] Moreover, oxidative damage to deoxyribonucleic acid (DNA) purines is significantly higher in FD patients, despite efficient DNA repair.[43]

Gb3 loading may also cause a deregulation of nitric oxide (NO) pathway, with down-regulation of endothelial NO synthase, up-regulation of inducible NO synthase and enhanced cyclooxygenase-2 expression in microvascular (but not in macrovascular) cardiac endothelial cells.[44] Moreover, FD causes increased nitrotyrosine expression in brain tissue from FD patients[45] and elevated levels of nitrotyrosine in an endothelial FD cell line and in the plasma of FD patients,[46] attributable to endothelial NO synthase deregulation and production of nitrogen reactive species.

Regarding cardiomyocytes oxidative damage, in a study involving 18 FD patients performing endomiocardial biopsies, immunohistochemistry has shown that inducible NO synthase and nitrotyrosine expression was increased in FD patients compared with hypertrophic cardiomyopathy due sarcomere protein gene mutations (HCM) and normal controls. Oxidative damage to DNA, assessed by immunostaining for 8-hydroxydeoxyguanosine, was also only identifiable in FD patients (25% of cardiomyocyte nuclei) and not in controls; cardiomyocytes apoptotic cell death was 187-fold greater in FD patients than in controls and only detected in nuclei with oxidative DNA damage.[47]

The disease pathophysiology is potentiated by ischaemia, which may occur even in the absence of significant epicardial coronary artery disease. Elliott P et al., in a study including 10 FD male patients (none had angiographically significant coronary artery disease), showed that resting and hyperaemic myocardial blood flow and coronary flow reserve were significantly reduced in FD patients, with comparable resting coronary reserve, attributable to abnormal microvascular function.[48] This might be due to increased susceptibility, for unknown reasons, of microvascular endothelial myocardial cells to deregulation of NO pathway,[44] increased oxygen demand of the hypertrophied muscle cells, decreased capillary density, increased diastolic filling pressures (impairing blood flow throughout the subendocardium) and sphingolipid storage within endothelial and vascular smooth muscle cells of small arterioles and capillaries.[5]

Furthermore, incubation of FD patients plasma with mouse vascular smooth muscle cells and neonatal cardiomyocytes, suggests that there are circulating growth-promoting factors inducing cell hypertrophy and proliferation, correlating with LV mass and carotid common artery intima-media thickness.[49]

Cellular dysfunction affects myofilaments and cardiomyocyte contractility; there is evidence of myofilament proteolysis (revealed by presence of degradation products of troponin I and desmin) and dislodgement and disarray (partly due to intracellular glycosphingolipids storage) and myofibrillolysis. In this context, it was reported that the active tension of cardiomyocytes was four times lower in Fabry cardiomyocytes compared to controls, and this correlated well with the extent of myofibrinolysis. Furthermore, the resting tension was six times higher than in controls, which was shown to correlate with tissue Doppler imaging (TDI) data, suggesting that increased stiffness of cardiomyocytes contributes to diastolic dysfunction in FD cardiomyopathy.[29]

Myofilament dysfunction, with impaired relaxation and contractility and increased cell stress may trigger, as hypothesized for HCM, expression of stress-responsive trophic and mitotic factors (in an attempt of structural and functional cell repair), a possible direct cause of myocyte hypertrophy and disarray and increased interstitial collagen synthesis (figure 1).[50]

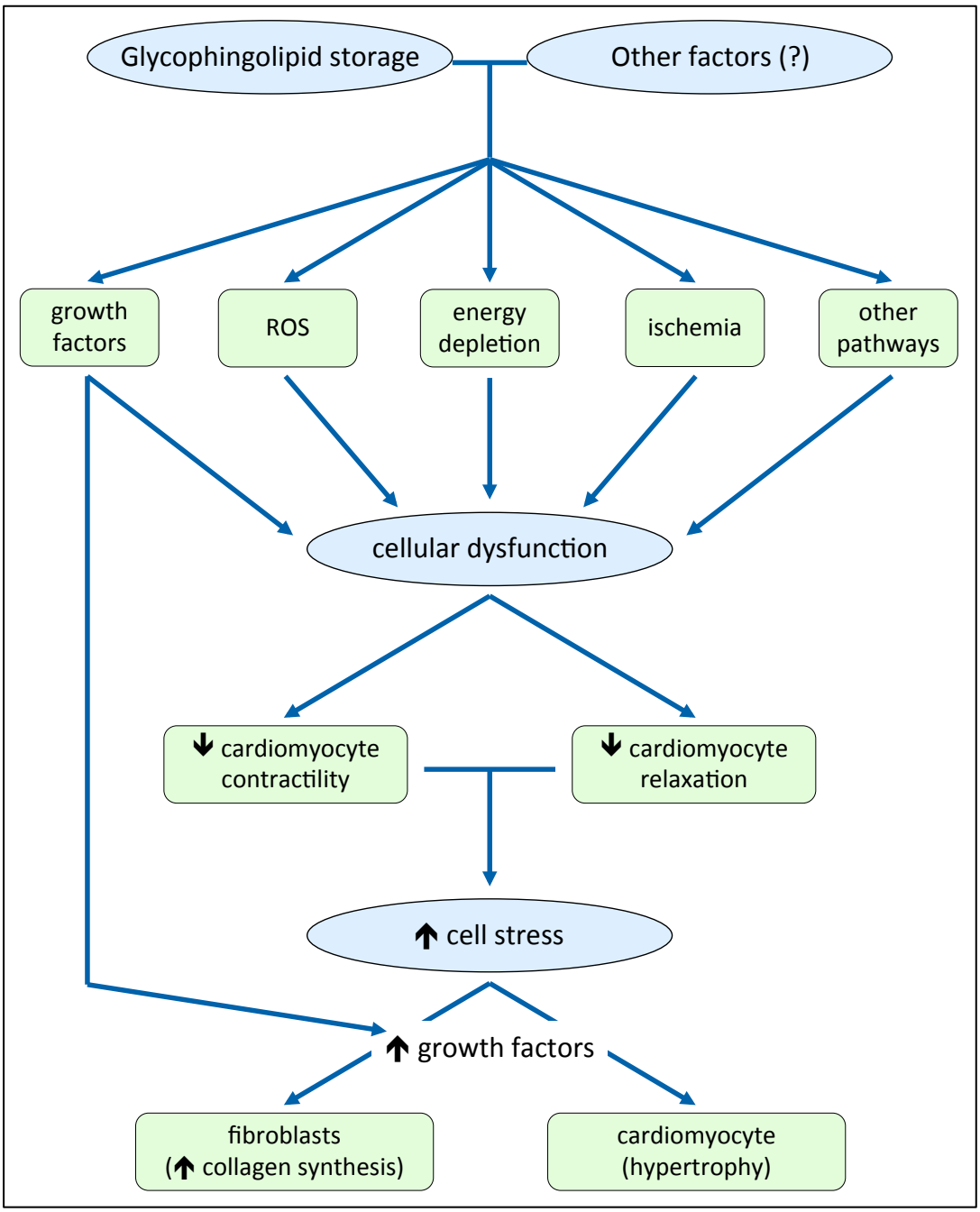


Figure 1: Pathophysiology of Fabry disease cardiomyopathy. Glycosphingolipid storage is probably the main trigger to the activation of several pathways, ending in cellular dysfunction and increased cell stress; this will promote increased collagen synthesis and cardiomyocyte hypertrophy. ROS: reactive oxygen species.

Histologically, the most prominent feature in patients with advanced FD cardiomyopathy is the severe cardiomyocyte hypertrophy, mainly attributable to intracytoplasmic glycosphingolipids storage, appearing as perinuclear vacuoles in light microscopy and as electron-dense deposits consisting of parallel or concentric lamellae in electron microscopy.[24, 29, 51-53] There is disarray and peripheral displacement of the myofibrils, with some of the fibres displaying signs of disintegration (being surrounded by small collections of foamy histiocytes).[24, 28, 29]

Myocardial fibrosis is also one of the hallmarks of FD cardiomyopathy. Fibrosis is unequally distributed along the LV wall, being more prominent within the mid-myocardial layers and the posterolateral segments of the LV[29, 51] and ranging from mild fibrosis (with fibres embedded in loose fibrous tissues) in the interventricular septum (IVS),[28, 53] to marked fibrosis (with almost no myocardial cells observed) in the base of the LV posterior wall (PW).[54] Moreover, fibrosis seems to be predominantly interstitial diffuse (with only focal areas of replacement fibrosis) and, in one study, it did not correlate with TDI measurements of systolic or diastolic LV function.[29, 54]

Furthermore, massive accumulation of lipids is also present in the conduction tissue, tricuspid and mitral valves and endothelium cells of the myocardial capillaries.[24, 55] In FD patients with attenuated phenotypes with predominant cardiac involvement, no lysosomal inclusions were observed in cardiac capillary endothelial cells.[54]

Concluding, Gb3 storage is probably the trigger for the activation of several signalling pathways leading to cellular dysfunction and, ultimately, to cardiomyocyte hypertrophy and fibrosis, in direct relationship with the clinical manifestations of LV hypertrophy and diastolic dysfunction.

1.2.2 *Nephropathy*

As mentioned above for cardiomyopathy, Gb3 storage also occurs in all renal cells, namely the endothelial, glomerular, interstitial and tubular cells, and the disease progression results in the development of glomerulosclerosis, tubular atrophy and interstitial fibrosis.[56, 57] However, the pathophysiology of FD nephropathy and the relationship between Gb3 storage and the aforesaid findings are not completely understood. Podocyte and endothelial damage, as well as other mechanisms may play an important role in FD nephropathy pathogenesis.

Though present in all glomerular cells, Gb3 storage is most abundant in podocytes.[57-60] Moreover, podocytes are unique neuron-like glomerular cells that are highly differentiated, with only limited potential of self-renewal, a fact that could make them particularly susceptible to damage by lysosomal storage disorders. Podocyte injury leads to loss of integrity of the glomerular filtration barrier and progression to chronic kidney disease.[61] Several pathways are certainly involved in podocytes damage, namely autophagy dysregulation, cytoskeleton disorganization (and podocyturia) and activation of fibrotic and inflammatory pathways.

Dysregulation of the autophagic pathway is one of the contributors to podocytes damage. *In vitro* studies in a human podocytes cell line, a FD phenotype was established using small hairpin ribonucleic acids (RNA) to reduce α-galactosidase A expression and activity. In these α-galactosidase A deficient podocytes, Gb3 accumulation was accompanied by an increase in autophagosomes and a down-regulation of mammalian target of rapamycin (mTOR) signalling cascade (a well-known inhibitor of autophagy).[62] This data was confirmed in kidney biopsies of 5 patients, showing increased number of autophagic vacuoles in FD kidney tissue (notably in podocytes), which decreased in renal biopsies performed after three years of ERT.[63] Sphingolipid storage may have a direct role in the dysregulation of the autophagic pathway, since ceramide is an activator of autophagy.[64]

Gb3 overload in podocytes may also interfere, by mechanical stress, with the distribution of synaptopodin, an actin-associated protein highly expressed in podocyte foot processes that is involved in cytoskeletal reorganization.[65] Moreover, Gb3 may interact with F-actin causing cell contraction, slit diaphragm widening and the coupling with integrins.[65, 66] Integrins, mainly $\alpha_v\beta_3$ (also known as vitronectin receptor), are essential to anchor podocytes to the basement membrane and their activation triggers podocyte contraction and migration and, ultimately, detachment from the glomerulus. In FD patients, increased urinary excretion of $\alpha_v\beta_3$ integrin was found in both classical and attenuated phenotypes; also its increased expression was observed in podocytes of the kidney tissue from a patient with a classical phenotype.[67] Therefore, integrin activation seems to be actively involved in podocytes detachment from the glomerular basement membrane in FD. This phenomenon will lead to higher levels of podocyturia, as previously shown in two studies with FD patients, one of them reporting a significant correlation

between podocyturia and albuminuria.[66, 68] Podocytopenia is the main consequence, and it is believed that when each glomerulus loses more than 40% of its 500 podocytes, it undergoes obliteration.[69]

In a human glomerular podocytes cell line, as lyso-Gb3 increases, there is activation of fibrotic pathways, leading to the expression and production of extracellular matrix proteins (fibronectin and collagen type IV [ColIV]), mediated by the transforming growth factor-β1 (TGF-β1) and Notch1 pathways. Paricalcitriol and calcitriol, vitamin D receptor activators, prevented the up-regulation of these mediators. This expansion of extracellular matrix is certainly involved in the glomerulosclerosis pathogenesis.[18, 70]

Inflammatory response is also up-regulated in FD glomerular podocytes, as shown by higher urinary excretion of CD80 in FD patients, as well as increased CD80 messenger RNA expression in podocytes cultured with lyso-Gb3.[71] CD80 (lymphocyte activation antigen 7-1) is normally located in antigen presenting cells (not expressed by normal podocytes) and modulates T helper and T cytotoxic cells activity.[72] Increased expression of CD74, a macrophage migration inhibitory factor receptor that regulates the expression of lethal cytokines, had also been shown.[18] Furthermore, Notch1 pathway, a key mediator of proinflammatory response, was up-regulated in a podocytes cell line cultured with lyso-Gb3, with increase expression of chemokines MCP-1 (Monocyte Chemoattractant Protein-1) and RANTES (Regulated on Activation, Normal T Cell Expressed and Secreted), mediated by the recruitment of the transcription of NFkB (nuclear factor kappa B). This data was confirmed in kidney biopsies from FD patients, with immunohistochemistry confirming Notch1 expression in glomerular podocytes and tubular cells.[70]

Vasculopathy is also certainly involved in FD nephropathy pathogenesis. As mentioned above for FD cardiomyopathy, production of ROS and dysregulation of NO pathway are major contributors to endothelial dysfunction.[41, 42, 44-46] The classical theory emphasized and suggested that kidney fibrosis and advanced kidney lesions were a consequence of ischaemic tissue damage, derived from microvascular endothelial disease and/or necrosis of vascular smooth muscle cells.[57, 60] This hypothesis was supported by pathological studies showing universal lipid deposits at a very early age, unlike the nonspecific changes (like glomerulosclerosis, tubular atrophy and interstitial fibrosis) which were age-related and firstly involving the vessels.[57] However, these are nonspecific features of advanced kidney disease of any aetiology and are generally minimal in

patients younger than 25 years old, suggesting that they are secondary features and not the early pathogenic event. Therefore, the current knowledge on the importance of podocyte injury in glomerulosclerosis pathogenesis indicates that vasculopathy is certainly involved in FD pathogenesis, but it is not the sole or even the main pathogenic mechanism.

Tubulointerstitial injury is also involved in FD nephropathy pathogenesis. Gb3 may have direct toxic effects in tubular cells, causing focal tubular atrophy and interstitial fibrosis. Although there is weak evidence supporting this mechanism, the finding of glomerular enlargement in the early stages of glomerular and tubular injury could corroborate that the glomeruli upstream of affected tubules may function poorly and that other glomeruli may undergo hypertrophy to compensate. Hyperfiltration in these glomeruli may trigger a secondary form of focal segmental glomerulosclerosis.[60] Diffuse involvement of interstitial cells, with electron dense inclusions, has also been reported.[57, 73] By causing tubular injury, proteinuria may have, as in other nephropathy models, a direct role in chronic kidney disease (CKD) progression and may provide a link between the aforesaid pathological processes and the development of tubulointerstitial disease.[74, 75]

These abovementioned effects of storage in all renal cells will give rise to progressive nephropathy, characterized by nonspecific degenerative lesions, namely mesangial widening, segmental and/or global glomerular sclerosis, tubular atrophy and interstitial fibrosis (figure 2).[56, 57, 60, 73, 76] These well known histological findings in advanced FD nephropathy contrast with the limited knowledge about the histology of patient with incipient nephropathy.

Lipid deposition related lesions are characteristic of FD nephropathy. As ceramide tails of glycosphingolipids are dissolved during paraffin embedding of kidney tissue, the most characteristic finding in routine light microscopy of kidney biopsies is vacuolization. This is particularly prominent in podocytes, parietal epithelial cells of Bowman's capsule and Henle's loop and distal tubular cells.[56, 57, 60, 76] In light microscopy, glycosphingolipids deposits are best assessed in toluidine blue stained semi-thin sections of glutaraldehyde / formaldehyde fixed and epoxy-embedded tissue. In the glomeruli the largest amount of lipid material is seen in podocytes followed by parietal epithelial, mesangial and glomerular endothelial cells.[57, 59, 77] Tubular inclusions are more prominent in distal

tubules, with relatively spared proximal tubules (mainly in females) and there is also peritubular capillary inclusions, including intimal and medial vascular inclusions, leading to severe medial injury and necrosis, with arterial hyalinosis.[57, 59, 77]

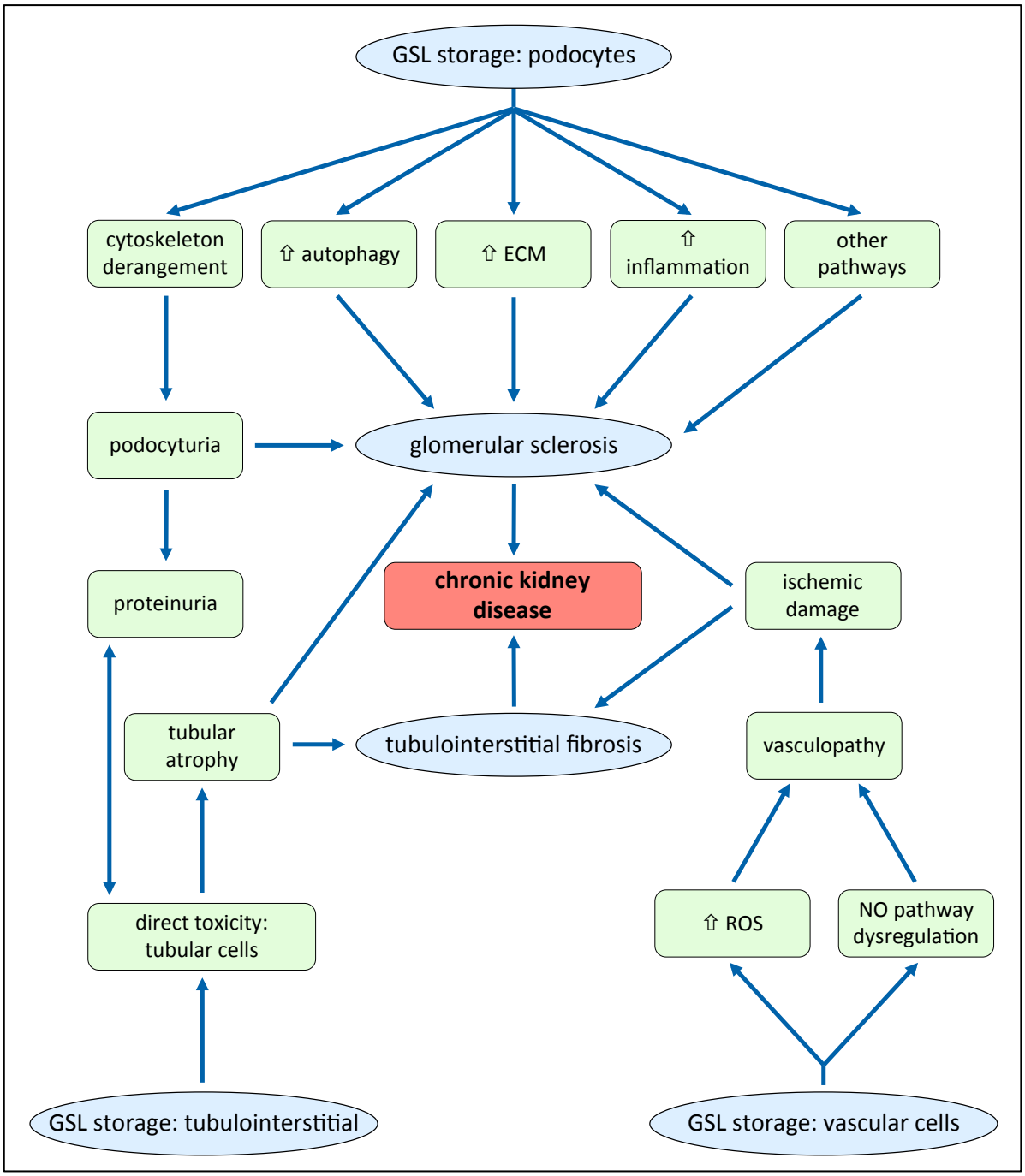


Figure 2: Pathophysiology of Fabry disease nephropathy. Glycosphingolipids storage in several kidney compartments is the main trigger to the activation of several pathways, ending in glomerular sclerosis and tubulointerstitial fibrosis; these are the main findings leading to progressive chronic kidney disease. ECM: extracellular matrix; GLS: glycosphingolipids; NO: nitric oxide; ROS: reactive oxygen species.

Under electron microscopy, electron dense cytoplasmic granules (inclusions) are found in all renal cells, but their size, shape, abundance and distribution vary from one type of cell to another. Though the inclusions are more abundant and larger in podocytes, Henle's loop and distal convoluted tubules, they are present in all glomerular and interstitial cells, proximal convoluted tubules and arterial endothelial and smooth muscle cells.[57, 76, 77] In contrast to the earlier reports showing normal podocytes' foot processes and glomerular basement membranes,[57] several recent publications show foot process effacement as an early finding in FD nephropathy.[58, 77-80]

These characteristic severe inclusions in podocytes and distal tubules, as well as segmental foot process effacement have been shown even at early stages of Fabry nephropathy, in paediatric and adult patients with minimal or no alterations in standard renal tests (namely, glomerular filtration rate, albuminuria or proteinuria).[57, 58, 77, 79-81] Moreover, mesangial and endothelial cells inclusions, as well as non-specific degenerative lesions, such as mesangial widening, glomerulosclerosis, tubulointerstitial fibrosis and arteriopathy have also been described in this group of patients.[57, 77, 80] This data confirms that clinically silent deposition of Gb3 begins in early childhood, long before overt FD nephropathy, with a wide variation in the individual progression of glomerulosclerosis and interstitial fibrosis and development of end-stage renal disease.[82]

However, the importance of these pathological findings as potential surrogate markers for the progression of renal dysfunction (glomerular filtration rate [GFR]) is uncertain and needs to be further studied longitudinally. In contrast to semi-quantitative scoring systems for intracellular Gb3 inclusions, using light microscopy, that has failed to show any correlation with age, proteinuria and GFR,[57, 59, 77, 83] a small study with 14 FD patients, using quantitative stereological electron microscopy methods, has shown significant correlations between podocyte Gb3 fractional volume of inclusions by cytoplasm or foot process width and age or proteinuria.[58] Contrariwise, some studies showed correlation between degenerative glomerular / tubulointerstitial lesions or arterial sclerosis scores and age or GFR.[57, 59, 83] Furthermore, under light microscopy, segmental and/or global glomerulosclerosis was found to be the only pathologic correlate of proteinuria at early stages of Fabry nephropathy.[84] Thus, chronic nonspecific glomerular and tubulointerstitial lesions seen in kidney biopsies seems to correlate better with the natural history and manifestations of FD nephropathy than renal

glycosphingolipids accumulation.

1.3 Clinical manifestations and natural history

1.3.1 *Cardiomyopathy*

As the disease progresses, heart involvement occurs in the great majority of FD patients, with virtually all patients presenting cardiac symptoms in the seventh decade of life.[10, 85] The incidence of cardiac symptoms seems to be the same in both genders, but with a delayed mean age of onset in female patients (about seven years difference between genders).[10, 85] The prevalence of cardiac events and interventions varies according to the study (given the distinct definitions of event), between 19 - 49% and 14 - 35% for male and female patients, respectively.[11, 82, 85] Cardiac events seem to be more prevalent and tend to occur at an earlier age in male patients.[11, 82, 85] Furthermore, probably reflecting the improvement in the management of renal disease in FD, the most recent studies have shown cardiovascular disease as the main cause of death in FD and a major determinant of overall disease prognosis.[86, 87]

In view of the ubiquitous deposition of Gb3 in all cardiac cells, cardiac manifestations / complications of FD include myocardial hypertrophy, impaired diastolic and/or systolic LV function, electrophysiological / conduction abnormalities, myocardial ischemia, valve disease and autonomic neuropathy.

LV hypertrophy is the hallmark of FD cardiomyopathy and the most common structural cardiac abnormality seen in FD.[85] It is characterized, in the early stages, by concentric LV remodelling (seen in approximately 50% of adolescents and children over the age of 10 years old), which progresses over time to hypertrophy.[88, 89] Concentric LV hypertrophy is the most typical pattern, but asymmetrical septal hypertrophy (more characteristically seen in HCM) is seen in 5-10% of the cases with severe cardiac involvement, usually associated to LV outflow tract obstruction.[88, 90, 91]

Hypertrophic changes are more prevalent, more severe and occur earlier in males than in females.[85, 88, 91] Its prevalence increases with age in both men and women (whether treated or not)[85, 88, 91, 92] and LV mass is inversely correlated with estimated GFR.[85] The presence of LV hypertrophy is associated with the presence of cardiac signs and symptoms, in particular dyspnoea, angina and palpitations.[85, 88] There is limited

longitudinal data describing the progression of LV hypertrophy in untreated patients, but in a study with serial echocardiograms in 39 male and 39 female untreated patients, the average increase in LV mass was significantly higher for male patients (4.1 ± 1.0g/$m^{2.7}$ per year against 2.3 ± 0.8g/$m^{2.7}$ per year in females), with a greater rate of progression when only patients with baseline LV hypertrophy were analysed.[91]

Cardiac hypertrophy is readily detectable by different techniques, namely electrocardiography, echocardiography and magnetic resonance imaging (MRI).

LV hypertrophy by voltage criteria and ST-segment abnormalities and T-wave inversion are some of the most common electrocardiographic abnormalities seen in FD.[88, 93] A significant correlation between Sokolow-Lyon voltage index or Romhilt–Estes scores and LV mass has been described, as well as a high prevalence of ST-segment and T wave abnormalities in patients with LV hypertrophy or remodelling (83-93%).[88, 93] Remarkably, repolarisation abnormalities may be seen in about a quarter of patients with normal LV morphology.[88, 93] Therefore, repolarisation abnormalities or LV hypertrophy by voltage criteria, in the absence of abnormal cardiac morphology, may be the first manifestation of Gb3 accumulation in cardiac tissue.

There are no echocardiographic pathognomonic features of FD cardiomyopathy, but, as noted above, it is characterized by an early stage of concentric remodelling (an increase in relative LV wall thickness, with normal LV mass), which progresses over time to overt symmetrical LV hypertrophy,[88, 89, 92] characteristically without resting LV outflow tract obstruction (except in a small proportion of patients with asymmetrical LV hypertrophy), with some patients presenting exercise-induced LV outflow tract obstruction.[88, 90, 91, 94] Probably due to the energy depletion in the cardiomyocytes, eccentric hypertrophy (indicated by an increase in end-diastolic LV diameter), may be seen in a few patients (less than 10%).[88, 91] Another typical aspect is the prominence of papillary muscles (present in up to 75% of patients).[95, 96]

As a lysosomal storage disorder, FD was misleadingly assumed to be a restrictive cardiomyopathy, because storage of Gb3 would lead to a stiff left ventricle with impaired passive diastolic filling (the hallmark of restrictive cardiomyopathy).[97-99] However, the hallmark of FD cardiomyopathy is LV hypertrophy (not present in most restrictive forms of cardiomyopathy) and severe diastolic dysfunction consistent with a restrictive filling pattern was very rare in several studies, with up to 100 FD patients.[88, 90, 100] Nevertheless,

mild diastolic dysfunction with impaired LV relaxation is encountered in a quarter to a third of patients and a smaller proportion (≈10%) has moderate diastolic dysfunction with a pseudonormal filling pattern.[90, 100] Conventional measures of LV systolic function (LV ejection fraction and LV fractional shortening) are usually within the normal range, regardless of whether or not the patient has LV hypertrophy.[88, 101, 102] Nonetheless, the sole small study evaluating natural history of systolic function in FD suggests that there is a gradual deterioration in LV fractional shortening.[101] Moreover, studies using more sophisticated non-invasive measures, like TDI and strain rate (SR) imaging, demonstrated reductions in systolic performance at a very early stage of disease progression.[103-108] Dyspnoea and other symptoms of heart failure are mainly caused by diastolic dysfunction (more rarely by systolic dysfunction) and occur in up to 30% of patients, with the same incidence in both genders.[85]

As myocardial lipid storage and fibrosis are hallmarks of FD cardiomyopathy, cardiac MRI provides complementary data to echocardiography through its ability to characterize myocardial tissue. Focal myocardial replacement fibrosis can be visualized by late gadolinium-contrast enhancement (LGE) in cardiac MRI.[109] LGE may be present in up to 50% of male and female patients and is located particularly in the posterolateral basal LV wall (usually sparing the subendocardial layers).[105, 106, 110-112] The reason for this distribution of LGE is unknown, but it has been hypothesized that FD cardiomyopathy might predispose the patient to subclinical myocarditis (causes a similar pattern of LGE) or, since LV work load is highest in the inferolateral wall, these segments may be prone to myocardial fibrosis development due to increased local wall stress.[105, 109] Female patients seem to have lower LGE areas and a higher probability of LGE presence with normal LV mass.[110, 113] Moreover, LGE correlates with abnormalities in regional myocardial function (assessed by SR or two-dimensional speckle-tracking imaging studies)[105, 114, 115] and is associated with a worse prognosis in terms of exercise capacity and risk for malignant ventricular arrhythmias.[106, 116]

The right ventricle (RV) is also affected by storage, with about 30-70% of patients presenting RV hypertrophy.[117-120] RV hypertrophy prevalence increases with age and appears similar in both genders, but the onset seems to occur later in females.[118, 119] RV hypertrophy occurs predominantly in association and is paralleled to the severity of LV hypertrophy.[117-120] However, in cardiac MRI, myocardial replacement fibrosis in the RV is

absent, even in the end-stage of the disease, as evidenced by two studies with up to 75 patients.[120, 121] Data on the impact of FD in RV systolic function is contradictory, but the regional function of RV lateral free wall (measured by RV longitudinal SR) seems reduced in patients with LV fibrosis.[117-120] Contrariwise, RV diastolic dysfunction seems frequent (mainly impaired RV relaxation) and predominantly affects patients with RV hypertrophy.[118, 119] Nonetheless, the clinical significance of RV dysfunction is believed to be low, as isolated right ventricular failure rarely complicates the course of the disease.

Up to 50% of the patients with FD, regardless of gender and especially those with LV hypertrophy complain of pain suggestive of myocardial ischemia.[82, 85, 88, 90] Nevertheless, the incidence of proven myocardial infarction or revascularization procedures is low, as well as epicardial coronary artery disease.[3, 82, 85] Thus, by the aforesaid mechanisms in cardiomyopathy pathophysiology, microvascular angina, in the setting of reduced coronary flow reserve, may be responsible for these symptoms in some patients;[48, 122, 123] vasospastic stimuli may also be involved.[90, 124] As mentioned, large vessel coronary artery disease is rarer, but the risk of death due to it should not be underestimated;[26] it may be more correlated to classic risk factors, such as arterial hypertension (HBP), dyslipidaemia and CKD and, in one series, despite a lack of significant large vessel disease, diffuse hypoechogenic plaques, assessed by intravascular ultrasound, were more common in FD compared to age-matched controls.[125]

Palpitations, the most frequent clinical symptom for underlying cardiac arrhythmias, are common in male and female patients with FD, but tend to start at an earlier age in males and its incidence is higher in patients with LV hypertrophy and increases with age.[82, 85, 126] Furthermore, arrhythmias are by far the most frequent cardiac event reported in FD.[82, 87] Supraventricular tachycardia is the most frequently encountered rhythm abnormality, mainly atrial fibrillation (paroxysmal or persistent / permanent), identified in almost 20% of patients in a cohort of 60 FD patients;[126] non-sustained ventricular tachycardia was recognized, during 24 hour Holter recordings, in 8.3% of the patients in the same series and are reported in cases of fatal malignant arrhythmias resistant to implantable cardioverter defibrillator.[126, 127] This higher risk of both atrial and ventricular arrhythmias is related to glycosphingolipid deposition, atrial dilatation and ischemia secondary to LV hypertrophy and fibrosis. A study including 73 patients reported an annual increase in cardiac fibrosis as the sole independent predictor

of malignant ventricular arrhythmias and non-sustained ventricular tachycardia and sudden cardiac death occurred only in the group of patients with LGE in cardiac MRI.[55, 116, 128]

Even the resting ECG is frequently abnormal. Early ECG manifestations of FD, occurring in younger patients with normal LV mass, include a short PR interval (observed in up to 40% of patients), which is likely due to accelerated atrioventricular (AV) nodal conduction rather than by accessory pathways.[90, 93, 126, 129, 130] With disease progression (development of LV hypertrophy) and/or age the conduction system dysfunction occurs and the PR interval may change and patients develop bundle branch block, AV conduction delay (which can progress to high-grade AV block) and progressive sinus node dysfunction.[88, 90, 126] Between 3% and 12.5% of patients require pacemaker implantation for complete heart block or symptomatic bradycardia, suggesting that bradyarrhythmias have a significant impact on the natural history of FD.[85, 126, 131]

FD patients may have dysfunction of autonomic cardiac control, but usually do not present clinically overt signs of orthostatic dysregulation. One of the first signs of cardiovascular system involvement in FD is depressed heart rate variability, which has been demonstrated in paediatric patients, mainly in boys, and may represent a pro-arrhythmogenic electrophysiological substrate.[89, 132] Moreover, most adult patients (mainly males) may present resting bradycardia and chronotropic incompetence, as well as reduced sympathetic activation and baroreflex sensitivity adjustment upon standing.[133, 134]

As in other LSDs, massive Gb3 accumulation in heart valve tissue and fibroblasts, with secondary fibrosis and calcification may cause valvular dysfunction.[24, 135] Changes are predominantly found in the left heart valves (mitral and aortic), probably due to the higher haemodynamic stress in this side of the heart.[88, 136] In early studies, mitral valve prolapse was said to be common in FD patients,[137, 138] but subsequent reports were not able to confirm this finding, perhaps due to the novel diagnostic criteria for identification of mitral valve prolapse.[88, 136] Nevertheless, minor structural abnormalities in both mitral and aortic valves are frequently noted, with no difference in incidence between genders.[85] Mitral valve seems to be the first and the most frequently affected by structural changes, mainly leaflet thickening and redundancy, often accompanied by hypertrophy and hyperechogenicity of the papillary muscles and mild valvular

regurgitation.[88, 136] Moreover, in patients with asymmetrical septal hypertrophy, the typical systolic anterior motion of the anterior mitral leaflet may contribute to mitral valve dysfunction.[90] Aortic valve abnormalities seems to appear at an older age[88] and the aortic root dilatation, found in the advanced stage of FD cardiomyopathy, may contribute to mild aortic valve regurgitation.[88, 136, 138, 139] Nonetheless, valve regurgitations are often mild and irrelevant in haemodynamic terms, hence the need for surgical valve repair is rare.[85, 88, 136, 140]

1.3.2 *Nephropathy*

Kidney involvement is common in FD disease, with a proportion of patients progressing to end-stage renal disease (ESRD).[82, 83, 141] In the past, before renal dialysis and transplant became widely available, renal failure was the main cause of death in male patients with FD.[10, 86] More recent studies have shown cardiovascular disease as the main cause of death. However, the majority of patients dying from cardiovascular events had previously received renal replacement therapy.[86, 87] Therefore, FD nephropathy remains as a major determinant of overall disease prognosis.

As noted above, accumulation of Gb3 occurs in all renal cells, with consequences on glomerular and tubular function, reflected in the clinical and laboratory manifestations of FD nephropathy: proteinuria, impaired GFR, HBP, tubular dysfunction and urine sediment abnormalities.

Most patients (up to 50%) with FD develop overt proteinuria (>300mg/day), which is more prevalent and more severe in males than in females, except for CKD stages 4/5, where the incidence and degree of proteinuria is similar for both genders.[82, 141, 142] Moreover, its prevalence and severity increases in accordance with CKD stage, and it is believed to directly contribute to the progression of FD nephropathy.[82, 141, 142] Proteinuria (>150mg/day) and albuminuria A2 (>30mg/day) are usually the first signs of renal involvement and may start as early as in the 2nd decade of life in both genders, but, before the age of 18 years old, they are presente only in a minority of patients (up to 15%) of both genders.[143-146] Thereafter, there is a progressive increase in the number of patients with proteinuria, with 50% of male patients developing proteinuria by the age of 35 years old, and all surviving patients presenting proteinuria by the age of 52 years.[83] Proteinuria seems to be often of glomerular origin (containing ≥ 50% albumin)[83] and

nephrotic range proteinuria (> 3.5g/day) is relatively uncommon, being present in 7.3 – 18% of male patients and in 3.6 – 7.5% of female patients, with a greater prevalence in more severe CKD stages.[83, 141, 142] Nonetheless, a full-blown nephrotic syndrome is not frequent, with only 21% of the patients with nephrotic range proteinuria presenting serum cholesterol > 200mg/dL.[83]

Progressive decline of GFR is a common complication of Fabry disease, with greater prevalence and decline in male compared with female patients.[82, 83, 142] As in other nephropathies, an initial hyperfiltration state of glomerular compensation may exist and mask the early impairment of renal function, until the damage of a critical number of nephrons. Actually, several studies in FD paediatric population showed that most children exhibited estimated GFR (estimated by Schwartz equation) higher than expected for healthy children and adolescents.[143, 145, 147] However, this data must be interpreted cautiously, as the Schwartz equation may overestimate GFR in patients without CKD, as described in a population of 30 paediatric patients with FD, with a mean difference of 18.0ml/min/1.73m^2 between estimated and measured GFR.[81] For adult populations, a similar issue is raised, as estimation of GFR may also be inaccurate for patients with higher GFR, mainly with Modification of Diet in Renal Disease (MDRD) study equation.[148] Nevertheless, there remain a few paediatric and adult patients with FD and true hyperfiltration based on measured GFR, but whether hyperfiltration presages a later fall in GFR is unknown.[81, 149]

Regardless of this hyperfiltration stage, only a minority (up to 3%) of FD patients have decreased GFR (< 90ml/min/1.73m^2) before the age of 18 years old (nonetheless, cases of ESRD have been reported as early as in second decade of life).[143, 145, 146, 150, 151] However, thereafter the rate of GFR decline is heterogeneous (with several factors affecting the rate of decline), but can be as high as 12.2ml/min/year in male patients with serum creatinine > 1.5mg/dL, with a mean time of progression to ESRD of 4 years (range: 1 to 13 years).[83] In a cohort of 105 classically affected males, 23% developed ESRD, at a median age of 47 years old, and with all the patients surviving to the age of 55 years old developing ESRD.[83] In larger cohorts, including male and female patients, with both classical and late-onset phenotypes, the prevalence of ESRD was 13.7 – 17.6% and 2.0 – 4.8%, for male and female patients, respectively, with a similar median age of development of ESRD for both genders (between the end of the fourth and the beginning

of the fifth decade of life).[82, 150] The incidence of ESRD was also markedly higher in male patients, regardless of the age group.[150]

As mentioned above, the progression of FD nephropathy is heterogeneous, with several factors influencing the rate of GFR decline, including gender, baseline CKD stage, degree of proteinuria and residual enzymatic activity. Regarding gender, there is a significant greater proportion of male patients achieving CKD stage ≥ 3 (22.2 - 28.4% versus 13.1% - 15.1% for male and female patients, respectively), with a higher mean age of female patients in all CKD stages, except in CKD stages 4 / 5, as well as a larger proportion of male patients with a loss of estimated GFR > 1ml/min/1.73m^2/year.[82, 141, 152] In a large cohort of FD patients, the rate of GFR decline in male patients who did not develop ESRD was −2.9ml/min/1.73m^2/year, compared with −1.02ml/min/1.73m^2/year for female patients.[82] The progression rate is also greater for patients presenting CKD stage ≥ 3 at the first evaluation, with a more than two-fold greater GFR decline, for both male and female patients.[82] Proteinuria also seems to play an important role in FD nephropathy progression, because for both genders, the proportion of patients with overt proteinuria (> 300mg/day) and its magnitude and the prevalence of nephrotic range proteinuria are higher with more advanced CKD stages.[82, 141] Moreover, comparing the GFR decline stratified by baseline proteinuria, higher baseline proteinuria levels were associated with more rapid GFR decline, but the patients with higher baseline proteinuria were also older and with lower baseline GFR.[82, 152] Notwithstanding this, in one study a regression model for GFR slope retained proteinuria as the most important indicator of renal disease progression in adult FD patients.[152] Therefore, there seems to be an influence of proteinuria in nephropathy progression, although the magnitude of this influence is not well established, as several studies, with both male and females patients, finding no relationship between the degree of proteinuria and the rate of GFR decline and, in one study, 11% of the male and 28% of the female patients with estimated GFR < 60ml/min/1.73m^2, presented no overt proteinuria.[15, 83, 141] In male patients, the presence of residual α-galactosidase A activity ≥ 1% in leucocytes also seems to be associated with better renal outcomes, with a significantly older age of achieving a serum creatinine > 1.5mg/dL, compared with the patients with undetectable α-galactosidase A activity.[83]

Data on HBP or blood pressure (BP) control in patients with FD is scarce. In a previous study, the prevalence of HBP was 30% in a cohort of male FD patients.[83]

However, contemporary studies, with larger cohorts, showed a greater overall prevalence of patients with systolic BP ≥ 130mmHg and/or diastolic BP ≥ 80mmHg, between 57.1% - 58% in males and 46.9% - 52.5% in females.[82, 153] This contrasts with a 44.2% prevalence of HBP among the general European population, although the threshold for diagnosis of HBP in this study (BP > 140/90mmHg) differs.[154] Moreover, the prevalence of HBP is significantly greater in FD patients with GFR < 60ml/min/1.73m^2 (64 - 80%), compared to FD patients with GFR ≥ 60ml/min/1.73m^2 (45 – 52%)[82, 141, 153] and, in one study, 65% of the hypertensive FD patients, the HBP appeared concurrently or after serum creatinine reached ≥ 1.5mg/dL.[83] Nevertheless, HBP seems less prevalent in FD with GFR < 60ml/min/1.73m^2 than in the general CKD population at comparable levels of GFR.[155] Besides, systolic BP positively correlates with proteinuria in adult male and female patients and diastolic BP correlates with GFR slope in adult male patients.[141, 152]

As noted above, tubular inclusions are more prominent in distal than in proximal tubules,[57, 77] but the aetiology of this preferential accumulation of Gb3 is not well understood. Therefore, distal tubular function is particularly affected, resulting in impaired urinary concentration and isosthenuria, which was demonstrated even in patients with normal GFR and may be the earliest apparent renal abnormality.[83, 156, 157] The involvement of distal tubules and collecting ducts may also result in nephrogenic diabetes insipidus, impaired tubular potassium reabsorption (and enhanced net potassium excretion) and defective renal acidifying mechanisms (with reduced net acid excretion).[156, 158, 159] Proximal tubule dysfunction has been reported more rarely, with reduced tubular reabsorption of glucose and amino acids and, occasionally, an overt Fanconi syndrome.[159, 160]

The accumulation of neutral glycosphingolipids in renal parenchyma is reflected in urine composition and sediment. Even at early stages of FD nephropathy, erythrocytes, leucocytes and hyaline and granular casts can be found in urine sediment.[161] The urine sediment may also contain oval fat bodies, which may be present either freely or within desquamated tubular / uroepithelial cells or cell fragments and represent lipid inclusions. Under polarised light microscopy, these oval bodies are birefringent, with the characteristic “Maltese cross” configuration, with lamellar appearance. In electron microscopy, they match the typical lamellar osmiophilic inclusions in urinary cells.[162, 163]

1.4 Treatment

As a multi-system condition, a comprehensive therapy for FD is warranted, not only including disease specific treatment, but also conventional medical treatment for complications and adjunctive therapies.[30, 164] Until recently the former was not available and its management mainly consisted of other treatment strategies. However, in 2001 a disease specific ERT was aprroved as the gold standard in the treatment of FD and, more recently, in 2017, European regulatory authorities approved the pharmacological chaperone migalastat for treatment of FD patients with amenable mutations.

In Europe, there are currently two commercially available ERT preparations, both based on the infusion of recombinant enzymes: agalsidase-α (Replagal®, Shire Pharmaceuticals, Lexington, Massachusetts), produced in a human cell line by gene activation and licensed at the dose of 0.2mg/Kg, every other week (EOW);[165] agalsidase-β (Fabrazyme®, Sanofi Genzyme, Cambridge, Massachusetts), produced in Chinese hamster ovary cells by recombinant techniques and licensed at the dose of 1mg/Kg, EOW.[166] Both products have the same primary amino acid sequence (equivalent to the wild-type human enzyme),[167] but there are several differences in post-translational modification, including glycosylation.[168, 169] Post-translational modifications may have profound effects on the conformation and physicochemical properties of proteins, and glycosylation is known to have an impact on the antigenicity of recombinant proteins.[170, 171]

Migalatat is currently available in Europe and it is a pharmacological chaperone, which binds to the active site of α-galactosidase A and stabilizes certain mutant enzymes (amenable mutations), thus facilitatng proper trafficking to lysosomes, where dissociation of migalastat allows α-galactosidase A to catabolize accumulated substrates.[172] This treatment is an orally administred small-molecule, with apprently higher volume of distribution than ERT and does not present the burden of immunogenicity associated with ERT.[173]

New treatment strategies are currently under development in clinical trials, namely substrate reduction therapy (lucerastat)[174] and modified forms of plant derived ERT (pegunigalsidase-α).[175]

1.4.1 *Cardiomyopathy*

The gold standards in the treatment of FD cardiomyopathy are the ERT and the

pharmacological chaperone migalastat, but adjunctive therapies directed at clinical manifestations (angina, arrhythmias, heart failure) and other cardiovascular risk factors are also paramount. Nowadays, with 15 years' experience of available ERT, there is increasing evidence supporting the efficacy of ERT in several cardiac endpoints, including histological, intermediate (clinical and imaging parameters) and "hard" outcomes (cardiac events and death).

Regarding cardiac Gb3 storage, in a randomized, double-blind, placebo-controlled clinical trial with agalsidase-α, there was, over a 6 month period, a mean reduction of approximately 20% in the myocardial Gb3 content in the active treatment group, compared with a mean increase of approximately 10% in the placebo group, but these findings did not reach statistical significance.[176]

In the phase III pivotal clinical trial of agalsidase-β, after a 20 weeks period, there was a significant decrease in the microvascular endothelial deposits score in the active treatment group, compared with the placebo group, with 67% of the patients reaching a 0 score; unfortunately, the cardiomyocyte storage was not described in this trial.[166] However, in the phase I/II agalsidase-β clinical trial, after 5 infusions, there was also a reduction in endothelial deposits, although the heart Gb3 concentration only decreased slightly in 4 out of 7 patients and Gb3 storage remained unchanged in the cardiomyocytes, pericytes and vascular smooth muscle.[177] Moreover, in the 60 months open-label extension study of the aforementioned agalsidase-β phase III clinical trial, in the eight patients consenting to biopsies at the end of the study, the clearance in endothelial cells was maintained, but there was no clearance of Gb3 in the cardiomyocytes.[178, 179] Persistent storage in cells other than vascular endothelial cells has also been identified in several case reports of patients treated with agalsidase-β.[26, 180] Notably, in a study with 5 patients treated with agalsidase-β for one year, the glycosphingolipids deposits appeared unmodified, including in endothelial cells; these patients were older than the ones previously described and probably had more severe cardiac involvement).[122] Thus, the available evidence suggests that ERT has, at least in some patients, limited access to non-vascular cardiac tissues and that the Gb3 clearance may be more difficult in older and more severe patients.

Several intermediate endpoints have been evaluated in response to ERT, namely cardiac structure and function or cardiac symptoms (angina and heart failure). In

summary, both ERT preparations have shown to improve myocardial remodelling and cardiac function, by decreasing LV wall thickness and mass[103, 106, 176, 181-190] or the progression of LV mass index[48, 191-194] and improving LV fractional shortening and strain.[103, 106, 187]

The effects of agalsidase-α in cardiac structure and function were evaluated in the two randomized, double-blind, placebo-controlled clinical trials, with significant reductions in the QRS complex duration[165] and LV mass index assessed by cardiac MRI,[176] compared to the placebo group, after 6 months of ERT, in a group of 14 and 7 male patients, respectively. In the open-label extension of the second trial, including 10 of the 14 recruited for the original trial, the LV mass remained stable over a 2 year follow-up period, but there were significant reductions in LV posterior wall (LVPW) and IVS thickness, suggesting cardiac remodelling during treatment.[176]

The effects of agalsidase-α in female patients have also been assessed in a 4 year, prospective, open-label study, with 36 patients, 69% of them with LV hypertrophy at baseline. There was a significant reduction in LV mass index (measured by echocardiography) after 12 months of treatment in both patients with and without LV hypertrophy at baseline (of greater extent and significance in patients with baseline LV hypertrophy), which was sustained throughout 4 years of treatment. Moreover, there was also a significant improvement in the New York Heart Association (NYHA) heart failure functional class.[181, 184]

A pooled and blinded analysis of all available echocardiograms from 4 industry-sponsored clinical studies (involving male and female patients), including the aforesaid clinical trial, confirmed the positive effect of agalsidase-α in patients with baseline LV hypertrophy, with a significant decrease in LV mass index over a period of 12 to 36 months (not observed in the group of patients with normal LV mass at baseline).[182] This data was confirmed in a single centre, based on long-term retrospective analysis of data from 45 patients, treated for approximately 10 years with agalsidase-α, showing a sustained significant decrease in LV mass index (maintained during the 10 years of treatment in male patients) in patients with baseline LV hypertrophy and a non-significant change in patients without baseline LV hypertrophy; this was accompanied by an improvement in heart failure and angina symptoms and stabilization of the ejection fraction.[183] The analysis of data extracted from the Fabry Outcome Survey (a registry /

database for FD patients who are receiving, or are candidates for, ERT with agalsidase-α) confirmed these results in larger cohorts of patients, with up to 5 years of follow-up and additionally revealed a significant increase in midwall fractional shortening of the LV.[185-187] It is worth mentioning that in all these studies, in only a small proportion of patients with established LV hypertrophy, the LV mass falls to normal range with ERT. Moreover, in one observational study with 76 patients, 33 of whom were treated with ERT (agalsidase-α), it was found that ERT significantly prevented, after a follow-up period of approximately 3 years, the appearance of TDI abnormalities in FD patients with no LV hypertrophy or TDI abnormalities at baseline, in comparison with the group of untreated patients.[195]

The effects of agalsidase-β in cardiac structure and function were not evaluated in the randomized, double-blind, placebo-controlled clinical trials. However, they have been reported in several non-controlled studies. Four prospective, open-label, non-controlled studies assessed the effects of agalsidase-β on LV mass by cardiac MRI; all evaluated small cohorts of patients (between 9 and 17 patients), included both male and female, and the mean follow-up periods were between 12 and 45 months.[103, 188, 191] These reports presented conflicting results, with three studies showing a significant decrease in LV mass[103, 188, 190] and one displaying no change in LV mass after 24 months of ERT.[191] Remarkably, in the study evaluating 17 patients (follow-up time: 12 months), 47% of these patients presented LGE in cardiac MRI at the study baseline and, in this patient subgroup, the decrease in LV mass was non-significant and the improvement of regional function was restricted to myocardial segments without LGE; moreover, the amount of LGE increased significantly during ERT, but no patient without LGE developed LGE during ERT.[190]

The effects of agalsidase-β in cardiac structure and function, by echocardiography, have also been evaluated in several small (up to 10 patients), short/medium-term studies (12-24 months), including patients of both genders. Significant decreases in LV mass have been reported in one study,[189] while other authors reported no significant changes in LV mass.[48, 192, 193] It worth noting that one of these studies was conducted in 9 FD patients undergoing dialysis, with a 24 months period of follow-up and, although a non-significant increase in LV mass was observed, there was an almost significant decrease in the mean slope of LV mass index progression, when comparing the 24 months before and after the

beginning of ERT.[193] Moreover, some authors reported improvements in diastolic function parameters[189] or in myocardial systolic strain (a more sensitive method to quantify regional myocardial function).[103, 190]

Long-term data on the benefits of agalsidase-β in cardiac structure have also been reported. In an open-label, non-controlled study based on 32 FD patients over a period of 3 years, a significant and sustained reduction in LV mass (measured by echocardiography) was observed within the follow-up period. However, comparing the subgroups of patients without fibrosis, with mild fibrosis and with severe fibrosis (assessed by cardiac MRI with no LGE, LGE in one segment and LGE in at least 2 segments, respectively) the reduction in LV mass was more pronounced in patients without fibrosis (although significant in all subgroups); moreover, an improvement in myocardial function (measured by myocardial systolic strain) and exercise capacity (obtained by bicycle stress exercise) was only evident in the subgroup of patients without fibrosis.[106]

In the long-term (10 years) analysis of 52 out of the 56 patients originally included in the pivotal phase III clinical trial of agalsidase-β, there was a slight and non-significant increase of LVPW and IVS thicknesses. However, it was more pronounced (though non-significant) in patients with high renal involvement (defined as proteinuria >0.5g/day or ≥50% sclerotic glomeruli at baseline) and there was a significant increase in wall thickness in patients starting ERT at an age ≥40 years old.[194] This influence of renal function in response to agalsidase-β had already been found in a previous study, where only the subgroup of patients with estimated GFR >90ml/min/1.73m^2 presented a significant decrease in LVPW thickness after 24 months of ERT; moreover, in the same study there was no improvement in the heart failure functional class.[196]

Limited data is available regarding “hard” outcomes, such as cardiac events and death, in patients treated with ERT. A composite of time to first clinical renal, cardiac or cerebrovascular events or death was the primary endpoint in a randomized, double-blind, placebo-controlled clinical trial with agalsidase-β. The analysis of protocol-adherent patients (adjusted for imbalanced proteinuria at baseline) favoured agalsidase-β in the primary endpoint, with a hazard ratio of 0.39 (95% confidence interval [CI]: 0.16 – 0.93). However, the difference in cardiac events (defined in the trial as myocardial infarction; new symptomatic arrhythmia requiring antiarrhythmic medication, pacemaker, direct current cardioversion or defibrillator implantation; unstable angina accompanied by

electrocardiographic changes resulting in hospitalization; worsening congestive heart failure requiring hospitalization) alone, although with a hazard ratio of 0.42, did not reach statistical significance. Moreover, patients with estimated GFR ≥55ml/min/1.73m^2 tended to have a strong treatment effect (on the primary endpoint), compared to patients with GFR <55ml/min/1.73m^2.[197] Furthermore, an analysis of the Fabry Registry (an international registry of FD patients), including 1044 patients treated with agalsidase-β, showed that the incidence rate of various clinical events was maximal in the first 6 months of ERT, significantly decreasing and remaining stable thereafter.[198] These results contrast with the findings published by the Würzburg Fabry Disease Centre (in Germany), where the event rate, after a follow-up period of at least 5 years, was not different between the group of patients treated with agalsidase-β and the untreated (natural history) group of the Fabry Registry.[199]

Regarding the evaluation of "hard" cardiac outcomes with agalsidase-α, only indirect comparisons have been done between Fabry Outcome Survey treated cohort (677 patients) and historical untreated cohorts. Moreover, only the results concerning the composite endpoints of time to or age at the first clinical event (renal, cardiac or cerebrovascular event or death) have been reported. Nonetheless, treatment appeared to delay the time to as well as the age at the first event.[200]

A direct comparison between ERT preparations in terms of major clinical events is currently underway, as part of the Canadian Fabry Disease Initiative. In the most recent published data from this study, there was no difference between ERT preparations in the percentage of patients meeting the primary composite clinical endpoint (consisting of renal, cardiovascular or cerebrovascular events and death), but a specific analysis of cardiac events was not reported.[201]

However, the effects of ERT on major clinical events, including cardiac events, are still debatable, given that in a comparison between the treated FD patients (including agalsidase-α and -β treated patients) and a natural history cohort (of untreated patients meeting criteria for ERT) in the Dutch FD population, there was no difference in the incidence of the first and second major clinical event. [202] Moreover, major cardiac events continued to occur in treated patients, mainly in patients with advanced disease at ERT beginning, namely patients with LV hypertrophy, severe cardiac fibrosis, impaired kidney function and heavy proteinuria.[187, 194, 196]

In summary, although longer follow-up studies are needed to confirm the published data and assess the effects of ERT on overall prognosis, the previous findings support that early treatment strategy may be more effective in preventing cardiac manifestations and major cardiac events, because patients with myocardial fibrosis or kidney dysfunction may have experienced irreversible changes in cardiac morphology and do not fully respond to ERT.

The pharmacologic chaperone migalastat has been studied in two phase III clinical trials, one in naive patients (comparison with placebo) and another in patients previously treated with ERT (comparison between switch patients and the ones mantained in ERT).[172, 203] Although the primary objective of both trials was renal histology / function, echocardiographic assessment was a secondary endpoint. In the placebo-controlled trial, there was a significant decrease in LV mass index in patients treated with migalastat for up to 24 months, with a trend toward a larger reduction in patients with LV hypetrophy at baseline.[172] In the switch trial, the patients switched from ERT to migalastat presented a significant decrease in LV mass índex, whereas in patients remaining on ERT there was a smaller, non-significant decrease. In the same trial the percentage of patients who experienced renal, cardiac or cerebrovascular events during the 18-month treatment period was non-sigficantly different between switch and ERT groups.[203]

1.4.2 *Nephropathy*

The treatment of FD nephropathy aims to slow CKD progression to a loss of GFR ≤-1.0ml/min/1.73m^2/year. However, in order to achieve such an objective several treatment strategies should be applied, namely: ERT / chaperones, antiproteinuric drugs and control of other potentially treatable factors that may contribute to progressive loss of GFR (HBP, dyslipidaemia and smoking). As for cardiomyopathy, with 15 years of available ERT, we have increasing evidence supporting the efficacy of ERT in several renal endpoints, including histological, intermediate (laboratorial parameters) and "hard" outcomes (renal events and death).

In the pivotal randomized, double-blind, placebo-controlled, phase III clinical trial of agalsidase-α, after 24 weeks of treatment there was a significant increase in the percentage of normal glomeruli and a significant decrease in the fraction of glomeruli with mesangial widening in the active treatment group, compared to the placebo

population. Furthermore, there was a significant decrease in glycolipid inclusion score within the vascular endothelium in the active treatment group, as well as a 21% non-significant decrease in kidney Gb3 content. However, there was no significant change in total score for tubulointerstitial pathology or for the total Fabry inclusion score after 24 weeks of treatment.[165]

In the agalsidase-β drug development, both phase I/II and phase III clinical trials had histological endpoints as the main efficacy measures.[166, 177] In a phase I/II open-label, dose-ranging study, there was mean decrease of 67.6% in kidney Gb3 concentration after 5 infusions, in 5 five patients treated with agalsidase-β in a doses ranging from 3mg/Kg, EOW to 3mg/Kg, every other day. Moreover, Gb3 accumulation in the endothelium of interstitial and glomerular capillaries seemed to decline, as well as in mesangial and cortical interstitial cells, but the Gb3 deposits in glomerular podocytes remained unchanged.[177] These results were confirmed in the randomized, double-blind, placebo-controlled, phase III clinical trial (recruiting 58 patients), with a mean decrease of 23.3% in Gb3 concentration in kidney tissues and a significant decrease in the inclusion score in the kidney microvascular capillary endothelial cells (with 69% of the patients reaching complete clearance) after 20 weeks of active treatment; after six months of an open-label extension study, 98% of the patients had complete clearance form kidney microvascular capillary endothelial cells.[166] These results were maintained at the end of the 54 months open-label, extension study of this clinical trial (in 8 patients consenting to a kidney biopsy), with complete clearance of the endothelial, mesangial and distal convoluted tubule / collecting duct cells. However, in this long-term period, only 70% of the patients reached complete clearance of interstitial cells and none of the patients achieved a zero score in noncapillary smooth muscle cells and podocytes; there was only a one-point reduction in 4 out of 6 patients, in podocyte Gb3 inclusion score.[178]

The long-term histological outcomes have also been evaluated in a small observational study, with 12 young patients (aged between 7 and 33 years old), 11 of whom were treated with ERT for at least 5 years. After 5 years of ERT there was complete clearance of inclusions in glomerular endothelial and mesangial cells in all patients. However, the clearance of inclusions in glomerular podocytes was inconsistent and partial clearance was only observed in patients treated with agalsidase-β at the licensed dose and agalsidase-α at the dose of 0.4mg/Kg, EOW; there was also a significant correlation

between the cumulative dose of ERT (regardless of the ERT preparation) received and the clearance of podocyte Gb3.[79] However, this study was carried out on a small number of patients and the clearance of Gb3 from podocytes might have been affected by heterogeneity between patients in the expression levels of receptors (mannose-6-phosphate receptor, sortilin and megalin) that are responsible for the uptake of recombinant α-galactosidase A into the lysosomes of podocytes;[204] thus, these results needs to be confirmed in a larger cohort of patients. Moreover, electron microscopy was able to detect a significant decrease (average 73%) in podocyte Gb3 content after 12 months of ERT with agalsidase-β in a cohort of 6 patients, which was not detectable by light microscopy inclusion scores.[205]

However, the importance of the finding of persistent podocyte Gb3 inclusions remains controversial, because its role as surrogate biomarker for the progression of renal dysfunction (GFR) in patients treated with ERT is not well established. Notwithstanding the significant correlation between the decrease in urinary albumin to creatinine ratio (ACR) and the decrease of podocyte Gb3 inclusion score in the aforementioned long-term evaluation of histological outcomes, the GFR remained stable in all patients, regardless of the decrease in podocyte Gb3 inclusions.[79] Similarly, in the 54 months extension study of the agalsidase-β phase III clinical trial, despite persistent inclusion in podocytes in the 6 evaluated patients, only in one patient was there progressive decline in GFR.[178] Moreover, as mentioned above, even in untreated patients there is no correlation between semi-quantitative scoring systems for intracellular Gb3 inclusions and GFR[57, 59, 77, 83] and only a small study, using electron microscopy, has shown significant correlations between podocyte Gb3 content and age or proteinuria.[58]

Regarding intermediate endpoints, ERT has been shown to slow or halt the deterioration of renal function and proteinuria in patients with mild to moderate renal impairment.[149, 165, 166, 178, 181, 183, 185, 187, 194, 196, 197, 199, 200, 206-209] The effects of agalsidase-α in renal function (GFR) was originally evaluated in the randomized, double-blind, placebo-controlled phase III clinical trial (including only male patients) and, after 6 months, there was a 3 fold greater (although non-significant) decrease in measured GFR (by inulin clearance) in the placebo group compared with the active treatment group. The change in estimated GFR by creatinine clearance was significantly different between groups, increasing and decreasing in the active treatment and placebo groups, respectively.[165] In

the 48 to 54 months extension, open-label study of this clinical trial, there was only a slight, though significant, decline in estimated GFR, which was mainly driven by the group of four patients with CKD stage ≥3 at baseline, where there was decrease at an average rate of 5.2ml/min/1.73m^2/year; there was no significant change in proteinuria during the follow-up period.[206]

In a pooled analysis of the three 6 months randomized, double-blind, placebo-controlled clinical trials (including the aforementioned clinical trial) with agalsidase-α and their, open-label, extension studies for up to 12 to 48 months, including 108 male FD patients, the mean decline in measured GFR was -2.9ml/min/1.73m^2/year (excluding hyperfiltrators: GFR >135ml/min/1.73m^2), significantly different from no change, but also much lower than the figure for placebo groups (-7.0ml/min/1.73m^2/year). There was no significant change in proteinuria during the follow-up, but the rate of GFR decline was much higher in patients with baseline proteinuria ≥1g/day (-6.4ml/min/1.73m^2), compared with the ones with proteinuria <1g/day (-2.1ml/min/1.73m^2) and also predicted by the baseline GFR.[149]

The effects of agalsidase-α in female patients were evaluated in the study described above with 36 women and 4 years follow-up. The estimated GFR remained unchanged during the entire follow-up, but there was a significant decrease in estimated GFR in the subgroup of patients who were hyperfiltrators (estimated GFR > 135ml/min/1.73m^2) at baseline and a significant increase in estimated GFR in the CKD stage 2 subgroup. In the overall cohort, the observed decrease in proteinuria was non-significant, but in the subgroup of 11 patients with proteinuria in excess of 300mg/day, mean protein excretion declined significantly after 4 years of treatment.[181]

The analysis of data extracted from the Fabry Outcome Survey registry confirmed these results in larger cohorts of patients, in progressively longer follow-up periods, the last one with a mean follow-up of 7.4 years, in a cohort of 208 treated adult patients. In the longer follow-up studies the annualized decline in estimated GFR was between -2.2 and -3.17ml/min/1.73m^2 and between -0.7 and -0.89ml/min/1.73m^2 for male and female patients, respectively and was partially driven by a greater decline in the hyperfiltrators subgroup; there was no significant changes in proteinuria, but the rate of estimated GFR decline was significantly greater in patients with higher baseline proteinuria (>1g/day).[185, 187, 207-209] In a recent report from the Fabry Outcome Survey, comparing treated patients

(mean follow-up 5.2years) with an untreated historical cohort, the rate of decline in estimated GFR was lower compared to untreated patients, regardless of the baseline GFR, proteinuria and gender.[200]

In the longer follow-up study (approximately 10 years) with agalsidase-α, in a cohort of 45 patients, the estimated GFR remained stable over the entire follow-up period, except for a significant improvement in estimated GFR in the first 3 to 5 years of ERT, in both male and female patients, with baseline estimated GFR < 90ml/min/1.73m^2.[183]

Regarding agalsidase-α dosing frequency, in a small cohort of 11 patients showing a continuous decline in estimated GFR ≥5ml/min/1.73m^2/year, switching from EOW to weekly dosing was found to significantly decrease the rate of decline of estimated GFR from -7.92 to 3.84ml/min/1.73m^2/year after up to 10 years of follow-up, with an estimated average delay to ESRD of 13.8 years.[210, 211]

The effects of agalsidase-β in renal function were also evaluated in the randomized, double-blind, placebo-controlled phase III clinical trial, in a cohort of 58 patients. Estimated GFR remained stable in both active treatment and placebo groups after 20 weeks of treatment, as well as in the six month, open-label, extension study.[166] Long-term follow-up of these patients has been reported after 54 months and 10 years of ERT.[178, 194] After 10 years of ERT (available data for 52 of the initially recruited 58 patients), there was a significant decline in estimated GFR, which was greater in patients with high renal involvement (defined as proteinuria >0.5g/day or ≥50% sclerotic glomeruli at baseline) than in the remaining patients: -6.82ml/min/1.73m^2/year and -1.89ml/min/1.73m^2/year, respectively;[194] proteinuria remained stable within the first 54 months of the extension study.[178] In the other randomized, double-blind, placebo-controlled trial with agalsidase-β, after a median follow-up of 18 months, the authors reported that estimated GFR and proteinuria did not change significantly in both active treatment and placebo groups.[197]

Two, open-label studies with agalsidase-β measured GFR by clearance of Technetium-99m. In a cohort of 25 patients, with a mean follow-up period of 23 months, there was a declining trend in GFR, but which was only significant in the subgroup of patients with measured GFR <90ml/min/1.73m^2; proteinuria was significantly higher in this subgroup of patients, but does not change significantly in any subgroup during the

follow-up period.[196] After at least 5 years of follow-up, in a cohort of 40 patients (including the 25 patients of the previous study), the mean decline in GFR was -2.3ml/min/year (-2.4 and -1.9ml/min/year in male and female patients, respectively).[199]

The analysis of a cohort of patients from the Fabry Registry, treated with agalsidase-β for at least 2 years showed that, in multivariate regression analysis, proteinuria ≥1g/g and a longer time from symptom onset and treatment were the most important factors associated with renal disease progression.[212]

The few studies evaluating "hard" outcomes were described in the treatment of FD cardiomyopathy, but often also evaluated renal events, mainly defined as a 33% increase in serum creatinine level or ESRD requiring long-term dialysis or kidney transplantation. In the aforementioned randomized, double-blind, placebo controlled clinical trial with agalsidase-β, with a primary endpoint of a composite of time to first renal, cardiac or cerebrovascular events or death, most events were renal, consisting in all cases of a 33% increase in serum creatinine levels. However, the difference between active treatment and placebo in renal events, although with a hazard ratio of 0.49, did not reach statistical significance. Moreover, proteinuria was significantly associated with any renal event (hazard ratio 1.4 [95% CI: 1.2 to 1.8].[197] None of the other studies reporting clinical events, previously described in the treatment of cardiomyopathy, described the effect of ERT specifically on renal outcomes, in comparison with no treatment.[199-202] However, renal manifestations are significant risk factors for any clinical event, namely: proteinuria, ≥50% sclerotic glomeruli and decreased GFR.[194, 196, 199]

To conclude, because of the heterogeneous disease presentation, no evidence is available to support the optimal timing of ERT. However, it appears that if a threshold of renal disease (CKD stage ≥3, proteinuria >1g/day and ≥50% of glomerular sclerosis) is exceeded, the benefits of ERT may be limited, with a self-propagation of end-stage pathology. Furthermore, these results emphasize the need to move towards combined therapy with ERT and antiproteinuric drugs.

Actually, as described, proteinuria is major risk factor for decline in GFR and major clinical events, but ERT does not seem to reduce proteinuria (mainly in male patients). Thus, antiproteinuric therapies may play a role in stabilization of kidney function. Nonetheless, the evidence supporting the use of angiotensin-converting enzyme inhibitors (ACEi) and/or angiotensin receptor blockers (ARB) in Fabry disease is scant. A

first, open-label study in a small cohort of patients treated with ACEi and/or ARB in conjunction with agalsidase-β, reported promising results with reductions in proteinuria and stabilization of estimated GFR (slope -0.23ml/min/1.73m^2/year) in a subgroup of 6 patients with CKD stage ≥3, within 30 months of follow-up.[213] However, these promising results have not been confirmed in a larger (24 patients, treated with agalsidase-β), multicenter study, with 21 months of follow-up. In this study, 18 out of 24 patients reached proteinuria <0.5g/g, with estimated GFR slopes significantly better than the six who did not achieve this value (-3.6 and -7.0ml/min/1.73m^2/year, respectively). Nevertheless, even in the subgroup of 18 patients with proteinuria <0.5g/g, 12 still progressed with an estimated GFR slope <-2ml/min/1.73m^2/year.[214]

The two phase III clinical trials with migalastat had as primary endpoints: clearance of Gb3 inclusions from the kidney interstitial capillaries and effect on renal function. In the placebo-controlled trial, after 6 months of migalastat, in the subgroup of patients with suitable mutations there was a significantly greater reduction in the mean number of Gb3 inclusion per kidney interstitial capillary than was in placebo group; moreover, there was a qualitative decrease in Gb3 in glomerular podocytes, endothelial cells and mesangial cells in 22%, 26% and 48% of the patients, respectively.[172] These data were further confirmed with a significant decrease in the quantitative analysis of Gb3 inclusions in podocytes after 6 months of migalastat.[215] Measured and estimated GFR remained essentially stable for up to 24 months of treatment.[172, 203]

2. Biomarkers

2.1 Definition and application in Fabry disease

Biological markers or biomarkers are defined, according to the National Institutes of Health Biomarkers Definitions Working Group, as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention".[216] Biomarkers may have greatest value in several clinical applications, namely: as a diagnostic tool, staging or classifying the extent of the disease, disease progression and prognosis, in prediction and monitoring of clinical response to an intervention and early efficacy and safety evaluation in clinical trials.[216] However, rigorous validation of the relationship between a proposed

biomarker and disease activity and outcome is of key importance.

Candidate biomarkers for LSDs are mainly analytes and imaging techniques. Analytes may range from simple metabolites to complex proteins and, for LSDs, can be divided into two categories: molecules that accumulate in tissues and body fluids directly due to enzymatic defect; and molecules produced by the cells in response to lysosomal storage.[217]

One of the most urgent needs in FD is for reliable and validated biomarkers, ideally measured by non-invasive testing. This urgency is mainly related to the characteristics of and pitfalls in FD: diagnosis, determination of phenotype (classical vs attenuated), monitoring and evaluation of treatment response.

The diagnosis of FD is based mainly on the enzymatic activity of α–galactosidase A and GLA gene sequencing, but both methods have limitations that need to be addressed. Due to random X chromosome inactivation, female patients may present significant residual enzymatic activity and about one third may have α–galactosidase A activity within the normal range for general population, thus the diagnosis can only be reliably performed by GLA sequencing.[218] Moreover, in male patients with residual enzymatic activity (> 5% of normal),[219, 220] definitive diagnosis of FD may only be confirmed by GLA gene mutation analysis.[221] However, GLA gene sequencing may not provide a definitive diagnosis for several reasons: most GLA mutations found in FD patients are novel / ”private”, there are various mutations of unknown significance and routine sequencing can only identify a mutation in 97% of male patients with FD.[221, 222] Hence, reliable biomarkers may help diagnosis in these situations.

Furthermore, FD is clinically heterogeneous (in presentation and rate of progression),[10, 15, 82, 83] with genetic factors and gender certainly contributing to this fact.[9, 14] However, the clinical picture may vary widely even in patients within the same family or with the same mutation, thus other genetic, epigenetic and environmental factors also contribute to clinical heterogeneity. Therefore, the identification of prognosis biomarkers and biomarkers with value determining the likely phenotype (classical vs attenuated) is paramount to the identification of patients at increased risk of a progressive disorder.

As previously described, although the optimal timing of ERT beginning is not known, increasing evidence suggests that an early treatment strategy may be more effective to prevent cardiac and renal manifestations and major clinical events. However,

currently the European recommendations for ERT initiation are based on functional or structural manifestations.[223] Nonetheless, as mentioned above, there is a long clinically silent period before overt major organ manifestations, characterized by histological changes (often irreversible lesions) or in the transcriptional profile, whose detection depends on invasive procedures. Consequently, identification of non-invasive biomarkers of pre-clinical involvement of the organs may have a profound impact on the treatment decisions.

Finally, FD is a relatively slowly progressive disorder, with major events occurring mainly in adulthood. This fact constitutes a major difficulty in the design of clinical trials,[165, 166, 176] because long follow-up periods are required to demonstrate the benefits of any therapeutic intervention. Therefore, the identification of surrogate biomarkers of response to treatment, with rigorous correlations with clinical outcomes, is essential in the evaluation of the therapeutic strategies that are available or under development.

2.2 Fabry disease biomarkers used in the clinical practice

There is currently no proper or well established / validated plasma or urinary biomarkers for FD. However, there are several biomarkers (imaging techniques and analytes [metabolites related to lipid abnormalities or proteins]) that are used in clinical practice with some limitations (a summary is shown in table 2). These biomarkers may reflect the total body burden of storage cells or manifestations in a particular organ.

2.2.1 *Total disease burden*

FD is characterized by disruption in glycosphingolipids metabolism, so lipid abnormalities have been studied as potential biomarkers. For a long time, the primary accumulating substrate Gb3 has been considered a surrogate marker for FD and its reduction in the endothelium has served as an indicator for the development and registration of ERT with agalsidase-β.[166, 177] Actually, Gb3 is not only found inside the cells, but it has been recognized for a long time that its concentration is increased in plasma and urine in FD male patients.[224] Plasma and urine Gb3 values are more strikingly elevated in FD males and plasma Gb3 is elevated in only a small percentage of female patients (15-30%), and urinary Gb3 may even be within the normal range in 8-13% and 12-20% of male and female patients, respectively.[225, 226] There is a correlation between

types of mutations and urinary Gb3 excretion, with patients with missense mutations, mainly those associated with late-onset phenotypes, presenting lower values, most of which within the normal range; in patients with mutations associated with an attenuated phenotype, plasma Gb3 is usually within the normal range, even in male patients.[226-228] Thus, the added value of Gb3 for diagnostic purposes is at least questionable.

Table 2: Fabry disease biomarkers used in clinical practice. ERT: enzyme replacement therapy; Gb3: globotriaosylceramide; eGFR: estimated glomerular filtration rate; PCR: protein to creatinine ratio; ACR: albumin to creatinine ratio; LV: left ventricular; MSSI: Mainz Severity Score Index; WML: white matter lesions; HR: hazard ratio; HCM: hypertrophic cardiomyopathy due sarcomere protein gene mutations; LGE: late gadolinium-contrast enhancement; MRI: magnetic resonance imaging; NT-proBNP: N-terminal fragment of the pro-hormone of brain natriuretic peptide; TDI: tissue Doppler imaging; LA: left atrial; FD: Fabry disease; CKD: chronic kidney disease.

Biomarker	Diagnosis	Clinical correlations	ERT monitoring
Gb3 (plasma / urine)	Not useful	Poor: • Plasma Gb3 with cerebral complications (♂) • Urinary Gb3 with eGFR, PCR and ACR	Decrease during ERT: • Correlation with clinical endpoints not established
Lyso-Gb3 (plasma)	Added value (cautious interpretations of results)	• ♀: LV mass and MSSI • ♂: WML	Decrease during ERT: • ♀: correlation with ↓ LV mass and HR to WML
Cardiac troponin	Differentiate HCM versus infiltrative cardiomyopathy	• LV wall thickness • LGE volume in cardiac MRI	Not useful: • High-sensitivity troponin T increases during ERT
NT-proBNP	Not evaluated	Diastolic dysfunction parameters and LV mass	Not evaluated
Echocardiogram (TDI)	Superior to conventional echocardiography in the detection of early cardiac involvement	LV wall thickness	ERT successfully prevented appearance of abnormal TDI velocities
Echocardiogram (speckle-tracking)	Better sensitivity than TDI in detecting early diastolic dysfunction	• LGE in cardiac MRI • Functional status	ERT improves: • Systolic strain and strain rate (?) • LA peak positive strain
Cardiac MRI (LGE)	Characteristic pattern of LGE distribution (distinguish from HCM)	LGE amount correlates with: • LV mass • Regional myocardial function • Risk of ventricular arrhythmias	Not useful: • LGE amount increase during ERT • Major predictor of response to ERT
Cystatin C	Better diagnostic accuracy than creatinine in detecting early renal involvement	Better correlation of cystatin C and creatinine-based equations with measured GFR (?)	More sensitive than creatinine in detecting minor decline in GFR during ERT (?)
Proteinuria Albuminuria	Earlier biomarker of FD nephropathy (limitations)	Good correlation with GFR decline (there are several patients with CKD stage ≥3 without overt proteinuria)	Does not respond to ERT

The correlation with clinical manifestations and its added value to monitor progression of FD is also poor. In a Dutch cohort of 63 male and female FD patients, there

was no correlation between plasma or urinary Gb3 with age, most of clinical parameters and total disease severity (measured by Mainz Severity Score Index [MSSI]);[229] however, plasma Gb3 was significantly higher in male patients with cerebral complications (compared with those without) and urinary Gb3 significantly correlates with estimated GFR, urine protein to creatinine ratio and ACR (in a cohort with male and female patients).[225, 230] Finally, there was significant decrease in plasma and/or urine Gb3 in patients treated under the clinical trials of both ERT preparations;[165, 166, 178, 206] however, the correlation between this decrease and the therapeutic outcome in terms of "intermediate" or "hard" endpoints is not established.[226, 228, 231] Moreover, in one study the occurrence of antibodies anti ERT was accompanied by a blunted decrease in urinary Gb3, but the clinical significance of this remains unclear.[232] The limitations of Gb3 in terms of predictive value for FD manifestations are not surprising, given that prominent Gb3 has been noted in placental tissues of FD male patients,[233, 234] with the onset of clinical complications occurring only several years later, thus, as mentioned above, other factors in addition to Gb3 may participate in pathogenesis.

Given the limitations of Gb3 as biomarker, research on Gb3 metabolites identified a product of Gb3 deacetylation, globotriaosylsphingosine (lyso-Gb3), which is hydrophilic and highly diffusible and whose plasma levels in FD patients are markedly increased (exceeding those of Gb3 by more than one order of magnitude and most prominent in male patients).[17] In this first study all male and female patients presented very high levels of plasma lyso-Gb3, except for a young (6 year-old) and asymptomatic female patient.[17] These results were confirmed in larger cohorts of patients (with up to 92 patients), showing that even male patients with attenuated phenotypes presented increased lyso-Gb3 levels (though in lower magnitude than patients with classical phenotype).[228, 235, 236] However, the discriminative power in male and female patients with mutations of unknown significance, namely p.R112H and p.P60L, seems limited, as lyso-Gb3 is not increased in this group of patients (even in patients with histological demonstration of Gb3 storage in podocytes).[235, 237] Nonetheless, recently nano-liquid chromatography-tandem mass spectrometry (a technique enabling the detection of extremely low concentrations of lyso-Gb3, with greater sensitivity than conventional techniques), in patients with mutations of unknown significance (p.R112H and p.M296I), demonstrated that lyso-Gb3 was lower than in classical and attenuated phenotypes FD patients having

other mutations, but higher than in those with functional variants (p.E66Q) and healthy subjects.[238] Urinary lyso-Gb3 performance as a diagnostic tool seems similar to that of plasma lyso-Gb3, with only a minority of patients excreting undetectable amounts and with patients presenting missense mutations having significantly lower excretion of lyso-Gb3.[230] Thus, lyso-Gb3 seems to be a promising diagnostic biomarker, with added value for diagnosis in specific situations, but evaluation of pathogenicity of mutations based solely in this parameter should be cautious.

Various correlations have been found between plasma lyso-Gb3 and clinical manifestations. In male patients, lyso-Gb3 does not increase with age (with markedly increased values even in neonates) and consequently does not correlate strictly with disease severity indexes or clinical manifestations, except for a significant correlation with the presence of white matter lesions.[17, 235] In contrast, in females lyso-Gb3 tends to increase progressively with age and significant correlations have been found between lyso-Gb3 and MSSI, LV mass and carotid intima media thickness.[17, 235, 239] There is no established correlations between plasma lyso-Gb3 and kidney function parameters, but a significant correlation between urinary lyso-Gb3 and urine protein to creatinine ratio or ACR has been observed; however, this correlation was performed in a cohort of male and female patients and no adjustment for gender was reported.[230] As lyso-Gb3 seems to be a risk factor directly implied in FD pathogenesis, lifetime exposure to plasma lyso-Gb3 (estimated product of lyso-Gb3 concentration by age) should correlate better with disease (mainly in male patients, showing very high values from birth) than the current lyso-Gb3 level. Therefore, both in male and female patients plasma lyso-Gb3 exposure is significantly correlated with disease severity and with the cold detection threshold and thermal sensory limen of the upper limb (both signs of characteristic FD small fibre neuropathy).[235, 240]

Lyso-Gb3 decreases significantly with ERT, but does not reach normal values, even in female patients, independently of ERT preparation.[17, 228, 236, 241] Moreover, in patients developing anti-ERT neutralizing antibodies, reduction of plasma lyso-Gb3 is relatively poor, mainly in patients treated with agalsidase-α (with a significant difference between patients with and without anti-drug antibodies).[17, 236, 241] The reduction in plasma lyso-Gb3 during ERT was found to correlate with correction of LV mass in female patients and with a lower hazard ratio for developing new cerebral white matter lesions in male and

female patients.[241] The potentially prognostic value of plasma lyso-Gb3 reduction during ERT for clinical outcome clearly needs further investigation in larger cohorts of FD patients.

Finally, as previously described, lyso-Gb3 may play a direct role in disease pathogenesis: it has the capacity to inhibit α-galactosidase A activity in "wild type" cells, contributing to the "cross-induction" of the defect in female patients;[17] "in vitro", it induces smooth muscle cell proliferation (directly contributing to FD vasculopathy);[17, 242] it induces expression and production of extracellular matrix proteins by podocytes, via the TGF-β1 and Notch1 pathways.[18, 70]

Research of potential protein biomarkers for FD showed a lack of prominent plasma protein abnormalities. However, several biomarkers mainly related to inflammation and endothelial dysfunction have been studied, but not widely validated and, consequently, are not currently used in clinical practice. A brief description of these protein biomarkers will be given in the section "Experimental biomarkers in Fabry disease".

2.2.2 *Fabry disease cardiomyopathy*

Currently, imaging techniques are the most commonly used biomarkers of cardiac injury in FD, but some biochemical biomarkers are also used in clinical practice.

Cardiac troponins are well-validated biomarkers of cardiomyocyte injury. New generation / high-sensitivity assays for cardiac troponin T enable the identification of minimal cardiac injury and have been associated with poor outcomes in cardiomyopathies other than ischemic heart disease.[243, 244] Two studies, evaluating the performance of troponin I as biomarker of FD cardiomyopathy have shown that this analyte was elevated (≥0.04ng/mL) in 21 – 37% of the patients, with a very high diagnostic accuracy for LV hypertrophy or LGE in cardiac MRI.[245, 246] High-sensitivity troponin T was evaluated in a large cohort of 75 patients and found to be elevated (>14ng/L) in 40% of the cohort, 97% of whom had evidence of LGE in cardiac MRI (LGE-positive myocardial volume was the only factor independently associated with troponin elevation in multivariate logistic regression analysis). In the same study, the correlation between troponin and IVS wall thickness was also significant and in a retrospective analysis, over a follow-up period of 3.9 years, patients with elevated troponin at baseline

had significantly increasing replacement fibrosis.[247] Moreover, high-sensitivity troponin T may help to differentiate between patients with HCM and infiltrative cardiomyopathy (amyloidosis and FD), which share phenotypic features, being significantly more elevated in patients with infiltrative aetiologies.[248]

Brain natriuretic peptide and the N-terminal fragment of its pro-hormone (NT-proBNP) have an established role in determining the diagnosis and prognosis of heart failure.[249, 250] NT-proBNP was evaluated as a biomarker of early cardiac involvement in FD in two relatively large cohorts of 89 and 117 FD patients. In both studies a significant correlation was found between NT-proBNP and parameters of diastolic dysfunction and LV wall thickness; moreover, it has good diagnostic accuracy in predicting abnormal echocardiographic findings and may be a sensitive marker in detecting early changes in cardiac involvement, such as diastolic dysfunction.[251, 252]

Nonetheless, cardiac imaging techniques are the mainstay in the assessment of FD cardiomyopathy. Conventional two-dimensional and Doppler echocardiography is the standard imaging tool in the identification and staging of cardiac involvement in FD disease, but it is not suitable to detect subtle myocardial dysfunction in the early course of FD cardiomyopathy.[138, 139] Therefore, advanced echocardiography techniques, like TDI and strain and SR speckle-tracking based echocardiography have been used in early cardiac involvement that precedes overt LV hypertrophy and appearance of replacement myocardial fibrosis.

There are several studies, such as in HCM and other inherited cardiomyopathy,[253] demonstrating the added value of TDI in the early detection of FD cardiomyopathy.[102, 104, 195, 254-257] In the first study on the evaluation of TDI in FD cardiomyopathy (10 FD patients with LV hypertrophy, 10 FD patients without LV hypertrophy and 10 healthy controls), it was reported that echocardiography did not show any difference between FD patients without LV hypertrophy and controls in conventional parameter of diastolic dysfunction, but there was a significant decrease in myocardial systolic and diastolic velocities (evaluated by TDI), even in FD patients without LV hypertrophy (although of lesser magnitude than in patients with overt LV hypertrophy). Furthermore, in the histological analysis, even in the FD group without LV hypertrophy, in some patients' interstitium was slightly widened due to intercellular fibrosis and there were also tiny areas of fibrous replacement.[104] These results were further confirmed in several small cohorts of patients

(with up to 19 FD patients).[102, 254, 255] Abnormalities of systolic velocities in the RV, measured by TDI have also been recently described.[258]

The sensitivity of various parameters to detect early diastolic dysfunction have been compared in two studies: a study with 81 echocardiographic examinations showed that early diastolic mitral annular velocity (E') measured by TDI has significantly better diagnostic accuracy to detect diastolic dysfunction (defined by the LV filling patterns obtained using standard Doppler indexes) than colour M-mode flow propagation velocity.[256] Another study including 59 FD patients (none of them under ERT) not only confirmed the results of impaired TDI parameters even in FD patients without LV hypertrophy (including myocardial systolic and diastolic velocities, but also a significant increase in isovolumic relaxation time [IVRT] and a significant decrease in isovolumic contraction time), but also demonstrated a significant inverse correlation between systolic mitral annular velocity (S') or E' and IVS or LVPW thickness and a significant direct correlation between IVRT and LV wall thickness. Moreover, comparison, between several TDI parameters, of the diagnostic accuracy to detect early preclinical cardiac involvement (defined as absent cardiac symptomatology and semiology and normal echocardiogram and ECG) was maximal for isovolumic contraction time (area under the curve [AUC] 0.97), but also significant for IVRT and E'.[257]

The effects of ERT in the prevention of early cardiac involvement (defined by the presence of TDI abnormalities) was evaluated in a study with 66 FD patients, 33 of whom were treated with agalsidase-α over a follow-up period of around 3 years. Agalsidase-α successfully prevented the appearance of abnormal TDI velocities in the group of 29 patients with normal echocardiograms at baseline (80% of the patients not under ERT progressed to abnormal TDI velocities during follow-up, whereas this progression only occurred in 33% of patients under ERT).[195] The appearance of TDI abnormalities before overt LV hypertrophy is not surprising: bioptic studies have shown ultrastructural evidence of myofibrillolysis correlated with a far lower active tension coupled with higher resting tension of cardiomyocytes in FD.[29] Furthermore, increased myocardial stiffness, due to mild interstitial fibrosis and tiny areas of replacement fibrosis has even been found in FD patients without LV hypertrophy, but has a negative effect on contraction and relaxation velocities.[104] Concluding, reduction in myocardial contraction and relaxation velocities in TDI are detectable before the development of LV hypertrophy or even

abnormalities in the traditional parameters of diastolic function and can enable the recognition of preclinical cardiac damage.

New tools like strain and SR speckle-tracking based echocardiography (a TDI-derived technique), which enables measurement of myocardial systolic and diastolic strains, seems superior and more sensitive than myocardial velocities (measured by TDI) in quantifying changes of myocardial systolic function, because it is less influenced by overall cardiac motion.[259] Decreased systolic strain and SR of the LV has been reported in FD patients, compared with healthy controls.[107, 108, 260, 261] These alterations in myocardial systolic strains are detectable even in the early stages of FD cardiomyopathy: in a study comparing 19 FD patients with LV hypertrophy, 21 FD patients without LV hypertrophy and 40 healthy controls, there was a significant reduction in both global systolic longitudinal and circumferential strains, as well as an absence of the normal regional base-to-apex circumferential strain gradient, even in the subgroup of patients without LV hypertrophy.[261] These results were further confirmed in another study comparing 22 FD with LV hypertrophy, 22 patients without LV hypertrophy and 22 controls.[107]

Decreased LV diastolic strain and SR was also reported in FD.[108, 260] In a cohort of 16 patients (9 of whom with LV hypertrophy), a significant decrease in longitudinal early diastolic SR and in SR during isovolumic relaxation period was reported, even in the subgroup of patients without LV hypertrophy (for SR during isovolumic relaxation period). Moreover, the sensitivity to detect diastolic dysfunction was higher with speckle-tracking imaging than with TDI early diastolic velocities, and the diastolic parameter considered the best predictor for FD was SR during isovolumic relaxation period (AUC 0.953, compared with 0.753 for E').[108] Left atrial (LA) systolic and diastolic strain and SR is also decreased in FD patients, with a significant decrease of systolic SR even in patients without LV hypertrophy.[262, 263]

Speckle-tracking based echocardiography may also be a non-invasive tool for detection of myocardial fibrosis in FD.[114, 190] In a study with 101 patients, global longitudinal systolic strain was lower in patients with LGE in cardiac MRI, compared with those without LGE, with a significant correlation between global longitudinal systolic strain and the amount of LGE; furthermore, segmental strain values were particularly decreased in the LGE positive basal posterior and lateral segments and the strain in these wall segments shows the best diagnostic accuracy to detect myocardial LGE (AUC

0.913).[114] Moreover, LV, RV and LA strains are inversely linked to the heart failure functional class[260] and LA strain parameters are associated with atrial fibrillation and stroke.[264]

The effect of ERT in myocardial systolic and diastolic strains is still controversial: in a study with 16 patients treated with agalsidase-β, there was a significant increase in both longitudinal and radial peak systolic SR and systolic strain after 12 months of treatment;[103] while in another study, for a cohort of 13 patients, treated with ERT for a mean period of 3.1 years, there was no change in global systolic longitudinal or circumferential strain.[261] The improvement in systolic SR may be influenced by the presence and amount of myocardial fibrosis at ERT initiation, with a study showing a significant increase in radial systolic SR occurring only in the subgroup of patients without fibrosis; systolic SR remained essentially unchanged in patients with mild fibrosis, but rather decreased in the severe fibrosis subgroup.[106] ERT also seems to improve LA function, by an increase in LA peak positive strain.[264]

Cardiac MRI plays a critical role in evaluating the differential diagnosis of cardiomyopathy and is the diagnostic standard for assessing cardiac morphology and function, with high spatial resolution and low observer variability, in patients with FD.[265] Cardiac MRI, as noted above in cardiomyopathy treatment, can reliably evaluate the beneficial effects of ERT on LV mass in FD patients.[103, 176, 188, 190, 191, 266] Furthermore, it is essential for the comprehensive segmental analysis of LV regional morphology and function in FD. In patients with LGE, the regional function is severely restricted, mainly in the segments displaying LGE, but also in the non-enhanced segments.[190] Likewise, segmental LV wall thickness is higher in patients with LGE and significantly correlates with the segmental volume of the LGE, and wall motion in patients with LGE is significantly impaired in some segments, compared with patients without LGE and the distribution of hypokinetic segments followed that of hypertrophic segments.[267] ERT, in a study with agalsidase-β administered for 12 months, seems to improve segmental LV wall thickness and wall motion (with a significant decrease in hypokinetic segments), but only in patients without LGE at ERT initiation.[267]

LGE imaging techniques using cardiac MRI is the gold standard for non-invasive detection of focal replacement fibrosis in the myocardium. The intercellular space is increased in the areas with replacement myocardial fibrosis and chelated gadolinium

diffuses into this space and is unable to cross the cell membrane; therefore, the distribution kinetic is slower and a higher relative concentration of gadolinium is found in myocardial areas with replacement fibrosis compared to unaffected myocardium.[110]

As noted above, between 31% and 77% of patients with FD disease may present LGE, with a characteristic mid-myocardial distribution (sparing the subendocardium) in the inferolateral basal and mid basal segments of the LV wall, that seems to be specific of FD cardiomyopathy (differentiating it from other causes of hypertrophic cardiomyopathy).[105, 106, 110-112, 190, 268] Additionally, in FD LGE was correlated with histologic findings (myocardial collagen deposition) from autopsy.[109] A greater proportion of male patients presents larger amounts of LGE in cardiac MRI;[110, 112] it was reported previously that only female patients might present LGE with normal LV mass (in one study, 10 out of the 19 female patients with LGE did not present LV hypertrophy),[113] but recent reports have demonstrated that a significant proportion of male patients without LV hypertrophy may also present LGE.[112, 269]

Several studies reported a significant correlation between the amount of LGE and LV mass[110, 112, 190, 267, 270] and, as mentioned above, LGE correlates with abnormalities in regional myocardial function assessed by speckle-tracking imaging studies.[105, 113-115, 190] However, the sensitivity of LGE to detect early cardiac involvement, as expected due to the identification of irreversible replacement fibrosis, is limited. In a study with 20 FD patients, none of the patients without LV hypertrophy, but with abnormal TDI velocities, presented LGE in cardiac MRI.[271] Moreover, in a study with 73 patients, followed for a mean period of 4.8 years, logistic regression analysis revealed that the annual increase in LGE during the follow-up was the only independent predictor of malignant ventricular arrhythmias (sudden cardiac deaths only occurred in the group of patients with LGE).[116]

ERT does not seem to have any effect on LGE: the amount of LGE significantly increased in patients treated for 12 months with agalsidase-β (no patients without LGE at baseline developed LGE during ERT) and LV mass only significantly decreased in patients without LGE at baseline.[190] These results were further confirmed with longer treatment periods, also showing some patients developing LGE even during treatment with ERT.[116] Moreover, as previously mentioned, the presence of LGE is one of the most important predictors of response to ERT in terms of myocardial function and exercise capacity.[106]

Nevertheless, LGE has several limitations as an imaging biomarker: it only detects

irreversible tissue damage with focal replacement fibrosis (with limited resolution: theoretical limit of about 0.2g, probably higher in the clinical setting of infarction studies);[272, 273] it may not detect early, potentially reversible, diffuse fibrosis;[274] although possible, there is no universally accepted technique to quantify fibrosis volume and the intra- and inter-observer variability is in the range of 20-40% of the LGE amount.[275, 276] These limitations may be paramount in FD: in a study with 20 FD patients, 10 with and 10 without LV hypertrophy, the interstitium was mildly widened in some patients without LV hypertrophy due to intercellular fibrosis and tiny areas of fibrous replacement were also detected in this subgroup of patients; in patients with LV hypertrophy the interstitium was moderately to severely increased because of scar tissue; however, none of these patients presented LGE in cardiac MRI, depicting the limited resolution and inability to detect diffuse interstitial fibrosis of LGE based techniques.[104]

A novel technique, T1 mapping, enables the measurement of native myocardial T1 (non-contrast myocardial T1) and T1 after administration of gadolinium contrast. Native T1 allows for a better characterization of the myocardium content, with increased values in the setting of fibrosis, oedema or amyloid deposits and decreased values in iron overload or lipid storage. Measurement of T1 with extracellular gadolinium based contrast agents gives additional information about the extracellular volume fraction, which is particularly valuable for diffuse diseases that are usually more difficult to detect using conventional LGE and has been studied for assessment of diffuse fibrosis, with good histological correlations.[274] However, in FD it has been more extensively studied as an imaging biomarker for early detection of cardiac involvement and for distinguishing FD from other aetiologies of concentric remodelling and hypertrophy (due to lipid storage, yielding low native T1 [correlative biopsy data is not yet available]), than for evaluation of diffuse fibrosis.[277-281]

In a cohort of 63 FD patients, low septal native T1 was evidenced in around 90% of the patients with LV hypertrophy and in 48% of the patients without LV hypertrophy (this subgroup had lower global longitudinal strain by speckle tracking and higher LV filling pressure). This data suggests that low T1 is a possible marker of cardiac involvement in early stages of hypertrophy and fibrosis and 4 phases of myocardial involvement were proposed: phase 1: no involvement; phase 2: low T1 and early myocardial dysfunction; phase 3: LV hypertrophy with low T1; phase 4: “pseudonormalization” of T1, fibrosis and

heart failure (as extensive fibrosis and scarring present high T1 values).[278]

2.2.3 *Fabry disease nephropathy*

Reproducible and accurate estimates of renal function are essential in the management of FD. There are limitations in all current equations to estimate GFR, but MDRD equation[282] is not validated in patients with higher GFR (knowing that an hyperfiltration stage have been described in FD nephropathy) and seems less accurate than Chronic Kidney Disease Epidemiology Collaboration (CKD-$EPI_{creatinine2009}$) equation.[148] Thus, CKD-$EPI_{creatinine2009}$ equation is the recommended equation to estimate GFR in adults with FD.[283, 284] However, given that measured GFR by iohexol plasma clearance and isotopic methods is more accurate, depending on local availability, the precise measurement of the GFR is recommended for FD patients if the estimated GFR is >60ml/min/1.73m^2.[284]

Cystatin C is a protease inhibitor, produced at a constant rate by all nucleated cells, freely filtered through the glomeruli and completely reabsorbed and broken down by the proximal tubules; therefore, its serum concentration is constant and it was proposed as a reliable marker of renal function.[285] Cystatin C has been compared with creatinine in the evaluation of renal function, in a cohort of 21 patients starting treatment with agalsidase-α, with a follow-up period of up to 4 years. During follow-up under ERT there was no significant change in creatinine or in creatinine-based estimated GFR, but there was a significant increase (just after only one year of ERT) in cystatin C and a concurrent decrease in estimated GFR by Hoek equation[286] (using cystatin C values). The authors concluded that cystatin C was an early marker for the decline of GFR, but no gold standard precise measurement of GFR was used to support this conclusion.[287] This data was corroborated by another study including 89 patients, showing that the diagnostic accuracy of cystatin C to detect mild renal damage and minor decreases in GFR was superior to diagnosis based on creatinine.[252]

Cystatin C-based estimation of GFR was compared with measured GFR in a relatively small validation study (including several cystatin and creatinine-based equations), including 136 GFR measurements in a population of 36 FD patients. In contrast to that previously mentioned, Hoek equation was less accurate than CKD-$EPI_{creatinine2009}$ equation in detecting GFR decline during ERT, but Stevens' equation[288] (a

creatinine and cystatin C-based formula) was the one that most closely approximated to the measured GFR.[289] However, Stevens' equation development was based on serum cystatin C assays in adults that were not traceable to standard reference material, and are no longer recommended by international CKD guidelines.[283]

Total urinary protein and albumin excretion can be considered as important biomarkers in FD nephropathy. As previously mentioned, proteinuria (>150mg/day) and albuminuria A2 are usually the first signs of renal involvement.[143-146] The sensitivity of albuminuria seems superior to that of total urinary protein excretion in detecting early renal involvement in FD.[141] Thus, albuminuria remains as the best existing marker to detect early renal involvement, but the relevance of albuminuria A2 as a biomarker is largely based on validation studies in the earlier stages of other nephropathy models (diabetic and, to a lesser extent, hypertensive renal disease). Moreover, in FD nephropathy albuminuria may not result only from glomerular damage of the filtration barrier, but also from tubular involvement with decreased reabsorption of filtered albumin.

However, the usefulness of albuminuria and/or proteinuria to identify incipient FD nephropathy is questionable. Though the significant correlation between urinary protein excretion rates and foot process width and fractional volume of Gb3 inclusions in the podocytes,[58] FD nephropathy, as noted above, is clinically silent for a long period and significant histological changes (not only the characteristic inclusions, but also nonspecific degenerative lesions, like mesangial widening, glomerulosclerosis, tubulointerstitial fibrosis and arteriopathy) may occur without pathological albuminuria and/or proteinuria.[57, 58, 77, 79-81] In incipient stages of FD nephropathy, tubular reabsorption of albumin may overcome its increased excretion, limiting the sensitivity of this biomarker.

Furthermore, proteinuria seems also to play an important role in FD nephropathy progression, even in patients treated with ERT, because it is an independent risk factor affecting the extent of renal decline and is one of the determinants of the success of ERT.[82, 141, 149, 152, 194, 196, 197, 207, 212] However, the magnitude of the influence of proteinuria in FD progression is not well established, as some studies find no relationship between the degree of proteinuria and the rate of GFR decline, and several FD patients with CKD stage ≥3 may not present overt proteinuria.[15, 83, 141] Finally, as already mentioned, ERT does not seem to reduce proteinuria (mainly in male patients), so this biomarker is not a

good biomarker of response to ERT; actually, reduction in proteinuria is the objective and guides the use of ACEi and ARB, indicating the effectiveness of these drugs.

2.3 Searching for new biomarkers

Searching for new biomarkers in Fabry disease is paramount: as an illustration, no proper or well-established plasma or urinary biomarkers are available in clinical practice to aid the diagnosis, early detection of major organ involvement, monitoring and evaluation of treatment response. Moreover, the sensitivity of imaging techniques for cardiac evaluation seems limited in the detection of early events in FD cardiomyopathy. However, biomarkers discovery remains a very challenging task due to the complexity of the samples (body fluids or tissues) and the wide dynamic range of molecule concentrations in a heterogeneous disease.

There are two main approaches to discover new plasma / urine biomarkers in FD: "angling", a one by one approach, which involves the study of candidate biomarkers (for example, tubular proteins or inflammatory mediators) where laboratory studies have suggested a pathological link or with proven value in a similar pathological model; "trawling", based on "omics" medicine, where biological fluid / tissue is screened for disease-associated molecules (for example, proteins or metabolites) using an array of technologies, predominantly based on mass spectrometry.

One by one approach has been widely used and several biomarkers with clear pathological correlation have been found; moreover, the interpretation of the data and correlation with the clinical variables / disease heterogeneity is easier with this approach. However, most of the serum and urine biomarker studies performed to date seem to have converged on a set of proteins and metabolites that are repeatedly identified in many studies and that represent only a small fraction of the entire proteome / metabolome, so the added value of the one by one approach in deepening knowledge about the disease pathophysiology is limited.[290]

In contrast, omics-based applications make available a formidable technological resource to further expand our knowledge on the complexities of human disease. However, if reliable and useful inferences with potential for translation into clinical practice are to be achieved, omics techniques require: understanding inherent biological variables, rigorous methodology and analytical chemistry tools, the use of

instrumentation that ensures high data quality, and consistent and transparent analysis of the generated data.[291] In proteomics and metabolomics there are several challenges and limitations that need to be overcome so as not lag behind and achieve clinical practice: complexity of proteome (large number of structural and biochemical differences of proteins), very wide range in protein concentration, complex sample preparation and data analysis (most reported biomarkers remain unidentified) and limited sensitivity. Moreover, capacity for quantitative measurements is not yet at a level required by routine diagnostics in a clinical setting. It is also unclear how clinicians will use this sensitive data since even small changes in physiology, such as food ingestion or going up a flight of stairs, can have significant impact on the metabolome.[292]

2.3.1 *Experimental biomarkers in Fabry disease*

Several protein and lipid experimental biomarkers are under investigation in Fabry disease, mainly related to inflammation, endothelial dysfunction, cardiac fibrosis, glomerulosclerosis and tubulointerstitial fibrosis. Furthermore, there are also few reports on proteomic and metabolomics analysis.

As mentioned above, FD is characterized by vasculopathy related to endothelial dysfunction and leucocyte activation, leading to a low grade inflammatory disorder.[293] Thus, few inflammatory biomarkers have been studied in FD patients: the classic marker of an inflammatory response, C-reactive protein, generated conflicting results in three large cohorts of FD patients, with two studies showing no increase and the other showing a marked increase, but not correlated with disease severity and not responsive to ERT.[294-296] Nonetheless, pro-inflammatory cytokines, including interleukin 6 (IL-6), are elevated in patients with FD cardiomyopathy and decrease during ERT (IL-6 decrease significantly correlated with the decrease in lyso-Gb3 and the improvement in LV mass);[297] Another study evaluating an IL-6 promoter polymorphism (c.-174G>C), with IL-6 C/C genotype associated with susceptibility of cerebral arteries to IL-6-mediated inflammatory damage, revealed that FD patients with CC genotype was associated, in both male and female patients, with a significant increase in neurological subscore and total MSSI and with all the 6 (out of 56) patients who had suffered a strike during follow-up with this same genotype.[294] Two other inflammatory markers, myeloperoxidase and chitotriosidase, are also significantly increased in FD males (but not in females): the former seems to predict

the risk of vasculopathy–related events, but does not decrease during ERT;[295] the second does not correlate with clinical manifestations, but significantly decreases during ERT (with an hampered response in patients developing neutralizing antibodies).[298]

In the same model of a systemic vasculopathy and prothrombotic state,[293, 299] several studies focused on the identification of biomarkers of coagulation and endothelial activation. Laboratory investigations in this field gave conflicting and disappointing results: first studies, in a cohort of 25 FD patients, showed a significant increase in soluble adhesion molecules (soluble intercellular adhesion molecule-1, soluble vascular cell adhesion molecule-1 (sVCAM-1) and soluble P-selectin), a significant decrease of the coagulation factor thrombomodulin (TM, an anti-thrombotic factor) and an increased expression of the integrin Macrophage-1 antigen (a cell surface adhesion molecule), consistent with a prothrombotic state.[293] However, in subsequent studies only a significant increase in sVCAM-1 was confirmed in one investigation and other studies reported only minimal and inconsistent abnormalities in markers of platelet and coagulation activation.[296, 300, 301] Endothelial-derived NO, produced by endothelium-derived NO synthase, is also a key regulator of endothelium and vessel wall function. Polymorphisms of the gene encoding the enzyme endothelium-derived NO synthase, associated with a higher risk for cardiovascular diseases in general population, influenced the LVPW thickness of the heart in one study with FD patients.[302] An increase in homocystein level was found in 2 separate studies (with 9 and 12 FD patients) and one of them reported that all the patients presenting cerebrovascular disease had hyperhomocysteinemia.[300, 301]

Finally, regarding the biomarkers related with vasculopathy, one study identified another soluble factor, apart from lyso-Gb3, promoting vascular smooth muscle cell proliferation, sphingosine-1 phosphate; its levels are significantly increased in male patients with FD (but not in females) and correlate with carotid artery intima-media thickness and LV mass index.[303]

Given the aforementioned limitations of albuminuria / proteinuria as biomarkers of renal involvement in FD, other biomarkers of glomerular and tubular injury have been studied. As previously described, the massive Gb3 storage in the podocytes translates into early podocytopathy, with increased podocyturia (podocyte count in urine sediments is a time-consuming procedure and it may be technically challenging to obtain reliable

data because the reading is observer-dependent). A few studies evaluated podocyturia in FD, showing that it is already increased in patients without albuminuria and overt nephropathy, but a significant correlation with albuminuria / proteinuria was inconsistent between the studies and an inverse correlation with estimated GFR was only found in one of them (and only in male patients), and the added value against albuminuria, in terms of diagnostic accuracy, nephropathy prognosis and response to ERT has not been established.[66, 68, 304, 305] Some tubular injury biomarkers have been also studied: a decreased urinary excretion of uromodulin has been reported and immunohistochemical analysis revealed aberrantly processed uromodulin, abnormal uromodulin localization and expression inversely proportional to the degree of storage (these abnormal patterns normalized under ERT in all patients).[306] In a cohort of 13 female patients under ERT for more than one year, a decrease in proximal tubular damage markers α1-microglobulin and retinol-binding protein excretion was found.[307] A significant increase in urinary excretion of bikunin, a serine protease inhibitor, has been reported in FD patients with renal impairment (but not in those without overt nephropathy); however, bikunin origin and the mechanisms by which its urine levels are elevated remains unclear and deserve further evaluation.[308]

Next to targeted analysis of potential lipids and proteins candidates, the search for biomarkers of FD has been extended to analysis of the entire metabolome and proteome. A metabolomics approach has been employed using tandem mass spectrometry and has identified several metabolites over-expressed in FD patients, all of them Gb3, lyso-Gb3 and galabiosylceramide isoforms / analogues. In plasma it was possible to identify analogues / isoforms of Gb3 and lyso-Gb3 (the magnitude of increase was lower for all of them, compared with lyso-Gb3), all of them significantly more elevated in male patients (compared with female patients) and most of them showing a significant decrease during ERT.[309, 310] Urinary metabolomics studies were able to show an increased excretion of lyso-Gb3 (whose differences relied mainly on the sphingosine moiety), Gb3 and galabiosylceramide isoforms / analogues, also with a more prominent increase in male patients and a significant decrease during ERT. Interestingly, all but one of the lyso-Gb3 analogues have relative concentrations that are higher than lyso-Gb3 (up to 17 times higher than lyso-Gb3) and the types and relative proportion of galabiosylceramide structural variants present some significant differences to their Gb3 counterparts, raising

some questions about possible different synthesis or degradation pathways.[311, 312] Despite the promising results of these studies, the candidate biomarkers still need to prove that they overcome the lyso-Gb3 limitations: the need to test mutations carriers known to cause little increase in metabolites concentration and correlation with clinical manifestations and treatment response.

The proteomic approach has also been applied in the search for biomarkers of FD in plasma, peripheral blood mononuclear cells and urine. The first study with plasma proteomics compared the plasma of 13 children with FD, prior and after ERT and was able to show a decrease in 5 proteins during treatment.[313] Subsequently, another study identified, in plasma, an eight-protein biomarker panel that was very specific and sensitive for diagnose of male FD patients; in female patients, a nine-biomarker panel of proteins was identified, with only three proteins (apolipoprotein E, haemoglobin α-2 [up-regulated in females and down-regulated in males, when compared to matched controls] and peroxiredoxin 2), common to both genders, suggesting a gender-specific alteration in plasma biomarkers in patients with FD.[314]

Proteomic analysis of peripheral blood mononuclear cells was performed in a small cohort of 8 FD patients and 6 controls and all proteins that were found to have significant expression differences (either up or down-regulation) play roles in mechanisms that may be associated with the pathophysiology of FD.[315]

Urinary proteome is the most extensively studied in FD, with a few studies with up to 23 patients reporting several proteins with altered expression.[316-320] The most consistent alterations found were the up-regulation of prostaglandin H2 D-isomerase and prosaposin (the latter including in paediatric, pre-symptomatic patients), both known to play roles in processes that might be involved in FD pathophysiology. These proteins also enabled discriminating female FD patients from controls and their levels significantly reduced during ERT[318-320] In another study with 35 treatment-naive female patients a diagnostic biomarker pattern (composed of 64 proteins) exhibited high diagnostic accuracy when validated in an independent FD cohort and remained highly specific when applied to cohorts with a variety of other renal, metabolic and cardiovascular diseases. Several of the identified diagnostic biomarkers showed correlation with disease severity and most of them responded to ERT (8 out of the 11 treated patients scored negative for FD in the diagnostic model).[317]

3. Circulating biomarkers of myocardial fibrosis

3.1 Myocardial fibrosis: relevance and available circulating candidate biomarkers

In the heart, a complex extracellular matrix (mainly composed of type I fibrillar collagen) connects cardiomyocytes, fibroblasts and vascular cells and is paramount for preserving structural integrity and plasticity of the heart. In the diseased heart, the matrix undergoes structural and subcellular changes that progressively influence heart function, playing a major role in progression of the cardiac disease, in a vicious circle.[321]

Myocardial fibrosis is characterized by dysregulated collagen turnover, with a predominance of increased synthesis of type I and type III collagen over an unchanged or decreased degradation.[322] Two distinct patterns of collagen can be distinguished in myocardial fibrosis: replacement fibrosis, which is focal and forms scars in order to replace dead cardiomyocytes; and interstitial / reactive fibrosis, which is diffuse and occurs in the interstitial space without notable cells loss.[323] In interstitial / reactive fibrosis the accumulation of type I collagen (highly cross-linked, large-diameter fibres) prevails over type III collagen (non-cross-linked, small-diameter fibres), which enhances myocardial stiffness and the resistance of collagen fibres to degradation by matrix metalloproteinases (MMP). Moreover, collagen alignment is disrupted, which impairs the transmission of the strength generated by the cardiomyocytes to the ventricular chamber and has a detrimental effect on myocardial contractility.[324, 325]

Therefore, the quantitative and qualitative aspects myocardial fibrosis, as evaluated in biopsy samples, disrupt myocardial architecture and have been shown to be associated with: mechanical impairment portrayed by increased LV stiffness and diastolic dysfunction and impaired LV contraction and systolic dysfunction;[326, 327] electrical instability leading to arrhythmias;[328, 329] and vasomotor dysfunction with impaired coronary blood flow.[330] Moreover, the amount of myocardial fibrosis, in biopsy samples, influence long-term prognosis in patients with heart failure, not only in terms of cardiac events, but also all-cause mortality[331, 332] and predict the effectiveness of long-term heart failure therapy.[333] Consequently, the assessment of myocardial fibrosis is crucial not only for better pathophysiological understanding of the clinical picture, but also to help in the determination of the prognosis and appropriate treatment.

In this setting, the search for biomarkers of structural myocardial remodelling and fibrosis has evolved at a remarkable pace in the last decade. However, histopathological analysis of endomyocardial biopsy specimens remains as the gold standard for diagnosis and assessment of cardiac fibrosis. Thus, any candidate biomarker of myocardial fibrosis must be compared with the gold standard and its blood levels should directly correlate with quantitative parameters used to define fibrosis in endomyocardial biopsy specimens, namely myocardial collagen volume fraction and the determination of the proportion occupied by either collagen type I or III fibres.[334] Moreover, due to the patchy distribution of myocardial fibrosis, the analysis of several tissue fragments is important for diagnostic accuracy and interpretation.

A number of molecules, detectable in either human serum or plasma, have been proposed as biomarkers of myocardial fibrosis, namely: molecules related to collagen metabolism (described in detail in the next sections), molecules related to the regulation of collagen turnover and molecules integrating cardiac stress injury, inflammation and fibrosis. However, in most cases validation against the histological gold standard is lacking or remains inconclusive (a summary is shown in table 3).[335, 336] Cardiac MRI is emerging as a technique that enables non-invasive evaluation of the myocardial interstitial space through the measurement of myocardial extracellular volume with T1 mapping. This measure seems to correlate with myocardial collagen volume fraction, but still requires methodological standardization and further investigation (if it can serve as a surrogate parameter of myocardial fibrosis for validation purposes).[337]

In recent years, interest / research on some of the presented candidate biomarkers had increased substantially. In brief, some of the results related to microRNAs and gallectin-3 are described as follows. MicroRNAs are small non-coding RNA molecules able to regulate gene expression at the post-transcriptional level and are key players in heart development and myocardial conditions.[338] Over the last few years, abundant literature has accumulated suggesting that aberrant expression of various microRNAs is crucial in cardiac fibrosis and heart failure. MicroRNAs may regulate fibrotic response via various distinct mechanisms: acting as an essential downstream intermediate in the signalling pathway; fine-tuning critical components of the signalling pathways; repressing or amplifying the fibrotic signal by participating in negative or positive feedback loops, respectively.[339] Myocardial fibrosis in patients with diverse cardiac disease is

accompanied by the increased expression of profibrotic microRNAs such as microRNA-21 and microRNA-499-5p[340, 341] and decreased expression of antifibrotic microRNAs such as microRNA-29, microRNA-122 and microRNA-133a.[342-344]

Table 3: Potential circulating biomarkers for assessment of cardiac fibrosis. HCM: hypertrophic cardiomyopathy due sarcomere protein gene mutations; ECM: extracellular matrix; RAAS: renin–angiotensin–aldosterone system (adapted from López B[336]).

Candidate biomarker	Role an correlation to fibrosis	Histological evidence
Related to collagen metabolism		
C-terminal propeptide of procollagen type I	Cleaved enzymatically from procollagen I (collagen biosynthesis)	Yes
N-terminal propeptide of procollagen type I	Cleaved enzymatically from procollagen I (collagen biosynthesis)	Unknown
N-terminal propeptide of procollagen type III	Cleaved enzymatically from procollagen III (collagen biosynthesis)	Yes
C-terminal telopeptide of collagen type I	Cleaved by matrix metalloproteinase-1 (collagen I degradation)	Inconclusive
Matrix metalloproteinase-1	Degrades collagens I, II, and III	No
Matrix metalloproteinase-2	Degrades collagens I, III, IV and V	Unknown
Matrix metalloproteinase-3	Degrades collagens II, III, IV, IX and X	Unknown
Matrix metalloproteinase-8	Degrades collagens I, II, and III	Unknown
Matrix metalloproteinase-9	Degrades collagens I, IV and V	Unknown
Tissue inhibitor of metalloproteinases-1	Inhibits several matrix metalloproteinases	No
Tissue inhibitor of metalloproteinases-4	Inhibits several matrix metalloproteinases	Unknown
Related to the regulation of collagen turnover		
microRNA-21	Correlation with fibrosis in aortic stenosis	Inconclusive
microRNA-29b	Correlation of plasma levels with hypertrophy and fibrosis in HCM, reduced cardiac expression	Unknown
microRNA-122	Targets and inhibits transforming growth factor-β1	Unknown
microRNA-133a	Regulatory effects on the expression of Col1α1	Unknown
microRNA-499-5p	Blocks cardiomyocyte differentiation	Unknown
Transforming growth factor-β1	Promotes myofibroblast transactivation and ECM synthesis, deactivates macrophages	Inconclusive
Growth differentiation factor-15	Predictor of atrial fibrosis	Inconclusive
Connective tissue growth factor	Modulating the activity of growth factors in the ECM	Inconclusive
Osteopontin	Matricellular protein involved in macrophage regulation	No
Osteoglycin	Regulate collagen assembly	Unknown
Syndecan-1	Regulates transforming growth factor-β1	Unknown
Syndecan-4	Regulates mechanical stress-induced cardiac fibroblast differentiation	Unknown
Integrating inflammation and fibrosis		
Galectin-3	Galactosamine binding protein associated with collagen deposition of fibroblasts	Inconclusive
Cardiotrophin-1	Cytokine associated with cardiac fibrosis	No
Soluble ST2	A "decoy" receptor for IL-33, inhibiting IL-33/ST2 signalling	Unknown
Mid-regional pro-atrial natriuretic peptide	Stable marker for the release of ANP, which induces natriuresis and inhibition of RAAS	Unknown
Myostatin	Activation of the TAK-1-MKK3/6-p38 signalling pathway	Unknown

Galectin-3 is a galactoside-binding protein that is released to the circulation by fibroblasts and inflammatory cells.[345, 346] Galectin-3 is up-regulated by angiotensin II and aldosterone[347, 348] and turns quiescent fibroblasts into active myofibroblasts producing structural matrix proteins.[346] In heart failure patients, serum levels of galectin-3 predict hospitalization for acute heart failure and death and have an inverse correlation with estimated GFR.[349, 350] Although serum levels of galectin-3 may be reliably assayed by a commercially available enzyme-linked immunosorbent assay (ELISA), recent reports showed that there is a significant correlation between myocardial galectin-3 expression and the histological amount of fibrosis in patients with idiopathic dilated cardiomyopathy (DC), but there are no correlations between the myocardial galectin-3 expression or the myocardial fibrosis volume and the serum levels of galectin-3, questioning the circulating galectin-3 as a reliable biomarker of myocardial fibrosis.[351, 352]

3.1.1 *Circulating candidate biomarkers related to collagen metabolism*

Molecules related to collagen metabolism are the best characterized group of circulating biomarkers of myocardial fibrosis. As mentioned above, myocardial collagen is primarily composed of collagen types I and III, the former prevailing in processes of interstitial myocardial fibrosis.[324] Fibrillar collagen types I and III synthesis and degradation, as well as its regulatory control mechanisms are similar and occur in the following steps: procollagen synthesis, post-synthetic procollagen processing, post-translational collagen cross-linking and collagen degradation (figure 3).

Collagen is synthesized as a procollagen molecule primarily in myocardial fibroblasts, beginning with the synthesis of procollagen α-chain monomeric proteins, which then form the triple-helical structure of a procollagen molecule.[353] Afterwards, it is secreted, as a soluble molecule with an amino (N) and carboxy (C) terminal propeptide attached, into the extracellular matrix space for sequential post-synthetic processing in order to create mature insoluble collagen fibril.[354-357] Removal of the terminal propeptides by specific proteinases is the initial of a number of essential steps of post-synthetic processing.

First, enzymatic cleavage of the C-terminal propeptide by bone morphogenic protein-1 (enhanced by procollagen C-proteinase enhancer) is required.[355, 358] A 100-KDa procollagen type I C-terminal propeptide (PICP) is released into the bloodstream after this

first enzymatic step during the synthesis of fibril-forming collagen type I. A stoichiometric ratio of 1:1 exists between the number of collagen type I molecules produced and that of PICP released, with PICP cleared from the blood by the liver.[359] The second enzymatic step is the cleavage of the N-terminal propeptide by the A Disintegrin-like and Metalloproteinase Domain with Thrombospodin Type Motif (ADAMST)-2/3 enzyme; however, the 70-KDa procollagen type I N-terminal propeptide (PINP), which is also cleared from the blood by the liver, is not completely cleaved during this cleavage and may remain in the final fibres, where they can be released during fibre degradation.[360, 361] During collagen type III processing, equivalent procollagen type III C-terminal propeptide and procollagen type III N-terminal propeptide (PIIINP) are released, but it is unknown if the former reaches the bloodstream.[336] Like PINP, the 42-KDa PIIINP is also not completely cleaved during the conversion of procollagen type III to collagen type III and is cleared from the blood via hepatobiliary elimination.[362, 363]

After the cleavage of C and N terminal propeptides, the resulting collagen molecules undergo covalent cross-linking to form insoluble fibrils; initially, cross-linking is formed spontaneously by oxidized reactive aldehydes on lysine and hydroxylysine catalysed by the enzymes lysil and hydroxylysyl oxidase, but additional non-enzymatic cross-links can be formed by glycation end product formation.[364-366] A number of matricellular proteins such as Secreted Protein Acidic and Rich in Cysteine (SPARC) and thrombospondin-2 are also critical in the regulation of procollagen processing by facilitating fibril assembly, formation and collagen deposition to insoluble ECM.[356, 357, 367]

Myocardial fibrillar collagen type I degradation begins with catalytic cleavage by MMP-1 (the rate-limiting step); MMP-1 cleaves all three alpha chains of collagen type I by hydrolysing a peptide bond following a glycine residue located at a distance of three-fourths of the collagen molecule length from the N terminus, resulting in 36-KDa and 12-KDa telopeptides (which maintain their helical structure). The larger telopeptide spontaneously denatures into non-helical gelatine derivatives, which are completely degraded by the gelatinases MMP-2 and MMP-9.[368] The final fragments of these derivatives or matrikines, like tripeptide glycyl-histidyl-lysine (GHL) may regulate collagen metabolism, stimulating: new collagen synthesis by fibroblasts and *in vitro* MMP-2 expression and secretion by fibroblasts.[369, 370] GHL can found in plasma, but its stoichiometry relative to the degradation of collagen type I is unknown.[371] The smaller

telopeptide, collagen type I C-terminal telopeptide (CITP), is released immunochemically intact into the blood stream (in a stoichiometric ratio of 1:1 between the number of collagen type I molecules degraded and that of CITP released) and cleared from the circulation via glomerular filtration.[372] MMPs are regulated by tissue inhibitor of metalloproteinases (TIMP), which bind to active MMPs and inactivate their protease activity, leading to a reduction in collagen degradation.[373]

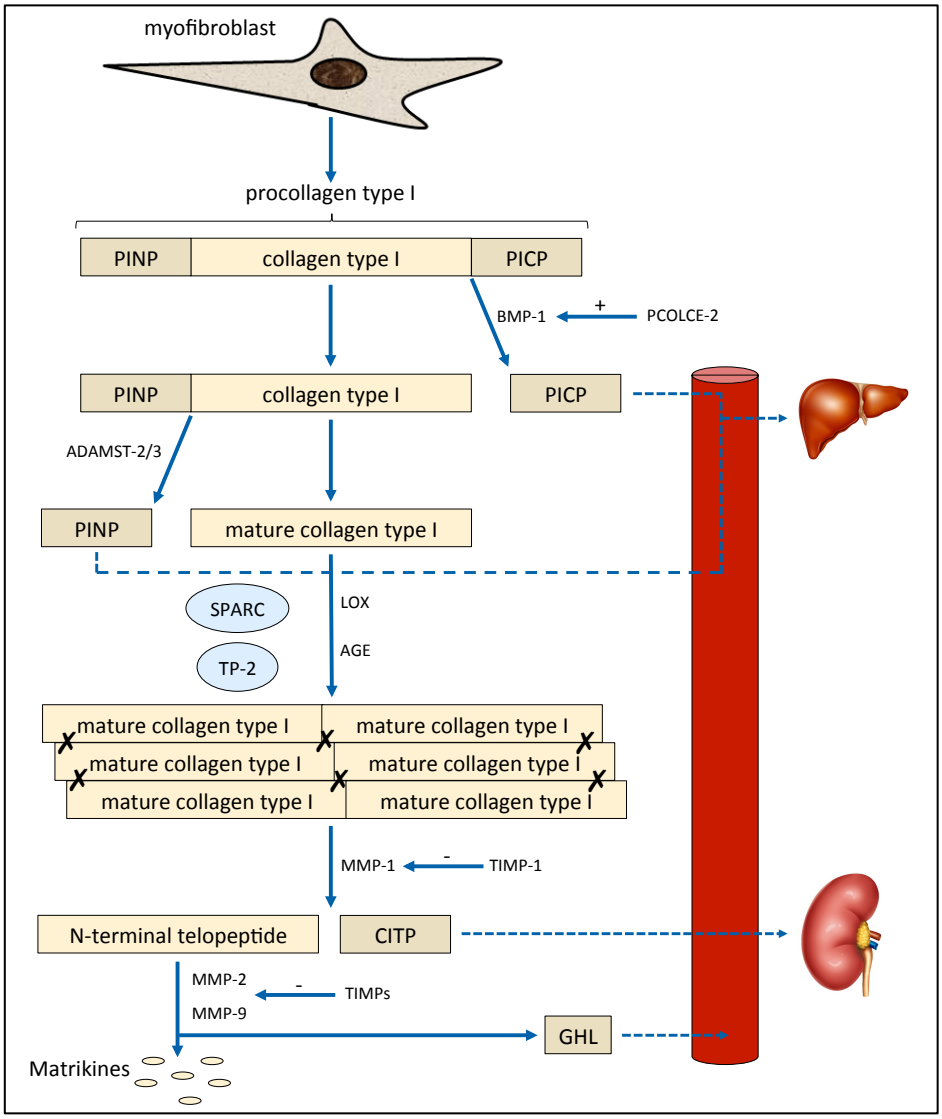


Figure 3: Steps involved in the synthesis and degradation of collagen type I fibrils. Several peptides released during this process reach the bloodstream and may be measured in serum. PINP: procollagen type I amino-terminal propeptide; PICP: procollagen type I carboxy-terminal propeptide; BMP: bone morphogenic protein; PCOLCE: procollagen carboxy-proteinase enhancer; ADAMST: a disintegrin-like and metalloproteinase domain with thrombospodin type motif; LOX: lysil oxidase; AGE: advanced glycation end product; SPARC: secreted protein acidic and rich in cysteine; TP: thrombospondin; MMP: matrix metalloproteinase; TIMP: tissue inhibitor of matrix metalloproteinases; CITP: collagen type I carboxy-terminal telopeptide; GHL: tripeptide glycyl-histidyl-lysine.

Each protein and peptide referred to above can be measured in the plasma / serum and can be used as a biomarker to assess collagen synthesis and degradation rates. Thus, PICP, PINP and PIIINP are candidate surrogate biomarkers of collagen type I and collagen type III synthesis and high serum levels may reflect ongoing tissue fibrosis.[374, 375] Instead, CITP, MMP-1, MMP-2, MMP-9 and TIMP-1 may be used as biomarkers related to the intensity of the degradation of collagen type I fibrils.[372] However, myocardial collagen content, composition and geometry are the result of the balance between synthesis and degradation of collagen, which determines its turnover (estimated at 80 to 120 days); thus, the ratio of propeptides to telopeptides reflects the collagen turnover rate, with higher ratios resulting in a net increase in collagen content and fibrosis.[376]

Nonetheless, as noted above, because biomarkers of collagen metabolism present in blood are not cardiac specific, the serum levels of any candidate biomarker of myocardial fibrosis must be compared with the gold standard quantitative measures of fibrosis in endomyocardial biopsy specimens.[334] The evidence supporting the association between biomarkers related to collagen metabolism and myocardial fibrosis is variable, according to the biomarker.[336] Among the many proposed circulating biomarkers of myocardial fibrosis (see Table 3), only 2 biomarkers related to collagen metabolism have been shown to be associated histologically to myocardial fibrosis: PICP and PIIINP.

PICP is the circulating biomarker most extensively validated against the histological gold standard. In a study with 65 hypertensive patients with LV hypertrophy (31 of them with heart failure), the concentration of peripheral blood PICP was significantly lower compared with that in the coronary sinus, but there was a significant direct correlation between peripheral and coronary PICP and between peripheral blood PICP and the collagen volume fraction or the extent of collagen type I deposition in the myocardium.[326] The direct correlation between peripheral blood PICP and collagen volume fraction of the myocardium was further confirmed in patients with hypertensive heart disease with and without heart failure, patients with chronic heart failure (of diverse aetiologies).[352, 377-379] Moreover, PICP levels and collagen fractional volume changed in parallel in response to diverse treatments: in one study torasemide was compared with furosemide in the ability to decrease myocardial fibrosis and collagen fractional volume, as well as PICP, which only decreased in the torasemide group, with a direct correlation between the change in both variables.[377] Another study showed that

after 12 months of treatment with losartan, there was a parallel significant decrease of collagen volume fraction and PICP in the subgroup of patients with severe fibrosis (>6% of the total myocardium), whereas in the subgroup of patients without severe myocardial fibrosis there was only a non-significant trend of decrease in both variables.[380] Similar results were found in patients with idiopathic DC treated with spironolactone for 12 months.[381] In this last study, a significant correlation between PICP to CITP ratio and collagen volume fraction was found, both before and after treatment with spironolactone.[381]

There is conflicting evidence supporting the correlation between serum PIIINP and the histological gold standard. In a study in 17 patients with DC, there was a significant correlation between PIIINP and myocardial collagen type III fractional area;[382] moreover, after 12 months of treatment with spironolactone, the reduction of the collagen volume fraction of the myocardium was paralleled by a reduction in serum PIIINP.[381] However, 2 further studies did not confirm this histological validation: in 38 patients with hypertensive cardiomyopathy and heart failure, a correlation between PIIINP and collagen volume fraction was not found (but the collagen type III fractional area was not analysed).[379] In another study with 39 patients with heart failure, there was no significant correlation between PIIINP and collagen volume fraction or collagen type III fractional area.[352]

The evidence regarding the relationship between CITP and myocardial fibrosis is controversial. In the aforementioned study with 17 patients with DC, a significant correlation was identified between CITP and myocardial collagen type I fractional area.[382] However, in another study in patients with idiopathic DC, CITP levels were lower in patients with severe fibrosis (compared with the remaining patients) and increased after 12 months of spironolactone, whereas collagen volume fraction in the myocardium decreased in the same subgroup of patients.[381] Moreover, in the previously mentioned study comparing torasemide and furosemide, CITP remained unchanged after treatment in both groups (collagen volume fraction significantly reduced in the torasemide group) and no correlation was found between serum CITP and collagen volume fraction of the myocardium.[377] Serum CITP was inversely associated with insoluble (cross-linked) collagen volume fraction in one study.[379]

Higher concentrations of MMP-1 and TIMP-1 were found in coronary sinus blood compared with peripheral blood, but a significant correlation between both sources of blood was found for the two molecules. Nonetheless, correlations between MMP-1 or TIMP-1 and myocardial fibrosis are intriguing, because no correlation was found between MMP-1 or TIMP-1 and collagen volume fraction in patients with hypertensive heart disease and heart failure; however, it is noteworthy that the patients with higher values of serum MMP-1 exhibited reduced levels of perimysial and endomysial collagen. This finding suggests that these molecules may be related more to the loss of the physiological mysial collagen scaffold than to the accumulation of pathologic non-mysial collagen.[383]

No data is available in the literature on the association between the remaining circulating candidate biomarkers related to collagen metabolism and myocardial collagen content.

Apart the histological validation of the candidate biomarkers, the interpretation of their serum levels may be complex due to several aspects influencing their clinical evaluation, namely: immunoassay method of detection, elimination of circulating biomarkers from the circulation, patient demographics, presence of comorbidities and effects of pharmacological treatment.

The abovementioned molecules may be measured in serum or plasma samples easily, reproducibly and inexpensively using commercially available ELISA or radioimmunoassay kits; however, several kits are available for the same biomarker (with various antibodies and standards) and it is important to: standardize these determinations before applying them to routine clinical practice and take this aspect into account when comparing results provided by different studies. Moreover, whereas most of the peptides generated during the processing of procollagen types I and III are found as one antigen form in serum, PINP appears in two different forms: one form corresponding to the whole propeptide and a smaller form that is the product of its degradation. Thus, it is important to use assays that can identify the intact molecule as the biomarker of interest.[384] Remarkably, all propeptide antigens are very stable in serum if samples are taken, handled and stored according to good clinical laboratory practice (there are a number of reports in the literature on the analysis of PINP, PICP or PIIINP in frozen samples that were collected several years previously).[385]

Other potentially confounding factors in the interpretation of blood concentrations of the biomarkers are: their rate of release, how they reach the blood (cardiac lymph versus venous drainage), their volume of distribution and the pathway of elimination.[363] PICP, PINP and PIIINP are cleared from the circulation by the liver and CITP is cleared through the kidneys, thus chronic liver insufficiency and CKD (GFR <50ml/min/1.73m^2) may cause increase in their serum concentrations, respectively.[359, 360, 362, 372] The mechanism for the clearance of MMPs and TIMPs in not fully known, but various pathways have been implied: own autoproteolysis, direct clearance via low-density lipoprotein-related scavenger receptor and entrapment in a complex with α_2-macroglobulin.[386-388]

Demographic / physiological parameters may also affect the serum concentration of some biomarkers: PICP is increased in infants, children and adolescents up to 18 years old (with a peak before 3 months of age) and correlates with growth velocity;[389] PIIINP and TIMP-1 increase both with age and body mass and the second biomarker is also higher in males.[390, 391]

Due to the ubiquitous distribution of collagen type I (bone, tendon, ligament, skin, cornea and others) and collage type III (skin, blood vessels and others), the lack of cardiac specificity of the circulating biomarkers related to collagen metabolism is one of the major concerns in their clinical applicability.[354] Taking into account this diversity of tissue sources of collagen biomarkers, change in their blood levels may be related not only to the myocardial collagen network, but also to alterations in other organs. Even in cardiovascular diseases, like HBP, arterial stiffness was directly correlated with serum PICP or CITP in elderly patients and inversely associated to plasma MMP-1. Although no cardiac parameters were included in a multiple regression analysis and no cardiac histological evaluation was performed, alterations in these circulating biomarkers may reflect disturbances of collagen metabolism that occur not just at the myocardial but also at the arterial wall level.[392, 393]

Moreover, the presence of concomitant non-cardiovascular diseases affecting collagen matrix turnover can also affect the circulating levels of these biomarkers and must be excluded in the patients under study. This issue is paramount concerning bone disorders, because bone is one of the main sources of collagen type I in the body. Metabolic bone diseases, like osteoporosis, increase collagen type I synthesis biomarkers

(PICP and PINP) and post-menopausal women present an increase of about 20% in PICP;[394, 395] osteolytic metastatic bone disease and multiple myeloma increased the biomarkers of collagen type I degradation, namely CITP.[396] To minimize this confounding factor, in one study evaluating collagen biomarkers in HCM, the investigators measured specific markers of bone metabolic activity (like bone-specific alkaline phosphatase [B-AP]) and normalized biomarkers to this analyte.[397] Chronic liver disease and CKD may also affect the levels of the biomarkers related to collagen metabolism, not only impairing their elimination, and also to the hepatic fibrosis (increases PIIINP and, to a lesser extent, PICP) and to the kidney-related bone disease (increases PICP and CITP).[398, 399] Other fibrogenic diseases, like diffuse fibrosing lung disease, may also increase the concentration of the metabolites related to collagen type I metabolism (PICP and CITP).[400] Finally, inflammatory (for example, rheumatoid arthritis) and endocrine (for example, thyroid disorders) diseases can also affect bone metabolism and consequently may increase collagen metabolism biomarkers.[401, 402]

As noted above, long-term pharmacological treatment of cardiovascular disorders may decrease collagen volume fraction of the myocardium and modify serum levels of the biomarkers of collagen metabolism: ACEi and ARB decreases the levels of PICP and the PICP to CITP ratio and increases the levels of CITP and MMP-1;[380, 403-406] aldosterone antagonists have a similar effect on PICP, PICP to CITP ratio and CITP, but a significant decrease in PIIINP was also reported.[381, 407-410] As previously described, treatment with torasemide also decreased PICP blood levels.[377] Moreover, CITP decreases in hypercholesterolaemic patients treated with statins.[411] The administration of bisphosphonates, glucocorticoids or hormone replacement therapy in post-menopausal women decreases PICP, PINP and CITP levels.[395, 412]

3.2 Biomarkers related to collagen metabolism in cardiac diseases: state of the art

3.2.1 *Fabry disease*

The body of knowledge about the circulating candidate biomarkers related to collagen metabolism in FD is limited. A study evaluating extracellular matrix turnover (MMP-9, TIMP-1 and TIMP-2), including 29 FD patients (15 males, 16 on ERT) and 21 controls, reported: significantly increased levels of MMP-9 in FD patients compared with

controls; significant inverse correlation between MMP-9 and endocardial or midwall fractional shortening of the LV (independent of age and sex); significant direct correlation between MMP-9 and QRS duration or total MSSI (but not MSSI cardiac subscore); and there was no correlation between MMP-9 and LV mass index or LV cavity dimension. No difference was found in levels of TIMP-1 and TIMP-2 between FD patients and controls.[413]

Another study based on 73 FD patients (57 receiving ERT), followed by a mean time of 4.8 years reported an increase in PICP, PIIINP and CITP, without any significant difference between treated and untreated FD patients or among patients with and without LGE in cardiac MRI; moreover, no significant change between baseline and follow-up measurements was found, regardless of ERT status.[116]

3.2.2 *Hypertrophic cardiomyopathy due sarcomere protein gene mutations*

HCM is caused by mutations in genes encoding sarcomere proteins, being the most common monogenic cardiac disorder (prevalence of 1:500 in the general population).[414] HCM share some features with FD cardiomyopathy, namely: they are both monogenic cardiac disorders (though with different patterns of inheritance);[415] heterogeneous phenotype, with mutation carriers not presenting overt hypertrophic phenotype (however at high risk of development), but often exhibiting early manifestations that precede the pathological remodelling of the overt disease (for example, impairment of LV relaxation);[416-418] histological evidence of diffuse interstitial and focal replacement myocardial fibrosis in overt disease (the latter emerging as LGE in cardiac MRI).[419, 420]

A few studies evaluated circulating biomarkers related to collagen metabolism in patients with HCM, not only their sensitivity to detect early cardiac involvement, but also their clinical correlations, prognostic value and response to treatments. A study evaluating 77 patients with pathogenic sarcomere mutations associated with HCM, 38 of them with overt LV hypertrophy (defined as LV wall thickness ≥12mm) and 39 without LV hypertrophy (though presenting lower average E' than healthy controls), reported a significant increase in PICP (biomarker of collagen type I synthesis) even in the subgroup of patients without overt LV hypertrophy (in comparison with healthy controls), although significantly lower than in subgroup of patients with overt LV hypertrophy. This significant increase remained after adjustment for bone turnover (ratio of PICP to B-AP). There was

no significant difference in circulating biomarkers of collagen degradation MMP-1, TIMP-1 and CITP (except a significant increase of MMP-1 in the subgroup of patients without LV hypertrophy in comparison with controls), but a progressive and significant increase in PICP to CITP ratio (reflects the balance between collagen synthesis and degradation) was observed, comparing controls and patients without LV hypertrophy, as well as patients without and with LV hypertrophy. In the same study 71% of the patients with overt LV hypertrophy presented LGE in cardiac MRI, whereas LGE was not evidenced in any patient without LV hypertrophy. However, there were no significant correlations between LGE and levels of PICP, CITP, MMP-1 or TIMP-1, and nor was PICP correlated significantly with natriuretic peptides, cardiac troponin or any echocardiographic variables.[397] In another study of 23 patients with established HCM and 16 patients at risk, a clear trend was identified of increased MMP-9 in the subgroup of patients at risk (compared with healthy controls), but it was only significant in patients with established HCM.[421]

No other studies evaluated patients without overt phenotype. Nonetheless, further studies confirmed increased levels in PICP and no change in CITP in patients with overt HCM. Moreover a significant decrease in MMP-1 and a significant increase in TIMP-1, MMP-2 and MMP-9 were reported in patients with established HCM.[422, 423] Conflicting results were reported by one study showing a non-significant increase in PICP and a significant decrease of CITP in patients with overt HCM;[424] another study described no clear difference between HCM and controls in the levels of PINP, CITP and PIIINP.[425]

However, some other studies demonstrated significant correlations between circulating collagen biomarkers and parameters of diastolic function: significant inverse correlation between PICP or PINP and the difference between the duration of transmitral forward wave (A dur) and the duration of the pulmonary venous retrograde wave (Ar dur); significant direct correlation between free MMP-1 or active MMP-2 and A dur – Ar dur; lower S' and higher E' and ratio of peak velocity of early LV filling (E) to E' (an indirect estimate of LV filling pressure) in patients with higher PINP to ICTP ratio.[426, 427] These results support the hypothesis that the observed increase in collagen I synthesis with a concurrent suppression of catabolic enzymes leads to diastolic dysfunction. One study evaluating diastolic dysfunction during exercise in patients with HCM was not able to identify any correlation with circulating biomarkers related to collagen metabolism.[424, 427]

A small number of studies showed significant correlations between PICP and IVS thickness or between PINP and maximal LV wall thickness and a significant inverse correlation between CITP and IVS wall thickness.[422, 424] In other studies, no correlation between collagen biomarkers and severity of LV hypertrophy or the amount of LGE (or the post-contrast myocardial T1 time in one study) in cardiac MRI was found.[423, 425, 426] Only in one study with 54 patients with overt HCM, was a significant correlation found between MMP-9 and the amount of LGE in cardiac MRI (and only in female patients).[428] Nonetheless, in one study based on 28 patients with HCM, 11 of them at an advanced stage characterized by LV wall thinning, LV dilatation and systolic dysfunction, a significant increase in MMP-2 and TIMP-2 was reported only in the subgroup of patients with advanced HCM (with no difference between the remaining patients and the controls); moreover, MMP-2 concentrations significantly increased as the NYHA functional class increased (another study further confirmed this inverse correlation between MMP-2, as well as MMP-9 and exercise capacity).[423, 429] An inverse correlation between CITP and NYHA class has been also reported.[422]

The correlation between the levels of the circulating biomarkers related to collagen metabolism and cardiac events, like malignant ventricular arrhythmias, is not well established, with two studies identifying a significant correlation between MMP-3 and ventricular arrhythmias (in multivariate regression analysis),[428, 430] while other authors were not able to show any correlation.[424]

In patients with HCM, data on the response of the circulating biomarkers related to collagen metabolism to treatments is sparse. In a small cohort of 23 patients, 11 treated with losartan and 12 non treated with ARB or ACEi, there was, after 12 months of treatment, a significant decrease in PICP in the subgroup of patients treated with losartan, while in the other subgroup it remained unchanged; in the same study PIIINP levels were not influenced by the treatment.[405]

3.2.3 *Dilated cardiomyopathy and hypertensive heart disease*

Non ischemic DC is characterized by LV dilatation and impaired systolic function, associated with profound morphological and histological changes, including myocardial fibrosis,[431] which may predispose patients to both atrial and ventricular arrhythmias.[432] A few studies have evaluated circulating biomarkers related to collagen metabolism in

patients with DC and the results are conflicting. Nevertheless, a consistent pattern of increased collagenase expression and activity, increased collagen type I degradation and a shift toward an increased synthesis of collagen type III was reported,[433-435] as evidenced by: one study recruiting 43 DC patients showed a non-significant trend of increased collagen synthesis (measured by PICP) and a significant increase in CITP, MMP-1 and TIMP-1;[436] other studies corroborated the increase in CITP and demonstrated a significant increase in PIIINP levels (even in the paediatric population).[382, 437] However, diverging results were found in another study recruiting 73 patients with mild to moderate DC (NYHA class I or II), where a significant decrease in PINP and PIIINP and a no significant difference in CITP were found in DC patients (in comparison with healthy controls). This finding was attributed by the authors to the mild phenotype and to the use of ACEi / ARB and aldosterone antagonists (as noted above, known to decrease collagen synthesis biomarkers). Moreover, in the same study, LA size was directly correlated with CITP or the PIIINP to PINP ratio and negatively correlated with PINP.[438]

PIIINP was also directly correlated with diastolic dysfunction in patients with DC, predicting mitral inflow pattern independently of the haemodynamic variables.[439] Regarding systolic function: free MMP-1 presented a direct correlation with LV end diastolic diameter and an inverse correlation with cardiac index, and CITP was directly correlated with LV end diastolic diameter;[436] PIIINP showed a significant direct and inverse correlations with NYHA functional classes and cardiac output, respectively.[382]

The prognostic value of circulating biomarkers related to collagen metabolism in patients with DC was highlighted in a study involving 70 patients during 12 months of follow-up. During this follow-up period 14 patients implanted cardiac devices for prevention of sudden cardiac death and the baseline levels of CITP, MMP-1 and TIMP-1 were significantly higher in this subgroup of patients (CITP showing better diagnostic accuracy to predict malignant arrhythmic event).[440] The prognostic significance of CITP, in terms of overall survival, was corroborated in another study.[382] Moreover, few studies identified PIIINP as a significant predictor of overall survival during follow-up.[382, 439, 441]

Hypertensive heart disease is one of the most common aetiological conditions predisposing to heart failure and is characterized by complex changes in myocardial structure: enhanced cardiomyocyte growth and apoptosis, accumulation of interstitial and perivascular collagen fibres and disruption of endomysial and perimysial collagen

network.[442] Several circulating biomarkers of myocardial remodelling have been studied in hypertensive heart disease, particularly those related to collagen metabolism.

As mentioned above, PICP is the sole circulating biomarker related to collagen metabolism has been consistently validated against the histological gold-standard (mainly in patients with hypertensive heart disease).[326, 352, 378] Several authors reported that serum PICP is increased in hypertensive patients (in comparison with normotensive patients).[326, 377, 378, 380, 404, 443-447] No significant differences in serum PICP were found between hypertensive patients with and without LV hypertrophy, but patients with overt heart failure presented significantly higher levels of PICP compared with the remaining patients.[326, 404, 446] Actually, PICP seems to be associated with increasing severity of myocardial fibrosis, as shown by its diagnostic accuracy to detect severe myocardial fibrosis in hypertensive patients with LV hypertrophy.[378]

The increase in PICP parallels the increase of LV stiffness and filling pressures, suggesting a correlation with diastolic dysfunction (due to accumulation of collagen fibres within the myocardium.[380, 447] Moreover, a significant correlation between serum PICP concentration and LV mass index has also been identified.[404, 443]

There is also histological validation that the decrease of PICP during anti-hypertensive treatments parallels the changes in the amount of myocardial fibrosis, with significant decreases in PICP reported in two studies, with a follow-up period of 12 months, of patients treated with losartan and torasemide.[377, 380]

An elevated serum concentration of PIIINP was also found in hypertensive patients, which was significantly higher in patients with heart failure, compared with asymptomatic patients.[443, 446, 448] PIIINP levels were also directly correlated with the severity of diastolic dysfunction,[443, 446] as well as with systolic dysfunction (measured by LV strain) and NYHA functional class.[449] Moreover, PIIINP decreased significantly to levels of normotensive population in patients treated with lisinopril.[443]

Regarding markers of collagen degradation, the serum MMP-1 toTIMP-1 ratio is abnormally increased in patients with hypertensive cardiomyopathy and heart failure and is abnormally decreased in both asymptomatic patients with and without LV hypertrophy.[383, 406] Furthermore, this ratio is higher in hypertensive patients with systolic heart failure than in patients with diastolic hear failure, and is associated with a decrease in ejection fraction and an increase in LV end-diastolic volume.[383] MMP-1 was also

identified as an independent predictor of cardiac events or death in a hypertensive population, with a median follow-up period of 5.5 years.[450]

There are few reports about CITP in hypertensive heart disease, but an increase in CITP levels was identified in hypertensive populations, which is of greater magnitude and directly correlated with systolic dysfunction (measured by LV strain) in patients with worse NYHA functional classes.[448, 449]

4. Urinary biomarkers of glomerular injury

4.1 Glomerular physiology: rational for the categorization of biomarkers

The glomerulus is the filtering unit of the kidney and a unique and specialized bundle of capillaries situated between two resistance vessels (contained within the Bowman's capsule), enabling the formation of primary glomerular filtrate. The glomerular filter is composed of various structures: endothelium, basement membrane, visceral epithelial cells (podocytes) and mesangium. The integrity of all these components is essential for normal charge and size selectivity of the glomerular filter, as demonstrated by the fact that disruption of any of these components leads to proteinuric disease states.[451]

The glomerular endothelium represents the first layer in the glomerular filtration barrier and is in direct contact with the blood; it is a flattened and fenestrated endothelium, characterized by the presence of individual fenestrae of the order of 70-100nm in diameter, permitting high flow of water and small solutes. It is covered by a glycocalyx that likely restricts the passage of large molecules and is important in charge selectivity (due to its negatively charged components), but our knowledge about its components and functions in humans is limited.[452] Moreover, the maintenance of normal endothelial structure seems to depend on a paracrine factor secreted by podocytes, the vascular endothelial growth factor (VEGF).[453]

The second layer of the filter is the glomerular basement membrane (GBM), which is thin (250 to 400nm) and formed by the fusion of basement membranes from both the endothelial and epithelial cells and contains, morphologically, a dense inner layer (*lamina densa*), flanked by the inner *laminae rara interna* and *rara externa*. It is a complex mash of extracellular proteins (ColIV, laminin, nidogen, fibronectin and proteoglycans),

providing structural support for the glomerular capillaries and harbouring ligands for receptors on the surface of the adjacent endothelial cells, podocytes and mesangial cells. Moreover, the GBM also contributes to glomerular charge and size selective permeability, restricting the passage of plasma proteins across the glomerular filtration barrier.[454] However, the exact mechanism by which GBM allows such high rates of water flow while restricting the flow of large molecules and yet remains functional and "unclogged" is unknown. Studies suggest that GBM acts more like a gel than a simple filter in that the size-selective properties of the glomerular filter are determined by permeation and diffusion properties of the GBM.[451]

The distal layer of the glomerular filter is composed of podocytes, which are both morphologically and functionally unique. Podocytes may serve as support to help sustain the integrity of the freestanding capillary loops, but their main function is related to filtration. Long intricate extensions, or primary processes, lead to secondary or foot processes, which form a complex interdigitating structure with the foot processes from adjacent podocytes. The serpentine cell–cell junction known as the slit diaphragm that bridges these podocytes can be considered a modified adherens junction with some unique additional molecular components and is essential for the normal function of the filtration barrier.[455] The normal structure and function of the podocytes and slit diaphragms depend on transmembrane proteins (like nephrin and podocin, which are podocyte limited in their expression),[456] proper function of the actin cytoskeletal apparatus[457] and efficient communication and signalling between slit diaphragm proteins and the cytoskeleton.[458] Moreover, the integrity of signalling pathways (like small GTPase signalling) and autophagy are critical to podocyte function.[459, 460]

The mesangium refers to the mesangial cells (third cell type in the glomerular tuft) and the matrix they produce. Mesangial cells provide support to the adjacent capillary loops; moreover, the mesangial processes are filled with bundles of actin and myosin-based microfilaments that extend to contact the GBM (in which they bind laminin) and seems to provide protection against glomerular pressure and may regulate glomerular capillary flow via contractile properties.[461, 462] The mesangial matrix produced by mesangial cells is composed of a diverse group of proteins, including ColIV, heparan sulphate proteoglycans, and elastic fibre proteins including fibronectin, laminin, entactin and fibrillin-1; accumulation of the mesangial matrix is a feature commonly observed in a

number of glomerular diseases.

The ultimate function of the glomerular filter is the production of the primary filtrate, according to the size and the charge selectivity of the filtered molecules. Studies using dextran molecules of varying sizes and charge demonstrated that neutral particles with a molecular radius 4.2nm are restricted from the urinary space, whereas anion particles 3.4 nm have a fractional clearance that approaches zero.[463]

The key features of glomerular damage in any nephropathy are the changes in glomerular permeability and structure that occur as a result of injury to any of the major components of the aforementioned components of the glomerular filtration barrier apparatus. Thus, urinary biomarkers of glomerular damage represent either increased permeability to plasma proteins or increased excretion of intra or extracellular proteins of the glomerular structures.[464, 465] Increased permeability may result from: loss of glomerular charge selectivity, loss of glomerular size selectivity or increased intragomerular pressure; for example, urinary excretion of albumin or transferrin are biomarkers of increasing permeability.[466, 467] Some biomarkers are constitutive elements of the glomerulus, whose excretion increase with glomerular damage, for example: nephrin and podocalyxin from the podocytes;[468] collagen and laminin from the mesangium and GMB;[469] VEGF from the endothelial cells.[470] Actually, proteins derived from the mesangium and GBM, like ColIV, laminin and fibronectin are high molecular weight proteins that are not normally filtered through the glomerulus; thus, their excretion in urine may reflect the synthesis / degradation in damaged kidneys and may be surrogate biomarkers of mesangial matrix expansion and GBM thickening.[469, 471]

4.2 Urinary transferrin excretion

4.2.1 *Rational and pathophysiology*

Transferrin is a plasma glycoprotein synthesized primarily in the liver, but also in the eyes, brain, testis and placenta.[472-475] Its half-life is approximately 7 days and its plasma levels are mainly regulated by iron availability (increasing in iron deficiency states), but are also influenced by physiological states, like pregnancy and ageing.[476, 477] Transferrin is metabolized in the liver and excreted by the kidney and the bowel mucosa.[477] Transferrin is the main iron-binding protein in plasma and carries ferric iron

from the intestine, reticuloendothelial system and liver parenchymal cells to all proliferating cells in the body.[477, 478] Furthermore, transferrin also has antimicrobial properties and regulates cell growth, proliferation and apoptosis.[479]

Transferrin has a molecular weight (76.5KDa) and radius (4.0nm) only slightly greater than albumin (66.5KDa and 3.6nm, respectively); moreover, It is less anionic than albumin with an isoelectric point that is one unit higher and therefore, expected to be filtered more readily through the glomerular barrier.[480, 481] However, glomerular sieving coefficients of endogenous plasma proteins, estimated in patients with Fanconi syndrome (characterized by a "knock-out" of renal tubular protein reabsorption, causing "tubular" proteinuria) is slightly greater for albumin than for transferrin.[482] After entering the glomerular filtrate, transferrin is retrieved by specific receptor-mediated uptake (through cubilin and transferrin receptors) in the kidney tubular system, mainly in the proximal tubules.[483, 484] In the proximal tubular cells, divalent metal transporter (DMT)-1, expressed in late endosomal / lysosomal membranes, processes the apically sequestered transferrin.[485] Resorption of transferrin in the tubular system seems to be important because: it is an essential growth factor in the development of kidney and differentiation of tubule and may be the major mechanism by which proximal tubule cells acquire the iron that they need.[485, 486] After resorption in the tubular system, the quantity of transferrin in normal urine is approximately 0.32 to 0.47mg/day.[487] It is noteworthy that there is no correlation between the quantity of transferrin in the blood and the urine.[488-490] Thus, transferrin is not only a biomarker of glomerular injury, but also of tubular proximal dysfunction.

Nonetheless, transferrin may also be a mediator of tubular toxicity; in the setting of increased glomerular permeability to transferrin, its concentration in tubules increases and the resorption and degradation capacity in the tubular proximal cells is exceeded, leading to rupture of the lysosomes and release of free iron and formation of free hydroxyl radicals in the tubular cells, triggering up-regulation of pro inflammatory and pro fibrotic cytokines, which causes mononuclear cells infiltrate, tubular and interstitial cells ischemia and, eventually, tubulointerstitial fibrosis.[490-492] This detrimental effect of increased urinary transferrin excretion (UTE) in tubular interstitial injury was evidenced in a study involving 45 type 2 diabetic patients, where the UTE was significantly higher in patients with albuminuria A2 with tubular interstitial lesions, in comparison with patients

with albuminuria A2 without lesions or patients with albuminuria A1; moreover, there was a significant correlation between UTE and the degree of interstitial fibrosis, tubular atrophy and inflammatory cells infiltrate in the interstitium.[493]

4.2.2 *Urinary transferrin excretion in renal diseases: state of the art*

UTE has been evaluated as biomarker of kidney dysfunction mainly in type 2 diabetes mellitus (T2DM) and diabetic nephropathy. In FD, it has been only measured in a small cohort of 17 female patients, 13 of them treated with ERT, where there was a non-significant trend of a decrease in UTE in treated patients after a mean follow-up period of 6 years; an opposite trend of an increase in UTE was observed in the untreated patients after a mean follow-up period of 5 years.[307]

However, FD nephropathy and diabetic nephropathy share some characteristics, as both are proteinuric nephropathies of metabolic origin, characterized by a progressive decrease of renal function to an end-stage CKD, requiring dialysis or transplantation. Although the metabolic environments of the two diseases are considerably different, in diabetes high glucose and in FD lyso-Gb3 and other mediators, both may result in recruitment of secondary mediators of injury on target organ cells, which will result in kidney fibrosis (to which ischemia is also a contributor in FD). In both diseases GBM and tubular basement membrane thickening depends on increased production of extra-cellular matrix by glomerular epithelial podocytes and tubular epithelial cells injured by the metabolic defect; this is followed by recruitment of activated fibroblasts, focal and segmental glomerular fibrosis and sclerosis and interstitial fibrosis.[494]

In T2DM several studies, based on up to 157 patients, showed an increase in UTE comparing patients with T2DM and healthy controls, even in diabetic patients with albuminuria A1 (between 21 - 61% of the normoalbuminuric patients presented an increase in UTE).[480, 488, 495-500] For example, in a study of 60 T2DM patients (30, 19 and 11 patients with albuminuria A1, A2 and A3, respectively), 33.3% of the normoalbuminuric patients presented high UTE, whereas all patients with albuminuria A2 and A3 presented high UTE.[488] In type 1 diabetes mellitus (T1DM), two studies demonstrated a significant increase of UTE in diabetic patients, in comparison with healthy controls. Moreover, one of the studies reported an increase in UTE in 77.8% of the normoalbuminuric patients and a significant correlation between UTE and albuminuria.[489, 501-503] Therefore, UTE may be

more sensitive than albuminuria to identify early diabetic nephropathy, as corroborated in a recent systematic review of the available evidence.[504]

A significant direct correlation between UTE and albuminuria was shown in several studies in patients with both T1DM and T2DM (documenting a similar glomerular leak and tubule handling for these two proteins),[488, 490, 496, 498, 499, 505-508] as well as a significant inverse correlation between UTE and creatinine clearance.[488] The correlation between the UTE and the degree of histological glomerular lesions was evaluated in a study involving 60 T2DM patients and the UTE showed a significant increase with respect to the progress of glomerular diffuse lesions; moreover, UTE was more sensitive than albuminuria in detecting early pathological alterations of diabetic nephropathy.[488]

The prognostic value of UTE was evaluated in longitudinal studies, where the risk of subsequent development of albuminuria A2, in normoalbuminuric patients with T2DM, was significantly greater in patients with increased UTE at baseline,[480, 509] with the largest study evaluating 117 normoalbuminuric T2DM patients, for a follow-up period of 5 years and showing that UTE was significantly higher (more than double) in the patients progressing to albuminuria A2 and that 35.3% of the progressors had increased UTE at baseline (versus only 9% of the non-progressors).[509]

The correlation between UTE and blood pressure was explored in several studies and a significant correlation seems to exist between UTE and systolic or diastolic blood pressure.[498, 510, 511] Even the diurnal changes in blood pressure significantly correlate with diurnal changes in UTE in normoalbuminuric diabetic patients (an influence not found for albuminuria). Additionally, in the same study, UTE was influenced (whereas albuminuria was not) by enhanced GFR after acute protein loading, which causes increased glomerular capillary pressure due to afferent arterioles dilation, mimicking diabetic intra-renal haemodynamics. Thus, UTE may be a more sensitive indicator of glomerular capillary pressure change than albuminuria in normoalbuminuric diabetic patients.[510] These findings were further corroborated by other studies showing a greater magnitude of increase in UTE (in comparison with urinary albumin excretion) after exercise.[507, 512] However, the effect of diabetes in UTE, in comparison with HBP, seems to be of greater magnitude, as demonstrated in a study with 270 patients with HBP, 180 of them with T2DM, where after controlling for systolic, diastolic and mean arterial blood pressures,

greater than expected proportions of diabetic subjects (than if they were just additive risk factors for UTE) were found to have elevated excretion ratios of transferrin.[513]

There is conflicting evidence concerning the correlation between UTE and the degree of glycaemic control (measured by glycated haemoglobin [HbA1c]) in diabetic patients, with one study showing a direct significant correlation between both variables,[488] while others not.[498, 499] Furthermore, the correlation between UTE and duration of diabetes was inconstant, with one study reporting a significant correlation,[496] but a few studies did not find any correlation.[498, 499] However, one study recruited 40 newly diagnosis diabetic patients starting hypoglycaemic agents and, after 12 weeks of follow-up, there was a significant decrease in HbA1c, as well as in albuminuria and UTE (at baseline 53% of the patients had elevated UTE, whereas after 12 weeks of treatment only 20% presented elevated UTE).[508]

There is little evidence about the effect of antiproteinuric treatment in UTE. However, in a small study based on 19 T2DM patients with high-normal / grade 1 HBP and 33 T2DM normotensive patients, the administration of an ARB for 18 months in the former group was associated to normalization of HBP after only 2 months of active treatment and to a stabilization in both albuminuria and UTE; in the group of 33 patients, not treated with ARB, there was a significant increase in both albuminuria and UTE after 18 months of follow-up.[514] In another cohort of 11 patients treated with ARB for 8 weeks, there was a significant reduction of UTE during the treatment period, whereas the albumin excretion remained unchanged.[515]

The evidence supporting the usefulness of UTE in patients with primary glomerulonephritis is scant in comparison with that available for diabetic nephropathy. However, the few published studies reported a significant direct correlation between UTE and albumin excretion.[516-518] Moreover, in a study of 17 patients with glomerulonephritis and albuminuria A2, 35 T2DM patients with albuminuria A2 and 38 healthy controls, the UTE was comparable between glomerulonephritis and T2DM subgroups and significantly higher than in the controls.[516]

4.3 Urinary collagen type IV excretion

4.3.1 *Rational and pathophysiology*

ColIV is a large, non-fibrillar and distinct form of collagen found only in basement membranes, constituting their major insoluble scaffold. ColIV consists of a family of six, genetically distinct isoforms: α1 to α6 chains, which associate to form distinct ColIV molecules (together with many additional components, like laminin and proteoglycans, highlighting the complexity and variability between basement membranes in different organs).[519] The epithelial cells produce ColIV as an approximately 400nm long monomer, composed of: large globular non-collagenous domain at the C terminus (NC1 domain); an extended collagenous domain of 350nm (monomers have numerous interruptions in their regular Glycine-X-Y sequence repeats, which make them more flexible than the monomers of the fibrillar collagens); a short non-collagenous N-terminal domain (7S domain). These monomers are assembled in trimers when their 3 α-chains come together through associations among their non-collagenous domains, followed by folding of the collagenous domains into triple helices; these newly assembled ColIV monomers do not undergo processing before incorporation into the basement membrane matrix.[520, 521] In the basement membrane matrix these triple-helical molecules further associate to form supramolecular networks by dimerization at the C terminus through NC1 domains and by formation of tetramers at the N terminus (7S domain).[522, 523] This protomeric network serves as a network scaffold for other basement membrane proteins, but ColIV may also bind various cells via surface receptors such as integrins.[524]

In the kidney, differences in ColIV composition may hint towards specific roles of specialized basement membranes.[525] In adults, the human renal tubular basement membrane that surrounds proximal tubules consists exclusively of α1 and α2 protomers, whereas distal tubules also contain some α3 through α6 chains.[526] In GBM, two networks with distinct chain composition have been identified: an α1 and α2 network assembled first in the embryonic glomerulus and a more cross-linked α3, α4 and α5 network (appearing after a developmental switch), characterized by loops and supercoiling that forms the GBM of the mature glomerulus in humans.[527, 528]

The specific enzymes responsible for basement membrane dissolution and the processing of ColIV are not well known, and nor is the route of elimination of the

products of its catabolism. MMP-2 and MMP-9 were initially called type IV collagenases due to their ability to degrade this abundant component of basement membranes *in vitro*.[529, 530] However, this concept has been challenged by the weak MMP-2 and MMP-9 collagenolytic activity *in vivo* and by the fact that the double-null mice for MMP-2 or MMP-9 remain viable, with no basement membrane defects.[531, 532] Besides, bioactive fragments released by the proteolytic cleavage of ColIV (called matricryptins) are involved in the regulation of several physiological and pathological processes such as development, angiogenesis, tumour growth and metastasis, with antiangiogenic and antitumoural activity.[533] Nevertheless, given the high molecular weight of ColIV trimers (approximately 540-KDa), they are not filtered through the glomerulus and their urinary excretion of ColIV seems to be only marginally affected by the serum levels; thus, since ColIV is the main component of GBM and mesangial matrix, its urinary excretion may reflect the rate of matrix turnover (degradation).[534-539]

The major characteristics of diabetic nephropathy include tubular hypertrophy, thickening of tubular basement membrane, interstitial fibrosis, GBM thickening, mesangial expansion and hypertrophy and accumulation of extracellular matrix proteins such as ColIV and fibronectin.[540] Thus, ColIV is one of mediators in diabetic nephropathy pathophysiology, contributing to the progression of glomerulosclerosis and tubulointerstitial fibrosis. Actually, several experiments have demonstrated that elevated glucose levels up-regulates transcription of TGF-β1 in multiple renal cell types, including mesangial and tubular cells.[541, 542]

In the glomeruli, TGF-β1 exerts pleiotropic effects, inducing hyperplasia and hypertrophy of mesangial cells and apoptosis and detachment of podocytes.[543, 544] Moreover, TGF-β1 increases deposition of extracellular matrix, not only by stimulating synthesis of individual matrix components (like fibronectin and ColIV), but also by blocking matrix degradation (decreasing the synthesis of proteases and increasing the levels of protease inhibitors).[542] As mentioned above, in FD there is also evidence of activation of the TGF-β1 pathway and increased synthesis of ColIV and fibronectin, as demonstrated in human podocytes cultured with lyso-Gb3.[18]

The role of the TGF-β1 pathway in tubulointerstitial fibrosis is not well established, but ColIV is one of the key mediators of fibrosis in the interstitial compartment and tubular atrophy. The TGF-β1 not only induces collagen production by interstitial

fibroblasts, but also promotes tubular epithelial cell injury and apoptosis; moreover, the TGF-β1 *per se* and changes in ColIV structure and architecture of tubular basement membrane promote epithelial-mesenchymal transdifferentiation of tubular epithelium into a more fibroblast-like phenotype, increasing the expression of collagen and fibronectin.[521, 541]

As the mesangial matrix and GBM seem to be the source of ColIV, it may reflect the mesangial expansion and GBM thickening observed in diabetic nephropathy, corroborated by histological correlation studies, showing significant correlations between urinary ColIV excretion and mesangial expansion or tubulointerstitial injury scores, as well between urinary ColIV excretion and glomerular or tubulointerstitial ColIV expression areas.[545, 546]

4.3.2 *Urinary collagen type IV excretion in renal diseases: state of the art*

For the aforementioned reasons, urinary ColIV excretion has been evaluated as a biomarker of diabetic nephropathy in numerous studies. In FD nephropathy there are no published studies evaluating the diagnostic and prognostic value of urinary ColIV excretion.

In patients with T2DM there are several reports, evaluating up to 698 diabetic patients, showing a significant increase in urinary ColIV in diabetic patients, in comparison with healthy controls.[469, 497, 534, 537-539, 546-554] Furthermore, a few studies have reported an increase in urinary ColIV even in normoalbuminuric diabetic patients, in comparison with healthy controls or the established normal range (between 26 - 49% of T2DM with albuminuria A1 presented an increase in urinary ColIV).[469, 497, 534, 536-538, 547, 549, 550, 552-556] The value of urinary ColIV as an early biomarker of diabetic nephropathy is clearly shown in a study based on 82 T2DM patients (with albuminuria A1 and normal urinary ColIV excretion), in a longitudinal study during 6 months. After the follow-up period there was a significant increase in both urinary ColIV and albuminuria, but while urinary ColIV was above the reference value in 62.2% of the patients at the end of the study, only 32.9% presented albuminuria A2; in the subgroup of patients with HBP at baseline, a significantly higher proportion of patients developed urinary ColIV or albumin above the reference range.[547] Moreover, high urinary ColIV excretion was also reported as early in normoalbuminuric patients with impaired glucose tolerance.[535]

However, in one study the urinary ColIV excretion was similar between healthy controls and normoalbuminuric T2DM patients.[546] Moreover, a variable percentage (between 33 - 42%) of patients with evidence of increased albuminuria did not show an increase in urinary ColIV excretion.[469, 536, 555]

A significant correlation between urinary ColIV excretion and albuminuria was found in most of the studies,[469, 534, 536, 537, 547, 550, 552-557] as well as with other mesangial matrix proteins excretion, like laminin.[469] In one study including T1DM and T2DM patients, a significant inverse correlation was found between the urinary ColIV and the reciprocal of serum creatinine (there was no correlation between albuminuria and reciprocal of serum creatinine), but there was no significant correlation between urinary ColIV and albuminuria.[558] This result was further confirmed in a few other studies with T2DM patients that demonstrated a significant inverse correlation between urinary ColIV and creatinine clearance or estimated GFR.[539, 546, 554]

Various authors have documented the prognostic value of urinary ColIV excretion: in a group of normoalbuminuric T2DM patients, after 1 year of follow-up, there was a significant increase in urinary ColIV in the patients progressing to albuminuria A2, whereas there was a significant decrease in urinary ColIV in the non-progressors;[536] another 1-year follow-up study showed that 76.8% of the normoalbuminuric T2DM patients in the high urinary ColIV group developed albuminuria A2, as opposed to 22.6% patients in the normal urinary ColIV group (in multivariate analysis urinary ColIV was an independent risk factor for progression to albuminuria A2);[556] in a large cohort of 254 T2DM patients (with albuminuria A1 and A2), after a median follow-up of 8 years, urinary ColIV was the strongest predictor (in multivariate regression analysis) of estimated GFR annual decline (albuminuria was not a significant predictor in the same model) and the patients above median cut-off level presented significantly higher decline in estimated GFR (in comparison with those below), regardless of the baseline albuminuria;[557] similar results were observed in a cohort of 30 patients with overt proteinuria, where after a follow-up period of 4.2 years, baseline urinary ColIV excretion was negatively correlated with the subsequent GFR change.[559]

The prognostic value of urinary ColIV was also corroborated in T1DM patients, in a study involving 231 patients (with albuminuria A1 or A2 at baseline), with a mean follow-up period of 7.4 years. Urinary ColIV excretion presented a significant inverse correlation

with estimated GFR annual change in multivariate regression analysis; moreover, in the subgroup of patients with albuminuria A1, only urinary ColIV (and not albuminuria) was significantly correlated with estimated GFR decline, and the rate of estimated GFR decline was significantly higher in patients presenting elevated urinary ColIV (in comparison with those with normal urinary ColIV).[560] Another study based on 225 normoalbuminuric T1DM patients, followed by a median period of 8.8 years, identified, in multivariate Cox proportional hazards analysis, high urinary ColIV as the only significant risk factor for development of albuminuria A2.[561]

Most studies reported no evidence of correlation between urinary ColIV and blood pressure, duration of diabetes or glycaemic control (mainly in multivariate regression analysis).[469, 536, 547, 550, 558] However, two studies reported a significant direct correlation between urinary ColIV and HbA1c and another between urinary ColIV and duration of diabetes.[534, 537, 549] Furthermore, another study reported a significant correlation between urinary ColIV and diastolic blood pressure, as well a significantly higher excretion of urinary ColIV in patients with HBP.[557]

Urinary ColIV excretion seems to be distinctively affected by different classes of agents for treatment of T2DM. In a study with T2DM patients (with albuminuria A2), comparing 13 patients treated with an agonist of glucagon-like peptide-1 with 18 patients treated with a sulfonylurea, after 16 weeks of treatment, there was a similar decrease in HbA1c in both groups, but only the subgroup of patients treated with an agonist of glucagon-like peptide-1 showed a significant decrease in albuminuria, urinary TGF-β1 and urinary ColIV.[562] A thiazolidinedione was also compared with a sulfonylurea in T2DM patients with albuminuria A2 or A3; as mentioned above, after 12 months of treatment, sulfonylurea did not influence albuminuria or urinary ColIV, but in thiazolidinedione group a significant decrease in albuminuria and urinary ColIV was only observed in the subgroup of patient with albuminuria A2.[563] However, in another study (recruiting T2DM patients with proteinuria > 500mg/day) a thiazolidinedione derivative significantly decreased proteinuria, urinary TGF-β1 and urinary ColIV, in comparison with placebo, after 12 weeks of treatment.[564]

The effect of antiproteinuric treatment in urinary ColIV excretion has only been evaluated in a few longitudinal studies, with different designs. One of them, in patients with T2DM, enrolled 43 patients to receive an ACEi and 88 control patients (not treated

with an ACEi), with a follow-up period of 24 months. In the control group urinary ColIV significantly increased time-dependently, whereas in the ACEi group there was no change, thus in the ACEi group the urinary ColIV was significantly lower than in the control group at 24 months.[565] Other study assigned 22 T2DM patients to receive an ARB for 6 months and, although a significant decrease in urinary TGF-β1 was noted, urinary ColIV was not significantly altered by the treatment.[566] Finally, a study compared the effects of ACEi and ARB, after a follow-up period of 11 months, and a significant decrease in albuminuria was found in both groups, but urinary ColIV only significantly decreased in the ARB group.[567]

Concerning patients with other non-diabetic renal diseases, there is little evidence supporting the use of urinary ColIV. In most of the studies urinary ColIV has been used for differential diagnosis between diabetic and non-diabetic nephropathies, because a significant increase in urinary ColIV was reported (in comparison with healthy controls), but the magnitude of the increase or the urinary ColIV to albuminuria ratio was significantly lower in comparison with the diabetic population.[469, 537] Moreover, in one of the studies there was no significant correlation between urinary ColIV excretion and albuminuria in patients with chronic glomerulonephritis.[537] These results were confirmed in a study with 527 biopsy-proven non-diabetic renal diseases, but this study highlighted that urinary ColIV was distinctively higher in the subgroups of patients with membranous nephropathy and anti-neutrophil cytoplasmic antibody-associated glomerulonephritis, in comparison with the other forms of non-diabetic renal disease (however, even in these subgroups the magnitude of increase was lower in comparison to the diabetic population).[568] Contrariwise, in another report the level of urinary ColIV was similar between healthy controls and patients with non-diabetic glomerular diseases.[546]

5. Urinary biomarkers of tubular injury

5.1 Tubular physiology and pathology: rational for the categorization of biomarkers

The kidney is the principal organ that maintains the amount and composition of extracellular fluid, essential for cell viability. Most of this function occurs in the kidney tubule, whose components are: proximal convoluted tubule, thin limbs of the loop of Henle, thin ascending limb of the loop of Henle and distal convoluted tubule.

Proximal convoluted tubule function is critical for body haemostasis, because it is a *tour de force* of reabsorption of the 160-170L of primary glomerular ultrafiltrate produced each day. The proximal tubule fulfils most of the reabsorptive role for water, sodium chloride and sodium bicarbonate, leaving the fine-tuning to the distal nephron; it also completes the reabsorption of glucose, amino acids, phosphate and citrate.[569] The proximal tubule also performs metabolic functions: conversion of vitamin D to active and inactive forms and gluconeogenesis.[570, 571] Normal proximal tubular function is essential for reabsorption of filtered proteins; these molecules are reabsorbed and catabolized to amino acids in the proximal tubular cells in complex transport processes, that are essential to provide the nitrogen and carbon skeleton required to support renal gluconeogenesis and ammoniagenesis; moreover, proximal tubular cells promote secretion of several organic compounds, including proteins, to the tubular lumen, by active transport mechanisms.[569]

The main function of the thin and thick portions of the loop of Henle is urinary concentration, promoting the generation of a gradient of increasing osmolality along the medulla; moreover, they also participate in calcium and magnesium homeostasis, bicarbonate and ammonium homeostasis and urinary protein composition.[572, 573] The distal convoluted tubule plays a critical role in sodium chloride reabsorption, potassium secretion and calcium and magnesium handling; furthermore, it has a unique capacity to adapt to changes in hormonal stimuli and the contents of the tubular lumen.[574]

In the pathogenesis of diabetic nephropathy, glomerular pathophysiology has attracted the attention of research for many years. However, the correlation between the degree of glomerular injury and GFR decline is far from perfect, and tubular atrophy and interstitial fibrosis are apparent before overt proteinuria and more clearly associated with the prognosis of kidney function.[575, 576] Thus, there is increasing research about the role of tubulointerstitial injury in the pathogenesis of diabetic nephropathy; hyperglycaemia, advanced glycosylation end products and oxidative stress are some of the triggering factors pathophysiological activating pathways, leading to tubular toxicity and interstitial fibrosis.[577-579] Early tubulointerstitial damage may be the trigger for subsequent glomerular injury in the hyperfiltration stages of diabetic nephropathy, known as the tubular hypothesis of glomerular filtration; moreover, tubular atrophy can promote increased intraglomerular pressure and subsequent glomerular injury.[580] Nonetheless,

proteinuria resulting from glomerular damage may promote direct toxicity to the tubular cells, in a vicious circle of progression in diabetic nephropathy.[581]

Therefore, searching for biomarkers reflecting tubular injury is paramount for early identification of tubulointerstitial pathology. There are two main categories of urinary markers of tubular damage: low molecular weight proteins and urinary enzymes, both characterizing tubular proteinuria. Low molecular weight proteins (lower than 40-KDa), like retinol-binding protein (RBP), α1-microglobulin (A1MG) or β2-microglobulin are freely filtered through the glomerular membrane and depend on proximal tubular cell integrity to be reabsorbed. Thus, in the context of proximal tubular injury, their reabsorption is impaired, increasing their urinary concentration.[582] Urinary enzymes, like N-acetyl-β-D-glucosaminidase (NAG) and alanine aminopeptidase (AAP) are high molecular weight proteins, so they are not filtered through the glomerulus; they originate in renal tubular cells (mainly located in brush-border membranes, lysosomes and cytoplasm) and are excreted in urine, increasing their secretion from tubular cells if the tubules are damaged.[583]

5.2 Urinary α1-microglobulin excretion

5.2.1 *Rational and pathophysiology*

A1MG is a low molecular weight (27-KDa) plasma glycoprotein, belonging to the lipocalin superfamily.[584] This glycoprotein is synthesized in the liver, by a unique gene encoding both A1MG and bikunin, which has no known relation to A1MG other than co-synthesis. Subtilisin-like proprotein convertases are involved in the intracellular cleavage of inactive precursor proteins, still in the hepatocyte.[585-587]

After secretion to blood, the protein exists in free form, as well as in a variety of high molecular weight complexes: approximately 50% forms a 1 to 1 complex with monomeric immunoglobulin A, approximately 7% are linked to albumin and 1% to prothrombin.[588, 589] Tissue distribution studies showed that A1MG is found in most tissues, with the exception of the central nervous system, but liver, plasma and kidney are major sites of localization (reflecting the major phases of the metabolism of A1MG).[587, 590] A1MG plays an important physiological role, because A1MG deficiency has not been reported in any species (suggesting that its absence is lethal); however, its function is not

well established, being certainly involved in immunosuppression and control of inflammation, as well as in protection against oxidative agents.[591]

Plasma levels of A1MG are apparently very stable, even in pathological conditions, like neoplasms, central nervous system disorders, infections and rheumatoid arthritis; thus, A1MG does not met the description of an acute phase reactant.[592, 593] Therefore, altered total plasma or serum A1MG concentration is usually related to impaired liver or kidney function; A1MG is synthesized in the liver, so severe liver insufficiency leads to a decrease in serum level; free A1MG is freely filtered through the glomerulus, so its plasma concentration is, in contrast to A1MG bound to immunoglobulin A, mainly determined by GFR, increasing proportionally to decreasing creatinine clearance.[587, 594]

As mentioned above, free A1MG is cleared from plasma by the kidney, where it passes relatively freely through the glomerular membranes out into the primary urine; afterwards it is 99% reabsorbed by the proximal tubules, via the endocytic receptor megalin, where catabolization occurs.[595, 596] Normal urine contains a very small amount of free A1MG and none of the complexes can be detected.[597-599] Therefore, in conditions with disturbed tubular function, reabsorption of A1M is reduced and increased amounts are found in urine, making it a marker for renal tubular disorders.

One of the advantages of A1MG as biomarker is its stability in human urine *ex vivo*, at different pHs.[600, 601] Furthermore, A1MG is stable in urine stored with or without preservative at room temperature for 7 days or for longer periods at -4, -20 and -70ºC and the addition of a preservative solution preserves A1MG at -20ºC for more than 12 months.[602, 603]

5.2.2 *Urinary α1-microglobulin excretion in renal diseases: state of the art*

Urinary A1MG excretion has been evaluated as a biomarker in several kidney disorders, including diabetic nephropathy. A small study in FD, recruiting 17 women (13 of them under ERT) reported, after a mean follow-up period of 6 years, a significant decrease in A1MG in the treated patients; an opposite trend of an increase in urinary A1MG was observed in the untreated patients after a mean follow-up period of 5 years.[307]

In patients with T2DM, several studies, evaluating up to 590 diabetic patients, reported higher urinary A1MG excretion in diabetic patients (increasing with the severity

of the nephropathy) than in healthy controls.[469, 578, 579, 604-610] In the subgroup of T2DM patients with albuminuria A1, there are reports showing an increase, in comparison with controls or reference values, of urinary A1MG in 12.3 – 33.6% of the patients,[558, 578, 579, 607, 608, 611, 612] but there is also studies showing similar levels in normoalbuminuric patients and healthy controls[605] and patients with albuminuria A2 or A3 (between 44.8 – 49.4%) presenting A1MG within the normal range.[469, 607] For example, in a study recruiting 590 patients with T2DM, urinary A1MG excretion increased according to the severity of albuminuria; however, 33.6% of the normoalbuminuric patients presented urinary A1MG above the reference values, although 44.8% of the patients presenting albuminuria A2 and A3 had urinary A1MG within the reference range (in the group of patients with normal A1MG, 27.6% had albuminuria A2 or A3).[607] One study, recruiting T1DM and T2DM patients and healthy volunteers, showed that urinary A1MG had a diagnostic accuracy of 89.0% in identifying diabetic patients.[613] These results suggest urinary A1MG as a marker of renal dysfunction that may complement markers of glomerular dysfunction such as albumin rather than outperform them.

These results were also corroborated in T1DM, in a few studies involving up to 285 patients (adults and children) there was a significant increase in urinary A1MG excretion in the diabetic population (in comparison with healthy controls),[614, 615] even in normoalbuminuric patients.[616-619]

Surprisingly, in a group of 53 T2DM patients with albuminuria A2, undergoing A1MG urinary measurement and kidney biopsy, the increase of urinary A1MG was significantly higher in the subgroup of patients with “typical” diabetic glomerulopathy, than in the subgroup of patients with absent or mild diabetic glomerular changes, associated with disproportionately severe tubulointerstitial lesions.[620]

The correlation between urinary A1MG excretion and biomarkers of glomerular damage seems significant, because only one study reported no correlation with albuminuria,[617] whereas others reported not only a correlation with albuminuria, but also with other biomarkers of glomerular injury (namely, UTE, urinary ColIV and podocyte nephrin).[469, 508, 578, 579, 607, 611, 614, 615, 619, 621] In one study, urinary A1MG was significantly correlated with a pathophysiological mediator of diabetic nephropathy, TGF-β1.[615] There is little evidence regarding the correlation between urinary A1MG excretion and GFR,

with two studies showing a significant inverse correlation with estimated GFR[578, 579] and another demonstrating no correlation between urinary A1MG and serum creatinine.[614]

There is conflicting evidence concerning the correlation between urinary A1MG and diabetes duration, with a few studies showing an increase according to diabetes duration,[607, 611, 614, 616, 618] while other not.[617] No correlation with blood pressure was shown in various studies.[607, 614] Only two studies reported higher values of urinary A1MG in male patients, as well as with increasing age.[607, 608]

A significant correlation between urinary A1MG levels and glycaemic control (measured by HbA1c) was reported in several studies.[508, 607, 611, 614, 615, 617, 618, 622] Moreover, in newly diagnosed T2DM patients, A1MG significantly decrease with the treatment with antidiabetic agents.[508, 618, 622] Different classes of antidiabetic agents seem to distinctively affect urinary A1MG excretion. In a study with normoalbuminuric T2DM patients, comparing 17 patients treated with a thiazolidinedione derivative with 17 patients treated with a sulfonylurea, urinary A1MG was elevated at the beginning of the study (and significantly correlated with albuminuria), with no difference between groups, whereas after 12 months of treatment urinary A1MG was significantly lower in the thiazolidinedione group compared to the sulfonylurea group.[623] These results were further confirmed in another study with the same classes of antidiabetic agents.[624]

Treatment with a calcium channel blocker or an ACEi for 12 months significantly decreased the urinary A1MG excretion in a cohort of 20 patients with T1DM and albuminuria A2.[625]

Several studies evaluated urinary A1MG as biomarker in other nephropathies, namely caused by intoxication with heavy metals, primary glomerulopathies, Balkan nephropathy and acute pyelonephritis and vesicoureteral reflux.[626, 627] Urinary A1MG has been used to identify early nephropathy due to occupational exposure to heavy metals, like cadmium, lead and mercury.[628-630] In a longitudinal study, evaluating 54 patients with various primary glomerulopathies, with a follow-up period of 49 months, urinary A1MG excretion had a significant correlation with biomarkers of glomerular damage (albuminuria and urinary immunoglobulin G excretion) and an inverse correlation with creatinine clearance; moreover, during follow-up urinary A1MG did not change significantly, but its change was significantly correlated with urinary immunoglobulin G.[631] Another study in patients with idiopathic membranous nephropathy demonstrated that

excretion of immunoglobulin G and A1MG was associated with the extent of tubulointerstitial damage, but only A1MG excretion was associated with global glomerular sclerosis and arteriolar hyalinosis; excretion of immunoglobulin G and A1MG had a predictive value for both remission and progression and was useful to identify patients at risk of progression and for whom immunosuppressive therapy is indicated.[632]

5.3 Urinary N-acetyl-β-D-glucosaminidase excretion

5.3.1 *Rational and pathophysiology*

NAG is a high molecular weight (140-KDa) lysosomal enzyme (also known as β-N-acetylhexosaminidase or β-hexosaminidase), belonging to the group of exoglycosidases (that catalyse sequential removal of terminal, non-reducing sugar units from the oligosaccharide component of a variety of large molecules).[633] This enzyme is ubiquitously synthesized and is a dimeric enzyme (composed of two polypeptide chains / subunits: α and/or β), thus three main NAG isoenzymes are recognized: A (heterodimeric αβ), B (homodimeric ββ) and S (homodimeric αα, which is unstable and has limited catalytic activity).[634] α and β subunits are encoded in different chromosomes and protein moiety is synthesized in rough endoplasmic reticulum as pre-propolypeptide α and β, afterwards undergoing post-translational modifications (glycosylation, phosphorylation, proteolysis, folding and formation of disulfide bonds) and dimerization, forming stable NAG isoenzymes that are delivered to lysosomes.[635]

The main function of NAG is the cleavage of glycosidic linkages of the non-reducing, terminal β-D-N-acetylglucosamine or β-D-N-acetylgalactosamine residues in glycolipids, glycoproteins and glycosaminoglycans; β-hexosaminidase deficiency impairs the hydrolysis of GM2 gangliosides and is the biochemical defect in GM2 gangliosidosis: Tay-Sachs disease (mutation in α subunit) and Sandhoff disease (mutation in β subunit).[636] Nowadays, NAG is being increasingly associated with other pathological conditions, like liver diseases, multiple sclerosis and neoplasms.[635]

In kidneys, NAG is distributed along the whole nephron, but with very high activity in lysosomes of proximal tubule (mainly the straight portion).[637] NAG's high molecular size precludes filtration through the glomerulus, even in glomerular diseases (analysis of isoenzymes of NAG demonstrated that the increased urinary excretion of this enzyme is

due to an increased release by the renal tubular cells and not to increased filtration across the damaged glomerular capillary wall), so urinary NAG is derived from the renal tubule.[633, 638] Isoenzyme A is a part of soluble intralysosomal compartment and is secreted in urine by exocytosis, thus its urinary activity reflects the secretory activity of tubular cells (functional isoenzymuria); isoenzyme B is intralysosomal and membrane bound and is released in urine together with disrupted lysosomal membranes, so its urinary activity reflects breakdown of tubular cells (lesional type isoenzymuria).[639] However, increased urinary NAG excretion may not only occur in tubular damage, but also in a situation of increased lysosomal turnover and may be a measure of tubular function.[640]

Due to aforementioned reasons, urinary NAG has emerged as a biomarker of tubular injury; moreover, it has other qualities as a biomarker: stability in urine and storage at 4ºC for 2 days or at -20ºC for several months without degradation.[633]

5.3.2 *Urinary N-acetyl-glucosaminidase excretion in renal diseases: state of the art*

Urinary NAG excretion was evaluated as a biomarker of tubular injury in several nephropathies, mainly in diabetic nephropathy. There is only unpublished evidence of increased urinary NAG excretion in FD.[641]

In patients with T2DM, a few studies, based on up to 672 patients, reported a significant increase in urinary NAG excretion, in comparison with healthy controls; moreover, this increase was progressive according to the increase in the severity of diabetic nephropathy and occurred even in normoalbuminuric patients (between 25 - 62% of the patients in this group had increased urinary NAG).[604, 605, 611, 642-654] For example, in one study with 70 T2DM patients, urinary NAG was the most sensitive biomarker and with best diagnostic accuracy to identify patients with early renal involvement (albuminuria A2).[642] However, two studies showed an increase of urinary NAG in T2DM patients, but not in normoalbuminuric patient, where NAG excretion was similar to that of healthy controls.[655, 656] Moreover, in another study comparing urinary NAG excretion between T2DM patients with glomerular hyperfiltration, T2DM patients with normal GFR and healthy controls, although a trend of its increased excretion in the two T2DM subgroups in comparison with the controls was observed, the difference was not statistically significant.[657]

Similar results were found in patients with T1DM, with studies based on up to 659 patients showing an increase, in comparison with healthy controls, in urinary NAG (with a progressive increase according to nephropathy severity), even in normoalbuminuric patients.[650, 658-661]

There is conflicting evidence regarding the correlation between urinary NAG and albuminuria / proteinuria or GFR. Most studies showed a significant correlation with albuminuria and/or a significant inverse correlation with estimated GFR / creatinine clearance;[642, 643, 646, 647, 649, 654, 661-665] however, two studies reported no significant correlation between urinary NAG and proteinuria or creatinine clearance;[657, 660]

The prognostic value of urinary NAG regarding nephropathy progression was established in several studies. A longitudinal study including 296 T1DM with albuminuria A2, followed for 2 years, reported that albuminuria A2 regressed to albuminuria A1 in 24% of the patients, progressed to albuminuria A3 in 12.5% and remained stable in the others. In comparison with the other subgroups, urinary NAG excretion was significantly lower in the regression subgroup and 39% of the patients who regressed to albuminuria A1 were in the lower quartile of urinary NAG excretion (with a significant and progressive decrease in this percentage across the patients in higher quartiles, even after adjustment for other variables).[658] Another two studies corroborated these results in other cohorts of T1DM patients, one of them not only showing higher urinary NAG excretion in patients progressing from albuminuria A1 to A2 or A3, but also identifying this biomarker as a significant predictor (with a slightly higher odds ratio than albuminuria) for development of albuminuria A2 or A3.[666, 667]

Several studies reported a significant correlation between urinary NAG and glycaemic control (HbA1c).[642-644, 646, 647, 649, 659, 660] Only 3 studies showed no correlation between urinary NAG and HbA1c.[648, 656, 663] Moreover, glycaemic control with diet was associated with a significant decrease in urinary NAG excretion.[646, 647, 668]

The correlation between urinary NAG and diabetes duration was inconsistent, with a few studies showing a significant correlation,[642] while others not.[660] One study showed that urinary NAG excretion rises in the third year of diabetes, plateaux until the tenth year and, afterwards, increases rapidly.[653]

In one study no difference in urinary NAG excretion was identified between patients with and without HBP,[669] while in another there was a significant correlation

between urinary NAG and blood pressure.[645] In a study comparing T2DM patients, HBP patients (without diabetes) and healthy controls, urinary A1MG was similar in patients with HBP and healthy controls.[652] Nonetheless, in another study patients with HBP had significantly higher NAG excretion than healthy controls.[670]

Urinary NAG excretion in primary glomerulopathies was evaluated in a study enrolling 136 patients with idiopathic membranous nephropathy, primary focal and segmental glomerulosclerosis or minimal change disease. A significant correlation was identified between urinary NAG and a biomarker of glomerular damage (urinary immunoglobulin G), and urinary NAG was significantly higher in patients with nephrotic range proteinuria, in comparison with the remaining patients. In 43 patients with nephrotic range proteinuria performing kidney biopsy, a non-significant increase in urinary NAG was found in patients with higher scores of tubulointerstitial damage. Furthermore, urinary NAG was the most significant predictor of remission, progression and response to therapy, after a mean follow-up of 42 months, in patients with idiopathic membranous nephropathy and primary focal and segmental glomerulosclerosis.[671] This increased urinary NAG excretion and the correlation with tubular atrophy and interstitial fibrosis was further confirmed in another study in patients with glomerulonephritis.[672] The prognostic value of urinary NAG to predict progression and remission in patients with idiopathic membranous glomerulonephritis was also confirmed in an additional study.[673] Another study showed a significant increase in urinary NAG, in comparison with controls, in patients with non-diabetic-CKD.[646]

In patients with tubular injury due to various insults, like drugs, heavy metals, rhabdomyolysis and poisoning by chemicals, there is an increase in urinary NAG; however, urinary NAG excretion seems less sensitive than low molecular weight protein biomarkers in the identification of tubular damage in such patients.[674] Increased urinary NAG excretion has been observed in some urological abnormalities in children, like hydronephrosis and vesicoureteral reflux.[675]

5.4 Urinary alanine aminopeptidase excretion

5.4.1 *Rational and pathophysiology*

AAP is a microsomal exopeptidase that catalyses the splitting of an N-terminal amino acid, mainly alanine, from a peptide, amide or arylamide. Human kidney AAP is a high molecular weight (240-KDa) glycoprotein, composed of two subunits. In urine AAP can be found in two different forms, a soluble and a particulate AAP, which can be separated by ultracentrifugation and electrophoresis.

AAP is localized on the tubules' brush border membrane, mainly in the proximal straight tubules; normally present in urine in small quantities, possibly owing to simple avulsion caused by laminar flow within the tubules. In injured tubules, brush border membrane may be damaged, losing microvilli; these alteration in the membrane causes an increase in urinary AAP activity originating from microsomes and cytosol.[676]

The determination of urinary AAP should account for a few characteristics: seems to undergo an infradian rhythm with a cycle of around 10 days;[677] has an inverse correlation with age, with higher activity in healthy children;[678, 679] no apparent gender difference in excretion when expressed in relation with urinary creatinine.[680]

The specific activity of AAP in urine may be measured by various methods: spectrophotometrically by using L-alanine-4-nitroanilide and its derivatives as the substrates, and kinetic fluorimetric assay with the substrate L-alanine-β-naphthylamide and ELISA.[681] AAP is stable for only 4 hours after urine collection, which limits its clinical utility; moreover, filtration through a chromatographic column is advisable to eliminate interfering substances including low molecular weight AAP inhibitors, like amino acids and ammonia.[682]

5.4.2 *Urinary alanine aminopeptidase excretion in renal diseases: state of the art*

The evidence supporting the utilization of AAP as a biomarker of tubular injury is limited in comparison to the previously mentioned biomarkers. In FD, in the literature review AAP has never been studied.

Several studies, recruiting T2DM and/or T1DM patients, reported an increase in urinary AAP excretion, in comparison with healthy controls;[604, 645, 646, 683-687] moreover, a progressive increase in AAP excretion according to the severity of the diabetic

nephropathy was identified in some studies[688] and/or an increase in normoalbuminuric patients (in up to 22.4% of these patients) was also demonstrated in few studies.[645, 684, 685]

However, in a study evaluating 30 T2DM male patients, 6 of them with albuminuria A2 and the remaining with albuminuria A1, urinary AAP excretion was similar comparing T2DM patients and healthy controls. Moreover, after 6 months of aerobic exercise 5 out of the 6 patients with albuminuria A2 regressed to albuminuria A1, but urinary AAP did not change.[689] Another study based on T1DM patients also showed no increase in urinary AAP in diabetic patients, in comparison with healthy controls.[690]

A few studies have demonstrated a direct correlation between urinary AAP and albuminuria / proteinuria,[685, 686] as well as an inverse correlation between AAP excretion and estimated GFR.[646, 685]

Concerning the correlation between urinary AAP and glycaemic control (HbA1c), various studies showed conflicting result, with some demonstrating a significant correlation,[688] while others not.[646, 684] Moreover, glycaemic control with diet did not change the urinary AAP excretion.[646] Two studies reported no significant correlation between AAP excretion and blood pressure.[684, 688]

In patients with primary glomerulonephritis, urinary AAP excretion was significantly increased, in comparison with healthy controls and correlated with histological tubular atrophy and interstitial fibrosis.[672] Nonetheless, in another study AAP excretion in controls was similar to that in patients with membranous glomerulonephritis, immunoglobulin A nephropathy and lupus nephritis.[683] A similar result was found in another study in patients with non-diabetic CKD.[646]

It was reported that occupational exposure to heavy metals (arsenic, cadmium, lead) and tobacco as well as exposure to low levels of environmental cadmium induces an elevation of AAP activity in the urine of copper smelter workers and the urine of studied residents, respectively.[691, 692]

Aims

Stemming from the urgent need for better and reliable biomarkers and bearing in mind the theoretical background established in the introduction, my main key goal is the identification of serum and urinary biomarkers to detect cardiac and renal involvement, respectively, in patients with FD.

Concerning serum biomarkers of cardiac injury / fibrosis, I will evaluate molecules related to collagen metabolism; urinary biomarkers, beyond urinary albumin excretion, of glomerular and tubular injury will be assessed for kidney injury.

For each biomarker I will try to establish its added value in three fields: identification of FD patients and distinction from healthy individuals; recognition of incipient organ involvement, in comparison with the current gold standards to assess early FD cardiac or renal damage; definition of the prognosis in terms of the cardiomyopathy or nephropathy progression.

Therefore, I define as primary endpoint comparison of the biomarkers between healthy controls and patients with FD.

As secondary endpoints I will: 1) compare the biomarkers among subgroups of Fabry disease patients with increasing severity of heart or kidney involvement; 2) evaluate the longitudinal variation of these biomarkers and their correlation with the progression of the organ involvement.

As exploratory endpoint, I will try to identify the effect of therapeutic interventions (ERT and inhibitors of the renin-angiotensin-aldosterone system) in the biomarkers.

To achieve these objectives, I will perform a two-year follow-up study, with annual evaluation of the biomarkers and the organ involvement. With this design I expect to establish the role and value of these biomarkers in the three aforementioned aspects.

The overarching hypothesis throughout this thesis is that the increase in these biomarkers heralds the development of functional or structural manifestations of Fabry disease and, consequently, can be a new paradigm in the identification of pre-clinical / silent involvement of the heart and the kidney in Fabry disease, with stronger evidence for earlier therapeutic intervention in order to prevent the later major organ events.

In particular, I hypothesise that the biomarkers related to collagen type I metabolism may rise in serum before the development of any other cardiac abnormalities (including the first signs of diastolic dysfunction) and may predict the appearance of overt cardiomyopathy. Moreover, I postulate that some of the studied urinary biomarkers may outperform albuminuria as early and prognostics biomarkers of renal involvement.

Materials and methods

1. Research design

Aiming the identification of biomarkers of FD cardiomyopathy and nephropathy, I developed two parallel studies, hereinafter designated as "cardiomyopathy study" and "nephropathy study", both designed as multicenter (international), longitudinal, prospective and diagnostic test studies.

FD patients and healthy controls were recruited in three centres: 1) Centro Hospitalar Lisboa Norte (Lisbon, Portugal); 2) Hospital Senhora da Oliveira (Guimarães, Portugal); 3) Royal Free London NHS Foundation Trust (London, United Kingdom), between February 2013 and June 2014. FD patients and healthy controls could be enrolled in both studies.

Both studies had the same prospective and longitudinal design: each recruited FD patient was evaluated at baseline (enrolment) and after one and two years; healthy controls were evaluated only twice (at recruitment and after one year).

In the cardiomyopathy study, I assessed biomarkers related to collagen metabolism (PICP, PINP, PIIINP, CITP, MMP-1, MMP-2, MMP-3 and TIMP-1) in an population of 60 FD patients and 20 healthy controls, according to predefined criteria described in the next section.

In the nephropathy study, I evaluated two biomarkers of glomerular damage (UTE, urinary ColIV excretion) and five biomarkers of tubular injury (urinary RBP excretion, urinary κ and λ free light chains [FLC] excretion, urinary A1MG excretion, urinary NAG excretion and urinary AAP excretion) in an estimated cohort of 75 FD patients and 25 healthy controls, whose inclusion and exclusion criteria will be described in the next section.

Given the limited knowledge about the studied biomarkers in FD, these longitudinal studies were preceded by two pilot / proof concept studies: a cardiomyopathy pilot study and nephropathy pilot study. In both studies 10 FD patients and 10 healthy controls were recruited and the corresponding aforesaid biomarkers were measured; only the differentially expressed biomarkers (between FD patients and healthy controls) were evaluated in the main longitudinal studies. FD patients recruited for the

pilot studies were also enrolled in the longitudinal studies. A schematic representation of the research design is given in Figure 4.

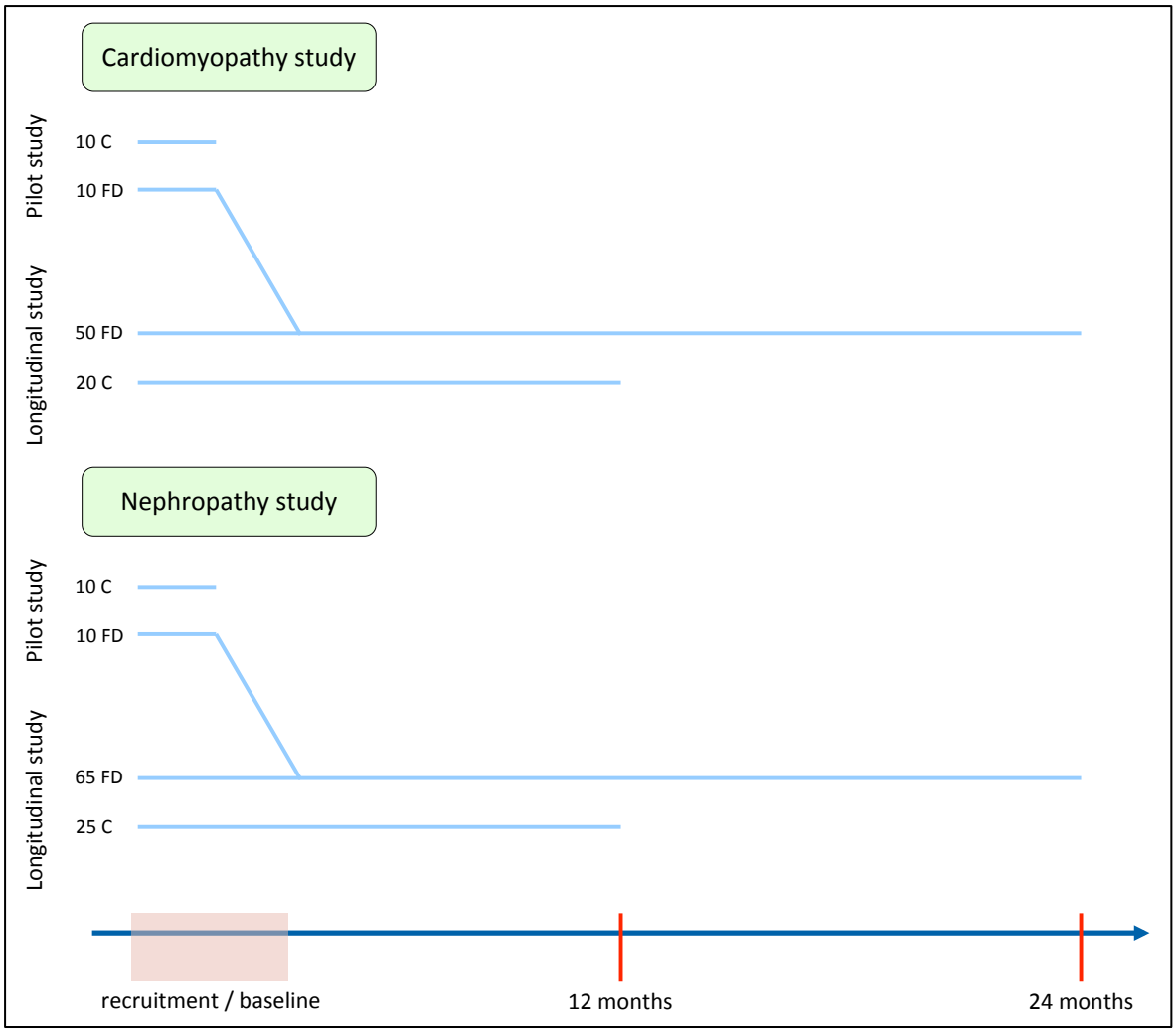


Figure 4: Research design. Two parallel studies were developed with a main objective of searching for new biomarkers of FD cardiomyopathy and nephropathy, where each FD patient or healthy control could be enrolled in any study. C: healthy controls; FD: Fabry disease patients.

According to the aims of the research and the presented design, I did not anticipate any major possible biases. Nonetheless, the possible biases were sorted out as following:

- selection bias: the selection of the patients with FD for the study. To avoid this bias, all the consecutive patients until the n defined for the study were enrolled.
- re selection bias: as consecutive patients were recruited, family groups attending together presented the same mutation, which may influence the results

of the biomarkers measurements (e.g. families presenting with mutations associated with attenuated phenotypes).

- information bias: the biomarkers measurements could be influenced by the severity of the organ involvement. To override this bias, those responsible for the laboratory analysis of the biological samples were blinded for any information about the patient or control.

2. Population

2.1 Cardiomyopathy study

The FD population was recruited according to the following inclusion and exclusion criteria. For FD patients the only inclusion criteria were diagnosis of FD and age ≥18 years old. FD was defined in males as low α-galactosidase A activity (<5% of the normal α-galactosidase A activity) and/or identification of a proven pathogenic mutation in the GLA gene and in females as the presence of a proven pathogenic mutation in the GLA gene.

The exclusion criteria defined for FD patients included any conditions, other than the usual manifestations of FD cardiomyopathy, potentially affecting cardiac collagen metabolism, as well as other diseases or medication, previously mentioned (introduction: section 3.1.1), affecting the serum levels of these biomarkers, namely:

- HCM or other cardiomyopathy;
- previous myocardial infarction;
- moderate or severe valvular heart disease;
- previous heart surgery;
- cardiac device implanted in the previous 6 months;
- surgery or major trauma within the previous 6 months;
- inflammatory or fibrotic diseases;
- active cancer;
- chronic liver disease;
- thyroid disorders;
- drugs affecting bone turnover: bisphosphonates, steroids and post-menopausal hormone replacement therapy.

The control group included healthy individuals, aged ≥18 years old, with a normal echocardiogram (as defined below) and not taking any drugs.

In order to enrol 60 FD patients representative of the entire spectrum of FD cardiomyopathy severity, the recruitment was made according to subgroups of increasing severity of FD cardiomyopathy (aiming at a specific number of patients), defined by echocardiogram:

- group 1: FD patients without any evidence of cardiac involvement: no LV hypertrophy or TDI abnormalities, as defined bellow (20 patients);
- group 2: FD patients with TDI abnormalities (defined by at least one of the following abnormalities: S' <6cm/s; E' <10cm/s, <8cm/s or <6cm/s at the septal corner of the mitral annulus, in patients ≤40 years old, between 41 and 60 years old and >60 years old, respectively; E' <14cm/s, <12cm/s or <6cm/s at the lateral corner of the mitral annulus in patients ≤40 years old, between 41 and 60 years old and >60 years old, respectively) and no LV hypertrophy (20 patients);
- group 3: FD patients with LV hypertrophy, defined as diastolic IVS or PW thickness ≥12 mm (20 patients).

The control group was age and sex-matched with the less severe FD subgroup (group 1).

As noted above, this longitudinal study was preceded by a pilot study, recruiting the following groups (enrolled according to the aforementioned inclusion and exclusion criteria):

- 10 healthy controls, with a normal echocardiogram (as defined previously), age and sex- matched with the following group;
- 10 FD patients with LV hypertrophy (as defined above) in echocardiogram.

2.2 Nephropathy study

In the nephropathy study, the inclusion criteria for FD patients were the same as for the cardiomyopathy study.

The exclusion criteria defined for the FD patients included other diseases with potential kidney involvement, namely:

- diabetes: defined as fasting glucose ≥126mg/dL or use of antidiabetic drugs;

- glomerular diseases: nephritic or nephrotic syndrome;
- immunoglobulin A nephropathy;
- systemic vasculitis;
- systemic lupus erythematosus;
- hepatitis C;
- amyloidosis;
- multiple myeloma.

The control group included healthy individuals, aged ≥18 years old, with a normal kidney function (defined as estimated GFR ≥60ml/min/1.73m^2 and ACR <30mg/g) and not taking any drugs.

To ensure the entire spectrum of severity of FD nephropathy, the recruitment of 75 FD patients was made according to subgroups of increasing severity of FD nephropathy (aiming at a specific number of patients), namely:

- group 1: FD patients without any evidence of kidney involvement: ACR <30mg/g and GFR ≥60ml/min/1.73m^2 (25 patients);
- group 2: FD patients with ACR 30-299mg/g and GFR ≥60ml/min/1.73m^2 (20 patients);
- group 3: FD patients with ACR ≥300mg/g and GFR ≥60ml/min/1.73m^2 (10 patients);
- group 4: FD patients with CKD stage ≥3 (according to Kidney Disease Improving Global Outcomes [KDIGO] guidelines),[283] defined as GFR <60ml/min/1.73m^2 (20 patients).

The control group was age and sex-matched with the less severe FD subgroup (group 1).

As mentioned above, this longitudinal study was preceded by a pilot study, recruiting the following groups (enrolled according to the aforementioned inclusion and exclusion criteria):

- 10 healthy controls, with a normal kidney function (as defined previously), age and sex- matched with the following group;
- 10 FD patients with GFR <60ml/min/1.73m^2.

3. Recruitment and compliance

The local or national Ethical Committees of each participating centre approved the study protocol and the study was conducted in accordance with this protocol and the ethical principles of the Declaration of Helsinki.

All consecutive patients until the n defined for the study were enrolled when they attended their care team for FD in their routine follow-up. Given that the study does not present any harm or scheduled visits outside the routine follow-up, I did not anticipate difficulties in enrolment of the eligible patients. Written informed consent was obtained from all participants before any study procedures.

Given that there was no recruitment of patients younger than 18 years old and with cognitive impairment or mentally ill, no specific arrangements were provided for these special populations.

The controls were identified and recruited at Centro Hospitalar Lisboa Norte (Lisbon) and Hospital Senhora da Oliveira (Guimarães), within the healthy population of health care providers.

As the patients were evaluated during their routine follow-up, I did not organize any specific arrangement to record the compliance of the patients. For patients missing their routine follow-up visits, the usual procedures of each hospital were performed to recall the patient.

4. Clinical data

4.1 General clinical data

As described in the research design, each patient had three scheduled visits: baseline and after one and two years. General clinical data was collected at baseline and after each visit, irrespective of the inclusion in a cardiomyopathy and/or nephropathy study, namely:

- identification: age at enrolment and gender;
- FD diagnosis: age at diagnosis, plasma α-galactosidase A activity and GLA gene mutation;
- current medications: ACEi, ARB, aldosterone antagonists and β-blockers;
- ERT: drug, dose and duration;

- FD clinical manifestations in the heart, kidney, central nervous system, peripheral and autonomic nervous system, gastro-intestinal system, eye, ear and skin, in order to calculate the severity indices: MSSI and Fabry International Prognostic Index (FIPI);[229, 693]
- cardiac laboratorial and imaging evaluation: NT-proBNP, echocardiogram and cardiac MRI (detailed in the next sections)
- kidney function tests: GFR, creatinine, albuminuria and proteinuria (detailed in the next sections)

Plasma collected during the research project was also used to measure plasma lyso-Gb3. Plasma lyso-Gb3 was quantified by liquid chromatography tandem-mass spectrometry (Agilent, ultra performance liquid chromatography [UPLC] triple quadrupole [QqQ] electrospray ionization [ESI]). The quantification was achieved by multiple reaction monitoring (MRM) of the transitions mass-to-charge ratios (m/z) 786,4→282,3 and, as internal standard 1-β-D-glucosylsphingosine (GSG), 460.5→280.3. The result was extrapolated from the calibration curve obtained from responses of calibrators of known concentrations versus the internal standard.

Data collected from the control group included only age at enrolment, gender and exclusion of comorbidities and medication.

4.2 Cardiomyopathy study

In the cardiomyopathy study, I compared the results of the biomarkers related to collagen metabolism (index tests) with the gold standard imaging techniques (reference tests) in the evaluation of cardiac function and structure in FD, namely echocardiogram and cardiac MRI. The usefulness of the biomarkers was tested in the identification of specific outcomes in the reference tests.

Healthy controls also performed echocardiograms to exclude any cardiac disorder and guarantee that they do not present any abnormality defined as an outcome. They did not perform cardiac MRI.

4.2.1 *Echocardiogram*

Echocardiogram (LV mass and TDI abnormalities) was used as one of the reference tests to compare with index tests, as it is considered the gold standard in the evaluation of early cardiac dysfunction in FD.

Data from the M-mode, 2-dimensional and Doppler transthoracic echocardiographic study was collected for each patient and control. FD patients' echocardiograms were executed in a Vivid 7 (General Electrics®) ultrasound system in all recruitment sites. The controls evaluation was performed in Vivid 7 (General Electrics®) or Toshiba Xario (model SSA-660A) ultrasound systems. The average of three cardiac cycles was used for measurement of cardiac dimensions, mitral inflow pattern, pulmonary veins flow and myocardial velocities at the corners of the mitral annulus.

LV dimensions (interventricular septum, posterior wall and LV end-diastolic diameter) were assessed from the long-axis view and ventricular mass was calculated according to the Devereux formula and normalized for height ($g/m^{2.7}$). LV hypertrophy was defined as LV mass ≥50$g/m^{2.7}$. Left atrium area was obtained in the apical four-chamber view and expressed in cm^2.

Mitral inflow pattern (rapid filling [E] and atrial contraction [A] peak velocities, E wave decelerating time, A dur and isovolumic relaxation time) were obtained at the mitral level by Doppler echocardiography in the apical four-chamber view. Retrograde flow velocity (Ar vel) and Ar dur were acquired from the pulmonary veins also by Doppler echocardiography in the same view.

Myocardial velocities during systole (S') and early diastole (E') were collected from TDI at lateral and septal corner of the mitral annulus in the apical four-chamber view. TDI abnormalities were defined as mentioned above. The ratio between E and E' average (septal and lateral) was used to estimate left ventricular end-diastolic pressure.

Echocardiographic studies were performed and reported by persons who were blinded to the measurement of collagen turnover biomarkers (index tests) and clinical data.

Since echocardiogram is an observer dependent technique, evaluation of inter-observer variability was anticipated. However, as the echocardiograms were executed during routine follow-up in echocardiography laboratories in each recruiting centre, by

various echocardiography technicians or cardiologists, it was not possible to test this variation and this is a limitation of the study.

4.2.2 *Cardiac magnetic resonance imaging*

Cardiac MRI was used as a reference test, as it is the gold standard technique for non-invasive detection of replacement myocardial fibrosis.

Data on cardiac MRI was collected only when it was performed as part of the follow-up protocol. Cardiac MRI studies were performed in a 3.0-T system (Philips® Intera). LGE is the standard for detection of focal myocardial replacement fibrosis and was defined in the study as greater than 2 standard deviations above the mean signal intensity of the distant myocardium. Due to limited access to the cardiac MRI images, I was not able to calculate the extent of LGE, in terms of total volume and as proportion of the total LV mass; this is another limitation of this research project.

4.2.3 *Outcomes*

To test the value of the biomarkers related to collagen metabolism in the identification of incipient FD cardiomyopathy, the correlation with cardiomyopathy severity and as a predictor of cardiomyopathy prognosis, several outcomes related to the reference tests were defined:

- comparison of the biomarkers with TDI abnormalities in the identification of patients with FD;
- correlation of the biomarkers with the severity of LV hypertrophy (defined as indexed LV mass in the echocardiogram) and of diastolic dysfunction (defined as TDI E' values in the echocardiogram);
- diagnostic accuracy of the biomarkers to identify TDI abnormalities (as previously defined), LV hypertrophy (as previously defined) and LGE in cardiac MRI;
- correlation of the longitudinal change of echocardiographic aforementioned measures of LV hypertrophy and diastolic dysfunction, with the biomarkers at the baseline;

- correlation of the variation of the biomarkers between baseline and 24 months of follow-up with the variation of the echocardiographic variables at the same time points;
- evaluation of the prognostic value of the biomarkers, expressed as their ability to identify patient progression in terms of LV hypertrophy and diastolic dysfunction along the observation period. LV mass progression was defined as an increase in LV mass ≥10g/$m^{2.7}$ between the first and last evaluation within the study. Diastolic dysfunction progression was defined as an increase in LV end-diastolic pressure (LVEDP), i.e. an increase ≥1.5 in the E to E' average ratio, associated with TDI abnormalities between baseline and last follow-up visit;
- exploratory sub-analysis by gender;
- exploratory sub-analysis of the effect of ERT or ACEi / ARB on biomarker longitudinal variation.

4.3 Nephropathy study

In the nephropathy study, I defined as the reference tests to compare with the results of the biomarkers of glomerular and tubular damage, the gold standards in the evaluation of FD nephropathy: GFR and albuminuria. The usefulness of the biomarkers was also tested in the identification of specific outcomes in the reference tests.

Healthy controls also underwent kidney evaluation, with the measurement of albuminuria and serum creatinine.

4.3.1 *Albuminuria*

Albuminuria (ACR) was used as one of the reference tests, as it is considered, as mentioned in the introduction, to be the gold standard and a sensitive marker of early renal dysfunction in FD nephropathy.

ACR was quantified from spot urine, in random urine samples. In FD patients this was determined by the available laboratory method in each recruiting centre (immunonephelometry methods). In the control group it was evaluated by immunoturbidimetry (Siemens Healthcare Diagnostics, Germany). Increased ACR was defined as ≥30mg/g, according to KDIGO guidelines.[283]

4.3.2 *Serum creatinine and glomerular filtration rate*

Glomerular filtration rate is the gold standard for diagnosis and staging of CKD. As mentioned in the introduction it may be measured or estimated according to serum creatinine and/or cystatin.

In FD patients, serum creatinine was assessed during routine follow-up in the Hospital laboratory by the modified Jaffe's method (photometric assay) and it was collected on the same day as the urine used to determine ACR and the biomarkers. In controls, serum creatinine was determined by a compensated kinetic Jaffé method (Siemens Healthcare Diagnostics, Germany). GFR was estimated by the CKD-EPI$_{creatinine2009}$ equation, according to KDIGO guidelines.[148, 283]

In a single recruiting centre GFR is often measured using Cr-51 EDTA clearance in FD patients. CKD staging by GFR categories, in agreement with KDIGO guidelines, was based on estimated GFR, except in patients for whom radioisotopic measurement of GFR was available.[283]

Laboratories assessing kidney function were blinded to the measurement of the biomarkers of glomerular and tubular injury (index tests) and clinical data.

4.3.3 *Outcomes*

To test the biomarkers correlation with incipient FD nephropathy and nephropathy severity and as predictors of nephropathy prognosis, several outcomes related to the reference tests were defined:

- comparison of biomarkers with ACR in the identification of patients with FD;
- correlation of the biomarkers with the severity of CKD (defined as the estimated GFR and the CKD stage);
- diagnostic accuracy of the biomarkers to identify CKD stage ≥2;
- correlation of the longitudinal change of GFR, with the concentration of the biomarkers at the baseline;
- correlation of the longitudinal variation of the biomarkers with the longitudinal variation of the GFR;
- definition of the prognostic value of the biomarkers, expressed as their ability to identify patient progression in terms of ACR and GFR. An ACR progressor was defined as a patient with an increase ≥1.5 times of ACR between its first and last

evaluation within the study, for at least ≥30mg/g or as a change between albuminuria A1 and A2 or A2 and A3. GFR progression was defined as a decrease ≥5ml/min/1.73m^2 in estimated GFR between baseline and last follow-up visit, for at least a GFR <90ml/min/1.73m^2;

- exploratory sub-analysis by gender;
- exploratory sub-analysis of the effect of ERT or ACEi / ARB on biomarker longitudinal variation.

4.4 Data handling and archiving

As an international, multicenter study, the collected data was handled according to the ethical requirements in the participating country, in order to protect personal data during transmission between the participating countries.

Patient personal data was anonymised and securely stored in filling cabinets in rooms with limited access. The electronic files of the processed data (all anonymised) were stored on the hospital computers where the study developed, with appropriate access controls to store the confidential research information. A numeric code was used to anonymise each recruited patients and the numeric code key was only known by the research team and securely stored on the hospital computers used in the research.

Anonymised data was appropriately transferred between the centres in Portugal and the United Kingdom for processing and statistical analysis. The data transferred by laptop computers was encrypted. An international carrier of biological products transported the anonymised biological samples to Portugal.

The research documents and files will be stored in each centre according to the requirements at each institution: for 20 years at the Royal Free London NHS Foundation Trust and for 5 years at both Portuguese centres. After this period the data will be removed from the computers using appropriate data destruction software.

5. Biomarkers assessment

5.1 Cardiomyopathy study

As described in the research design, to assess myocardial collagen turnover, I measured biomarkers related to collagen metabolism, either of collagen synthesis (PICP,

PINP, PIIINP) or degradation (CITP, MMP-1, MMP-2, MMP-3 and TIMP-1). Moreover, we calculated the PICP to CITP ratio as an index reflecting the balance between collagen synthesis and degradation.

As mentioned above, collagen type I is also a major component of bone, which may be a clue in the interpretation of the blood concentration of propeptides and telopeptides of collagen type I when assessing myocardial fibrosis. Therefore, to minimize this confounding factor, I also measured markers of bone synthesis (B-AP) and resorption (type 5 tartrate-resistant acid phosphatase [TRAP5b]) and the PICP to B-AP and the CITP to TRAP5b ratios were assumed as markers of collagen type I synthesis and degradation adjusted for bone turnover, respectively.

I also anticipated that another confounding factor could be kidney function, because, as noted above, it is the main route for CITP clearance and patients with moderate or severe CKD present altered bone turnover. As renal involvement is one of the predominant features of FD, adjustment for GFR was taken in consideration in the statistical analysis.

Given that there are no validated reference values for the tested biomarkers, the upper limit of a 95% confidence interval for the mean of the control group was assumed to be the upper limit of the reference value.

5.1.1 *Biological samples*

Peripheral venous blood samples were obtained less than 6 months apart from the echocardiogram (during the routine blood sampling of the patients with FD). Blood samples were collected in ethylenediamine tetra-acetic acid (EDTA) and serum separator chemistry (containing a separator gel) tubes, spun within 120 minutes after phlebotomy (2500rpm, during 10 minutes) and plasma and serum was separated and stored immediately stored at -20°C, in Eppendorf tubes.

Frozen stored tubes were transported to the laboratory at the same temperature conditions in dry ice and thawed and mixed thoroughly just prior to the assay. The serological assays were performed within less than 12 months after blood sample collection.

5.1.2 *Serological tests*

In brief, TRAP5b was evaluated by an ELISA method, according to manufacturer instructions and measured at 405 nm (ids, Immunodiagnostic Systems, UK); MMP-1 and MMP-2 were evaluated by an ELISA method, according to manufacturer instructions and measured at 450 nm (SunRed®, Shanghai, PRC); B-AP and PICP were evaluated by an ELISA method, according to manufacturer instructions and measured at 405 nm (QUIDEL Corporation, San Diego, USA); CITP was evaluated by an electro-chemiluminescence immunoassay (ECLIA) in a COBAS e411 instrument (Roche® Diagnostics GmBH, Mannheim; Germany). Duplicate determinations were made for each individual and the average result considered. Laboratory determinations were blinded to clinical data and cardiac assessments (reference tests).

PICP was measured in serum, by an ELISA method (QUIDEL Corporation®, Hannover, Germany), according to manufacturer instructions. The micro ELISA strip plate is pre-coated with human PICP monoclonal antibody and the kit uses p-nitrophenyl phosphate substrate to measure the immune complex obtained during the reaction. Final colorimetric reaction is measured by photometry at 405 nm. In this assay system, the assay sensitivity of PICP was over 0.2ng/mL; coefficients of variations of intra-assay and inter-assay were 5.5% and 7.2%, respectively.

CITP was measured in serum, by an ECLIA method (Roche® Diagnostics GmBH, Mannheim; Germany) in an automated analyser COBAS e411 instrument, according to manufacturer instructions. The detection of the marker in the samples is made using human CITP monoclonal antibody. In this assay system the minimum detectable concentration of CITP was over 0.01ng/mL. The intra-assay and inter-assay precision of the method were 4.6% and 4.7%, respectively.

MMP-1 was also measured in serum, in an assay using a double-antibody sandwich ELISA (SunRed® Biotechnology Company), according to the instructions of the manufacturer. The micro ELISA strip plate is pre-coated with human MMP-1 monoclonal antibody and the kit uses biotin-streptavidin-HRP technology to measure the immune complex obtained during the reaction. Final colorimetric reaction is measured by photometry at 450 nm. In this assay system, the assay sensitivity of MMP-1 was over 0.207ng/mL; coefficients of variations of intra-assay and inter-assay were <10% and <12%, respectively.

MMP-2 was measured in serum, also by a double-antibody sandwich ELISA assay (SunRed® Biotechnology Company), according to the instructions of the manufacturer. The micro ELISA strip plate is pre-coated with human MMP-2 monoclonal antibody and the kit uses biotin-streptavidin-HRP technology to measure the immune complex obtained during the reaction. Final colorimetric reaction is measured by photometry at 450 nm. In this assay system, the assay sensitivity of MMP-2 was over 10.016ng/mL; coefficients of variations of intra-assay and inter-assay were <9% and <12%, respectively.

B-AP was measured in serum, by ELISA methodology (QUIDEL Corporation®, Hannover, Germany) according to the instructions of the manufacturer. The micro ELISA strip plate is pre-coated with human B-AP monoclonal antibody and the kit uses p-nitrophenyl phosphate substrate to measure the immune complex obtained during the reaction. Final colorimetric reaction is measured by photometry at 405 nm. In this assay system, the minimum detectable concentration of B-AP was over 0.34ng/mL. The intra-assay and inter-assay precision of the method were 6% and 8%, respectively.

TRAP5b was measured in serum, by an ELISA assay (ids, Immunodiagnostic Systems®, United Kingdom), according to the manufacturer instructions. The micro ELISA strip plate is pre-coated with human TRAP5b monoclonal antibody and the kit uses p-nitrophenyl phosphate substrate to measure the immune complex obtained during the reaction. Final colorimetric reaction is measured by photometry at 405 nm. In this assay system, the limit of TRAP5b quantification is over 0.5U/L. The intra-assay and inter-assay precision of the method were <9.6% and <9.2%, respectively.

5.2 Nephropathy study

As mentioned in the research design, I measured two biomarkers of glomerular damage, one related to increased permeability to plasma proteins (UTE) and the other associated to mesangial and GBM expansion (urinary ColIV excretion); I also evaluated three biomarkers of tubular injury, one low molecular weight protein (urinary A1MG excretion) and two urinary enzymes (urinary NAG excretion and urinary AAP excretion).

All biomarkers were measured in spot, random collected urine; this urine was taken from the sample used to determine ACR. All biomarkers urine concentrations were normalized for the urinary creatinine concentration.

Given that there are no validated reference values for the tested biomarkers, the upper limit of a 95% confidence interval for the mean of the control group was assumed to be the upper limit of the reference value.

5.2.1 *Biological samples*

Urine was collected in the routine follow-up visits, on the same day as the blood for determination of serum creatinine or, when this was the case, on the same day as the measurement of GFR by radioisotopic method. Urine was collected in a tube without any conservative treatment and immediately stored at -20º, without any processing.

Frozen stored tubes were transported to the laboratory at the same temperature conditions in dry ice and thawed and mixed thoroughly just prior to the assay. The serological assays were performed within less than 12 months after urine sample collection.

5.2.2 *Serological tests*

In brief, transferrin, ColIV, A1MG and AAP were evaluated by an ELISA method, according to manufacturer instructions and measured at 450 nm (SunRed®, Shanghai, PRC); NAG was evaluated by a colorimetric assay at 580 nm (Roche® Diagnostics GmBH, Mannheim; Germany). Duplicate determinations were made for each individual and mean results are presented. Laboratory determinations were blinded to clinical data and renal function assessment (reference tests).

Transferrin was measured in urine, by a double-antibody sandwich ELISA assay (SunRed® Biotechnology Company), according to the instruction of the manufacturer. The micro ELISA strip plate is pre-coated with human transferrin monoclonal antibody and the kit uses biotin-streptavidin-HRP technology to measure the immune complex obtained during the reaction. Final colorimetric reaction is measured by photometry at 450 nm. In this assay system, the assay sensitivity of transferrin was over 0.014mg/L; coefficients of variations of intra-assay and inter-assay were <10% and <12%, respectively.

ColIV was measured in urine, by a double-antibody sandwich ELISA assay (SunRed® Biotechnology Company), according to the instructions of the manufacturer. The micro ELISA strip plate is pre-coated with human ColIV monoclonal antibody to the 7S domain of ColIV molecule and the kit uses biotin-streptavidin-HRP technology to measure

the immune complex obtained during the reaction. Final colorimetric reaction is measured by photometry at 450 nm. In this assay system, the assay sensitivity of ColIV was over 0.108μg/mL; coefficients of variations of intra-assay and inter-assay were <10% and <12%, respectively.

A1MG was measured in urine, by a double-antibody sandwich ELISA assay (SunRed® Biotechnology Company), according to the instructions of the manufacturer. The micro ELISA strip plate is pre-coated with human A1MG monoclonal antibody and the kit uses biotin-streptavidin-HRP technology to measure the immune complex obtained during the reaction. Final colorimetric reaction is measured by photometry at 450 nm. In this assay system, the assay sensitivity of A1MG was over 4.118ng/mL; coefficients of variations of intra-assay and inter-assay were <10% and <12%, respectively.

NAG was measured in urine, by a colorimetric assay (Roche®, Diagnostics GmBH). 3-Cresolsulphonphthaleinyl-N-acetyl-β-D-glucosaminide, sodium salt, is hydrolyzed by NAG (present in the sample), releasing 3-resolsulfonphthalein, sodium salt (3-cresol purple), which is measured by photometry at 580 nm. In this assay system, the limit of quantification of NAG was 1.64 U/L. The intra-assay and inter-assay precision of the method were <4.1% and <11.3%, respectively.

AAP was measured in urine, by a double-antibody sandwich ELISA assay (SunRed® Biotechnology Company), according to the instructions of the manufacturer. The micro ELISA strip plate is pre-coated with human AAP monoclonal antibody and the kit uses biotin-streptavidin-HRP technology to measure the immune complex obtained during the reaction. Final colorimetric reaction is measured by photometry at 450 nm. In this assay system, the assay sensitivity of AAP was over 0.204ng/mL; coefficients of variations of intra-assay and inter-assay were <10% and <12%, respectively.

6. Statistical analysis

There was no formal sample size calculation in the cardiomyopathy and nephropathy studies due to various reasons: rarity of FD, difficulty to match the patients with a rare disease with the predefined subgroups and the presence of several exclusion criteria. Therefore, I estimated sample size based on the potentially recruitable number of patients. Nonetheless, for comparisons including all groups, with about 20 to 25 subjects per group, I estimated a power of ~90% to detect differences among groups, if

the within-group standard deviation (SD) was no more than twice the SD between the groups. For correlations, using all the FD patients (n between 60 and 75), I had at least ~90% power to detect even small correlations ($\rho^2 \sim 0.2$) as significant.

Statistical analysis was performed with SPSS® (Statistical Package for the Social Sciences, version 24) software. Categorical variables were expressed as a number and percentage and continuous variables as medians and interquartile range (IQR). Normal distribution of continuous variables was tested using the Shapiro-Wilk test.

For categorical variables, the Qui-square or Fisher exact tests were used to compare the variables' distribution between the groups.

For continuous variables, comparison of means / medians was performed using Student t-test for variables that followed a normal distribution and Mann–Whitney test / related samples Wilcoxon signed rank test for variables that did not. Related samples tests were only used for comparison between age and sex-matched controls and group 1 and longitudinal measurements for each patient (these respect the structure of the data collection and also have more power) in both cardiomyopathy and nephropathy studies.

If the qualitative variable had more than 2 categories, an ANOVA test (post-hoc analysis with Bonferroni correction) was used for variables with normal distribution, and a Kruskal–Wallis test was used for those without.

Due to the skewed distribution of all the studied biomarkers (index and reference tests), to evaluate the correlation between the biomarkers and the quantitative variables, Spearman's correlation coefficient was determined. In the case of correlation between normally distributed variables, Pearson's correlation coefficient was determined. For easier visualisation of correlation in skewed distributions, a log or power scale was used in scatter plot graphs, depending on the direction of the skewness.

In order to construct a regression model to quantify an outcome variable (for example, LV mass), all potentially related variables were correlated, using Spearman's rank correlation coefficient, with the outcome variable (in univariate analysis). The variables showing significant correlation (probability value < 0.05) were entered into the multivariate analysis. For multivariate analysis, stepwise regression combining both forward selection and backward elimination was used. A cut-off limit to remain in the model was set to an F statistic probability value of < 0.05. Collinearity diagnostics were applied to detect related variables in the model.

I also evaluated the diagnostic accuracy of the studied biomarkers to detect predefined outcomes, like CKD stage ≥2, by calculating the sensitivity (S), specificity (Sp), positive predictive value (PPV), negative predictive value, likelihood ratio for a positive test result, likelihood ratio for a negative test result (LR-), diagnostic odds ratio (DOR), accuracy, Youden's index and the AUC from receiver operating characteristic (ROC) curves (I determined the confidence intervals of AUC [nonparametric method], with values between 1 [perfect test] and 0.5 [useless test]).

In the follow-up / prognostic analysis, I categorized the investigated biomarkers into quartiles to evaluate relationships between levels of the markers and the odds of progression, using contingency tables.

Given that this is a preliminary study, it is not powered for sub-group comparisons, such as sub-analysis by sex or age group. Nonetheless, I performed some of these comparisons in an exploratory way, both by calculating the corresponding descriptive statistics and some inferential analysis (for example, 2-way ANOVA with interactions). Analysis of covariance (ANCOVA) was used to explore the effect of treatment on the longitudinal variation of biomarkers, adjusting for clinical meaningful variables as covariates.

For all comparisons and correlations a probability value of < 0.05 was considered significant.

Results

1. Cardiomyopathy study

1.1 Pilot study

1.1.1 *Population characteristics*

As mentioned above, for the pilot study 10 patients were recruited with FD and LV hypertrophy (as previously defined) and 10 sex and age-matched healthy controls. Population characteristics are given in table 4. The median age at diagnosis of FD was 45.5 years old, 30% were taking renin-angiotensin-aldosterone axis inhibitors (ACEi, ARB and aldosterone antagonists) and 70% of them had been receiving ERT for a median time of 8.2 years. All patients in the FD group were non-relatives and only two had the same pathogenic mutation (p.N215S).

Table 4: Cardiomyopathy pilot study: population characteristics. FD: Fabry disease; ERT: enzyme replacement therapy; RAAi: renin-angiotensin-aldosterone axis inhibitors; IQR: interquartile range.

	Control group (n=10)	**FD group** (n=10)
Categorical variables (n (%))		
Sex (female)	5 (50.0)	5 (50.0)
ERT (yes)	-	7 (70.0)
RAAi (yes)	-	3 (30.0)
Continuous variable (median (IQR))		
Age (years)	57.0 (14.0)	59.5 (14.0)
Age at diagnosis (years)	-	45.5 (19.0)
Time in ERT (years)	-	8.2 (7.9)

As per inclusion criteria, all patients in the FD group presented LV hypertrophy, as opposed to the control group. Therefore, FD group had a significantly higher LV wall thickness and LV mass, lower systolic and early diastolic myocardial velocities and higher estimated left ventricular end-diastolic pressure (E/E') (table 5).

1.1.2 *Biomarkers related to collagen metabolism*

As compared with PICP concentration in controls, PICP concentration in the FD group was significantly elevated (a 69% increase, p=0.019); a significant difference that remained even after adjustment for bone turnover (table 6). The other biomarkers of collagen type I and type III synthesis did not differ significantly between healthy controls and FD patients.

Table 5: Cardiomyopathy pilot study: echocardiographic characteristics of the study population.

Expressed as median (IQR); IVS: diastolic interventricular septum thickness; PW: diastolic left ventricular posterior wall thickness; LVMi: left ventricular mass index; S': systolic mitral annular myocardial velocity; E': early diastolic mitral annular myocardial velocity; E/E': ratio of early mitral inflow velocity to the average early diastolic myocardial velocity.

	Control group	FD group	p value
dIVS (mm)	9.05 (1.47)	13.95 (2.54)	<0.001
dLVPW (mm)	9.00 (1.85)	11.96 (2.39)	0.001
LVMi ($g/m^{2.7}$)	34.99 (11.30)	66.46 (9.28)	<0.001
S' septal (cm/s)	7.5 (3.0)	5.0 (3.3)	0.027
S' lateral (cm/s)	9.0 (5.5)	6.5 (1.8)	0.023
E' septal (cm/s)	10.1 (4.4)	5.0 (1.8)	<0.001
E' lateral (cm/s)	12.0 (4.6)	7.5 (5.3)	0.001
E/E'	6.21 (2.80)	11.90 (10.97)	0.029

CITP, a product of collagen type I degradation, was non-significantly different between both groups, even after adjustment for bone turnover. However, a trend for a decrease in enzymes involved in collagen degradation in the FD group was identified (that was statistically significant for MMP-2).

Table 6: Cardiomyopathy pilot study: biomarkers related to collagen metabolism. Expressed as median (IQR); PICP: procollagen type I carboxy-terminal propeptide; B-AP: bone-specific alkaline phosphatase; PINP: procollagen type I amino-terminal propeptide; PIIINP: procollagen type III amino-terminal propeptide; CITP: collagen type I carboxy-terminal telopeptide; TRAP5b: type 5b tartrate-resistant acid phosphatase; MMP: matrix metalloproteinase; TIMP-1: tissue inhibitor of metalloproteinase type I.

	Control group	FD group	p value
Biomarkers of collagen synthesis			
PICP (ng/mL)	141.8 (115.3)	238.3 (207.9)	0.019
PICP:B-AP	14.2 (5.4)	25.2 (27.6)	0.004
PINP (ng/mL)	37.8 (42.8)	44.5 (35.1)	0.297
PINP:B-AP	3.7 (2.5)	5.6 (3.6)	0.016
PIIINP (ng/mL)	3.3 (1.5)	2.9 (0.9)	0.297
Biomarkers of collagen degradation			
CITP (ng/mL)	0.18 (0.29)	0.29 (0.36)	0.387
CITP:TRAP5b	0.08 (0.09)	0.14 (0.20)	0.085
MMP-1 (ng/mL)	9.3 (2.1)	8.5 (1.9)	0.073
MMP-2 (ng/mL)	345.9 (58.1)	277.4 (119.8)	0.029
MMP-3 (ng/mL)	14.3 (1.7)	12.8 (3.4)	0.075
TIMP-1 (ng/mL)	76.3 (17.5)	79.7 (32.0)	0.710

The PICP to CITP ratio (figure 5), reflecting the balance between collagen type I synthesis and degradation, tended to be higher in the FD group in comparison with the control group (a 28% increase, p=0.730); this difference was of greater magnitude, although non-significant, after adjustment for bone turnover (a 33% increase, p=0.254).

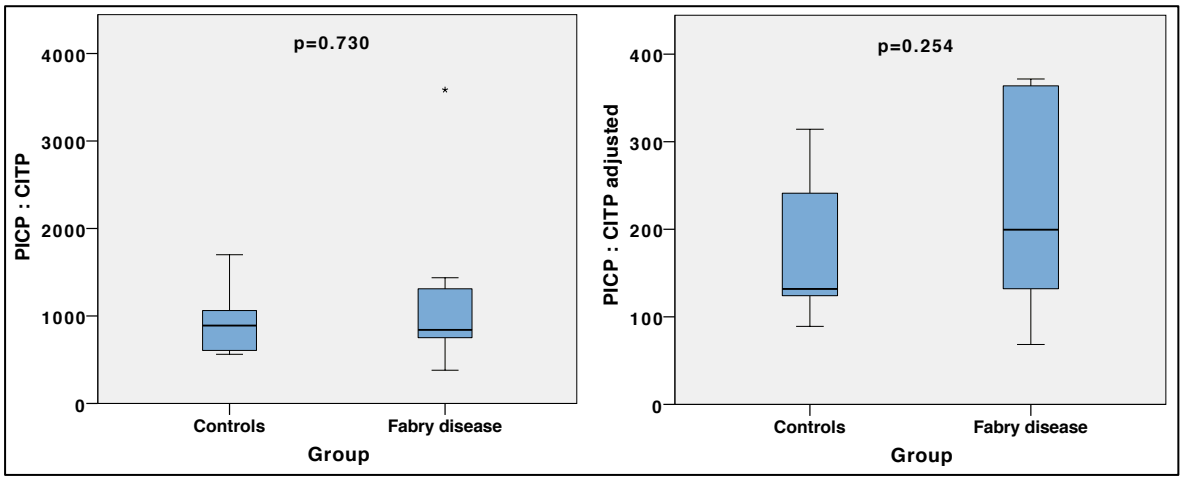


Figure 5: Cardiomyopathy pilot study: balance between collagen synthesis and degradation in healthy controls and FD patients. Left image: non-adjusted for bone turnover; right image: adjusted for bone turnover. PICP: procollagen type I carboxy-terminal propeptide; CITP: collagen type I carboxy-terminal telopeptide.

Based on the results of the pilot study, I selected the following biomarkers related to collagen metabolism to be evaluated in the entire cohort of recruited patients: PICP, CITP, MMP-1 and MMP-2.

1.2 Population characteristics

From February 2013 to June 2014, the planned 60 FD patients (20 in each subgroup) and 20 controls (age and sex-matched with subgroup 1) were recruited (study flow diagram in figure 6).

Population characteristics are given in table 7. In the 60 FD patients group, 61.7% were female (significantly less females in subgroup 3 [patients with established LV hypertrophy]), with a median age of 44.0 years old, ranging from 20 to 78 years old (significantly older, comparing the most severe subgroup 3 with the less severe subgroup 1 [patients with normal echocardiogram]); 65.0% of the patients were receiving ERT (no significance between subgroups; 94.9% under agalsidase-α, 5.1% under agalsidase-β), with a significantly older age for ERT initiation comparing group 3 to 1. The use of ACEi / ARB (48.3% of the entire cohort) and β-blockers was also not significantly different between subgroups; there was only one patient within the entire cohort taking AA. There was no significant difference between the subgroups in the percentage of patients with HBP, as well as in the NYHA class of heart failure. As expected, the severity indexes (MSSI

and FIPI) were also significantly higher in the most severe subgroup, as well as plasma lyso-Gb3 and NT-proBNP; in contrast, estimated GFR was significantly lower in the subgroup 3. Plasma α-galactosidase A activity was similar between FD subgroups, both in male and in female patients.

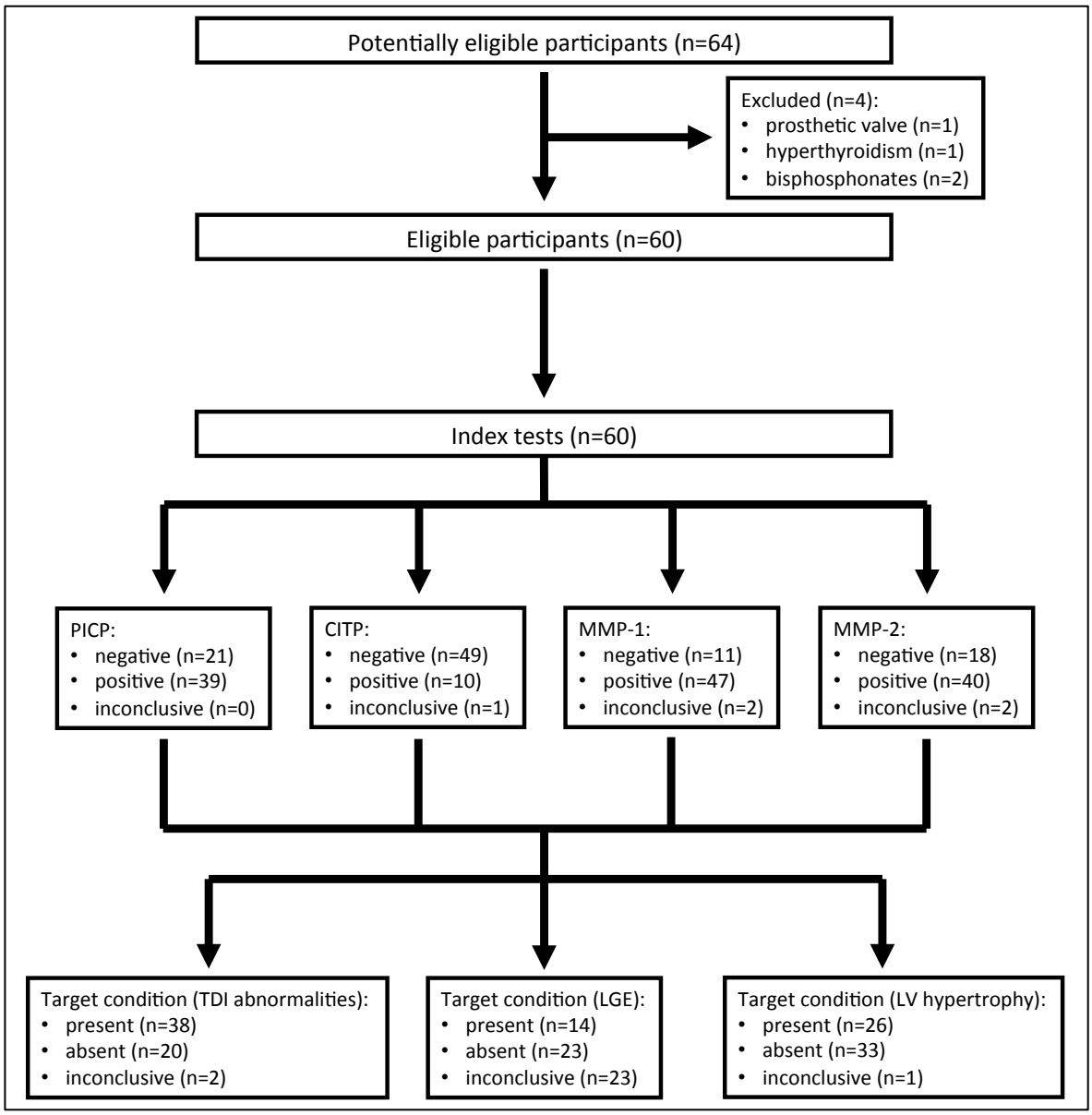


Figure 6: Cardiomyopathy study: study flow diagram. PICP: procollagen type I carboxy-terminal propeptide; CITP: collagen type I carboxy-terminal telopeptide; MMP: matrix metalloproteinase; TDI: tissue Doppler imaging; LGE: late gadolinium enhancement; LV: left ventricular.

Altogether 28 different GLA pathogenic mutations were identified (table 8), with p.N215S and p.F113L (two mutations associated with attenuated / late-onset phenotypes with predominant cardiac involvement) accounting for 31.7% of the patients; the remainder mutations are usually associated with classical phenotype.

Table 7: Cardiomyopathy study: clinical characteristics of the study population. HBP: arterial hypertension; ERT: enzyme replacement therapy; ACEi: angiotensin converting enzyme inhibitors; ARB: angiotensin II receptor blockers; MSSI: Mainz Severity Score Index; FIPI: Fabry International Prognostic Index; α-gal: α-galactosidase; NT-pro BNP: N-terminal of propeptide of brain natriuretic peptide; eGFR: estimated glomerular filtration rate.

	FD cohort (n=60)	FD subgroups		
		1 (n=20)	**2** (n=20)	**3** (n=20)
Categorical variables (n (%))				
Sex (female) ₸	37 (61.7)	14 (70.0)	18 (90.0)	5 (25.0)
HBP (yes)	25 (41.7)	8 (40.0)	10 (50.0)	7 (35.0)
HF class (0 / I / II)	39 / 6 / 14	15 / 3 / 2	14 / 2 / 4	10 / 2 / 8
ERT (yes)	39 (65.0)	14 (70.0)	8 (40.0)	17 (85.0)
ACEi / ARB (yes)	29 (48.3)	9 (45.0)	10 (50.0)	10 (50.0)
β-blockers (yes)	11 (18.3)	3 (15.0)	3 (15.0)	5 (25.0)
Continuous variables (median (IQR))				
Age (years) ζ	44.0 (23.0)	40.5 (21.0)	48.5 (18.0)	59.5 (13.0)
Age at diagnosis (years) §	41.5 (22.0)	34.5 (24.0)	44.0 (15.0)	50.5 (35.0)
Age at ERT initiation (years) *	49.0 (17.1)	38.1 (25.4)	52.4 (17.0)	49.4 (19.9)
Time in ERT (years)	6.3 (8.4)	4.8 (8.0)	5.9 (7.6)	8.2 (9.1)
MSSI °	18.5 (21.0)	11.0 (11.0)	12.5 (18.0)	31.0 (16.0)
FIPI °	2.0 (3.0)	1.0 (2.0)	1.0 (3.0)	4.0 (2.0)
Plasma lyso-Gb3 (nmol/L) **	9.1 (16.6)	3.6 (27.3)	8.3 (9.9)	18.8 (45.1)
Plasma α-gal A ♀ (nmol/h/mL)	4.3 (4.1)	5.1 (2.8)	3.5 (4.5)	3.8 (.)
Plasma α-gal A ♂ (nmol/h/mL)	0.11 (0.3)	0.14 (0.50)	0.06 (.)	0.20 (0.30)
NT-proBNP (ng/mL) ζ	142.0 (334.0)	40.0 (122.0)	129.5 (166.0)	940.7 (1602)
eGFR (ml/min/1.73m^2) ζ	93.0 (51.0)	99.5 (53.0)	101.0 (91.0)	67.5 (51.0)

₸ $p < 0.01$ for difference in the distribution of categorical variable between subgroups.
ζ $p < 0.01$ for difference between group 1 and 3.
§ $p < 0.05$ for difference between group 1 and 2.
* $p < 0.05$ for difference between group 1 and 3.
° $p < 0.01$ for difference between group 3 and all other groups.
** $p < 0.05$ for difference between group 2 and 3.

There were no significant echocardiographic differences between the control group and FD subgroup 1 (table 9). As per definition of FD subgroups, LV wall thickness and mass were significantly higher in FD subgroup 3, in contrast to TDI velocities and estimated LV end-diastolic pressure which were significantly higher in FD subgroup 1.

Table 8: Cardiomyopathy study: mutation frequency. * Mutations presented by only one patient in the study

Mutation	n	%
p.N215S	10	16.7
p.F113L	9	15.0
p.G35E	7	11.7
c.700_702del	2	3.3
p.R227X	2	3.3
p.C52G	2	3.3
p.L166P	2	3.3
p.N42V	2	3.3
p.R342Q	2	3.3
unknown	2	3.3
other*	20	33.3

A gadolinium-based contrast cardiac MRI result was available for 37 (61.7%) FD patients (9, 13 and 15 patients in subgroups 1, 2 and 3, respectively). The percentage of patients with LGE was 0%, 15.4% and 80.0% in FD subgroups 1, 2 and 3, respectively.

Table 9: Cardiomyopathy study: echocardiographic characteristics of the study population. Expressed as median (IQR); dIVS: diastolic interventricular septal thickness; dLVPW: diastolic left ventricular posterior wall thickness; LVMi: left ventricular mass indexed for height; LA: left atrium; E/A: left ventricular rapid filling (E wave) / atrial contraction (A wave) peak velocities; DT: E wave decelerating time; S': systolic myocardial velocity measured at the mitral annulus; E': early diastolic myocardial velocity measured at the mitral annulus; E/E': left ventricular rapid filling velocity / average (septal and lateral) early diastolic myocardial velocity measured at the mitral annulus.

	Controls	FD subgroups		
		1	2	3
dIVS (mm) °	9.0 (2.0)	9.0 (2.0)	9.0 (2.2)	14.5 (4.0)
dLVPW (mm) °	9.0 (1.0)	9.0 (3.0)	8.9 (2.8)	12.1 (4.5)
LVMi ($g/m^{2.7}$) °	39.3 (10.9)	35.2 (9.7)	39.5 (19.6)	63.3 (10.6)
LA area (cm^2) °	-	17.0 (4.6)	18.9 (5.6)	23.3 (10.2)
E/A ratio	-	1.47 (0.85)	1.25 (0.87)	0.98 (0.95)
DT (ms) *	-	184.0 (73.0)	230.0 (62.0)	249.5 (25.0)
S' septal (cm/s) ₸	8.0 (2.0)	8.0 (2.0)	6.0 (2.0)	6.0 (2.0)
S' lateral (cm/s) ₸	9.5 (5.0)	11.0 (3.0)	7.0 (3.0)	6.5 (3.0)
E' septal (cm/s) ζ	11.5 (5.0)	11.5 (3.0)	7.0 (4.0)	5.0 (2.0)
E' lateral (cm/s) ₸	15.5 (6.0)	14.5 (4.0)	9.0 (4.0)	8.0 (4.0)
E/E' ₸	6.3 (2.1)	6.0 (2.3)	9.4 (5.0)	11.8 (6.5)

° $p < 0.01$ for difference between group 3 and all other FD groups.
* $p < 0.01$ for difference between group 1 and 3.
₸ $p < 0.01$ for difference between group 1 and all other FD groups.
ζ $p < 0.01$ for difference between all FD groups.

1.3 Biomarkers related to collagen metabolism at baseline

1.3.1 *Concentration in healthy controls and Fabry disease patients*

Collagen type I synthesis biomarker (PICP) was increased in FD patients (table 10 and figure 7). As compared with PICP levels in controls, PICP levels in FD subgroup 1 were significantly elevated, a 61% increase (p=0.006). Comparing FD subgroups, PICP levels were significantly higher in FD subgroup 3 (p=0.001). The significant results comparing FD subgroup 1 and controls and FD subgroups remained with the PICP to B-AP ratio.

Collagen type I degradation biomarker CITP and the enzymes involved in its degradation (MMP-1 and MMP-2) levels were similar between the control group and FD subgroup 1. The difference between these two subgroups attained statistical significance only after adjustment for bone degradation, with the CITP to TRAP5b ratio (p=0.011). Moreover, there was a significant trend of a decreased activity of MMP-1 as severity

increased. Comparison of CITP between controls and FD subgroups after adjustment for estimated GFR retrieved similar results.

Table 10: Biomarkers related to collagen metabolism in controls and FD subgroups. PICP: procollagen type I carboxy-terminal propeptide; B-AP: bone-specific alkaline phosphatase; CITP: collagen type I carboxy-terminal telopeptide; TRAP5b: type 5b tartrate-resistant acid phosphatase; MMP: matrix metalloproteinase.

	Controls	FD subgroups			p value*	p value∓
		1	2	3		
PICP (ng/mL)	107.3 (39.3)	148.5 (129.0)	115.7 (88.8)	219.0 (98.4)	0.006	0.001
PICP/B-AP	10.1 (4.3)	16.2 (10.3)	12.7 (9.1)	19.4 (24.1)	0.003	0.001
CITP (ng/mL)	0.34 (0.20)	0.25 (0.27)	0.20 (0.19)	0.29 (0.23)	0.687	0.236
CITP/TRAP5b	0.11 (0.07)	0.16 (0.10)	0.11 (0.07)	0.13 (0.06)	0.011	0.186
MMP-1 (ng/mL)	16.8 (26.3)	13.5 (19.3)	14.1 (4.3)	9.6 (4.6)	0.159	0.002
MMP-2 (ng/mL)	440.3 (541.0)	341.8 (849.5)	356.5 (187.0)	318.7 (101.9)	0.872	0.108

* controls versus FD disease subgroup 1; ∓ between FD subgroups.

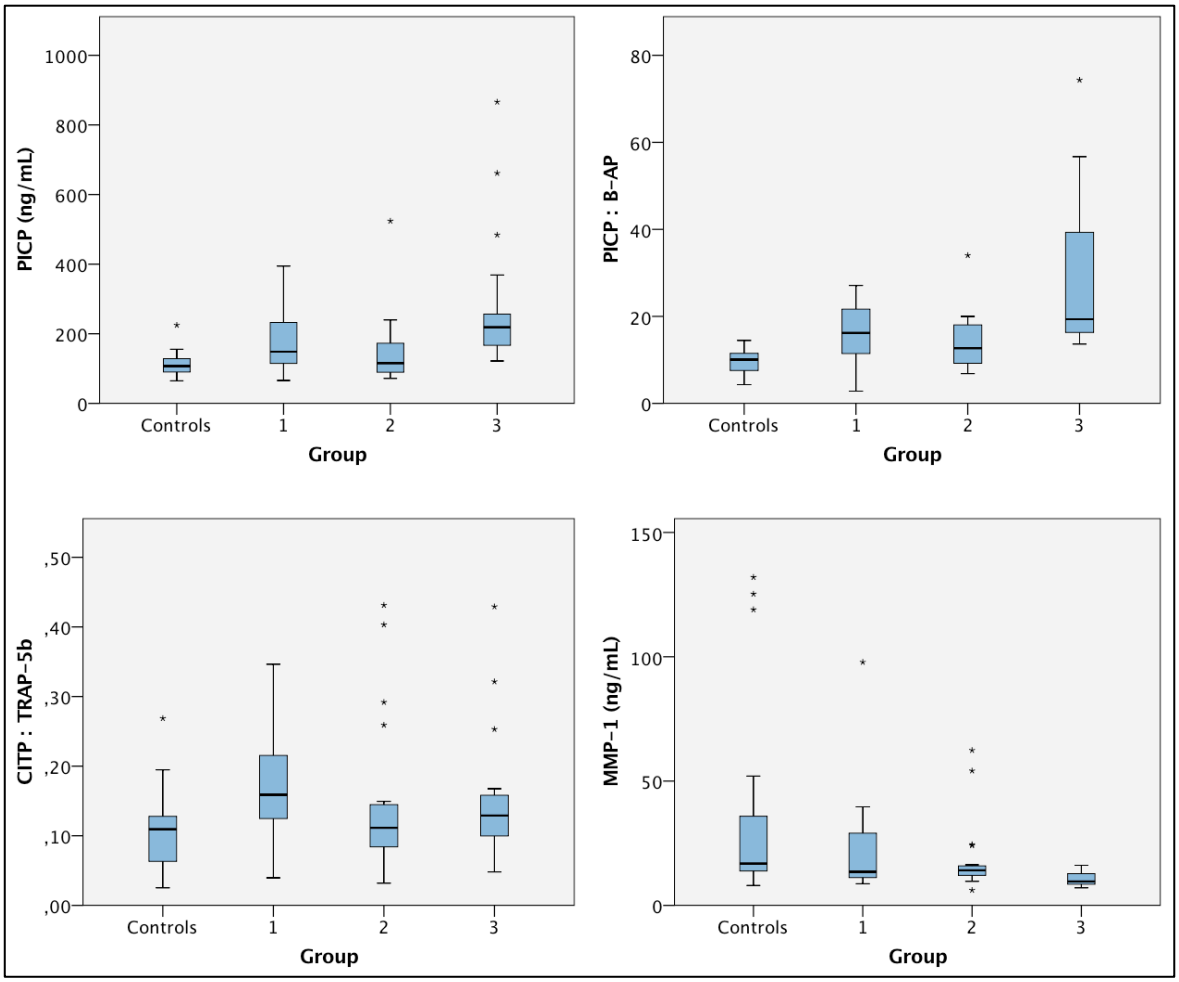


Figure 7: Biomarkers related to collagen metabolism with significant differences in controls and FD patient subgroups. Upper left image: PICP; upper right image: PICP to B-AP ratio; lower left image: CITP adjusted for bone turnover; lower right image: MMP-1. PICP: procollagen type I carboxy-terminal propeptide; B-AP: bone-specific alkaline phosphatase; CITP: collagen type I carboxy-terminal telopeptide; TRAP5b: type 5b tartrate-resistant acid phosphatase; MMP: matrix metalloproteinase.

The PICP to CITP ratio evaluation revealed a clear trend (only significant after adjustment for bone turnover [p=0.012]) for prevalent collagen type I synthesis over degradation within the subgroups of FD patients with greater severity (figure 8).

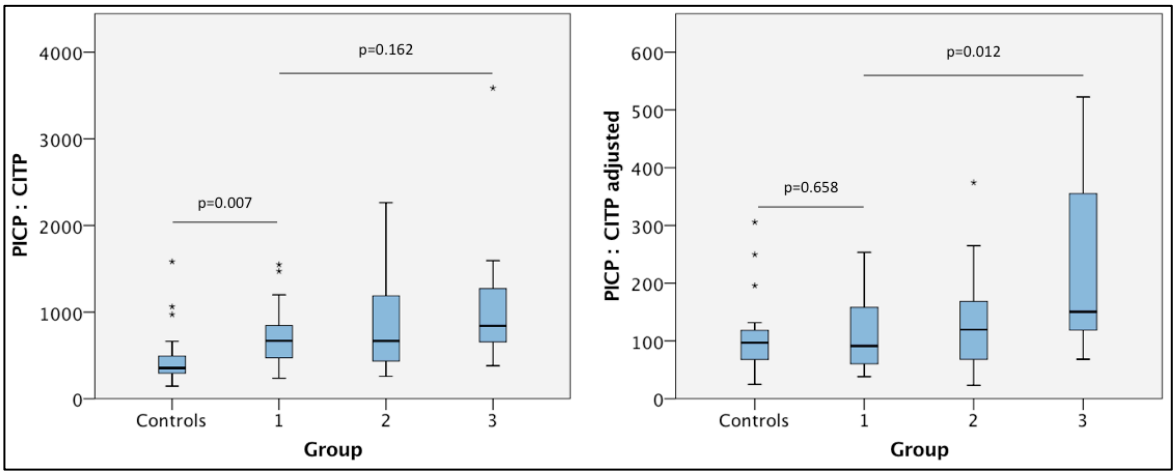


Figure 8: Balance between collagen synthesis and degradation in healthy controls and FD patient subgroups. Left image: non-adjusted for bone turnover; right image: adjusted for bone turnover. PICP: procollagen type I carboxy-terminal propeptide; CITP: collagen type I carboxy-terminal telopeptide.

Correlation between PICP to CITP ratio and MMP-1 reflects the influence of higher collagenase activity balancing the PICP to CITP ratio towards collagen type I degradation and lesser collagen type I deposition. Accordingly, we identified a significant inverse correlation between the PICP to CITP ratio and MMP-1 activity before (ρ=-0.348; p=0.008) and after (ρ=-0.322; p=0.015) adjustment for bone turnover (figure 9).

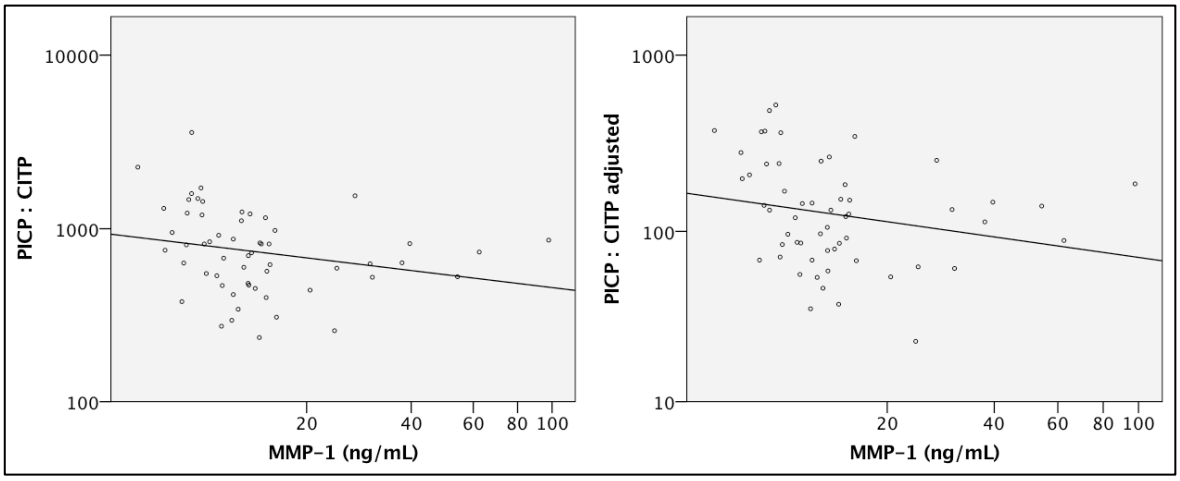


Figure 9: Correlation between balance collagen synthesis and degradation and MMP-1 activity. Left image: non-adjusted for bone turnover; right image: adjusted for bone turnover. PICP: procollagen type I carboxy-terminal propeptide; CITP: collagen type I carboxy-terminal telopeptide.

1.3.2 *Correlation with echocardiographic variables*

For the entire FD cohort, a significant direct correlation between the biomarker of collagen type I synthesis (PICP) and both LV wall thickness and mass (table 11 and figure 10) was found (PICP with LV mass: ρ=0.378, p=0.003). This significant correlation remained after adjustment for bone turnover (PICP to B-AP ratio with LV mass: ρ=0.313, p=0.016). No correlation was found between the biomarker of collagen type I synthesis and the parameters related to diastolic function.

Table 11: Correlation between biomarkers related to collagen metabolism and cardiac imaging parameters. Mean difference for categorical variables; ρ for continuous variables; PICP: carboxy-terminal propeptide of procollagen type I; B-AP: bone-specific alkaline phosphatase; CITP: carboxy-terminal telopeptide of type I collagen; TRAP: type 5b tartrate-resistant acid phosphatase; MMP: matrix metalloproteinase; dIVS: diastolic interventricular septal thickness; dLVPW: diastolic left ventricular posterior wall thickness; LVMi: left ventricular mass index; LA: left atrium; DT: E wave decelerating time; S': systolic myocardial velocity measured at the mitral annulus; E': early diastolic myocardial velocity measured at the mitral annulus; E/E': left ventricular rapid filling velocity / average (septal and lateral) early diastolic myocardial velocity measured at the mitral annulus; MRI: magnetic resonance imaging; LGE: late gadolinium enhancement.

	PICP (ng/mL)	**PICP:B-AP**	**CITP** (ng/mL)	**CITP:TRAP**	**MMP-1** (ng/mL)	**MMP-2** (ng/mL)
Echocardiogram						
dIVS (mm)	0.438*	0.378*	0.178	-0.017	-0.519*	-0.300*
dLVPW (mm)	0.322*	0.234	0.204	0.043	-0.438*	-0.294*
LVMi ($g/m^{2.7}$)	0.378*	0.313*	0.197	0.013	-0.484*	-0.235
LA area (cm^2)	0.173	0.066	0.092	0.080	-0.129	-0.239
DT (ms)	0.103	0.059	-0.024	-0.187	-0.294*	-0.145
S' septal (cm/s)	-0.023	0.071	0.020	-0.046	0.354*	0.188
S' lateral (cm/s)	-0.029	-0,017	-0.063	0.083	0.230	0.127
E' septal (cm/s)	-0.125	-0.113	0.130	0.139	0.354*	0.288
E' lateral (cm/s)	-0.037	0.000	0.127	0.234	0.280*	0.200
E/E'	0.105	0.005	0.027	-0.082	-0.097	0.087
Cardiac MRI						
LGE (yes)	88.8*	12.5*	0.066	-0.024	-5.27	-239.3

* $p < 0.05$

For biomarkers of collagen type I degradation, there was a significant inverse correlation between the enzyme MMP-1, involved in collagen type I cleavage, and LV thickness and mass parameters (MMP-1 with LV mass: ρ=-0.484, p<0.001). A significant inverse correlation with LV wall thickness, but not with LV mass was found for MMP-2.

Furthermore, a significant direct correlation was found for the MMP-1 and echocardiographic parameters of diastolic dysfunction, namely early diastolic mitral velocities measured at the septal (ρ=0.354, p=0.016) and lateral (ρ=0.280, p=0.042) corners of the mitral annulus.

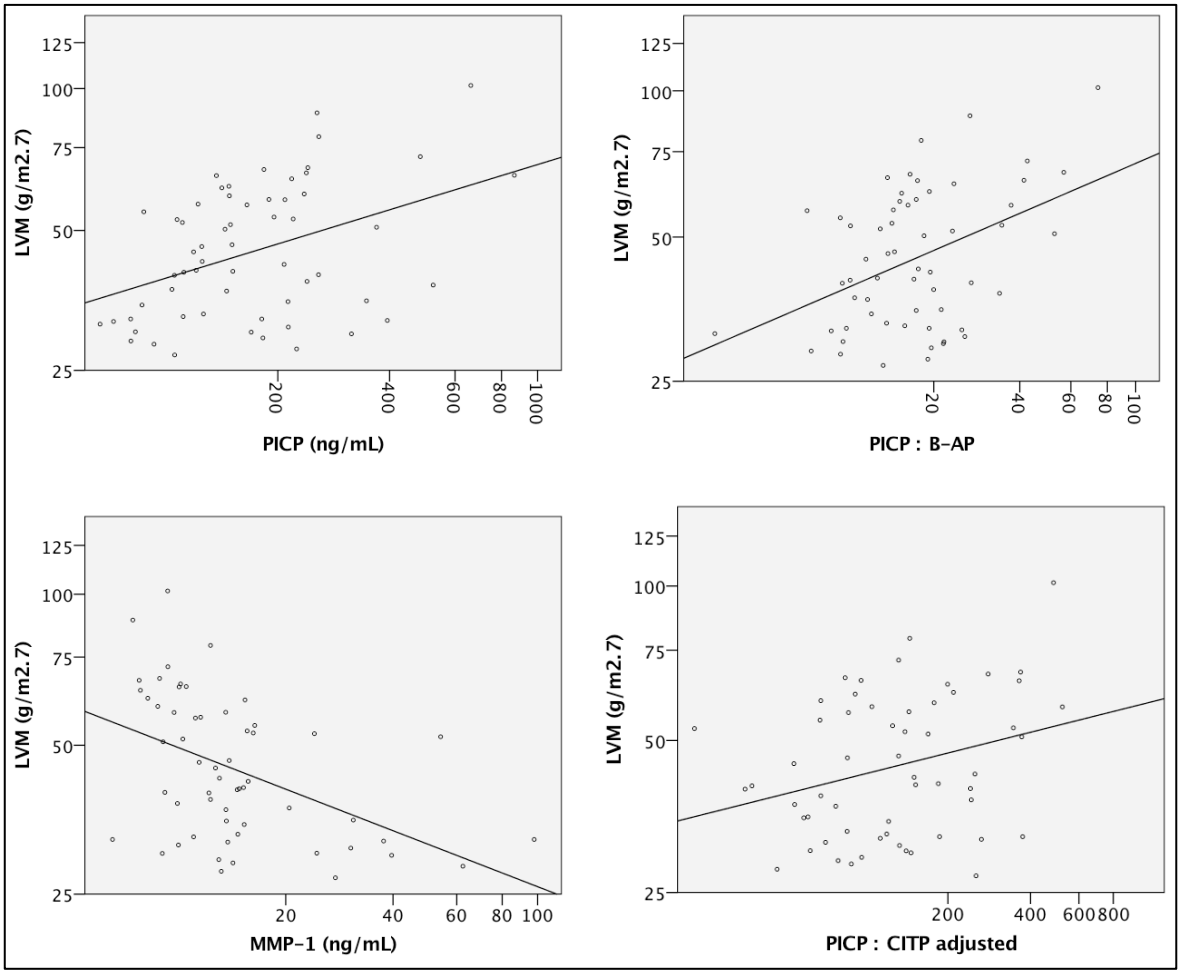


Figure 10: Correlation various biomarkers related to collagen metabolism and LV mass. Left upper image: PICP; right upper image: PICP adjusted for bone turnover; left lower image: MMP-1; right lower image: PICP to CITP ratio. A logarithmic scale was used for better visualization, due to the skewed distribution of the variables; LVM: left ventricular mass; PICP: procollagen type I carboxy-terminal propeptide; B-AP: bone-specific alkaline phosphatase; MMP: matrix metalloproteinase; CITP: collagen type I carboxy-terminal telopeptide.

1.3.3 *Correlation with late gadolinium enhancement*

There was a clear trend of higher values of collagen type I synthesis and decreased activity of the enzymes involved in collagen type I cleavage in patients with LGE in cardiac MRI (table 11), but the difference between patients with and without LGE was only significant for PICP, even after adjustment for bone turnover (for PICP to B-AP ratio: mean difference 12.5, a 74% increase for LGE positive group, p=0.01) (figure 11).

After adjustment for bone turnover, there was an almost significant correlation between PICP to CITP ratio and LV mass (ρ=0.260, p=0.051) (figure 10). Furthermore, a significant difference between LGE negative and positive patients was found for this ratio (figure 11).

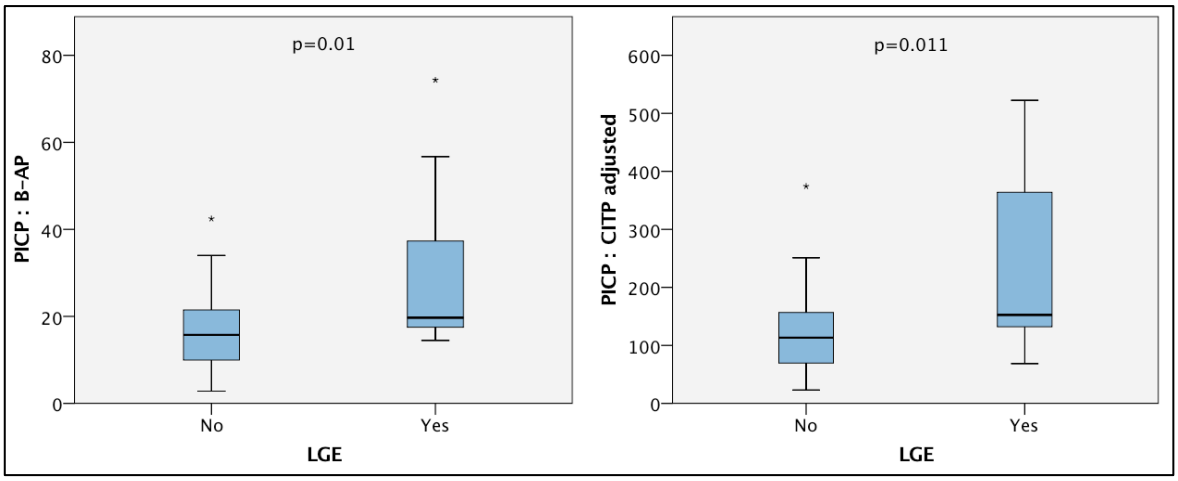


Figure 11 Significant differences in collagen biomarkers between patients with and without LGE in cardiac MRI. Left image: PICP to B-AP ratio; right image: PICP to CITP ratio adjusted for bone turnover. PICP: procollagen type I carboxy-terminal propeptide; B-AP: bone-specific alkaline phosphatase; CITP: collagen type I carboxy-terminal telopeptide; LGE: late gadolinium enhancement; MRI: magnetic resonance imaging.

1.3.4 *Correlation with other variables*

For the rest of the variables not directly related to cardiac imaging (including demographic, diagnosis, severity and treatment related), a consistent and significant correlation was found between the biomarker of collagen type I synthesis (PICP) and the disease severity indexes (MSSI and FIPI) as well as plasma lyso-Gb3 in female patients (table 12), which remained after adjustment for bone turnover; PICP was also significantly higher in male patients.

I also identified a significant inverse correlation between MMP-1 and severity indexes (MSSI and FIPI) or NT-proBNP; this biomarker related to collagen type I degradation, also presented a significant inverse correlation with age or age at diagnosis and direct correlation with estimated GFR.

In order to understand the effect of each variable as a predictor of PICP or MMP-1, I performed a multivariate regression analysis including the variables significantly correlated with each of these biomarkers (table 13). After adjustment for other variables, LV mass remained the only variable significantly correlated with PICP. Multivariate regression analysis did not identify any variable significantly correlated with MMP-1; however, LV mass was the predictor variable with lowest p value in terms of correlation.

Table 12: Influence of other variables in biomarkers of collagen type I turnover. Mean difference for categorical variables; ρ for continuous variables; PICP: carboxy-terminal propeptide of procollagen type I; B-AP: bone-specific alkaline phosphatase; CITP: carboxy-terminal telopeptide of type I collagen; TRAP: type 5b tartrate-resistant acid phosphatase; MMP: matrix metalloproteinase; α-gal: α-galactosidase; MSSI: Mainz Severity Score Index; FIPI: Fabry International Prognostic Index; NT-pro BNP: N-terminal of propeptide of brain natriuretic peptide; eGFR: estimated glomerular filtration rate; ERT: enzyme replacement therapy; ACEi: angiotensin converting enzyme inhibitors; ARB: angiotensin II receptor blockers.

	PICP (ng/mL)	**PICP:B-AP**	**CITP** (ng/mL)	**CITP:TRAP**	**MMP-1** (ng/mL)	**MMP-2** (ng/mL)
Demographic						
Sex (female)	-48.9*	-7.5*	-0.11*	-0.02	5.7	-55.6
Age (years)	0.077	0.071	-0.153	-0.175	-0.334*	-0.277*
Diagnosis						
Age at diagnosis (years)	-0.059	0.009	-0.142	-0.100	-0.308*	-0.309*
Plasma α-gal A ♀ (nmol/h/mL)	-0.318	-0.444*	0.028	0.029	0.071	-0.017
Plasma α-gal A ♂ (nmol/h/mL)	0.120	0.145	0.012	0.035	-0.121	-0.055
Disease severity						
MSSI	0.394*	0.287*	0.194	0.189	-0.392*	-0.222
FIPI	0.258*	0.141	0.158	0.043	-0.374*	-0.211
Plasma lyso-Gb3 ♀ (nmol/L)	0.402*	0.463*	0.225	0.255	-0.123	0.094
Plasma lyso-Gb3 ♂ (nmol/L)	0.240	0.095	0.171	0.308	0.037	0.266
NT-pro BNP (ng/mL)	0.289	0.489*	-0.163	-0.084	-0.533*	-0.262
eGFR (ml/min/1.73m^2)	-0.122	-0.081	-0.060	-0.059	0.289*	0.262*
Treatment						
ERT (yes)	13.6	3.8	0.03	-0.01	-1.0	-56.3
Age at ERT initiation (years)	-0.187	-0.139	-0.217	-0.218	-0.217	-0.220
Time in ERT (years)	0.204	0.110	-0.213	-0.288	-0.014	0.146
ACEi / ARB (yes)	-27.0	-1.8	-0.03	-0.04	-8.2	-173.4
β-blockers (yes)	6.3	0.2	-0.03	-0.00	4.7	67.5

* $p < 0.05$

1.4 Association between predictive variables and clinical endpoints

1.4.1 *Predictors of left ventricular mass*

In order to evaluate clinical and laboratorial variables predicting LV mass, variables with significant correlation with LV mass in univariate analysis were included in a multivariate regression model (table 14).

Table 13: Multivariate regression analysis of predictive variables affecting PICP or MMP-1. PICP: carboxy-terminal propeptide of procollagen type I; MMP: matrix metalloproteinase; LV: left ventricular; MSSI: Mainz Severity Score Index; FIPI: Fabry International Prognostic Index; eGFR: estimated glomerular filtration rate.

	PICP (multivariate)		**MMP-1** (multivariate)	
	β	p value	β	p value
LV mass (g/m$^{2.7}$)	**0.366**	**0.041**	-0.253	0.209
Sex	-0.078	0.576		
Age (years)			-0.303	0.215
Age at diagnosis (years)			0.055	0.791
MSSI	0.358	0.075	-0.022	0.983
FIPI	-0.214	0.293	0.119	0.613
eGFR (ml/min/1.73m^2)			0.064	0.720

In this model only two variables retained statistical significance: PICP to B-AP ratio and age, with the former expressing a better association with LV mass (β=0.919; p<0.001).

Table 14: Predictive model of LV mass by univariate and multivariate regression analysis. LV: left ventricular; PICP: carboxy-terminal propeptide of procollagen type I; B-AP: bone-specific alkaline phosphatase; CITP: carboxy-terminal telopeptide of type I collagen; TRAP5b: type 5b tartrate resistant acid phosphatase; MMP: matrix metalloproteinase; ERT: enzyme replacement therapy; α-gal: α-galactosidase; MSSI: Mainz Severity Score Index; FIPI: Fabry International Prognostic Index; NT-pro BNP: N-terminal of propeptide of brain natriuretic peptide; eGFR: estimated glomerular filtration rate.

	LV mass (univariate)		**LV mass** (multivariate)	
	R	p value	β	p value
PICP	0.413	0.001	0.081	0.418
PICP:B-AP	0.510	<0.001	**0.919**	**<0.001**
CITP	0.170	0.202		
CITP:TRAP5b	0.016	0.906		
MMP-1	-0.339	0.010	-0.066	0.646
MMP-2	-0.285	0.032	0.016	0.910
PICP:CITP	0.217	0.102		
PICP:CITP adjusted	0.379	0.004	0.035	0.751
Age	0.486	<0.001	**0.392**	**0.010**
Age at diagnosis	0.274	0.035	0.066	0.644
Age at ERT initiation	0.310	0.058		
Time in ERT	0.320	0.050	-0.094	0.288
Plasma α-gal A ♀	-0.027	0.906		
Plasma α-gal A ♂	-0.004	0.987		
MSSI	0.631	<0.001	0.041	0.686
FIPI	0.658	<0.001	-0.149	0.417
Plasma lyso-Gb3	0.409	0.009	-0.012	0.898
NT-proBNP	0.414	0.040	0.028	0.759
eGFR	-0.388	0.003	-0.057	0.645

1.4.2 *Diagnostic accuracy to identify Fabry Disease*

I used the diagnostic accuracy of each biomarker to identify FD in order to compare the S of serum biomarkers with the one of the gold standards, TDI abnormalities, to identify incipient FD cardiomyopathy (table 15).

Sp and positive predictive value PPV were influenced by the inclusion criteria in the control group (absence of TDI abnormalities in echocardiogram) and the definition of the reference range of biomarkers according to the control group, overestimating both measures of diagnostic accuracy.

Nonetheless, the S and global diagnostic accuracy (measured by AUC in ROC) were higher for PICP to B-AP ratio than for E' average imaging biomarker of incipient FD cardiomyopathy, in distinguishing patients with FD from healthy controls (figure 12).

Table 15: Diagnostic accuracy of serum and imaging biomarkers to identify Fabry Disease. S: sensitivity; Sp: specificity; PPV: positive predictive value; NPV: negative predictive value; LR+: likelihood ratio for a positive test result; LR-: likelihood ratio for a negative test result; DOR: diagnostic odds ratio; A: accuracy; YI: Youden's index; AUC: area under the curve; CI: confidence interval; LV: left ventricular; S': systolic myocardial velocity measured at the mitral annulus; E': early diastolic myocardial velocity measured at the mitral annulus; PICP: carboxy-terminal propeptide of procollagen type I; B-AP: bone-specific alkaline phosphatase; CITP: carboxy-terminal telopeptide of type I collagen; TRAP5b: type 5b tartrate-resistant acid phosphatase; MMP: matrix metalloproteinase.

	S	Sp	PPV	NPV	LR+	LR-	DOR	A	YI	AUC (95% CI)
LV mass	0.44	0.89	0.93	0.33	4	0.63	6.30	0.54	0.33	0.626 (0.500-0.753)
S' average	0.30	1	1	0.29	NA	0.70	NA	0.43	0.30	0.331 (0.194-0.468)
E' average	0.65	1	1	0.47	NA	0.35	NA	0.73	0.65	0.199 (0.097-0.302)
PICP	0.65	0.75	0.89	0.42	2.6	0.47	5.57	0.68	0.40	0.765 (0.657-0.873)
PICP:B-AP	0.80	0.75	0.91	0.56	3.2	0.27	12	0.79	0.55	0.845 (0.762-0.928)
CITP	0.17	0.79	0.71	0.23	0.81	1.05	0.77	0.32	-0.04	0.407 (0.255-0.559)
CITP:TRAP5b	0.49	0.79	0.88	0.34	2.33	0.65	3.62	0.57	0.28	0.652 (0.514-0.789)
PICP:CITP	0.58	0.84	0.92	0.39	3.63	0.5	7.25	0.64	0.42	0.775 (0.644-0.907)
PICP:CITP adj	0.38	0.84	0.88	0.31	2.38	0.74	3.26	0.49	0.22	0.614 (0.479-0.750)
MMP-1	0.81	0.5	0.82	0.48	1.62	0.38	4.27	0.73	0.31	0.298 (0.155-0.442)
MMP-2	0.69	0.5	0.80	0.36	1.38	0.62	2.22	0.64	0.19	0.310 (0.186-0.434)

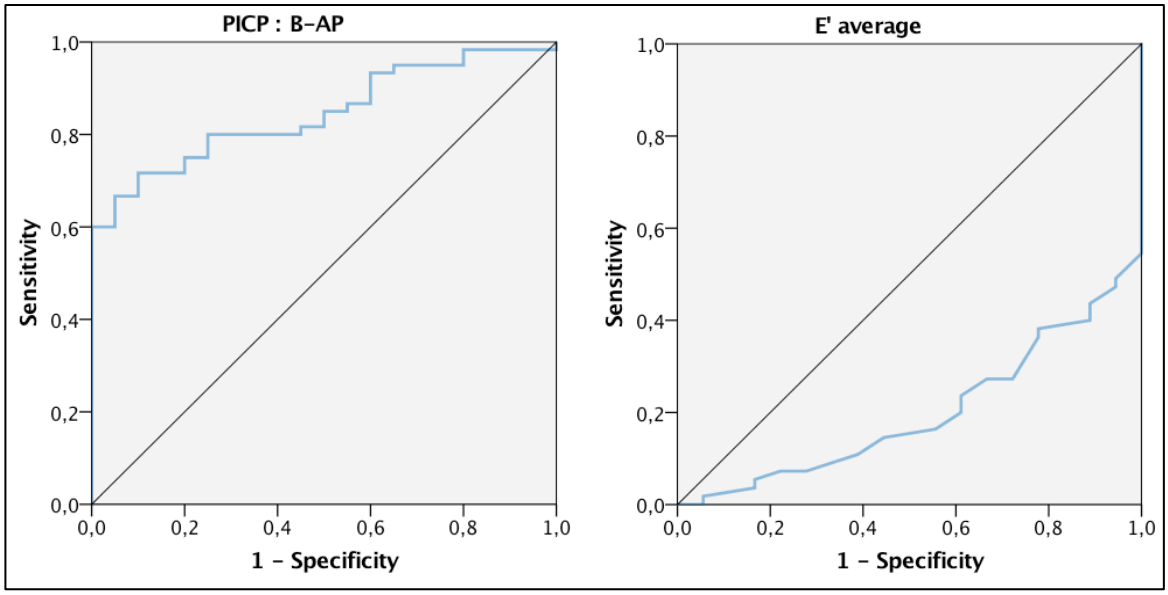


Figure 12: Global diagnostic accuracy to identify Fabry disease. Left image: PICP to B-AP ratio; right image: E' average. PICP: procollagen type I carboxy-terminal propeptide; B-AP: bone-specific alkaline phosphatase; E': early diastolic myocardial velocity measured at the mitral annulus.

1.4.3 *Diagnostic accuracy to identify tissue Doppler imaging abnormalities*

Biomarkers related to collagen metabolism did not present diagnostic accuracy to identify S' abnormalities (as previously defined), a measure of systolic myocardial velocity, being useless biomarkers, as expressed by the AUC of ROC (table 16). However, decreased MMP-1 had a good S (0.89) and a DOR of 2.1 to detect decreased S', but the

AUC of ROC was not significantly different from 0.5 (useless test). Both lyso-Gb3 in female patients and NT-proBNP showed a significant global diagnostic accuracy to identify S' abnormalities.

Table 16: Diagnostic accuracy of blood and imaging biomarkers to identify decreased S' in patients with FD. S: sensitivity; Sp: specificity; PPV: positive predictive value; NPV: negative predictive value; LR+: likelihood ratio for a positive test result; LR-: likelihood ratio for a negative test result; DOR: diagnostic odds ratio; A: accuracy; YI: Youden's index; AUC: area under the curve; CI: confidence interval; LV: left ventricular; S': systolic myocardial velocity measured at the mitral annulus; PICP: carboxy-terminal propeptide of procollagen type I; B-AP: bone-specific alkaline phosphatase; CITP: carboxy-terminal telopeptide of type I collagen; TRAP5b: type 5b tartrate-resistant acid phosphatase; MMP: matrix metalloproteinase; NT-pro BNP: N-terminal of propeptide of brain natriuretic peptide; eGFR: estimated glomerular filtration rate.

	S	Sp	PPV	NPV	LR+	LR-	DOR	A	YI	AUC (95% CI)
LV mass	0.82	0.75	0.58	0.91	3.28	0.24	14.0	0.77	0.57	0.810 (0.697-0.924)
PICP	0.59	0.35	0.28	0.67	0.91	1.17	0.77	0.42	-0.06	0.485 (0.322-0.648)
PICP:B-AP	0.82	0.23	0.31	0.75	1.06	0.78	1.35	0.40	0.05	0.563 (0.401-0.726)
CITP	0.19	0.85	0.33	0.72	1.27	0.95	1.31	0.66	0.04	0.472 (0.301-0.643)
CITP:TRAP5b	0.4	0.51	0.24	0.69	0.82	1.18	0.70	0.48	-0.09	0.477 (0.299-0.654)
PICP:CITP	0.56	0.4	0.27	0.70	0.93	1.1	0.86	0.45	-0.04	0.516 (0.317-0.714)
PICP:CITP adj	0.4	0.65	0.3	0.74	1.14	0.92	1.24	0.58	0.05	0.578 (0.390-0.767)
MMP-1	0.88	0.23	0.32	0.82	1.14	0.52	2.10	0.42	0.11	0.346 (0.177-0.515)
MMP-2	0.69	0.33	0.30	0.72	1.03	0.94	1.1	0.44	0.02	0.428 (0.257-0.599)
Lyso-Gb3 ♀	-	-	-	-	-	-	-	-	-	0.770 (0.584-0.956)
Lyso-Gb3 ♂	-	-	-	-	-	-	-	-	-	0.600 (0.249-0.951)
NT-proBNP	-	-	-	-	-	-	-	-	-	0.832 (0.658-1.000)
eGFR	-	-	-	-	-	-	-	-	-	0.370 (0.220-0.520)

Likewise, the global diagnostic accuracy to identify a decreased E' (a sensitive marker of diastolic dysfunction), was non-significant for all biomarkers related to collagen metabolism (table 17). However, both the marker of collagen type I synthesis (adjusted for bone turnover) and MMP-1 showed good sensitivity to detect the decreased E', but as they seem more sensitive to detect very early changes related to FD cardiomyopathy, they exhibited low specificity. Nonetheless, the DOR of MMP-1 to detect diminished E' was 4.67. In the same way, both lyso-Gb3 in female patients and NT-proBNP showed significant global diagnostic accuracy to identify E' abnormalities.

1.4.4 *Diagnostic accuracy to identify left ventricular hypertrophy*

Both the biomarker of collagen type I synthesis (PICP) and the enzyme MMP-1 had a significant global diagnostic accuracy to identify patients with LV hypertrophy, mainly

driven by a good S (0.85 and 0.92, respectively) and the applicability to rule out LV hypertrophy (LR- 0.29 and 0.30, respectively (table 18).

Table 17: Diagnostic accuracy of blood and imaging biomarkers to identify decreased E' in patients with FD. S: sensitivity; Sp: specificity; PPV: positive predictive value; NPV: negative predictive value; LR+: likelihood ratio for a positive test result; LR-: likelihood ratio for a negative test result; DOR: diagnostic odds ratio; A: accuracy; YI: Youden's index; AUC: area under the curve; CI: confidence interval; LV: left ventricular; E': early diastolic myocardial velocity measured at the mitral annulus; PICP: carboxy-terminal propeptide of procollagen type I; B-AP: bone-specific alkaline phosphatase; CITP: carboxy-terminal telopeptide of type I collagen; TRAP5b: type 5b tartrate-resistant acid phosphatase; MMP: matrix metalloproteinase; NT-pro BNP: N-terminal of propeptide of brain natriuretic peptide; eGFR: estimated glomerular filtration rate.

	S	Sp	PPV	NPV	LR+	LR-	DOR	A	YI	AUC (95% CI)
LV mass	0.57	0.89	0.91	0.52	5.18	0.48	11.16	0.68	0.46	0.772 (0.649-0.894)
PICP	0.65	0.40	0.67	0.38	1.08	0.88	1.23	0.56	0.05	0.511 (0.353-0.669)
PICP:B-AP	0.78	0.2	0.64	0.33	0.98	1.1	0.91	0.58	-0.02	0.504 (0.345-0.663)
CITP	0.14	0.75	0.5	0.33	0.56	1.15	0.48	0.36	-0.11	0.415 (0.252-0.577)
CITP:TRAP5b	0.32	0.3	0.44	0.21	0.46	2.27	0.20	0.31	-0.38	0.363 (0.204-0.522)
PICP:CITP	0.64	0.5	0.70	0.43	1.28	0.72	1.77	0.59	0.14	0.606 (0.454-0-757)
PICP:CITP adj	0.4	0.7	0.7	0.4	1.33	1	1.56	0.51	0.1	0.633 (0.483-0.783)
MMP-1	0.89	0.37	0.73	0.64	1.41	0.30	4.67	0.71	0.26	0.371 (0.216-0.527)
MMP-2	0.67	0.32	0.65	0.33	0.99	1.03	0.92	0.55	-0.01	0.436 (0.270-0.601)
Lyso-Gb3 ♀	-	-	-	-	-	-	-	-	-	0.824 (0.656-0.991)
Lyso-Gb3 ♂	-	-	-	-	-	-	-	-	-	0.300 (0.000-0.604)
NT-proBNP	-	-	-	-	-	-	-	-	-	0.845 (0.679-1.000)
eGFR	-	-	-	-	-	-	-	-	-	0.419 (0.257-0.581)

Both lyso-Gb3 in female patients, NT-proBNP and estimated GFR evinced a significant global diagnostic accuracy to identify LV hypertrophy. However, the global diagnostic accuracy of PICP and MMP-1 to identify LV hypertrophy was lower than that of TDI abnormalities and NT-proBNP (figure 13).

1.4.5 *Diagnostic accuracy to identify late gadolinium enhancement*

The AUC for LV mass to identify LGE in cardiac MRI was larger than the AUC of the biomarkers related to collagen metabolism. Nonetheless, the global diagnostic accuracy of PICP and of the balance between collagen synthesis and degradation was also significant (table 19 and figure 14).

The most remarkable finding was the 100% S of biomarkers related to collagen type I synthesis (PICP and PICP to B-AP ratio) and MMP-1 to detect LGE, with a corresponding LR- of 0. However, the specificity of both biomarkers to detect LGE was low.

Table 18: Diagnostic accuracy of biomarkers to identify LV hypertrophy in patients with FD. LV: left ventricular; S: sensitivity; Sp: specificity; PPV: positive predictive value; NPV: negative predictive value; LR+: likelihood ratio for a positive test result; LR-: likelihood ratio for a negative test result; DOR: diagnostic odds ratio; A: accuracy; YI: Youden's index; AUC: area under the curve; CI: confidence interval; S': systolic myocardial velocity measured at the mitral annulus; E': early diastolic myocardial velocity measured at the mitral annulus; PICP: carboxy-terminal propeptide of procollagen type I; B-AP: bone-specific alkaline phosphatase; CITP: carboxy-terminal telopeptide of type I collagen; TRAP5b: type 5b tartrate-resistant acid phosphatase; MMP: matrix metalloproteinase; NT-pro BNP: N-terminal of propeptide of brain natriuretic peptide; eGFR: estimated glomerular filtration rate.

	S	Sp	PPV	NPV	LR+	LR-	DOR	A	YI	AUC (95% CI)
S' average	0.58	0.91	0.82	0.75	6.44	0.46	14	0.77	0.49	0.157 (0.050-0.263)
E' average	0.91	0.52	0.57	0.89	1.90	0.17	11.16	0.68	0.43	0.155 (0.053-0.257)
PICP	0.85	0.52	0.58	0.81	1.77	0.29	5.84	0.66	0.37	0.674 (0.537-0.810)
PICP:B-AP	0.88	0.27	0.49	0.75	1.21	0.44	2.87	0.54	0.15	0.660 (0.518-0.801)
CITP	0.16	0.85	0.44	0.57	1.07	0.99	1.07	0.55	0.01	0.565 (0.416-0.713)
CITP:TRAP5b	0.42	0.47	0.37	0.52	0.79	1.23	0.63	0.45	-0.11	0.470 (0.317-0.623)
PICP:CITP	0.68	0.48	0.5	0.67	1.31	0.67	2	0.57	0.16	0.587 (0.437-0.736)
PICP:CITP adj	0.46	0.67	0.5	0.63	1.39	0.81	1.69	0.58	0.13	0.658 (0.514-0.802)
MMP-1	0.92	0.27	0.48	0.82	1.26	0.30	4.12	0.54	0.19	0.280 (0.139-0.421)
MMP-2	0.71	0.33	0.44	0.61	1.06	0.88	1.21	0.49	0.04	0.436 (0.284-0.587)
Lyso-Gb3 ♀	-	-	-	-	-	-	-	-	-	0.758 (0.546-0.970)
Lyso-Gb3 ♂	-	-	-	-	-	-	-	-	-	0.422 (0.116-0.729)
NT-proBNP	-	-	-	-	-	-	-	-	-	0.827 (0.654-0.999)
eGFR	-	-	-	-	-	-	-	-	-	0.342 (0.201-0.484)

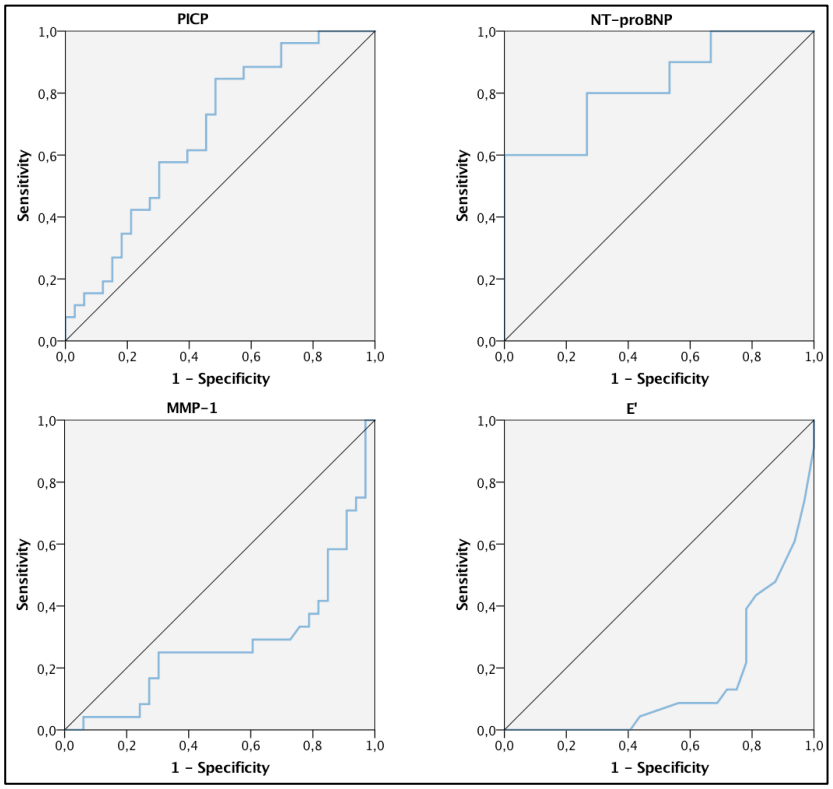


Figure 13: Global diagnostic accuracy to identify LV hypertrophy. Left upper image: PICP; right upper image: NT-proBNP; left lower image: MMP-1; right lower image: E'. PICP: procollagen type I carboxy-terminal propeptide; NT-proBNP: N-terminal of propeptide of brain natriuretic peptide; MMP: matrix metalloproteinase; E': early diastolic myocardial velocity measured at the mitral annulus.

Table 19: Diagnostic accuracy of blood and imaging biomarkers to identify LGE in patients with FD. LGE: late gadolinium enhancement; S: sensitivity; Sp: specificity; PPV: positive predictive value; NPV: negative predictive value; LR+: likelihood ratio for a positive test result; LR-: likelihood ratio for a negative test result; DOR: diagnostic odds ratio; A: accuracy; YI: Youden's index; AUC: area under the curve; CI: confidence interval; LV: left ventricular; S': systolic myocardial velocity measured at the mitral annulus; E': early diastolic myocardial velocity measured at the mitral annulus; PICP: carboxy-terminal propeptide of procollagen type I; B-AP: bone-specific alkaline phosphatase; CITP: carboxy-terminal telopeptide of type I collagen; TRAP5b: type 5b tartrate-resistant acid phosphatase; MMP: matrix metalloproteinase; NT-pro BNP: N-terminal of propeptide of brain natriuretic peptide; eGFR: estimated glomerular filtration rate.

		S	Sp	PPV	NPV	LR+	LR-	DOR	A	YI	AUC (95% CI)
LV mass		0.71	0.65	0.56	0.79	2.03	0.45	4.69	0.68	0.36	0.835 (0.708-0.962)
S' average		0.46	0.74	0.5	0.71	1.77	0.73	2.43	0.64	0.2	0.268 (0.096-0.439)
E' average		1	0.39	0.46	1	1.64	0	NA	0.6	0.39	0.178 (0.039-0.317)
PICP		1	0.43	0.52	1	1.75	0	NA	0.65	0.43	0.730 (0.569-0.890)
PICP:B-AP		1	0.30	0.47	1	1.43	0	NA	0.57	0.30	0.752 (0.596-0.907)
CITP		0.23	0.78	0.38	0.64	1.05	0.99	1.08	0.58	0.01	0.580 (0.383-0.778)
CITP:TRAP5b		0.38	0.59	0.36	0.62	0.93	1.05	0.90	0.51	-0.03	0.472 (0.278-0.666)
PICP:CITP		0.69	0.48	0.43	0.73	1.33	0.65	2.06	0.56	0.17	0.639 (0.454-0.824)
PICP:CITP adj		0.62	0.74	0.57	0.77	2.38	0.51	4.53	0.69	0.36	0.756 (0.594-0.918)
MMP-1		1	0.18	0.44	1	1.22	0	NA	0.5	0.18	0.393 (0.205-0.580)
MMP-2		0.71	0.36	0.42	0.67	1.11	0.81	1.43	0.5	0.07	0.451 (0.259-0.644)
Lyso-Gb3	♀	-	-	-	-	-	-	-	-	-	0.714 (0.288-1.000)
	♂	-	-	-	-	-	-	-	-	-	0.571 (0.223-0.920)
NT-proBNP		-	-	-	-	-	-	-	-	-	0.742 (0.513-0.970)
eGFR		-	-	-	-	-	-	-	-	-	0.266 (0.097-0.435)

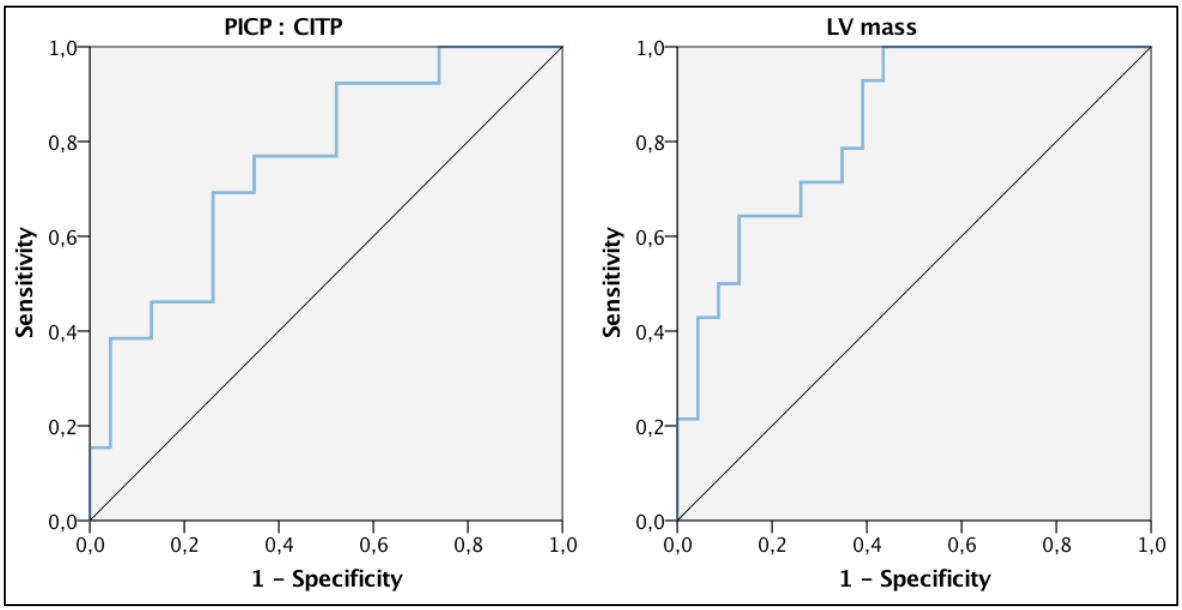


Figure 14: Global diagnostic accuracy to identify LGE. Left image: PICP to CITP ratio; right image: LV mass. PICP: procollagen type I carboxy-terminal propeptide; NT-proBNP: N-terminal of propeptide of brain natriuretic peptide; MMP: matrix metalloproteinase; E': early diastolic myocardial velocity measured at the mitral annulus.

1.5 Biomarkers related to collagen metabolism in the longitudinal study:

1.5.1 *Compliance and population characteristics*

During the longitudinal follow-up period the compliance with clinical assessments was high (figure 15), with all the recruited patients and 55 patients (91.7%) attending their appointments after 12 months and 24 months, respectively. In the last visit 4 patients re-scheduled their visits and one patient changed follow-up to another hospital.

Biomarkers were measured in 78.3% and 68.3% of the patients, after 12 or 24 months, respectively. The main reason for no sampling for biomarker assessment was the re-scheduling of appointments; as the research team was unaware of the patient's presence the samples were not collected; a few samples were also inadvertently wasted.

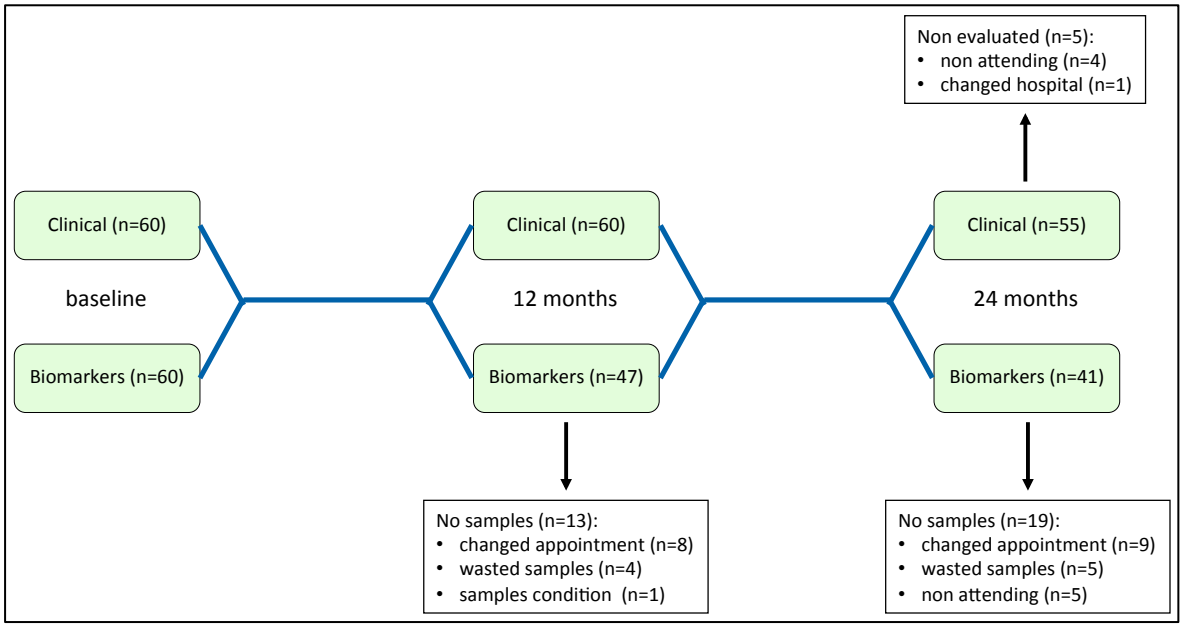


Figure 15: Cardiomyopathy study: flow diagram of longitudinal evaluation.

Variables related to cardiomyopathy severity (heart failure functional class and NT-proBNP) remained essentially stable during the 2 year follow-up period (table 20). Likewise, severity indexes and kidney function did not change significantly during follow-up, as well as the percentage of patients treated with ACEi or ARB and β-blockers. Six patients (10%) started ERT, 4 (6.7%) patients started and one stopped ACEi or ARB, one patient started and 2 stopped β-blockers and one patients started aldosterone antagonists during the study.

Table 20: Cardiomyopathy study: longitudinal variation of disease severity, kidney function and treatment. MSSI: Mainz Severity Score Index; FIPI: Fabry International Prognostic Index; HF: heart failure; NT-proBNP: amino-terminal fragment of the pro-hormone of brain natriuretic peptide; eGFR: estimated glomerular filtration rate; ERT: enzyme replacement therapy; ACEi: angiotensin converting enzyme inhibitors; ARB: angiotensin II receptor blockers.

	baseline (n=60)	**12 months** (n=60)	**24 months** (n=55)	**p value**		
				0 versus 12	12 versus 24	0 versus 24
Severity (median/IQR)						
MSSI	18.5 (21.0)	20.5 (19.0)	19.5 (21.0)	0.445	0.455	0.622
FIPI	2.0 (3.0)	2.0 (3.0)	2.0 (2.0)	0.871	0.415	0.560
HF class	0.0 (1.0)	0.0 (2.0)	0.0 (1.0)	0.048	0.186	1.000
NT-proBNP (ng/mL)	142.0 (334.0)	87.0 (222.0)	97.0 (539)	0.906	0.246	0.535
eGFR (ml/min/1.73m^2)	93.0 (51.0)	92.0 (46.0)	94.5 (53.0)	0.005	0.019	0.788
Treatment (n/%)						
ERT (yes)	39 (65.0)	43 (71.7)	40 (72.7)	0.125	0.500	0.031
ACEi / ARB (yes)	29 (48.3)	32 (53.3)	30 (54.5)	0.375	1.000	0.625
β-blockers (yes)	11 (18.3)	10 (16.7)	8 (14.5)	1.000	1.000	1.000

Despite a significant increase in diastolic IVS thickness during the 24 month period (table 21), diastolic LVPW thickness and LV mass remained essentially stable during this same time. The remaining variables related to diastolic function also remained unchanged during follow-up, except for a significant reduction in the E wave decelerating time.

Analysing indexed LV mass and the E to E' average ratio (an estimate of LV end-diastolic pressure) by FD subgroups, for both variables there was no significant change between baseline and 24 months follow-up in any subgroup of FD patients (figure 16).

Table 21: Cardiomyopathy study: longitudinal variation of echocardiographic variables. Expressed as median (IQR); dIVS: diastolic interventricular septal thickness; dLVPW: diastolic left ventricular posterior wall thickness; LVMi: left ventricular mass indexed for height; LA: left atrium; E/A: left ventricular rapid filling (E wave) / atrial contraction (A wave) peak velocities; DT: E wave decelerating time; S': systolic myocardial velocity measured at the mitral annulus; E': early diastolic myocardial velocity measured at the mitral annulus; E/E': left ventricular rapid filling velocity / average (septal and lateral) early diastolic myocardial velocity measured at the mitral annulus.

	baseline (n=60)	**12 months** (n=51)	**24 months** (n=48)	**p value**		
				0 versus 12	12 versus 24	0 versus 24
dIVS (mm)	10.1 (4.2)	11.0 (5.3)	12.0 (4.5)	0.009	0.206	0.006
dLVPW (mm)	10.0 (3.8)	10.0 (5.0)	10.2 (3.9)	0.220	0.581	0.284
LVMi (g/m$^{2.7}$)	42.9 (25.5)	52.6 (33.0)	51.3 (28.5)	0.110	0.278	0.056
LA area (cm^2)	18.9 (7.3)	20.1 (7.2)	20.7 (6.8)	0.861	0.190	0.185
E/A ratio	1.29 (0.91)	1.23 (0.85)	1.16 (0.69)	0.705	0.150	0.109
DT (ms)	225.0 (68.0)	215.5 (95.0)	202.5 (70.0)	0.557	0.147	0.001
S' septal (cm/s)	6.5 (2.0)	7.0 (3.0)	7.0 (2.0)	0.383	0.981	0.189
S' lateral (cm/s)	8.0 (4.0)	8.0 (4.0)	7.0 (4.0)	0.434	0.993	0.583
E' septal (cm/s)	7.0 (6.0)	8.0 (4.0)	7.0 (5.0)	0.897	0.165	0.457
E' lateral (cm/s)	10.0 (6.0)	10.0 (5.0)	9.0 (4.0)	0.405	0.720	0.583
E/E'	8.80 (5.72)	8.95 (5.24)	9.71 (6.12)	0.110	0.789	0.261

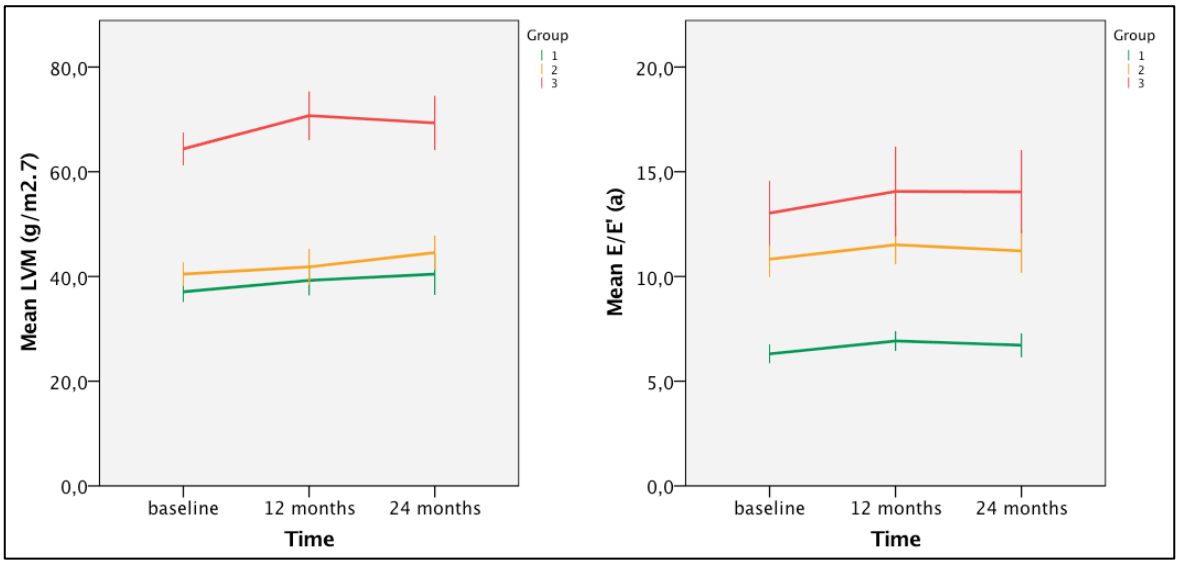


Figure 16: Cardiomyopathy study: longitudinal variation of indexed LVM and E/E' average by FD subgroups. Left image: LVM; right image: F/F' average. Errors bars: ±standard error; LVM: left ventricular mass; E/E' (a): left ventricular rapid filling velocity / average (septal and lateral) early diastolic myocardial velocity measured at the mitral annulus; FD Fabry disease.

According to the definition of indexed LV mass (increase ≥10g/m$^{2.7}$ in indexed LV mass between baseline and 24 months) or LVEDP (increase ≥1.5 in E to E' average ratio, associated with TDI abnormalities [pursuant to the definition in the methods section] upon follow-up) progressors, mentioned above in the methods section, 28.3% and 31.1% of the FD patients presented LV mass and LVEDP progression, respectively (table 22). There was no significant difference in the proportion of LV mass or LVEDP progressors across the FD subgroups.

Table 22: Patients progressing according to LVM and LVEDP criteria in the entire FD cohort and by subgroups. LVM and LVEDP progressors were defined in the methods sections. LVM: left ventricular mass; LVEDP: left ventricular end-diastolic pressure; FD: Fabry disease.

	LVM		LVEDP	
	non progressors	progressors	non progressors	progressors
FD cohort	33 (71.7)	13 (28.3)	31 (68.9)	14 (31.1)
Subgroup 1	12 (80.0)	3 (20.0)	12 (85.7)	2 (14.3)
Subgroup 2	11 (68.8)	5 (31.2)	12 (70.6)	5 (29.4)
Subgroup 3	10 (66.7)	5 (33.3)	7 (50.0)	7 (50.0)

1.5.2 *Variation in controls and Fabry disease patients*

There was no significant change in any biomarker in the control group between baseline and 12 months, except for a significant decrease of the biomarker of collagen type I degradation, even after adjustment for bone turnover (table 23).

Table 23: Cardiomyopathy study: longitudinal variation of biomarkers in control group. Expressed as median (IQR); PICP: procollagen type I carboxy-terminal propeptide; B-AP: bone-specific alkaline phosphatase; CITP: collagen type I carboxy-telopeptide; TRAP5b: tartrate-resistant acid phosphatase; MMP: matrix metalloproteinase.

	baseline (n=20)	**12 months** (n=20)	**p value**
PICP (ng/mL)	107.3 (39.3)	113.4 (39.1)	0.053
PICP/B-AP	10.1 (4.3)	10.5 (4.2)	0.122
CITP (ng/mL)	0.34 (0.20)	0.14 (0.09)	0.002
CITP/TRAP5b	0.11 (0.07)	0.06 (0.02)	0.009
MMP-1 (ng/mL)	16.8 (26.3)	16.2 (38.3)	0.744
MMP-2 (ng/mL)	440.3 (541.0)	433.0 (676.3)	0.647

No significant change was identified in biomarkers concentration between baseline and 24 months, except for CITP adjusted for bone turnover, which significantly decreased (table 24). Also, MMP-1 increased significantly at first follow-up, but returned to close to baseline values after 24 months.

Table 24: Cardiomyopathy study: longitudinal variation of biomarkers in the entire FD cohort. Expressed as median (IQR); FD: Fabry disease; PICP: procollagen type I carboxy-terminal propeptide; B-AP: bone-specific alkaline phosphatase; CITP: collagen type I carboxy-terminal telopeptide; TRAP5b: type 5b tartrate-resistant acid phosphatase; MMP: matrix metalloproteinase.

				p value		
	baseline (n=60)	**12 months** (n=47)	**24 months** (n=41)	0 versus 12	12 versus 24	0 versus 24
PICP (ng/mL)	158.1 (124.7)	192.5 (112.2)	182.1 (98.3)	0.386	0.623	0.841
PICP/B-AP	17.2 (9.6)	19.3 (14.2)	18.6 (11.9)	0.926	0.369	0.871
CITP (ng/mL)	0.23 (0.20)	0.21 (0.10)	0.27 (0.23)	0.332	0.166	0.640
CITP/TRAP5b	0.14 (0.10)	0.13 (0.07)	0.11 (0.10)	0.502	0.575	0.007
MMP-1 (ng/mL)	12.7 (5.9)	14.1 (0.32)	11.4 (10.3)	0.002	0.175	0.573
MMP-2 (ng/mL)	331.2 (149.7)	349.8 (113.0)	372.4 (105.1)	0.743	0.768	0.627

Even when analysing all biomarkers by FD subgroups, with few exceptions, there was no significant change after the follow-up period in any subgroup of patients (figure 17). There was only a significant increase in PICP to B-AP ratio in the subgroup 2 of patients with FD and a significant decrease in CITP adjusted for bone turnover in the less severe subgroup of FD patients. *Per se*, PICP to CITP ratio did not show a significant change during follow-up period (figure 18); however, adjusted for bone turnover, variation during follow-up revealed a significant increase after 24 months due to a significant increase in this ratio in subgroups 1 and 2 of FD patients.

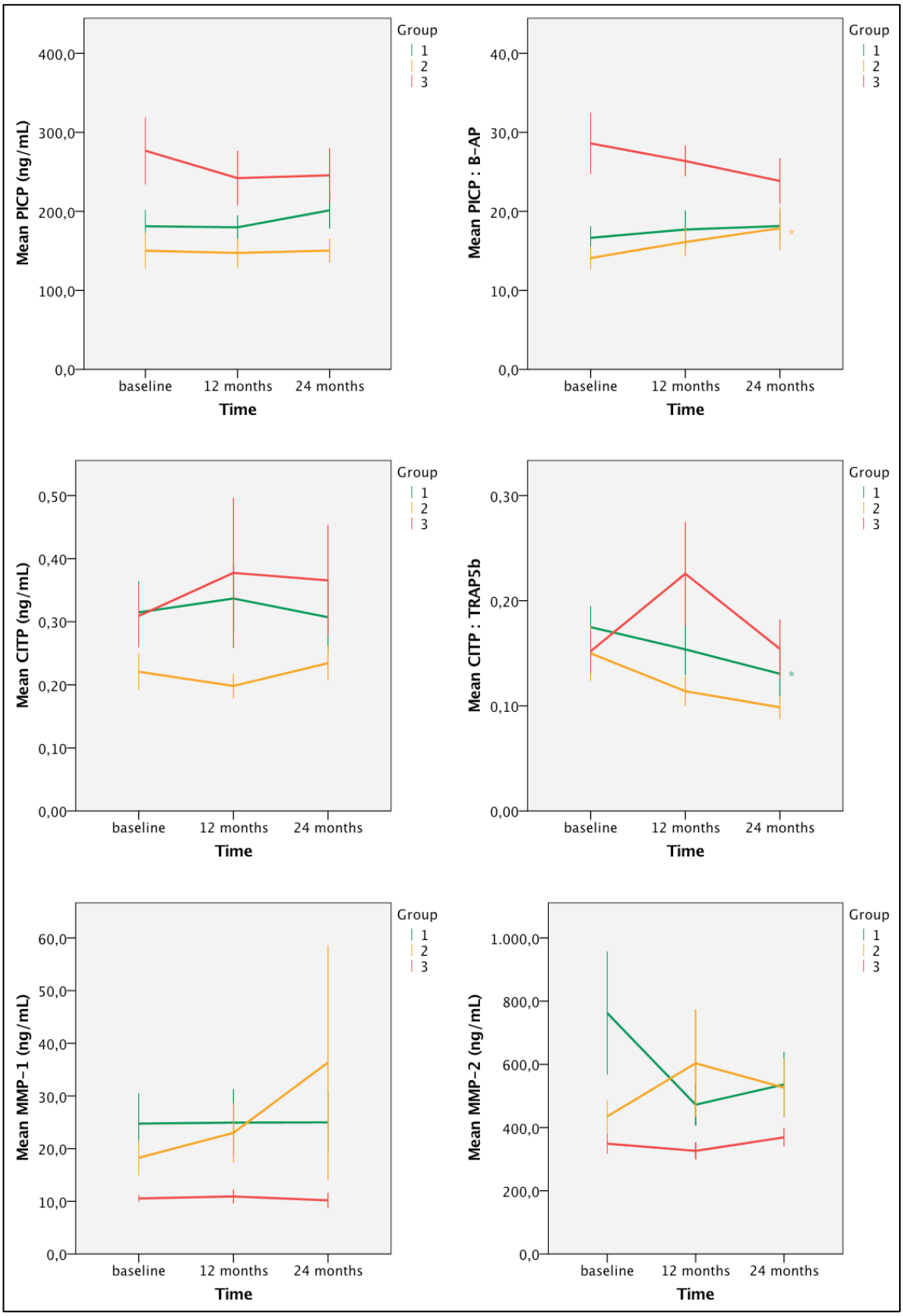


Figure 17: Cardiomyopathy study: longitudinal variation of biomarkers by subgroups of FD patients. Errors bars: ±standard error; * p<0.05 for variation between baseline and 24 months; FD: Fabry disease; PICP: procollagen type I carboxy-terminal propeptide; B-AP: bone-specific alkaline phosphatase; CITP: collagen type I carboxy-terminal telopeptide; TRAP5b: type 5b tartrate-resistant acid phosphatase; MMP: matrix metalloproteinase.

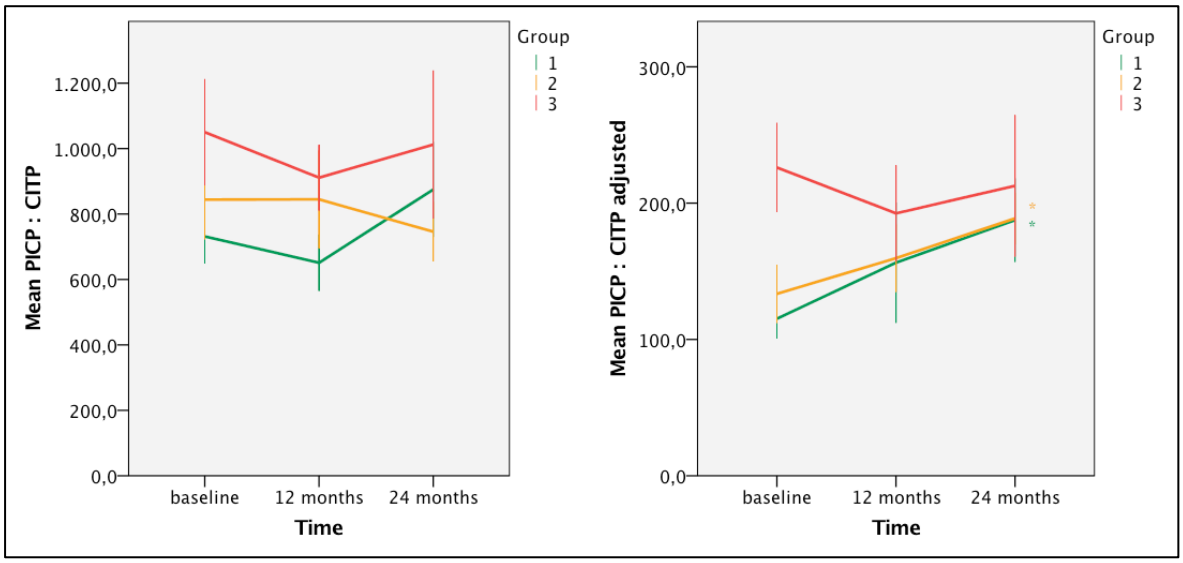


Figure 18: Cardiomyopathy study: longitudinal variation of balance between collagen type I synthesis and degradation by subgroups of FD patients. Left image: non-adjusted for bone turnover; right image: adjusted for bone turnover. Errors bars: ±standard error; * p<0.05 for variation between baseline and 24 months; FD: Fabry disease; PICP: procollagen type I carboxy-terminal propeptide; CITP: collagen type I carboxy-terminal telopeptide.

1.5.3 *Baseline biomarkers concentration and risk of progression*

To assess the effect of baseline concentration of each biomarker in the indexed LV mass and LVEDP progression status during follow-up, I divided the biomarkers into quartiles and compared the distribution of non-progressors and progressors between the quartiles.

There was no significant difference in the distribution of indexed LV mass non-progressors and progressor between the quartiles for all studied biomarkers related to collagen metabolism (table 25); however, there was a clear trend of a greater proportion of progressors in the higher quartiles of PICP (one patient in the fourth quartile of PICP concentration at baseline has an odds ratio (OR) of 7.86 [95% CI: 0.75-82.13] to be progressor, in comparison with a patient in the first quartile). Contrariwise, there was a clear trend of a smaller proportion of LV mass progressors in the higher quartiles of MMP-1 distribution (OR 0.40 [95% CI: 0.07-2.34] to be a progressor comparing the fourth and first quartiles).

Regarding LVEDP progression, I identified a significant difference in the distribution of non-progressors and progressors across the MMP-1 quartiles, with a significant protective OR ratio of 0.58 (95% CI: 0.36-0.94) to be a progressor, comparing

patients in the fourth and first quartiles of MMP-1 distribution. A similar protective OR of 0.07 (95% CI: 0.01-0.75) was found for MMP-2.

Table 25: Distribution of LVM or LVEDP progressors according to quartiles of the baseline concentration of the biomarkers. * for the distribution of non-progressors and progressors across the quartiles; ** of being a progressor comparing fourth to first quartile; LVM: indexed left ventricular mass; LVEDP: left ventricular end-diastolic pressure; OR: odds ratio; NP: non-progressor; P: progressor; Q: quartile; PICP: procollagen type I carboxy-terminal propeptide; B-AP: bone-specific alkaline phosphatase; CITP: collagen type I carboxy-terminal telopeptide; TRAP5b: type 5b tartrate-resistant acid phosphatase; MMP: matrix metalloproteinase.

		LVM		p value*	OR**	LVEDP		p value*	OR**
		NP (%)	P (%)			NP (%)	P (%)		
PICP	Q1	91.7	8.3	0.157	7.86 (0.75-82.13)	90.9	9.1	0.053	4.29 (0.37-50.20)
	Q2	81.8	18.2			36.4	63.6		
	Q3	92.3	7.7			76.9	23.1		
	Q4	58.3	41.7			70.0	30.0		
PICP / B-AP	Q1	90.9	9.1	0.657	4.44 (0.42-47.50)	77.8	22.3	0.494	0.70 (0.08-6.22)
	Q2	83.3	16.7			58.3	41.7		
	Q3	83.3	16.7			58.3	41.7		
	Q4	69.2	30.8			83.3	16.7		
CITP	Q1	76.9	23.1	0.796	1.25 (0.20-7.96)	45.5	54.5	0.279	0.21 (0.03-1.47)
	Q2	83.3	16.7			66.7	33.3		
	Q3	90.9	9.1			81.8	18.2		
	Q4	72.7	27.3			80.0	20.0		
CITP / TRAP5b	Q1	84.6	15.4	0.175	2.36 (0.31-17.85)	53.8	46.2	0.518	0.44 (0.08-2.44)
	Q2	100.0	0.0			75.0	25.0		
	Q3	72.7	27.3			85.7	14.3		
	Q4	70.0	30.0			72.7	27.3		
MMP-1	Q1	66.7	33.3	0.303	0.40 (0.07-2.34)	58.3	41.7	0.033	0.58 (0.36-0.94)
	Q2	72.7	27.3			45.5	54.5		
	Q3	91.7	8.3			75.0	25.0		
	Q4	92.3	7.7			100.0	0		
MMP-2	Q1	75.0	25.0	0.795	0.39 (0.07-2.13)	41.7	58.3	0.095	0.07 (0.01-0.75)
	Q2	75.0	25.0			72.7	27.3		
	Q3	90.0	10.0			72.7	27.3		
	Q4	85.7	14.3			90.9	9.1		
PICP / CITP	Q1	90.0	10.0	0.886	2.70 (0.24-30.85)	81.8	18.2	0.574	2.81 (0.42-18.74)
	Q2	76.9	23.1			72.7	27.3		
	Q3	81.8	18.2			55.6	44.4		
	Q4	76.9	23.1			61.5	38.5		
PICP / CITP adj	Q1	88.9	11.1	0.954	1.78 (0.13-23.52)	90.0	10.0	0.338	3.86 (0.33-45.57)
	Q2	76.9	23.1			70.0	30.0		
	Q3	84.6	15.4			53.8	46.2		
	Q4	81.8	18.2			70.0	30.0		

Comparing the baseline concentration of biomarkers between indexed LV mass progressors and non-progressor (table 26), there was no significant difference for any of the studied biomarkers; however, there was a clear trend of a higher PICP concentration (mean: 297.6 versus 188.6ng/mL) and a lower MMP-1 concentration (mean: 15.0 versus 20.2ng/mL).

Concerning the comparison of the baseline biomarker concentration between LVEDP progressors and non-progressors, it was significantly lower both for MMP-1 (mean: 10.0 versus 18.7ng/mL) and MMP-2 (mean: 302.3 versus 571.4ng/mL). Furthermore, there was a clear trend of a higher PICP to CITP ratio in patients presenting LVEDP progression (mean: 1203.3 versus 802.4).

Table 26: Comparison of the baseline concentration of the biomarkers between LVM or LVEDP non-progressors and progressors. Expressed as median (IQR); LVM: indexed left ventricular mass; LVEDP: left ventricular end-diastolic pressure; NP: non-progressor; P: progressor; PICP: procollagen type I carboxy-terminal propeptide; B-AP: bone-specific alkaline phosphatase; CITP: collagen type I carboxy-terminal telopeptide; TRAP5b: type 5b tartrate-resistant acid phosphatase; MMP: matrix metalloproteinase.

	LVM		p value	LVEDP		p value
	NP	P		NP	P	
PICP (ng/mL)	151.2 (111.2)	238.7 (217.8)	0.077	182.3 (114.3)	149.3 (117.2)	0.961
PICP/B-AP	17.0 (9.7)	20.0 (28.7)	0.114	17.6 (10.7)	16.2 (5.3)	0.462
CITP (ng/mL)	0.21 (0.20)	0.21 (0.34)	0.623	0.24 (0.21)	0.18 (0.18)	0.094
CITP/TRAP5b	0.12 (0.10)	0.16 (0.21)	0.130	0.14 (0.11)	0.10 (0.15)	0.167
MMP-1 (ng/mL)	13.5 (6.6)	9.9 (4.0)	0.134	13.5 (6.6)	10.6 (4.2)	0.026
MMP-2 (ng/mL)	333.5 (214.4)	325.8 (163.4)	0.603	345.0 (198.4)	285.6 (109.9)	0.023
PICP/CITP	743.0 (671.0)	817.4 (965.2)	0.464	624.4 (744.3)	894.0 (780.0)	0.066
PICP/CITP adjusted	132.6 (125.9)	114.2 (231.5)	0.943	123.5 (141.7)	144.0 (133.3)	0.300

1.5.4 *Correlation between variations of biomarkers and left ventricular mass*

Indexed LV mass variation significantly correlated with PICP variation (ρ=0.492; p=0.001), even after adjustment for bone turnover (table 27). The variation of the remaining biomarkers did not correlate significantly with LV mass, but there was a clear trend of an inverse correlation between CITP variation adjusted for bone turnover and LV mass variation (ρ=-0.314; p=0.066) (figure 19). There was no significant correlations between the variation of biomarkers and the variation of LVEDP.

Table 27: Correlation between the variation of the biomarkers and LVM or LVEDP variation. LVM: indexed left ventricular mass; LVEDP: left ventricular end-diastolic pressure; Δ: variation; PICP: procollagen type I carboxy-terminal propeptide; B-AP: bone-specific alkaline phosphatase; CITP: collagen type I carboxy-terminal telopeptide; TRAP5b: type 5b tartrate-resistant acid phosphatase; MMP: matrix metalloproteinase.

	LVM variation		LVEDP variation	
	ρ	p value	ρ	p value
Δ PICP	0.492	0.001	0.163	0.328
Δ PICP/B-AP	0.341	0.036	0.171	0.335
Δ CITP	-0.081	0.637	0.216	0.234
Δ CITP/TRAP5b	-0.314	0.066	0.164	0.378
Δ MMP-1	0.266	0.112	-0.063	0.728
Δ MMP-2	-0.185	0.273	-0.068	0.709
Δ PICP/CITP	0.300	0.076	-0.115	0.530
Δ PICP/CITP adjusted	0.178	0.307	-0.336	0.065

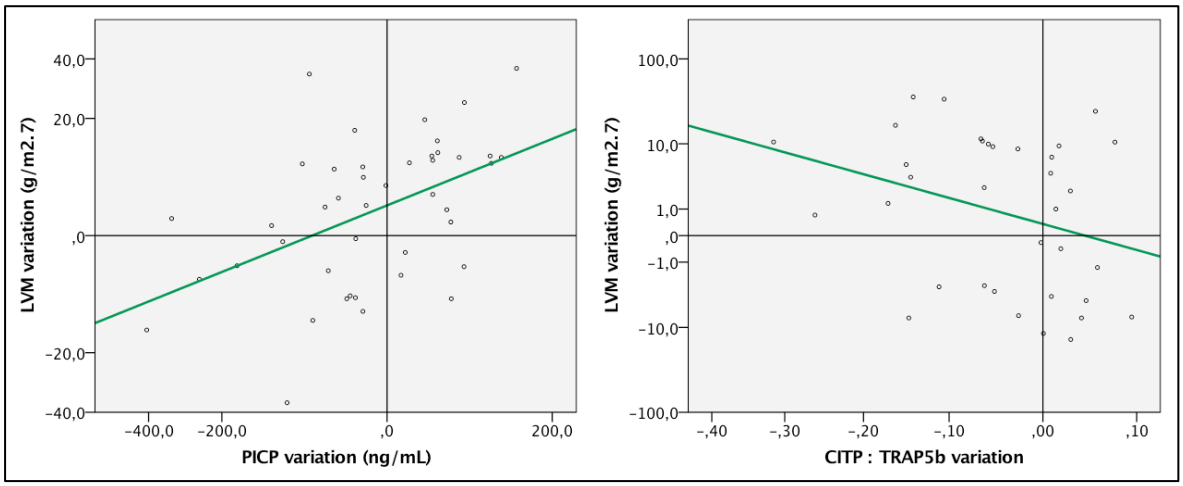


Figure 19: Correlation between LVM variation and PICP (left image) or CITP/TRAP5b (right image) variation. LVM: indexed left ventricular mass; PICP: procollagen type I carboxy-terminal propeptide; CITP: collagen type I carboxy-terminal telopeptide; TRAP5b: type 5b tartrate-resistant acid phosphatase.

1.5.5 *Longitudinal variation and risk of progression*

Comparing LV mass or LVEDP non-progressors and progressors, the variation of the biomarkers during the follow-up was only significantly higher in progressors for PICP concentration (even after adjustment for bone turnover), but only for LV mass progressors (table 28). Nonetheless, there was also a clear trend of significantly higher PICP concentration in LVEDP progressors (mean difference between progressors and non-progressors: 34.08ng/mL; p=0.071).

Table 28: Comparison of the variation in the concentration of the biomarkers between LVM or LVEDP non-progressors and progressors. Expressed as median (IQR); LVM: indexed left ventricular mass; LVEDP: left ventricular end-diastolic pressure; NP: non-progressor; P: progressor; Δ: variation; PICP: procollagen type I carboxy-terminal propeptide; B-AP: bone-specific alkaline phosphatase; CITP: collagen type I carboxy-terminal telopeptide; TRAP5b: type 5b tartrate-resistant acid phosphatase; MMP: matrix metalloproteinase.

	LVM		p value	LVEDP		p value
	NP	P		NP	P	
Δ PICP (ng/mL)	-7.6 (60.9)	20.6 (95.6)	0.001	-11.2 (49.5)	19.9 (95.4)	0.071
Δ PICP/B-AP	-1.76 (6.70)	7.53 (14.14)	0.018	-1.49 (9.74)	2.65 (8.08)	0.204
Δ CITP (ng/mL)	-0.00 (0.17)	-0.02 (0.17)	0.727	-0.04 (0.22)	0.05 (0.08)	0.106
Δ CITP/TRAP5b	-0.00 (0.09)	-0.07 (0.15)	0.187	-0.04 (0.17)	0.01 (0.13)	0.486
Δ MMP-1 (ng/mL)	-1.28 (7.22)	1.20 (2.64)	0.104	-0.08 (5.76)	-0.66 (5.83)	0.875
Δ MMP-2 (ng/mL)	32.0 (215.0)	-26.0 (138.8)	0.194	20.8 (219.4)	34.8 (91.3)	0.433
Δ PICP/CITP	-144.6 (455.9)	143.0 (339.0)	0.403	49.3 (487.5)	-82.1 (509.5)	0.912
Δ PICP/CITP adjusted	34.8 (92.3)	50.7 (167.1)	0.970	68.5 (113.1)	14.1 (79.8)	0.056

There was a significant difference in the distribution of indexed LV mass non-progressors and progressors between the quartiles of PICP (even after adjustment for

bone turnover) (table 29). Likewise, there was a significant higher proportion of progressors in the higher quartile of variation of PICP concentration, in comparison with the first quartile: OR 25.00 (95% CI: 1.80-346.69).

The distribution of the variation of all studied biomarkers, between LVEDP non-progressors and progressors, was non-statistically significant.

Table 29: Distribution of LVM or LVEDP progressors according to quartiles of the variation in the concentration of the biomarkers. * for the distribution of non-progressors and progressors across the quartiles; ** of being a progressor comparing fourth to first quartile; LVM: indexed left ventricular mass; LVEDP: left ventricular end-diastolic pressure; OR: odds ratio; NP: non-progressor; P: progressor; Δ: variation; Q: quartile; PICP: procollagen type I carboxy-terminal propeptide; B-AP: bone-specific alkaline phosphatase; CITP: collagen type I carboxy-terminal telopeptide; TRAP5b: type 5b tartrate-resistant acid phosphatase; MMP: matrix metalloproteinase.

		LVM		p value*	OR**	LVEDP		p value*	OR**
		NP (%)	P (%)			NP (%)	P (%)		
Δ PICP	Q1	90.9	9.1	0.008	25.00 (1.80-346.69)	77.8	22.2	0.147	3.50 (0.43-28.45)
	Q2	91.7	8.3			91.7	8.3		
	Q3	58.3	41.7			55.6	44.4		
	Q4	28.6	71.4			50.0	50.0		
Δ PICP / B-AP	Q1	77.8	22.2	0.011	8.17 (1.03-64.94)	100.0	0.0	0.264	1.60 (0.94-2.74)
	Q2	91.7	8.3			66.7	33.3		
	Q3	85.7	14.3			50.0	50.0		
	Q4	30.0	70.0			62.5	37.5		
Δ CITP	Q1	66.7	33.3	1.000	0.57 (0.07-4.64)	100.0	0.0	0.037	1.33 (0.89-1.99)
	Q2	75.0	25.0			75.0	25.0		
	Q3	70.0	30.0			33.3	66.7		
	Q4	77.8	22.2			75.0	25.0		
Δ CITP / TRAP5b	Q1	66.7	33.3	0.101	0.57 (0.07-4.64)	77.8	22.2	0.747	1.17 (0.12-10.99)
	Q2	50.0	50.0			80.0	20.0		
	Q3	100.0	0.0			55.6	44.4		
	Q4	77.8	22.2			75.0	25.0		
Δ MMP-1	Q1	88.9	11.1	0.362	4.80 (0.39-59.90)	85.7	14.3	0.591	2.40 (0.17-34.93)
	Q2	81.8	18.2			54.5	45.5		
	Q3	55.6	44.4			75.0	25.0		
	Q4	62.5	37.5			71.4	28.6		
Δ MMP-2	Q1	70.0	30.0	0.236	0.39 (0.03-4.80)	88.9	11.1	0.541	3.20 (0.23-45.19)
	Q2	50.0	50.0			57.1	42.9		
	Q3	90.0	10.0			60.0	40.0		
	Q4	85.7	14.3			71.4	28.6		
Δ PICP / CITP	Q1	90.0	10.0	0.419	3.00 (0.22-40.93)	62.5	37.5	0.914	0.48 (0.06-3.99)
	Q2	66.7	33.3			62.5	37.5		
	Q3	55.6	44.4			71.4	28.6		
	Q4	75.0	25.0			77.8	22.2		
Δ PICP / CITP adj	Q1	66.7	33.3	0.740	0.80 (0.09-6.85)	71.4	28.6	0.072	0.71 (0.45-1.14)
	Q2	88.9	11.1			37.5	62.5		
	Q3	70.0	30.0			77.8	22.2		
	Q4	71.4	28.6			100.0	0.0		

Comparing the indexed LV mass change according to variation quartiles, PICP showed a progressive positive variation of LV mass across the increasing quartiles of its distribution (p=0.034 for interquartile difference) (figure 20). Moreover, PICP variation

increased progressively between non-progressing patients, patients with only LVEDP progression, patients with only LV mass progression and patients presenting LVEDP and LV mass progression (p=0.01).

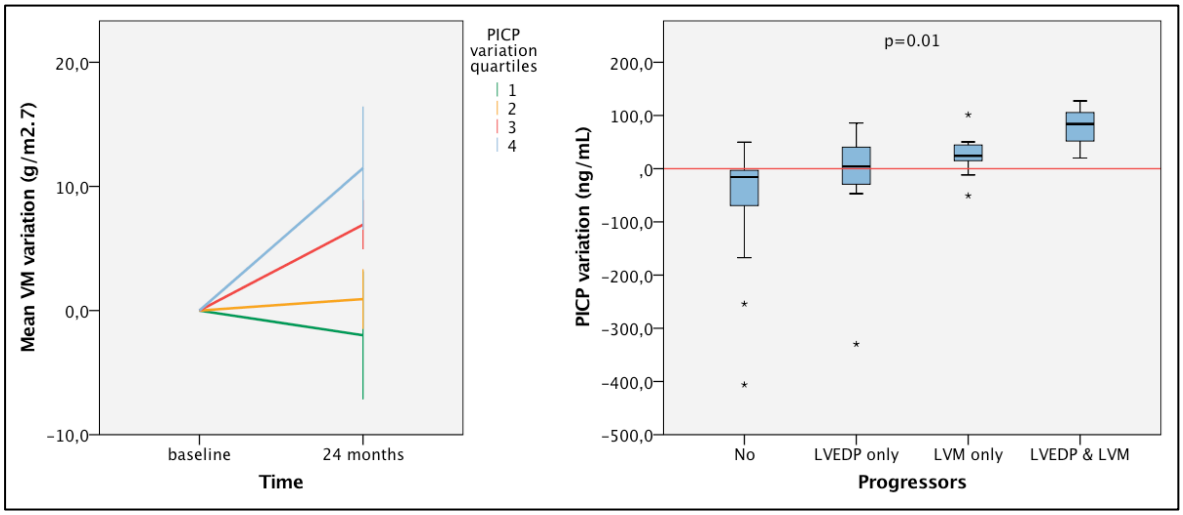


Figure 20: Longitudinal variation of LVM according to PICP variation quartiles (left image) and comparison of PICP concentration variation (right image) between subgroups of progression. Right image - from left to right: non-progressors, only LVEDP progressor patients, only LV mass progressor patients and patients presenting LVEDP and LV mass progression. LVM: indexed left ventricular mass; LVEDP: left ventricular end-diastolic pressure; PICP: procollagen type I carboxy-terminal propeptide.

Only the higher quartile of variation of PICP was associated with a significantly higher risk of an individual patient being a progressor of LV mass and/or LVEDP: fourth quartile of PICP variation (adjusted for bone turnover) gives an OR of 14.00 (95% CI: 1.54-127.23) to be a progressor, in comparison with the first quartile (table 30).

Table 30: Distribution of progressors (LV mass and/or LVEDP) and non-progressors in the first and fourth quartiles of biomarkers variation. * for the distribution of non-progressors and progressors between quartiles 1 and 4; ** of being a progressor comparing fourth to first quartile; OR: odds ratio; NP: non-progressor; P: progressor; Q: quartile; Δ: variation; PICP: procollagen type I carboxy-terminal propeptide; B-AP: bone-specific alkaline phosphatase; CITP: collagen type I carboxy-terminal telopeptide; TRAP5b: type 5b tartrate-resistant acid phosphatase; MMP: matrix metalloproteinase.

	Q1		Q4		p value*	OR**
	NP (%)	P (%)	NP (%)	P (%)		
Δ PICP	72.7	27.3	22.2	77.8	0.070	9.33 (1.19-72.99)
Δ PICP/B-AP	77.8	22.2	20.0	80.0	0.023	14.00 (1.54-127.23)
Δ CITP	66.7	33.3	66.7	33.3	1.000	1.00 (0.14-7.10)
Δ CITP/TRAP5b	55.6	44.4	55.6	44.4	1.000	1.00 (0.16-16.42)
Δ MMP-1	77.8	22.2	50.0	50.0	0.335	3.50 (0.43-28.45)
Δ MMP-2	60.0	40.0	62.5	37.5	1.000	0.90 (0.13-6.08)
Δ PICP/CITP	60.0	40.0	55.6	44.4	1.000	1.20 (0.19-7.44)
Δ PICP/CITP adjusted	44.4	55.6	71.4	28.6	0.358	0.32 (0.04-2.62)

1.5.6 *Longitudinal variation: influence of gender and treatment*

Gender was not a significant risk factor to be an indexed LV mass or a LVEDP progressor (table 31).

Table 31: Progressing and non-progressing Fabry disease patients: analysis by gender. LVM: indexed left ventricular mass; LVEDP: left ventricular end-diastolic pressure; NP: non-progressors; P: progressors; OR: odds ratio.

	LVM			LVEDP		
	NP (%)	P (%)	OR	NP (%)	P (%)	OR
Females	67.9	32.1	0.60	63.0	37.0	0.49
Males	77.8	22.2	(0.15-2.36)	77.8	22.2	(0.13-1.89)

However, this exploratory analysis by gender was hampered by the small sample of patients classified as progressors. However, in males the difference in baseline concentration of PICP to B-AP ratio and PICP to CITP ratio adjusted for bone turnover, between indexed LV mass non-progressors and progressors has became significant (table 32). I also noticed a significant difference in PICP concentration at baseline between LV mass non-progressors and progressors in females. The significant difference baseline MMP-1 and MMP-2 concentrations at baseline between LVEDP non-progressors and progressors only remained significant for female patients and solely for MMP-1.

Table 32: Comparison of the baseline concentration of the biomarkers between LVM or LVEDP non-progressors and progressors: stratification by gender. Expressed as median (IQR); LVM: indexed left ventricular mass; LVEDP: left ventricular end-diastolic pressure; NP: non-progressor; P: progressor; PICP: procollagen type I carboxy-terminal propeptide; B-AP: bone-specific alkaline phosphatase; CITP: collagen type I carboxy-terminal telopeptide; TRAP5b: type 5b tartrate-resistant acid phosphatase; MMP: matrix metalloproteinase.

		LVM			LVEDP		
		NP	P	p value	NP	P	p value
PICP (ng/mL)	♀	124.6 (94.2)	238.7 (340.0)	0.033	111.5 (102.7)	146.0 (133.2)	0.315
	♂	210.5 (107.5)	225.0 (.)	0.673	211.0 (104.9)	193.0 (123.4)	0.671
PICP/B-AP	♀	13.7 (10.1)	18.5 (28.5)	0.148	15.8 (9.6)	16.3 (13.3)	0.725
	♂	18.4 (10.1)	47.0 (.)	0.049	22.3 (10.7)	15.9 (3.5)	0.111
CITP (ng/mL)	♀	0.18 (0.17)	0.21 (0.44)	0.211	0.21 (0.15)	0.12 (0.14)	0.126
	♂	0.29 (0.24)	0.26 (.)	0.412	0.29 (0.23)	0.25 (0.35)	0.903
CITP/TRAP5b	♀	0.10 (0.10)	0.23 (0.21)	0.018	0.13 (0.11)	0.08 (0.19)	0.225
	♂	0.14 (0.14)	0.11 (.)	0.456	0.14 (0.14)	0.12 (0.12)	0.428
MMP-1 (ng/mL)	♀	13.6 (9.6)	10.3 (4.4)	0.178	14.1 (6.9)	10.0 (4.4)	0.010
	♂	12.4 (7.0)	9.0 (.)	0.399	11.8 (9.2)	11.7 (4.2)	0.750
MMP-2 (ng/mL)	♀	348.6 (198.4)	329.0 (214.3)	0.864	356.5 (190.1)	316.6 (115.9)	0.108
	♂	328.1 (357.4)	275.7 (.)	0.399	333.2 (480.5)	256.4 (146.3)	0.111
PICP/CITP	♀	820.4 (681.3)	817.4 (1071.8)	0.980	599.2 (726.1)	1187.5 (958.9)	0.018
	♂	636.3 (292.2)	1063.5 (.)	0.371	636.3 (852.7)	701.8 (381.3)	0.821
PICP/CITP adj	♀	133.2 (157.4)	90.2 (58.5)	0.162	119.5 (78.4)	178.0 (223.0)	0.071
	♂	132.1 (131.1)	445.1 (.)	0.037	150.6 (184.5)	138.1 (60.4)	0.571

The significant difference in longitudinal variation of PICP concentration between LVM non-progressors and progressors remained significant in the sub-analysis by gender in both females and males (table 33). The variation of all other biomarkers, in sub-analysis by gender, was no different between non-progressors and progressors.

Table 33: Comparison of the variation in the concentration of the biomarkers between LVM or LVEDP non-progressors and progressors: stratification by gender. Expressed as median (IQR); LVM: indexed left ventricular mass; LVEDP: left ventricular end-diastolic pressure; NP: non-progressor; P: progressor; Δ: variation; PICP: procollagen type I carboxy-terminal propeptide; B-AP: bone-specific alkaline phosphatase; CITP: collagen type I carboxy-terminal telopeptide; TRAP5b: type 5b tartrate-resistant acid phosphatase; MMP: matrix metalloproteinase.

		LVM			LVEDP		
		NP	P	p value	NP	P	p value
Δ PICP (ng/mL)	♀	-14.5 (87.2)	24.3 (78.0)	0.031	-9.7 (58.6)	20.6 (106.6)	0.115
	♂	-11.4 (68.8)	35.5 (72.2)	0.007	-11.2 (80.31)	-3.2 (.)	0.734
Δ PICP/B-AP	♀	-1.8 (4.2)	6.7 (12.6)	0.048	-0.8 (9.8)	-0.4 (8.0)	0.614
	♂	-2.1 (11.3)	9.5 (.)	0.310	-2.8 (12.0)	5.1 (.)	0.157
Δ CITP (ng/mL)	♀	0.00 (0.18)	0.04 (0.17)	0.682	-0.03 (0.22)	0.03 (0.17)	0.586
	♂	-0.01 (0.26)	-0.08 (.)	0.918	-0.09 (0.25)	0.06 (.)	0.117
Δ CITP/TRAP5b	♀	-0.03 (0.11)	-0.09 (0.12)	0.263	-0.04 (0.18)	0.00 (0.15)	0.823
	♂	0.02 (0.12)	-0.06 (.)	0.224	-0.03 (0.16)	0.08 (.)	0.037
Δ MMP-1 (ng/mL)	♀	-2.03 (8.03)	1.58 (6.00)	0.061	0.89 (7.58)	-0.76 (4.76)	0.633
	♂	-0.62 (6.71)	1.20 (.)	0.612	-1.16 (5.96)	0.77 (.)	0.814
Δ MMP-2 (ng/mL)	♀	22.2 (177.8)	-26.3 (123.2)	0.172	18.4 (178.8)	25.9 (96.0)	0.838
	♂	54.9 (476.6)	19.9 (.)	0.735	20.8 (898.3)	72.9 (.)	0.346
Δ PICP/CITP	♀	-192.0 (543.9)	139.8 (301.1)	0.092	-109.9 (487.5)	-82.1 (821.8)	0.849
	♂	-37.8 (388.0)	231.8 (.)	0.495	151.1 (613.7)	-100.4 (.)	0.296
Δ PICP/CITP adj	♀	48.7 (59.1)	58.4 (107.6)	0.944	78.5 (86.2)	34.1 (177.7)	0.052
	♂	0.2 (105.3)	-48.3 (.)	0.644	39.2 (179.1)	-5.4 (.)	0.602

This study was not designed to access the effect of ERT or anti-proteinuric agents (ACEi or ARB) on the biomarkers. However, this issue was approached as an exploratory endpoint. All comparative analysis has been adjusted for indexed LV mass variation during follow-up.

After adjustment, all biomarkers related to collagen type I synthesis showed a trend for a decrease in patients under ERT, a difference that was significant for PICP after adjustment for bone turnover, which increased in untreated patients and decreased in those treated (table 34). The biomarkers related to collagen type I degradation and the enzymes involved in its degradation presented a non-significant trend of increase / less evident decrease in treated patients. The effect of ACEi / ARB treatment on the variation of the biomarkers concentration was less evident, without any consistent variation in treated patients.

Table 34: Cardiomyopathy study: comparison of the variation in the concentration of the biomarkers between patients under or not under ERT or ACEi / ARB. Expressed as mean (standard error); ERT: enzyme replacement therapy; ACEi: angiotensin converting enzyme inhibitors; ARB: angiotensin receptor blockers; Δ: variation; PICP: procollagen type I carboxy-terminal propeptide; B-AP: bone-specific alkaline phosphatase; CITP: collagen type I carboxy-terminal telopeptide; TRAP5b: type 5b tartrate-resistant acid phosphatase; MMP: matrix metalloproteinase.

	ERT		p value	ACEi / ARB		p value
	No	Yes		No	Yes	
Δ PICP (ng/mL)	-1.8 (22.7)	-32.2 (18.7)	0.308	-18.2 (20.4)	-21.8 (21.4)	0.904
Δ PICP/B-AP	3.43 (2.53)	-3.82 (2.15)	0.036	-0.03 (2.42)	-1.60 (2.56)	0.663
Δ CITP (ng/mL)	-0.01 (0.04)	-0.00 (0.03)	0.950	-0.00 (0.03)	-0.00 (0.04)	0.986
Δ CITP/TRAP5b	-0.07 (0.02)	-0.03 (0.02)	0.295	-0.07 (0.02)	-0.02 (0.02)	0.131
Δ MMP-1 (ng/mL)	-1.8 (13.4)	16.4 (12.0)	0.318	16.5 (12.1)	-1.9 (13.5)	0.323
Δ MMP-2 (ng/mL)	-156.0 (128.2)	-54.1 (114.6)	0.557	-142.4 (115.5)	-45.6 (129.3)	0.583
Δ PICP/CITP	-50.1 (113.9)	32.9 (98.6)	0.586	71.3 (100.0)	-90.5 (109.0)	0.284
Δ PICP/CITP adjusted	66.0 (28.1)	14.3 (24.9)	0.179	58.0 (25.1)	10.7 (28.2)	0.221

Due to the reduced number of patients starting ERT or ACEi / ARB during the study, the comparison between patients starting these treatments, or not, was clearly underpowered. However, patients starting ERT presented a significant decrease in PICP to B-AP ratio, in comparison with patients non-starting ERT (table 35).

Table 35: Cardiomyopathy study: comparison of the variation in the concentration of the biomarkers between patients starting ERT or ACEi / ARB, or not, during the study. Expressed as mean (standard error); ERT: enzyme replacement therapy; ACEi: angiotensin converting enzyme inhibitors; ARB: angiotensin receptor blockers; Δ: variation; PICP: procollagen type I carboxy-terminal propeptide; B-AP: bone-specific alkaline phosphatase; CITP: collagen type I carboxy-terminal telopeptide; TRAP5b: type 5b tartrate-resistant acid phosphatase; MMP: matrix metalloproteinase.

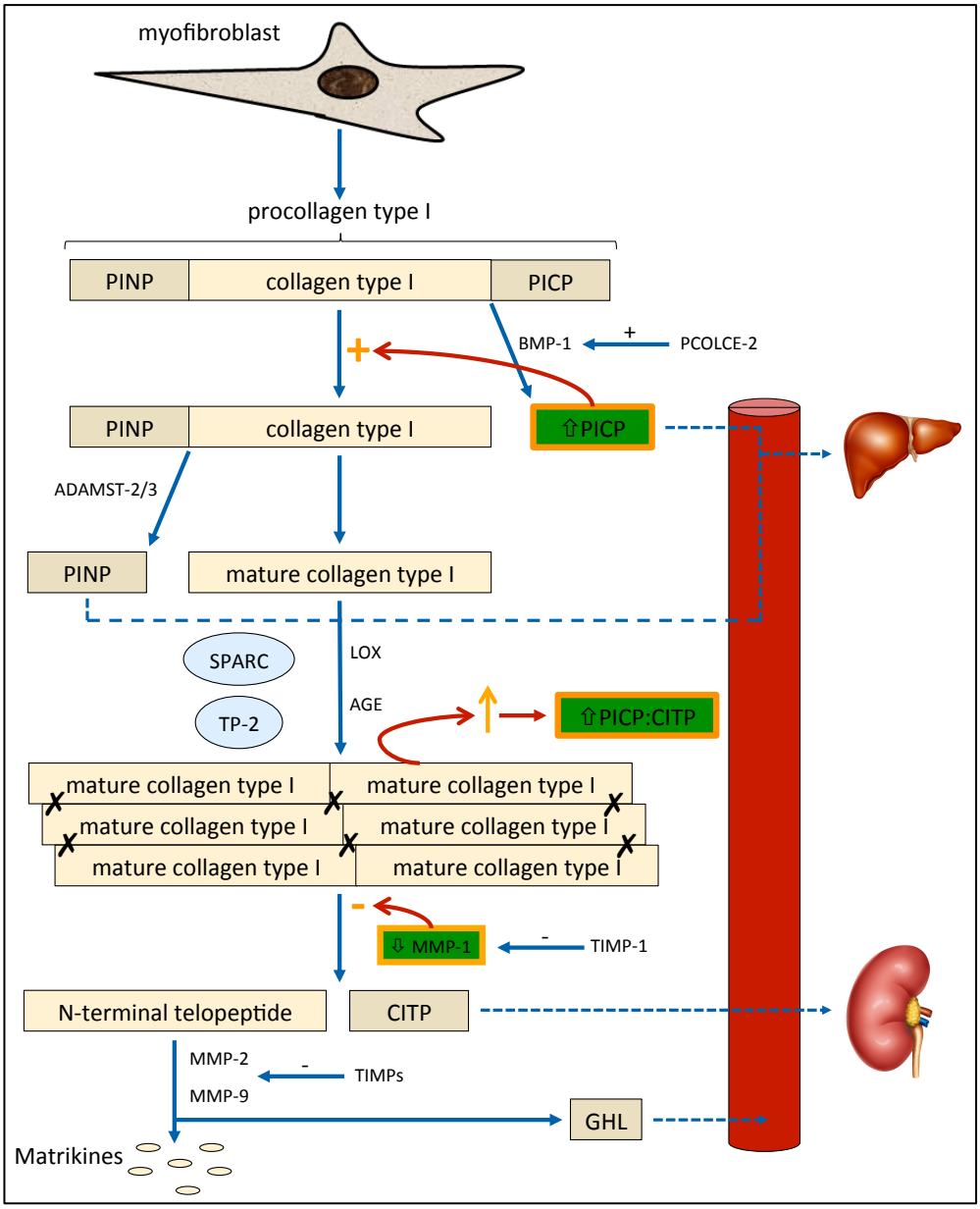
	Starting ERT		p value	Starting ACEi / ARB		p value
	No	Yes		No	Yes	
Δ PICP (ng/mL)	-27.0 (25.7)	-38.7 (34.8)	0.792	-46.4 (23.2)	-36.0 (61.2)	0.877
Δ PICP/B-AP	3.09 (2.35)	-6.40 (3.04)	0.028	-3.12 (1.85)	-7.07 (4.62)	0.446
Δ CITP (ng/mL)	-0.02 (0.05)	0.03 (0.07)	0.587	0.01 (0.04)	-0.12 (0.10)	0.261
Δ CITP/TRAP5b	-0.06 (0.04)	-0.08 (0.05)	0.835	-0.05 (0.03)	-0.18 (0.07)	0.114
Δ MMP-1 (ng/mL)	-0.09 (2.02)	1.67 (2.61)	0.604	19.97 (18.07)	3.79 (45.19)	0.748
Δ MMP-2 (ng/mL)	-180.4 (166.6)	-115.7 (215.1)	0.816	-125.6 (114.2)	-234.3 (285.5)	0.733
Δ PICP/CITP	-14.4 (181.6)	-285.4 (292.8)	0.443	-103.2 (146.6)	453.8 (427.5)	0.236
Δ PICP/CITP adjusted	77.1 (23.6)	49.7 (34.1)	0.534	48.6 (24.0)	118.3 (69.9)	0.359

1.6 Discussion

1.6.1 *Increased collagen type I synthesis*

PICP levels suggest an increased myocardial collagen type I synthesis in FD patients with established LV hypertrophy and, to a lesser extent, in FD patients with normal echocardiogram or with isolated TDI abnormalities (figure 21). Biomarkers related

to collagen type I synthesis also had the highest S and global diagnostic accuracy to detect FD, confirming the added value of these biomarkers in detection of incipient FD cardiomyopathy.



<u>Figure 21:</u> Main findings of cardiomyopathy study. A significant increase in PICP (biomarker of collagen type I synthesis) and a significant decrease in MMP-1 (biomarker related to collagen type I degradation) was found; when collagen type I build up, in patients with left ventricular hypertrophy, a significant increase in PICP to CITP ratio was identified. PINP: procollagen type I amino-terminal propeptide; PICP: procollagen type I carboxy-terminal propeptide; BMP: bone morphogenic protein; PCOLCE: procollagen carboxy-proteinase enhancer; ADAMST: a disintegrin-like and metalloproteinase domain with thrombospodin type motif; LOX: lysil oxidase; AGE: advanced glycation end product; SPARC: secreted protein acidic and rich in cysteine; TP: thrombospondin; MMP: matrix metalloproteinase; TIMP: tissue inhibitor of matrix metalloproteinases; CITP: collagen type I carboxy-terminal telopeptide; GHL: tripeptide glycyl-histidyl-lysine.

These results remained after adjustment for bone synthesis (with the ratio to B-AP) confirming the myocardial origin of the excessive collagen type I synthesis (rather than higher bone metabolic activity). This correction for bone activity is important mainly in post-menopausal women, because the concentration of collagen type I synthesis biomarkers, including PICP, is higher by about 20% in this population (even in women without evidence of osteoporosis).[394, 395] This correction in female patients may explain the increase in the magnitude of the difference between genders in PICP levels after adjustment for bone turnover (expectable given the gender unbalance in the most severe subgroup of FD patients, with only 25% female). Furthermore, given that, unexpectedly, a greater proportion of female patients progressed either in indexed LV mass or in LVEDP during the follow-up period, the adjustment for bone turnover was paramount for an accurate interpretation of the results regarding the prognostic value of the biomarkers.

Elevation of PICP levels in FD patients with normal echocardiograms suggests that it may be a serologic marker of risk detectable before the earlier echocardiographic signs of cardiac dysfunction. Moreover, it hints that the stimulus for myocardial fibrosis is an early event in FD cardiomyopathy (probably directly related to the disease pathophysiology), instead of a secondary event due to mechanical stress or ischemia at a microcirculatory level.

In HCM, as mentioned above, a pathological model with similarities with FD cardiomyopathy, a few studies reported an increase in serum PICP concentration in patients with overt cardiomyopathy (defined by the presence of LV hypertrophy).[422, 423] Furthermore, one study of genotyped patients investigated the role of the biomarkers related to collagen metabolism to detect early cardiac involvement. The authors compared biomarkers of collagen metabolism between mutation carriers with and without LV hypertrophy and mutation-negative normal controls and reported elevated serum levels of PICP even in mutation carriers without LV hypertrophy (that remained after adjustment for bone turnover), further supporting this profibrotic state preceding overt cardiomyopathy development.[397] Moreover, a histological study in cats with pre-clinical HCM has shown an increase in myocardial collagen deposition, compared with healthy controls.[694] Comparable results were found in hypertensive heart disease, with hypertensive patients evidencing increased PICP concentration, even lacking overt cardiomyopathy.[326, 446]

GLA knockout mice ("Fabry mice") display mild accumulation of glycosphingolipids, but do not develop significant cardiomyopathy, so this animal model does not help in understanding the pathophysiological mechanism of early cardiomyopathy.[695] However, studies in murine models of HCM have shown an up-regulation of several extracellular matrix proteins (connective tissue growth factor and POSTN) in the very early stage of the disease (pre-hypertrophic stage, with normal histologic findings).[696-699] Both proteins are regulated by TGF-β1 pathway,[700, 701] which is activated in several HCM murine models, with increased values in the pre-fibrotic stages and required for fibrosis development (its inhibition reduces fibrosis).[696, 698]

In FD, there are no experiments evaluating the direct toxic effects of Gb3 and lyso-Gb3 in cardiomyocytes or cardiac fibroblasts, but increasing evidence in various cell lines suggests that these metabolites may exert a direct pro-fibrotic stimulus. Actually, lyso-Gb3 promotes: proliferation of smooth muscle cells in culture;[17] production of extracellular matrix proteins (ColIV and fibronectin) in human glomerular podocytes, via the activation of TGF-β1 and Notch 1 pathways;[18, 70] activation of TGF-β1 pathway and expression of epithelial-mesenchymal transition markers both in proximal tubular epithelial or mesangial cell lines;[702] inhibition of the growth of fibroblasts, as well as their differentiation into myofibroblasts.[703] All these findings support our observations that up-regulation of cardiac fibrogenesis is an early event and directly related to the disease pathophysiology, serving as rational for the results found in this study and for the usefulness of biomarkers related to collagen type I synthesis in incipient FD cardiomyopathy.

The correlations between serum PICP concentration and variables related to demographics, diagnosis, severity and treatment were often non-significant, which suggests that this biomarker is related to FD cardiomyopathy and not to overall disease burden. However, there are a few exceptions: PICP presented a significant correlation with severity indexes (MSSI and FIPI), but both indexes present variables directly related to cardiac involvement[229, 693] and cardiomyopathy is a well-known determinant of overall disease prognosis and burden.[10, 86, 87] Furthermore, PICP concentration significantly correlated with plasma lyso-Gb3 in female patients, an expectable result given the significant direct correlation identified between plasma lyso-Gb3 and LV mass in heterozygotes.[17, 235] Therefore, the significant correlations between PICP and these

variables seems related to FD cardiomyopathy severity, as confirmed by the multivariate regression analysis showing that the sole significant predictor of PICP was LV mass.

1.6.2 *Collagen type I degradation: matrix metalloproteinases down-regulation*

In contrast to the evidence of increased collagen type I synthesis, there was no increase in the biomarker related to collagen type I degradation (CITP), even after adjustment for bone turnover or estimated GFR (the main route of CITP elimination is the kidney and impairment of glomerular function may increase serum CITP concentration). Nonetheless, I found a significant decrease in the activity of MMP-1 (as well as a non-significant clear trend of a decrease in MMP-2) across the subgroups of increasing severity of FD cardiomyopathy (figure 21).

Serum MMPs concentrations did not differ significantly between healthy controls and FD patients in the less severe subgroup. However, decreased MMP-1 or MMP-2 concentrations had better sensitivity to diagnose FD than the standard echocardiographic measurements used in the identification of early cardiomyopathy (TDI abnormalities). Thus, a decrease in serum MMPs seems to occur early in the development of FD cardiomyopathy and they may be good biomarkers of incipient cardiomyopathy.

As an index of degree of coupling between the collagen type I synthesis and degradation, the PICP to CITP ratio (only after adjustment for bone turnover) was significantly higher in the subgroup of patients with established LV hypertrophy (compared with the two less severe subgroups) and inversely correlated with MMP-1 activity (figure 21). The previous findings support the hypothesis of coupling between a decrease in MMP-1 activity and a higher balance between collagen type I synthesis and degradation; in other words, collagen type I seems to build up when there is suppression of MMPs activity.

Similar results were reported in HCM, with two studies showing a significant decrease in MMP-1 in patients with overt HCM and one study displaying a significant increase in PICP to CITP ratio, even in the early phases of HCM (patients with sarcomere mutation, without LV hypertrophy).[397, 422, 423] The suppression of MMPs as a physiopathological mechanism for cardiac fibrosis has been previously described in HCM. Münch et al. found an inverse correlation between MMP-1 or MMP-2, in patients with established HCM, and the amount of cardiac fibrosis (measured by the quantity of LGE in

cardiac MRI), but this correlation was only significant for MMP-2 in females patients.[428] Lombardi et al. also established a significant direct correlation between difference of A dur and Ar dur and free MMP-1 or active MMP-2, suggesting that diastolic function is impaired when collagen type I catabolic enzyme activity decreases.[426]

Moreover, a study in a mice model, supports MMP-2 as a mediator of metabolic cardioprotection, because the MMP-2 knockout mice developed a greater extent of cardiac hypertrophy and presented up-regulation of fibrosis markers after infusion of angiotensin II, when compared with wild-type mice.[704] Two other examples corroborate the deleterious effect of MMP-2 suppression: functional genetic polymorphisms, which increase MMP-2 gene expression, reportedly protect against cardiac remodelling in the hypertensive subject, preventing an increase in LV end-diastolic diameter and indexed LV mass;[705] a rare pan-ethnic genetic disease of deficiency in human MMP-2 enzyme activity affects some Saudi Arabian, Indian and Turkish families with congenital heart disease, including atrial and ventricular septal defects.[706]

The multiple pathways involved in MMPs suppression are not well understood, but recent studies imply aldosterone-induced TIMP-1 expression, acting mainly through MMP-1 inhibition increases net cardiac collagen content.[707] Furthermore, microRNA-214 (an anti-fibrotic microRNA) may play an important role in MMP-1 regulation, as shown by Dong et al. demonstrating that suppression of MMP-1 in cardiac fibroblasts, induced by angiotensin II, could be reversed after administration of pre-miR-214 (this also decreased TIMP-1 and TGF-β).[708]

Therefore, based on these data, I can hypothesize that: in incipient FD cardiomyopathy, increased collagen synthesis is balanced by degradation (limiting fibrogenesis), but when collagen type I synthesis exceeds the degradation, there is deposition of collagen in the myocardium and LV hypertrophy; the suppression of MMPs may be another mechanism of myocardial collagen type I build-up.

As expected, MMP-1 also presented a significant inverse correlation with both severity indexes (MSSI and FIPI), probably due to the aforesaid explanation (cardiac variables in both indexes).[229, 693] A significant inverse correlation with NT-proBNP was also found, presumed in the setting of the correlation between NT-proBNP and FD cardiomyopathy severity.[251, 252] Furthermore, serum MMP-1 also presented a significant inverse correlation with age, which I assumed as a confounding factor, because age

correlates directly with LV mass in FD patients;[85, 88, 91, 92] this hypothesis is further supported by previous reports showing a significant increase in MMP-1 with ageing.[709, 710] Therefore, the significant inverse correlations between MMP-1 and these variables seem related to FD cardiomyopathy severity, with multivariate regression analysis showing that indexed LV mass presented the strongest correlation with MMP-1, although it was non-significant.

1.6.3 *Prognostic value of biomarkers: correlation with cardiac imaging*

The identification of prognostic biomarkers is paramount, as shown in this study: indexed LV mass and LVEDP remained essentially stable during the follow-up period, but about 30% of the patients progressed in LV mass and/or LVEDP, according to the definitions in the study. Thus, biomarkers identifying this subgroup of patients are essential for a personalized follow-up.

In this study, I selected intermediate / surrogate imaging markers to define prognosis, because the relatively small cohort and short follow-up time hindered the use of "hard" outcomes, such as arrhythmias and cardiac death, to define prognosis. However, there is evidence showing that patients with LV hypertrophy have higher prevalence of heart failure and arrhythmias, so LV mass may be an acceptable surrogate marker of clinical meaningful progression.[85, 88]

This study demonstrated the prognostic value of biomarkers related to collagen type I synthesis. I was not only able to show a significant difference in serum PICP concentration between the subgroups of FD patients (with higher values in the most severe subgroup), but also a direct correlation with LV mass (PICP to B-AP ratio remained as the most significant predictor of LV mass in multivariate regression analysis) and a high S to detect LV hypertrophy. Furthermore, although PICP did not significantly change during the follow-up period (in accordance with the relative stability in heart structure and function in this cohort of FD patients), there was a non-significant clear trend of higher serum PICP (even after adjustment for bone turnover) concentration at baseline in patients presenting subsequent indexed LV mass progression, as well as a non-significant higher proportion of LV mass progressing patients in the fourth quartile of baseline PICP distribution.

Nonetheless, the longitudinal variation of PICP emerged as the most important prognostic marker: its variation not only presented a significant inverse correlation with indexed LV mass variation (that remained after adjustment for bone turnover), but PICP variation was significantly higher in LV mass progressors, with a significant OR of 25.0 to be a progressor in patients in the fourth quartile of PICP variation distribution, in comparison with patients in the first quartile. Furthermore, PICP presented a clear non-significant trend of higher variation in LVEDP progressors and a significant difference in LV mass variation, with progressively higher increases in LV mass across the increasing quartiles of PICP variation. These findings of longitudinal evolution strongly suggest a pathophysiological link between myocardial collagen type I deposition and increase in LV mass.

Previous studies in hypertensive heart disease also identified a significant correlation between serum PICP concentration and indexed LV mass.[404, 443] In contrast, studies in HCM provided conflicting results regarding correlation with echocardiographic imaging, with two studies reporting no correlation between PICP and severity of LV hypertrophy[397, 426] and one study evidencing significant correlation between PICP and IVS thickness.[422] Morphological studies in HCM elucidating relative contribution of fibrosis to the magnitude of hypertrophy and heart weight found clear, but weak correlations.[711, 712]

In FD, the main determinant of LV mass seems to be cardiomyocytes hypertrophy, as the magnitude of the increase of fibrotic tissue in endomyocardial tissue was not as high as the cardiomyocyte area and glycosphingolipids vacuoles.[29] However, in view of the present results I can hypothesize that cardiomyocyte hypertrophy and collagen type I deposition are parallel pathophysiological pathways, activated by a common mechanism: α-galactosidase A *per se* and accumulating substrates (Gb3 and lyso-Gb3). This hypothesis is supported by the aforementioned experiments showing that lyso-Gb3 is not only certainly involved in cardiomyocyte hypertrophy, but also promotes activation of profibrotic pathways and justifies the evident predictive value of collagen type I synthesis biomarker to LV mass.[18, 70, 702]

In contrast to a study in HCM showing a significant correlation between serum PICP concentration and diastolic function, I find no significant correlation between PICP and any of the measurements related to diastolic function in FD.[426]

I was also able to show the prognostic added value of serum MMP-1 and MMP-2 concentrations. MMP-1 was not only inversely correlated with LV mass and wall thickness, but had a high sensitivity to detect LV hypertrophy, as well as to rule out this condition, given the low negative LR. MMP-1 also exhibited a direct correlation with TDI abnormalities (either systolic or diastolic). MMP-2 only inversely correlates with LV wall thickness.

These findings and those previously described for serum PICP concentration might indicate that increased collagen synthesis and inhibition of collagenolysis are syntropic in the process of LV hypertrophy and further support the role of MMPs inhibition in diastolic function impairment, as previously described for HCM.[426]

The prognostic value of MMP-1 and MMP-2 suppression in diastolic dysfunction progression was clearly indicated by the findings of significantly lower baseline MMPs concentrations in patients subsequently progressing in LVEDP, with patients in the fourth quartile of MMPs baseline distribution being significantly "protected" from progressive diastolic dysfunction. Longitudinal evolution of MMPs did not correlate with the variation in diastolic dysfunction. Progressive suppression of collagenolysis in patients with increasing LV hypertrophy was also supported by the clear trend of an inverse correlation between variation of CITP (adjusted for bone turnover) and variation of LV mass. In the same way, the balance between collagen type I synthesis and degradation (PICP to CITP ratio) presented an almost significant correlation with LV mass and a clear trend of higher value in LVEDP progressors.

As expected, PICP was the only biomarker with significant difference between LGE positive and negative FD patients, as it is a surrogate biomarker of myocardial collagen type I synthesis. Furthermore, PICP was already elevated in FD patients with normal echocardiograms and without LGE. Accordingly, the PICP to CITP ratio (adjusted for bone turnover) was also significantly higher in patients presenting LGE in cardiac MRI, further supporting that myocardial collagen type I bulking occurs when the synthesis exceeds the degradation capacity.

Both increased serum PICP concentration or decreased serum MMP-1 concentration presented an S of 100% to detect LGE in cardiac MRI and, consequently, a negative LR of 0. This finding is of paramount clinical importance, because, according to these results, in patients with normal serum PICP and MMP-1 concentrations LGE may be

ruled out, precluding the need for a cardiac MRI; moreover it may have an important prognostic value that must be further investigated, as the presence of LGE is associated with worse prognosis in terms of exercise capacity and risk for malignant ventricular arrhythmias.[106, 116]

Previous results in HCM showed no significant correlation between collagen type I metabolism biomarkers and the amount of LGE in cardiac MRI.[397, 423, 425] These results are certainly related to the limited resolution of LGE to detect focal replacement fibrosis[272, 273] and inability to detect early diffuse interstitial fibrosis.[274] However, sensitive ELISA assays may detect very small amounts of circulating PICP and certainly provide a more sensitive index of fibrosis and reflect subtle changes in myocardial composition, non-detectable by cardiac MRI.

A novel cardiac MRI technique, T1 mapping (assessing T1 relaxation times), may overcome the inability of LGE to detect diffuse interstitial fibrosis. High T1 relaxation times are observed in diffuse fibrosis, protein deposition and water in oedema. Low T1 values are seen in iron or lipid deposition.[713] However, in FD cardiomyopathy, cardiac lipid storage is a hallmark and T1 mapping has been studied as an imaging biomarker for early detection of cardiac involvement (with low native T1).[278-280] Thus, the capacity of T1 mapping to detect early diffuse fibrosis may be impaired due to low native T1 (fibrosis may only be detected in the "pseudo normalization" stage, when the amount of fibrotic tissue is enough to overcome the low native characterizing FD cardiomyopathy.) and needs further evaluation.

1.6.4 *Biomarkers related to collagen metabolism: influence of therapeutics*

This study was not designed to access the effect of ERT on the biomarkers, but given the trend of a higher proportion of patients on ERT in the more severe subgroups, it is reasonable to assume that this is the reason why the concentration of biomarkers at baseline was similar between treated and untreated patients.

However, in an exploratory analysis, adjusted for variation in indexed LV mass, I evaluated the effect of patients under / starting ERT on the variation of the biomarkers related to collagen metabolism. Regarding the biomarkers related to collagen type I synthesis, patients under ERT presented a clear trend of a greater decrease in PICP than

the untreated patients, a difference that became significant after adjustment for bone turnover (with treated patients presenting a progressive decrease and the untreated ones an increase). Moreover, patients starting treatment during the follow-up period presented a decrease in PICP to B-AP ratio, whereas the patients that remained untreated showed an increase in the same ratio.

Patients under ERT also presented a trend in the variation of the biomarkers related to collagen type I degradation that was in agreement with an increase in collagenolysis, namely: a smaller decrease in serum CITP (adjusted for bone turnover) and MMP-2 concentrations and an increase in MMP-1 concentration in treated patients, in comparison with untreated patients. All these findings suggest that ERT may affect myocardial collagen type I metabolism, preventing its synthesis and increasing its degradation, probably limiting myocardial collagen type I build-up- This hypothesis deserves detailed study and may be of paramount interest in clinical practice.

There is no evidence about the effect of ERT on myocardial collagen content, because the pathological studies evaluating the effects of ERT on cardiac morphology and Gb3 inclusions did not report the effect on myocardial fibrosis. Nevertheless, ERT does not appear to have an effect on LGE, with two studies reporting an increase in the amount or *de novo* development of LGE in treated patients.[116, 190] However, as mentioned above, LGE only detects focal replacement (irreversible) and not interstitial diffuse (reversible) myocardial fibrosis;[274] thus, a response of focal fibrosis to ERT is not expected, as it is composed of relatively large areas of organized collagen fibres.

Instead, interstitial diffuse fibrosis may be reversible and susceptible to the influence of ERT, through the effects of enzyme deficiency correction and Gb3 clearance in the suppression of the activated profibrotic pathways that are involved in FD pathophysiology. Little evidence supports this hypothesis, but in a human kidney tubular cell line treated with Gb3, where there was an up-regulation of TGF-β1 pathway and activation of an epithelial-mesenchymal transition phenotype (characterized by a decrease in E-cadherin and an increase in N-cadherin and α-smooth muscle actin); when this cell line was treated with recombinant α-galactosidase A, it prevented the activation both of TGF-β1 pathway and epithelial-mesenchymal transition phenotype.[702] Thus, in the setting of the present results, the effect of ERT on the transcription profile and protein synthesis in cardiomyocytes should be further evaluated.

The effect of ACEi / ARB on the biomarkers related to collagen metabolism was less evident. Nonetheless, there was a trend, of smaller magnitude, of a decrease in PICP and PICP to B-AP ratio in patients treated with these agents. The effect in the enzymes related to collagen type I degradation was less consistent.

However, in hypertensive heart disease, there is evidence that serum PICP concentration decreases with treatment with anti-hypertensive drugs (ARB and loop diuretics) and that this decrease was accompanied by a histological validation of parallel changes in the amount of myocardial fibrosis.[377, 380]

In HCM, there is only one study showing a decrease in serum PICP concentration in the group of patients treated with an ARB, whereas in the group of patients not treated with an renin-angiotensin-aldosterone axis antagonist serum PICP concentration remained unchanged; however, a histological validation of a parallel response in the myocardial collagen content was not performed.[405]

In FD cardiomyopathy, the lack of expressive results may be related to the activation of distinct pathophysiological pathways, but the possibility that studies in larger cohorts and for a longer follow-up period may reveal an effect in these biomarkers of myocardial fibrosis must be kept in mind.

1.6.5 *Collagen type I biomarkers: previous findings in FD cardiomyopathy*

To my knowledge, only two studies have evaluated cardiac extracellular matrix turnover in FD. As previously mentioned, Shah et al. reported increased levels of MMP-9, with significant correlation with MSSI and inverse correlation with midwall fractional shortening of the LV.[413] In contrast to the identified decrease in MMP-1 and MMP-2 with increasing cardiomyopathy severity, MMP-9 has been identified in HCM as a profibrotic marker.[428, 714]

Krämer et al. reported an increase in three biomarkers of collagen metabolism (PICP, ICTP and PIIINP), compared with healthy historical controls. However, no difference in these markers, between patients with and without fibrosis in the cardiac MRI was observed, additionally no significant change between baseline and follow-up measurements was found, regardless of ERT status. The authors explained this finding assuming a systemic fibrotic state in FD, involving the heart, the kidney and other organs. Nevertheless, a sub-analysis by gender and adjustment for bone turnover was not

performed, which would had been crucial, since usually female patients have a milder phenotype, but the rise of collagen markers due to bone turnover can misleadingly over-estimate myocardial fibrosis. Moreover, the previously described limitations of LGE techniques to detect cardiac fibrosis certainly influenced the results.[116]

1.6.4 *Difficulties and limitations*

A few difficulties emerged during the study design, related to the epidemiology of FD, because as a rare disease, it is difficult to recruit a cohort large enough to allow sub-analysis and to detect small differences, which may be of clinical importance. During the study I recruited a cohort of the initially planned size, but the recruitment period had to be longer than planned (not affecting the longitudinal follow-up period). Ideally, a longer follow-up period would be desirable for a more accurate analysis of the prognostic value of the biomarkers, but due to constrains of the timeline in the research and doctoral program and in the setting of a long recruitment period, a 24 months longitudinal evaluation was the possible timeline.

The multicenter design of the study generated some difficulties, due to the organization of patients collecting samples at various centres at the same time, which caused some loss of data during follow-up, but did not hamper accurate statistical analysis. I did not experience difficulties in the laboratorial tasks and interpretation of the results.

This study has, *per se*, several limitations, namely: i) theabsence of histological correlation, which could be useful to biomarker validation, but endomiocardial biopsy is an invasive and potentially harmful technique; ii) the available cohort size and short time of follow-up, hindering an accurate definition of the prognostic value of the studied biomarkers; iii) the study was not designed to access the effect of treatments in the biomarkers, so this was just an exploratory analysis; iv) the laboratory standardization of these biomarkers' assays is an urgent need to improve their clinical application; v) no correlation with speckle-tracking analysis of echocardiograms, since this was not available in the majority of the cohort and no quantification of the LGE area, although no correlation was identified in previous studies; vi) no correlation with T1 mapping cardiac MRI techniques.

2. Nephropathy study

2.1 Pilot study

2.1.1 *Population characteristics*

As noted above, for the pilot study I recruited 10 patients with FD and CKD stage ≥3 (as previously defined) and 10 sex and age-matched healthy controls. Population characteristics are given in table 36. The median age of diagnosis of FD was 54.0, 70% were taking ACEi or ARB, 20% were on β-blockers and 90% of them were on ERT, for a median time of 8.7 years.

All patients in the FD group were non-relatives with seven distinctive pathogenic mutations (three have p.N215S, two have p.R227X and the others have unique mutations).

Table 36: Nephropathy pilot study: population characteristics. FD: Fabry disease; ERT: enzyme replacement therapy; RAAi: renin-angiotensin-aldosterone axis inhibitors; IQR: interquartile range.

	Control group (n=10)	**FD group** (n=10)
Categorical variables (n / %)		
Sex (female)	4 (40.0)	4 (40.0)
ERT (yes)	-	9 (90.0)
RAAi (yes)	-	7 (70.0)
β-blocker (yes)	-	2 (20.0)
Continuous variable (median / IQR)		
Age (years)	61.5 (25.0)	61.5 (25.0)
Age at diagnosis (years)	-	54.0 (31.0)
Time in ERT (years)	-	8.7 (3.3)

As per definition of the groups, FD group had a significantly higher serum creatinine and lower estimated GFR than the control group (table 37). A wide range of ACR values were observed in the FD patients, with one patient with ACR within the normal range, 3 with ACR 30 - 300mg/g and 6 patients with ACR >300mg/g; ACR was significantly higher in the FD group.

2.1.2 *Novel biomarkers of glomerular or tubular damage*

The studied glomerular damage biomarkers (UTE and urinary ColIV excretion) were both increased in FD group (table 38). As compared with UTE in controls, transferrinuria in the FD group had a 231% increase, p=0.001. Likewise, the urinary ColIV excretion was significantly higher in patients with FD (a 230% increase, p=0.001).

Table 37: Nephropathy pilot study: kidney function in study population. Expressed as median (IQR); ACR: urinary albumin to creatinine ratio; eGFR: estimated glomerular filtration rate.

	Control group	FD group	p value
ACR (mg/g)	4.1 (6.3)	342.6 (404.9)	<0.001
Creatinine (mg/dL)	0.72 (0.41)	1.48 (0.65)	0.001
eGFR (ml/min/1.73m^2)	84.0 (18.0)	40.5 (19.0)	<0.001

Urinary RBP and FLC κ and λ concentrations were bellow the limit of detection of the assay in controls and in some of the FD patients, so they were excluded from the statistical analysis. The other tubular injury biomarkers (urinary A1MG, NAG and APP excretion) were all significantly increased in FD group, in comparison with the control group (table 38).

For the freely filtered protein A1MG, as compared with urinary A1MG excretion in controls, it was significantly increased in FD patients (a 86% increase, p=0.006). For the urinary enzymes secreted by the tubules, I also found a significant difference between FD and control groups: for NAG a 119% increase, p=0.001; for AAP a 105% increase, p=0.005.

Table 38: Nephropathy pilot study: novel biomarkers of glomerular or tubular damage. Expressed as median (IQR); UTE: urinary transferrin excretion; ColIV: collagen type IV; A1MG: α1-microglobulin; NAG: N-acetyl-β-D-glucosaminidase; AAP: alanine aminopeptidase.

	Control group	FD group	p value
Biomarkers of glomerular damage			
UTE (mg/g)	0.42 (0.29)	1.21 (0.65)	<0.001
Urinary ColIV excretion (μg/g)	3.55 (1.46)	11.06 (12.47)	<0.001
Biomarkers of tubular damage			
Urinary A1MG excretion (μg/g)	203.1 (112.6)	441.6 (192.9)	0.006
Urinary NAG excretion (U/g)	11.8 (17.4)	30.3 (14.0)	0.001
Urinary AAP excretion (μg/g)	6.49 (5.84)	14.36 (5.03)	0.004

Based on the results of the pilot study, I selected the urinary excretion of the following biomarkers to be evaluated in the entire cohort of recruited patients: UTE, ColIV, A1MG, NAG and AAP.

2.2 Population characteristics

From February 2013 to June 2014, 78 FD patients (subgroups: 1 - 25 patients; 2 - 22 patients; 3 - 10 patients; 4 - 21 patients) and 25 controls (age and sex-matched with subgroup 1) were recruited (study flow diagram in figure 22).

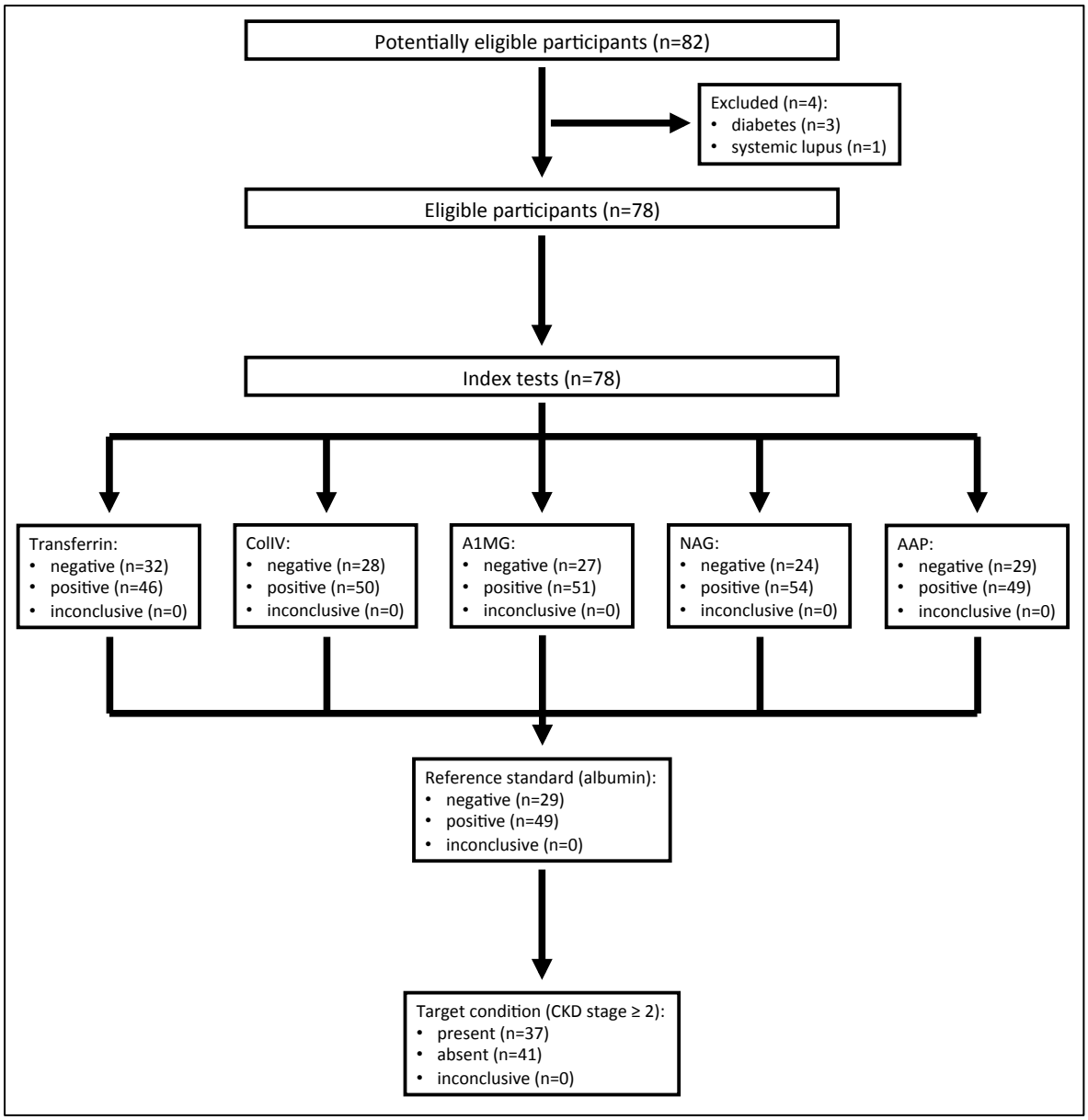


Figure 22: Nephropathy study: study flow diagram. ColIV: collagen type IV; A1MG: α1-microglobulin; NAG: N-acetyl-β-D-glucosaminidase; AAP: alanine aminopeptidase; CKD: chronic kidney disease.

Population characteristics are given in table 39. In the 78 FD patient group, 56.4% were females (distribution non-significantly different between subgroups), with a mean age of 50.0 years old, ranging from 19 to 80 years old (significantly older, comparing the most severe subgroup 4 [patients with CKD stage ≥3] with the two less severe subgroups); 69.2% of the patients were on ERT (significantly more in subgroup 4; 94.4% under agalsidase-α, 5.6% under agalsidase-β), with a significantly older age for ERT initiation comparing group 4 to 1; the use of renin-angiotensin-aldosterone axis blockers (48.7% of the entire cohort) was also significantly higher in the most severe subgroup. HBP was

identified in 42.3% of the patients, significantly more in the most severe subgroup. As expected, the severity indexes (MSSI and FIPI) were also significantly higher in the most severe subgroup. Plasma lyso-Gb3 and α-galactosidase A activity were similar between FD subgroups, both in male and female patients.

Table 39: Nephropathy study: clinical characteristics of the study population. FD: Fabry disease; HBP: arterial hypertension; ERT: enzyme replacement therapy; ACEi: angiotensin converting enzyme inhibitors; ARB: angiotensin II receptor blockers; MSSI: Mainz Severity Score Index; FIPI: Fabry International Prognostic Index; α-gal: α-galactosidase.

	FD cohort	FD subgroups			
	(n=78)	**1** (n=25)	**2** (n=22)	**3** (n=10)	**4** (n=21)
Categorical variables (n (%))					
Sex (female)	44 (56.4)	18 (72.0)	14 (63.6)	4 (40.0)	8 (38.1)
HBP (yes)ǂ	33 (42.3)	4 (16.0)	9 (40.9)	3 (30.0)	17 (81.0)
ERT (yes)*	54 (69.2)	15 (60.0)	13 (59.0)	6 (60.0)	20 (95.2)
ACEi / ARB (yes)ǂ	38 (48.7)	8 (32.0)	8 (36.4)	4 (40.0)	18 (85.7)
β-blockers (yes)	16 (20.5)	2 (8.0)	3 (13.6)	3 (30.0)	8 (38.1)
Continuous variables (median (IQR))					
Age (years)ζ	50.0 (20.0)	43.0 (21.0)	48.5 (13.0)	59.0 (16.0)	62.0 (24.0)
Age at diagnosis (years)	42.0 (24.0)	35.0 (27.0)	42.5 (17.0)	53.5 (32.0)	49.0 (44.0)
Age at ERT initiation (years) §	48.1 (17.3)	42.9 (25.2)	44.0 (16.2)	50.7 (17.1)	53.5 (25.0)
Time in ERT (years)	4.9 (7.6)	3.9 (8.4)	4.8 (5.4)	4.2 (6.7)	8.5 (8.9)
MSSI°	21.0 (20.0)	10.0 (12.0)	20.5 (18.0)	31.0 (11.0)	32.0 (10.0)
FIPI**	2.0 (3.0)	1.0 (1.0)	2.0 (2.0)	4.0 (1.5)	5.0 (2.0)
Plasma lyso-Gb3 (nmol/L)	9.58 (15.33)	6.03 (12.88)	9.22 (14.36)	11.79 (71.50)	17.79 (36.60)
Plasma α-gal A ♀ (nmol/h/mL)	4.3 (4.0)	6.6 (5.0)	3.8 (7.2)	4.2 (.)	4.5 (4.7)
Plasma α-gal A ♂ (nmol/h/mL)	0.11 (0.30)	0.15 (0.50)	0.16 (0.80)	0.10 (0.20)	0.20 (0.30)

ǂ $p < 0.01$ for difference in the distribution of categorical variable between subgroups.
* $p < 0.05$ for difference in the distribution of categorical variable between subgroups.
ζ $p < 0.01$ for difference between group 1 and 4; $p < 0.05$ for difference between group 2 and 4.
§ $p < 0.05$ for difference between group 1 and 4.
° $p < 0.01$ for difference between group 1 and all other groups and also between group 2 and 4.
** $p < 0.01$ for difference between group 1 and all other groups and also between group 2 and 4; $p < 0.05$ for difference between group 2 and 3.

Altogether, 35 different GLA pathogenic mutations were identified (table 40), with p.N215S and p.F113L, two mutations associated with attenuated / late-onset phenotypes with predominant cardiac involvement, accounting for 34.6% of the patients; the remaining mutations are usually associated with classical phenotype.

Table 40: Nephropathy study: mutation frequency. * Mutations presented by one patient within the study

Mutation	n	%	Mutation	n	%	Mutation	n	%
p.N215S	18	23.1	c.802-3_802-2delCA	2	2.6	p.N42V	2	2.6
p.F113L	9	11.5	p.C52G	2	2.6	p.R342Q	2	2.6
p.G35E	5	6.4	p.I317T	2	2.6	p.T410I	2	2.6
p.R227X	3	3.8	p.L166P	2	2.6	unknown	2	2.6
c.700_702del	2	2.6	p.L372P	2	2.6	other*	23	29.5

There was a slight, but significant difference in kidney function tests between the control group and FD subgroup 1 (table 41), with higher albuminuria and serum creatinine and lower GFR in FD patients. However, all these results were within the reference interval in patients of the FD subgroup 1. As per definition of FD subgroups, estimated GFR was significantly lower in FD subgroup 3, in contrast to albuminuria, that was significantly higher in subgroups 3 and 4.

Table 41: Nephropathy study: kidney function in the population of the study. Expressed as median (IQR); ACR: urinary albumin to creatinine ratio; eGFR: estimated glomerular filtration rate.

	Controls	FD subgroups			
		1	2	3	4
ACR (mg/g) *	3.9 (4.4)	9.6 (14.2)	88.2 (108.2)	614.8 (454.7)	206.2 (515.4)
Creatinine (mg/dL) ₸	0.62 (0.25)	0.76 (0.23)	0.76 (0.27)	0.87 (0.21)	1.53 (0.63)
eGFR (ml/min/1.73m^2) ₸	119.0 (20.0)	105.0 (30.0)	102.0 (22.0)	87.5 (28.0)	39.0 (25.0)

* $p < 0.01$ for difference between controls and group 1, between group 1 and 3 or 4 and also between group 2 and 3; $p < 0.05$ for difference between group 2 and 4.
₸ $p < 0.05$ for difference between controls and group 1; $p < 0.01$ for difference between group 4 and all other groups.

2.3 Glomerular and tubular damage biomarkers at baseline

2.3.1 *Concentration in healthy controls and Fabry disease patients*

Glomerular and tubular damage biomarkers were all increased in FD patients, in comparison with healthy controls (table 42 and figure 23). A significant increase in both glomerular damage biomarkers was evidenced, even in the less severe subgroup of FD patients (normoalbuminuric patients, without evidence of kidney involvement), with an increase of 132% (p=0.018) for UTE and of 120% (p=0.040) for urinary ColIV excretion, comparing this subgroup and the controls.

Table 42: Studied biomarkers of kidney injury in controls and FD subgroups. Expressed as median (IQR); UTE: urinary transferrin excretion; ColIV: urinary collagen type IV excretion; A1MG: urinary α1-microglobulin excretion; NAG: urinary N-acetyl-β-D-glucosaminidase excretion; AAP: urinary alanine aminopeptidase excretion.

	Controls	FD subgroups				p value*	p value₸
		1	2	3	4		
UTE (μg/g)	0.81 (0.62)	0.99 (2.10)	1.09 (0.94)	0.95 (0.94)	1.26 (1.16)	0.018	0.45
ColIV (mg/g)	5.09 (5.54)	7.92 (14.28)	10.88 (8.31)	7.53 (9.76)	9.75 (11.86)	0.040	0.53
A1MG (mg/g)	298.3 (260.9)	489.2 (1121.9)	570.5 (549.7)	580.7 (767.9)	522.9 (360.1)	0.009	0.91
NAG (U/g)	8.72 (5.66)	10.79 (17.91)	13.44 (8.37)	25.04 (23.04)	22.02 (19.22)	0.037	0.023
AAP (U/g)	4.98 (7.13)	13.07 (20.46)	13.02 (15.98)	16.68 (12.73)	13.37 (9.25)	0.009	0.97

* controls versus FD disease subgroup 1; ₸ between FD subgroups.

Likewise, there was a significant increase in all tubular injury biomarkers even in the subgroup 1 of FD patients, with increases of 206% (p=0.009), 87% (p=0.037) and 162%

(p=0.009) for urinary A1MG, NAG and AAP, respectively, comparing this subgroup and the controls. However, a significant progressive increase between FD subgroups of increasing severity was only found for urinary NAG excretion.

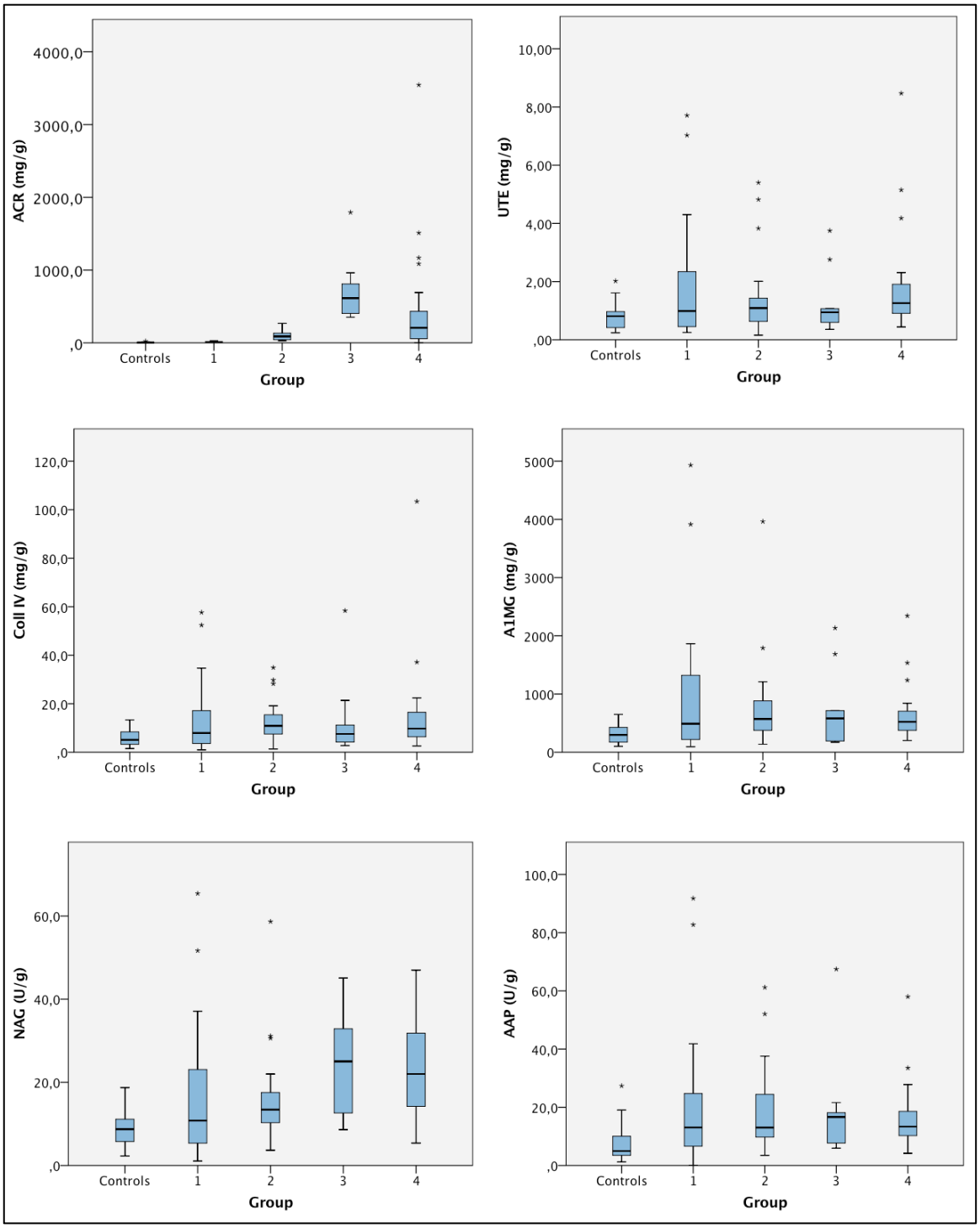


Figure 23: Glomerular and tubular damage biomarkers in controls and FD patient subgroups.

ACR: urinary albumin to creatinine ratio; UTE: urinary transferrin excretion; ColIV: urinary collagen type IV excretion; A1MG: urinary α1-microglobulin excretion; NAG: urinary N-acetyl-β-D-glucosaminidase excretion; AAP: urinary alanine aminopeptidase excretion.

2.3.2 *Correlation with urinary albumin excretion*

For the entire FD cohort, the correlation between ACR and the novel biomarkers (table 43) was stronger for urinary NAG excretion (ρ=0.447, p<0.001) (figure 23), although also significant for UTE and urinary ColIV excretion.

Table 43: Correlation between novel biomarkers of kidney injury and ACR for all FD cohort and subgroup 1. ACR: urinary albumin to creatinine ratio; UTE: urinary transferrin excretion; ColIV: urinary collagen type IV excretion; A1MG: urinary α1-microglobulin excretion; NAG: urinary N-acetyl-β-D-glucosaminidase excretion; AAP: urinary alanine aminopeptidase excretion.

	all FD cohort		Subgroup 1	
	ρ	p value	ρ	p value
UTE	0.240	0.034	0.487	0.013
ColIV	0.302	0.007	0.464	0.019
A1MG	0.184	0.107	0.457	0.022
NAG	0.447	< 0.001	0.586	0.002
AAP	0.173	0.141	0.238	0.252

If only the FD patients of subgroup 1 were considered (patients without any evidence of renal involvement) the correlations with ACR would be even stronger, also with a higher correlation coefficient for urinary NAG excretion (ρ=0.586, p=0.002), but also significant for UTE, urinary ColIV excretion and urinary A1MG excretion. Several normoalbuminuric patients had biomarkers concentrations above the assumed reference value (upper limit of a 95% confidence interval for the mean of the control group); for example for NAG, 48% of the normoalbuminuric patients had NAG excretion above the reference range (figure 24 and figure 25).

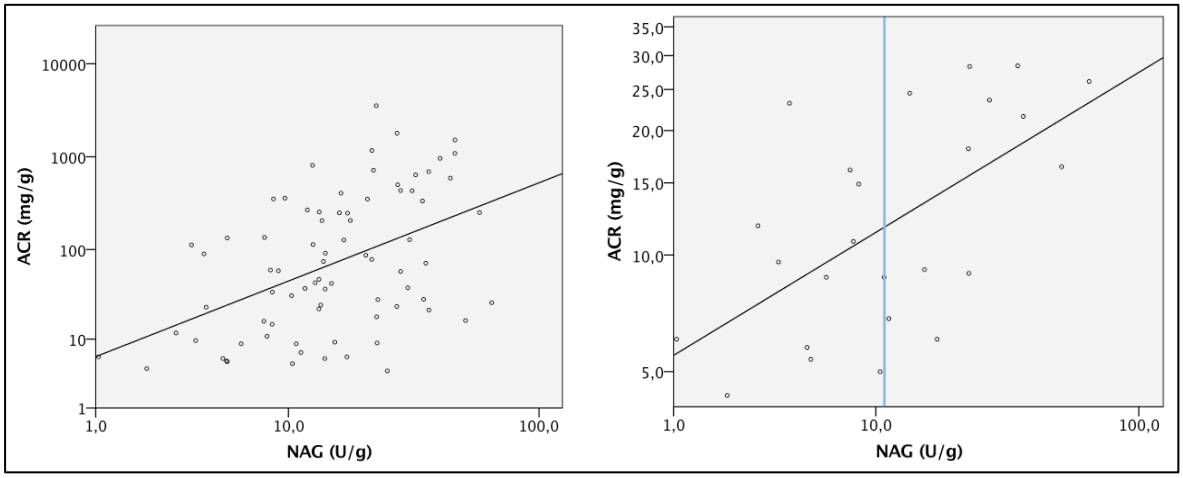


Figure 24: Correlation between NAG and ACR. Left image: entire FD cohort; right image: subgroup 1 of FD patients. Vertical line in right image represents the upper limit of the reference value; ACR: urinary albumin to creatinine ratio; NAG: urinary N-acetyl-β-D-glucosaminidase excretion; FD: Fabry disease.

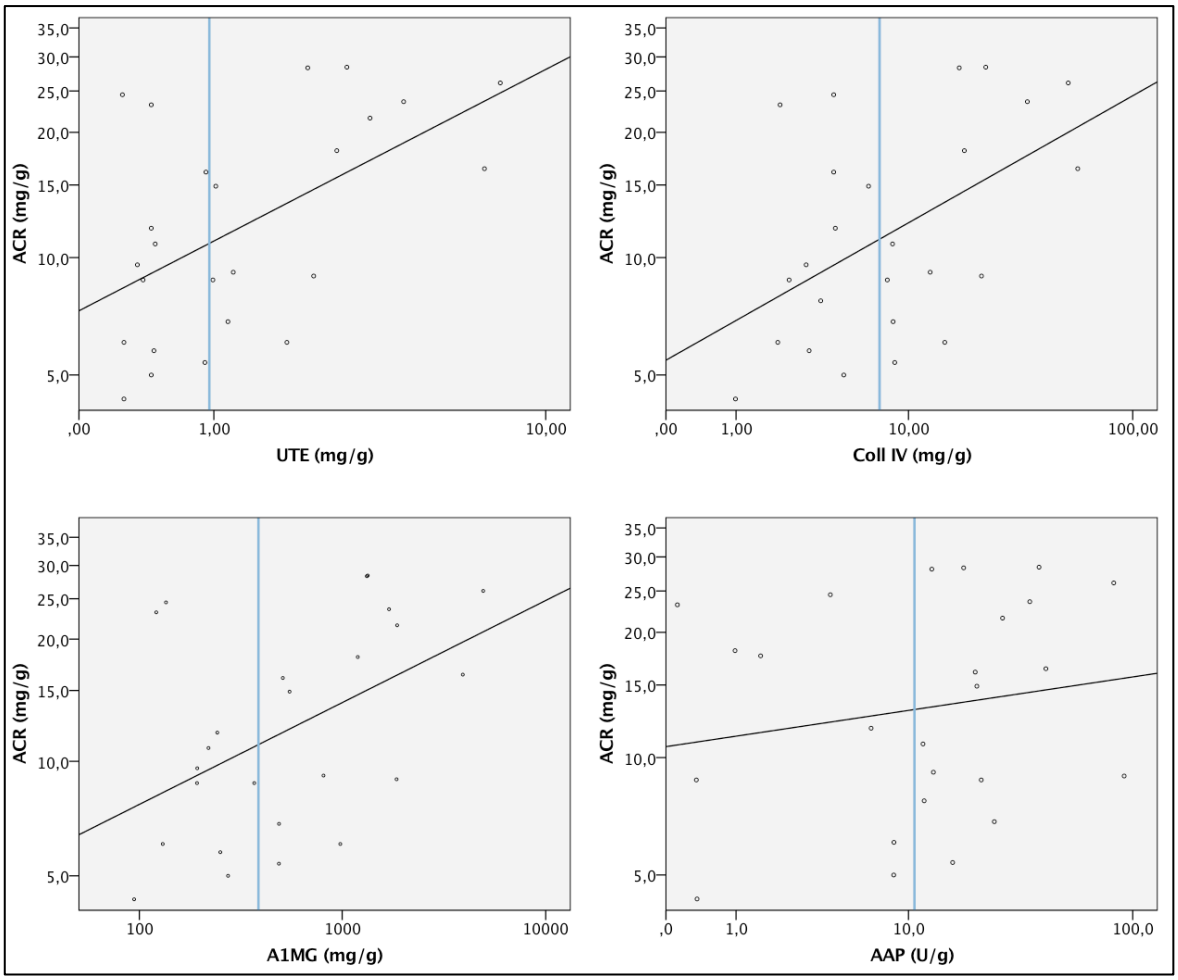


Figure 25: Subgroup 1: correlation between ACR and novel biomarkers. Left upper image: UTE; right upper image: ColIV; left lower image: A1MG; right lower image: AAP. Vertical line in right image represents the upper limit of the reference value; ACR: urinary albumin to creatinine ratio; UTE: urinary transferrin excretion; ColIV: urinary collagen type IV excretion; A1MG: urinary α1-microglobulin excretion; AAP: urinary alanine aminopeptidase excretion.

2.3.3 *Correlation with estimated glomerular filtration rate*

For the entire FD cohort, I found significant negative correlations between estimated GFR and ACR, UTE, urinary Col IV or urinary NAG (table 44). Furthermore, the correlation between urinary NAG and estimated GFR (ρ=-0.420; p<0.001) was stronger than the correlation between ACR and estimated GFR (ρ=-0.374; p=0.001) (figure 26).

In FD subgroup 1 (patients without evidence of FD nephropathy), all biomarkers but urinary AAP had better correlation with estimated GFR than ACR, with the strongest correlation for urinary NAG excretion (ρ=-0.668; p<0.001), in comparison with a ρ=-0.416, p=0.039 for ACR (table 44 and figure 26).

Table 44: Correlation between novel biomarkers of kidney injury and estimated GFR for all FD cohort and subgroup 1. GFR: glomerular filtration rate; FD: Fabry disease; ACR: urinary albumin to creatinine ratio; UTE: urinary transferrin excretion; ColIV: urinary collagen type IV excretion; A1MG: urinary α1-microglobulin excretion; NAG: urinary N-acetyl-β-D-glucosaminidase excretion; AAP: urinary alanine aminopeptidase excretion.

	all FD cohort		Subgroup 1	
	ρ	p value	ρ	p value
ACR	-0.374	0.001	-0.416	0.039
UTE	-0.305	0.007	-0.537	0.006
ColIV	-0.351	<0.001	-0.493	0.012
A1MG	-0.215	0.058	-0.544	0.005
NAG	-0.420	<0.001	-0.668	<0.001
AAP	-0.019	0.872	-0.167	0.424

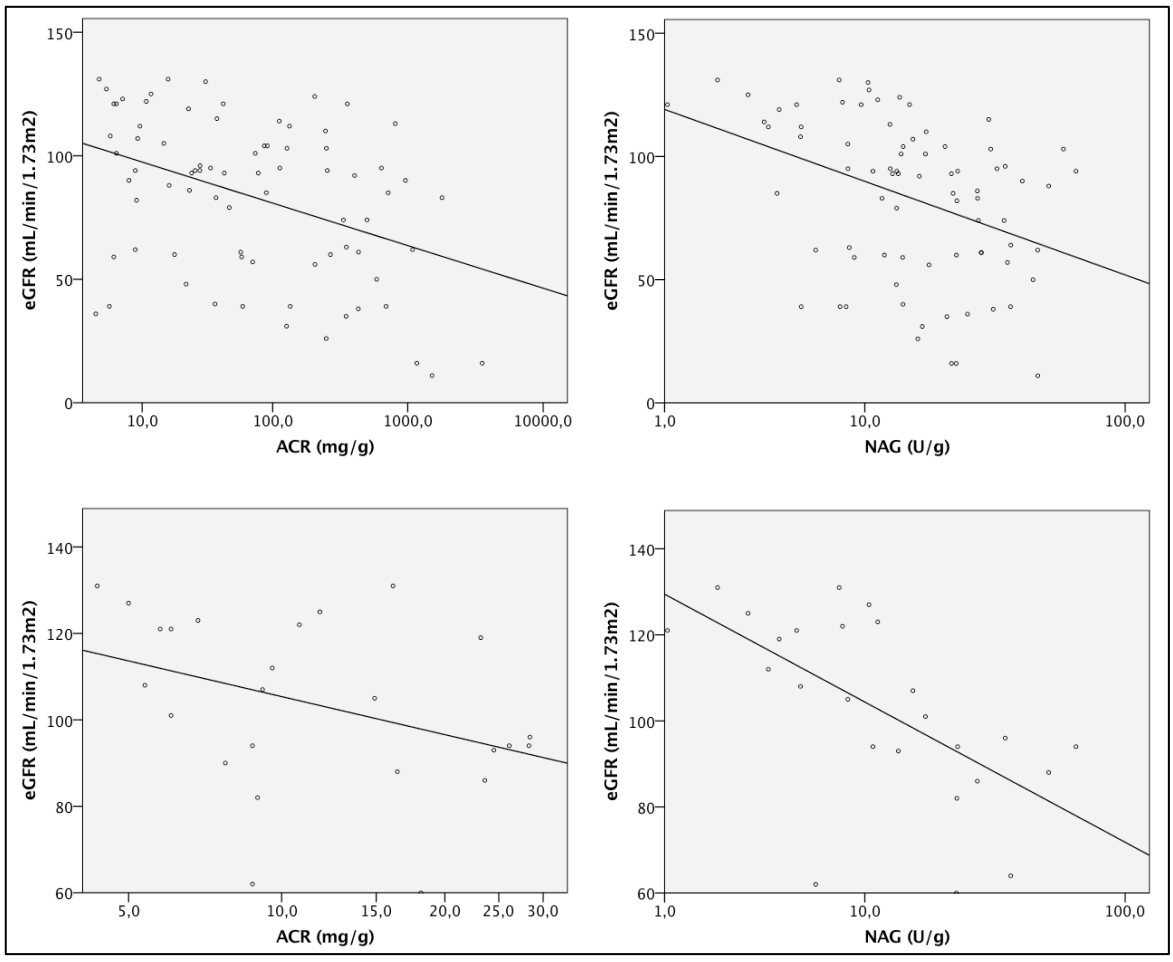


Figure 26: Correlation between eGFR and ACR or urinary NAG excretion. Left upper image: ACR (entire FD cohort); right upper image: NAG (entire FD cohort); left lower image: ACR (subgroup 1 of FD patients); right lower image: NAG (subgroup 1 of FD patients). eGFR: estimated glomerular filtration rate; ACR: urinary albumin to creatinine ratio; NAG: urinary N-acetyl-β-D-glucosaminidase excretion; FD: Fabry disease.

A significant increase of the biomarkers, according to CKD stage, was only found for NAG (p=0.014). Notably, a similar increase was not found for albuminuria (p=0.068) (table 45 and figure 27).

Table 45: Biomarkers in patients with FD according to CKD stage. CKD: chronic kidney disease; FD: Fabry disease; ACR: urinary albumin to creatinine ratio; UTE: urinary transferrin excretion; ColIV: urinary collagen type IV excretion; A1MG: urinary α1-microglobulin excretion; NAG: urinary N-acetyl-β-D-glucosaminidase excretion; AAP: urinary alanine aminopeptidase excretion.

	CKD stage			p value
	1	2	≥3	
ACR (mg/g)	34.2 (121.2)	53.0 (372.3)	170.6 (390.9)	0.068
UTE (µg/g)	0.92 (0.97)	1.08 (1.85)	1.26 (1.16)	0.067
ColIV (mg/g)	8.4 (10.1)	11.0 (13.4)	9.8 (11.9)	0.561
A1MG (mg/g)	468.2 (647.5)	715.2 (867.9)	522.9 (360.1)	0.080
NAG (U/g)	12.6 (11.1)	22.3 (16.0)	22.0 (19.2)	0.014
AAP (U/g)	13.2 (14.6)	14.5 (17.9)	13.4 (9.2)	0.955

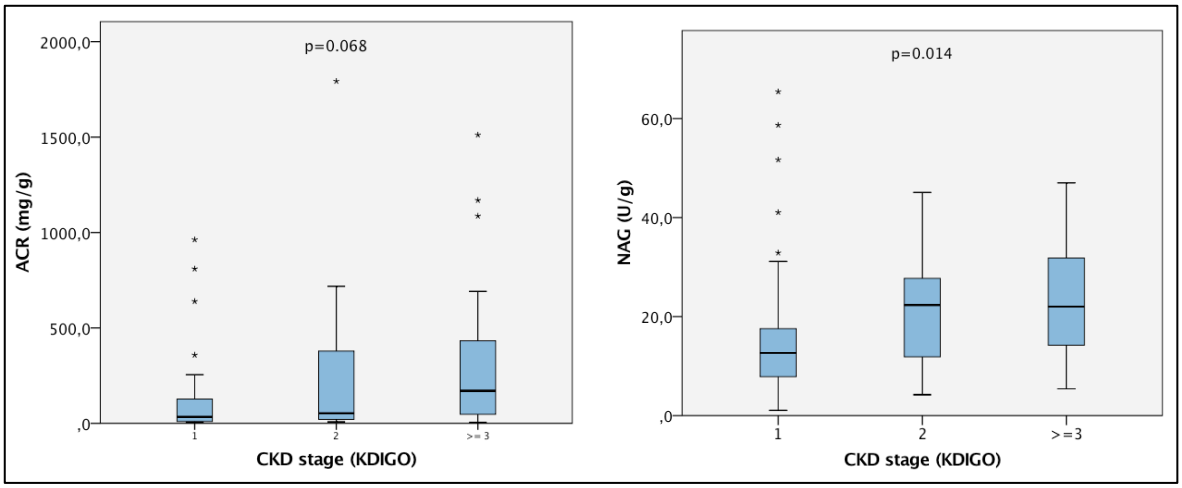


Figure 27: Boxplot comparing ACR and urinary NAG excretion between CKD stages. CKD: chronic kidney disease; ACR: urinary albumin to creatinine ratio; NAG: urinary N-acetyl-β-D-glucosaminidase excretion.

2.3.4 *Correlation with other variables*

For other variables (including demographic, diagnosis, severity and treatment related), not directly related to kidney function, a significant correlation was found between ACR or urinary NAG excretion and disease severity (both MSSI and FIPI scores) (table 46).

Moreover, there was a significant correlation between NAG and age (ρ=0.365; p=0.001). However, the significant inverse correlation between estimated GFR and NAG (ρ=-0.355; p=0.002) remained significant after adjustment for age. Other correlations were less consistent and reliable.

Table 46: Influence of other variables in biomarkers of kidney injury. Mean difference for categorical variables; ρ for continuous variables; ACR: urinary albumin to creatinine ratio; UTE: urinary transferrin excretion; ColIV: urinary collagen type IV excretion; A1MG: urinary α1-microglobulin excretion; NAG: urinary N-acetyl-β-D-glucosaminidase excretion; AAP: urinary alanine aminopeptidase excretion; α-gal: α-galactosidase; MSSI: Mainz Severity Score Index; FIPI: Fabry International Prognostic Index; ERT: enzyme replacement therapy; ACEi: angiotensin converting enzyme inhibitors; ARB: angiotensin II receptor blockers.

	ACR (mg/g)	**UTE** (µg/g)	**ColIV** (mg/g)	**A1MG** (mg/g)	**NAG** (U/g)	**AAP** (U/g)
Demographic						
Sex (female)	-11.1*	0.14	0.36	340.3	1.65	3.28
Age (years)	0.197	0.192	0.120	0.181	0.305*	-0.033
Diagnosis						
Age at diagnosis (years)	0.113	0.000	0.055	0.078	0.125	-0.094
Plasma α-gal A ♀ (nmol/h/mL)	-0.042	-0.094	-0.023	-0.085	-0.165	0.027
Plasma α-gal A ♂ (nmol/h/mL)	-0.046	0.330	0.387*	0.438*	0.205	0.309
Disease severity						
MSSI	0.579*	0.145	0.097	0.046	0.329*	-0.048
FIPI	0.514*	0.153	0.148	0.059	0.309*	0.013
Plasma lyso-Gb3 ♀ (nmol/L)	0.390*	0.027	-0.016	-0.043	0.065	-0.091
Plasma lyso-Gb3 ♂ (nmol/L)	0.074	-0.420	-0.415	-0.556*	-0.112	-0.478
Treatment						
ERT (yes)	149.8	0.41	3.14	-90.6	1.97	-0.24
Age at ERT initiation (years)	0.021	0.082	0.123	0.142	0.141	-0.070
Time in ERT (years)	-0.057	0.240	0.066	0.197	0.287*	0.051
ACEi / ARB (yes)	173.9	0.20	3.06	-71.9	1.65	0.20
β-blockers (yes)	-83.0	-0.05	-1.54	-185.7	-1.98	-5.35

* $p < 0.05$

Remarkably, a significant difference between male and female patients was only observed for ACR. In female patients, there was only a significant correlation between estimated GFR and urinary ColIV or NAG (table 47).

Table 47: Correlation of biomarkers with ACR and eGFR stratified by gender. ACR: urinary albumin to creatinine ratio; eGFR: estimated glomerular filtration rate; UTE: urinary transferrin excretion; ColIV: urinary collagen type IV excretion; A1MG: urinary α1-microglobulin excretion; NAG: urinary N-acetyl-β-D-glucosaminidase excretion; AAP: urinary alanine aminopeptidase excretion.

		ACR		**eGFR**	
		ρ	p value	ρ	p value
ACR	♀	-	-	-0.219	0.153
	♂	-	-	-0.468	0.005
UTE	♀	0.221	0.150	-0.245	0.109
	♂	0.264	0.131	-0.384	0.025
ColIV	♀	0.340	0.024	-0.340	0.007
	♂	0.328	0.059	-0.385	0.013
A1MG	♀	0.191	0.214	-0.176	0.252
	♂	0.327	0.059	-0.307	0.077
NAG	♀	0.410	0.006	-0.412	0.005
	♂	0.628	<0.001	-0.421	0.013
AAP	♀	0.150	0.330	0.099	0.521
	♂	0.350	0.058	-0.220	0.244

2.4 Association between predictive variables and clinical endpoints

2.4.1 *Predictors of estimated glomerular filtration rate*

Multivariate regression analysis was performed to evaluate the urine biomarkers as predictors of estimated GFR (table 48). No significant difference between genders was identified for estimated GFR (mean difference 8.39ml/min/1.73m^2 between female and male patients, p=0.311).

As expected, age was identified as the most significant predictor of eGFR, so the multivariate regression was weighted for age and identified urinary NAG excretion as the only significant predictor of estimated GFR (β-0.296, p=0.009).

Table 48: Predictive model of eGFR by univariate and multivariate regression analysis. An algorithmic transformation of variables with non-normal distribution was performed for correlation analysis; eGFR: estimated glomerular filtration rate; ACR: urinary albumin to creatinine ratio; UTE: urinary transferrin excretion; ColIV: urinary collagen type IV excretion; A1MG: urinary α1-microglobulin excretion; NAG: urinary N-acetyl-β-D-glucosaminidase excretion; AAP: urinary alanine aminopeptidase excretion.

	eGFR (univariate)		**eGFR** (multivariate)	
	R	p value	β	p value
Log ACR	-0.353	0.002	-0.178	0.815
Log UTE	-0.231	0.042	0.055	0.608
Log ColIV	-0.226	0.021	0.214	0.530
Log A1MG	-0.149	0.193		
Log NAG	-0.391	<0.001	**-0.296**	**0.009**
Log AAP	-0.049	0.670		
Age	-0.629	<0.001		

2.4.2 *Diagnostic accuracy to identify Fabry Disease*

I used the diagnostic accuracy of each biomarker to identify FD in order to compare the S of serum biomarkers with the gold standard ACR in the identification of incipient FD nephropathy (table 49).

Specificity (Sp) and positive predictive value (PPV) were influenced by the inclusion criteria in the control group (albuminuria A1) and the definition of the reference range of biomarkers according to the control group, overestimating both measures of diagnostic accuracy (mainly for ACR).

Nonetheless, the sensitivity to identify FD was highest for urinary NAG excretion; moreover, except for the AUC for albuminuria (influenced by the definition of the control group), which was higher than 0.9, the AUC for tubular injury biomarkers was slightly larger than those of the novel glomerular biomarkers (figure 28). Furthermore, plasma

lyso-Gb3 did not significantly correlate with estimated GFR in female (ρ=-0.373; p=0.051) and male (ρ=0.240; p=0.370) patients.

Table 49: Diagnostic accuracy of eGFR and urinary biomarkers to identify Fabry Disease. For this purpose, abnormal eGFR was defined as CKD stage ≥2; S: sensitivity; Sp: specificity; PPV: positive predictive value; NPV: negative predictive value; LR+: likelihood ratio for a positive test result; LR-: likelihood ratio for a negative test result; DOR: diagnostic odds ratio; A: accuracy; YI: Youden's index; AUC: area under the curve; CI: confidence interval; eGFR: estimated glomerular filtration rate; ACR: urinary albumin to creatinine ratio; UTE: urinary transferrin excretion; ColIV: urinary collagen type IV excretion; A1MG: urinary α1-microglobulin excretion; NAG: urinary N-acetyl-β-D-glucosaminidase excretion; AAP: urinary alanine aminopeptidase excretion.

	S	Sp	PPV	NPV	LR+	LR-	DOR	A	YI	AUC (95% CI)
eGFR	0.53	0.88	0.93	0.37	4.42	0.53	8.12	0.61	0.41	0.196 (0.102-0.291)
ACR	0.63	1	1	0.46	NA	0.37	NA	0.72	0.63	0.945 (0.903-0.987)
UTE	0.59	0.72	0.87	0.36	2.11	0.57	3.70	0.62	0.31	0.694 (0.584-0.803)
ColIV	0.64	0.68	0.86	0.38	2	0.53	3.79	0.65	0.32	0.706 (0.603-0.810)
A1MG	0.65	0.68	0.86	0.39	2.03	0.51	4.01	0.66	0.33	0.743 (0.645-0.842)
NAG	0.69	0.72	0.89	0.43	2.46	0.43	5.79	0.70	0.41	0.757 (0.663-0.851)
AAP	0.66	0.76	0.89	0.43	2.75	0.45	6.21	0.66	0.42	0.763 (0.655-0.871)

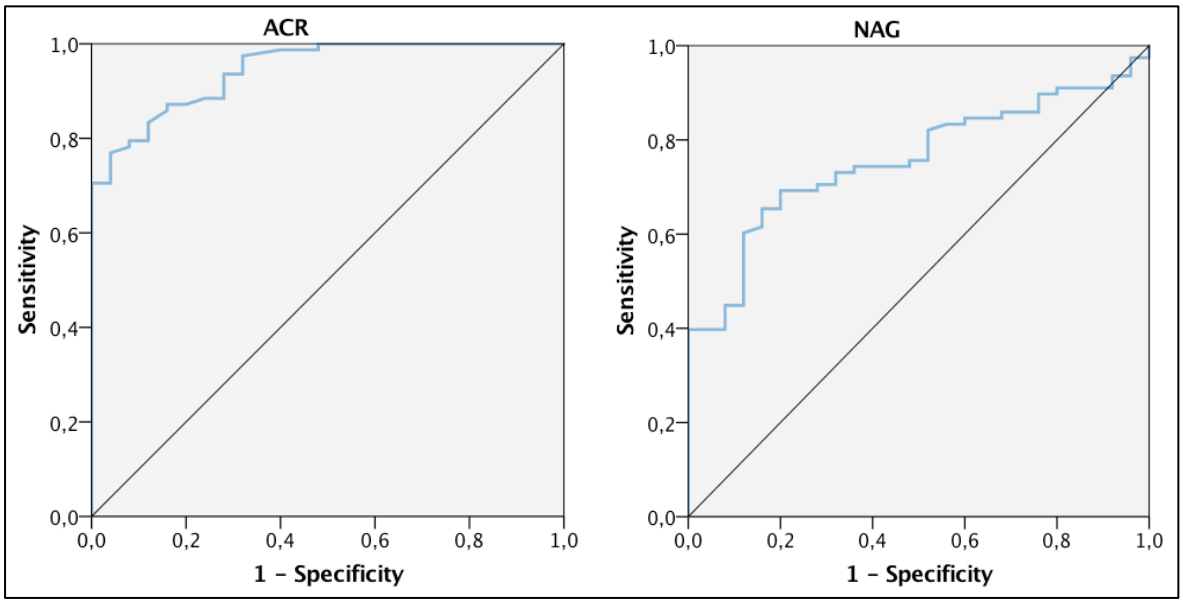


Figure 28: Global diagnostic accuracy to identify Fabry disease. Left image: ACR; right image: NAG. ACR: urinary albumin to creatinine ratio; NAG: urinary N-acetyl-β-D-glucosaminidase excretion.

2.4.3 *Diagnostic accuracy to identify chronic kidney disease stage ≥2*

Urinary NAG excretion presented the highest S (0.83) and global diagnostic accuracy (AUC=0.708) to identify CKD stage ≥2 (table 50 and figure 29); nevertheless, its DOR was not higher than the relatively lower Sp (related to its high S to detect incipient

FD nephropathy in stage 1 of CKD). Lyso-Gb3 did not demonstrate a significant global diagnostic accuracy to identify CKD stage ≥2 neither in female, nor in male patients.

Table 50: Diagnostic accuracy of biomarkers to identify CKD stage ≥2. CKD: chronic kidney disease; S: sensitivity; Sp: specificity; PPV: positive predictive value; NPV: negative predictive value; LR+: likelihood ratio for a positive test result; LR-: likelihood ratio for a negative test result; DOR: diagnostic odds ratio; A: accuracy; YI: Youden's index; AUC: area under the curve; CI: confidence interval; ACR: urinary albumin to creatinine ratio; UTE: urinary transferrin excretion; ColIV: urinary collagen type IV excretion; A1MG: urinary α1-microglobulin excretion; NAG: urinary N-acetyl-β-D-glucosaminidase excretion; AAP: urinary alanine aminopeptidase excretion.

		S	Sp	PPV	NPV	LR+	LR-	DOR	A	YI	AUC (95% CI)
ACR		0.71	0.46	0.59	0.59	1.31	0.63	2.05	0.59	0.17	0.653 (0.531-0.774)
UTE		0.70	0.53	0.61	0.63	1.49	0.57	2.59	0.62	0.23	0.653 (0.529-0.776)
ColIV		0.71	0.43	0.58	0.57	1.25	0.67	1.84	0.58	0.14	0.570 (0.441-0.700)
A1MG		0.77	0.46	0.59	0.67	1.43	0.50	2.86	0.62	0.23	0.625 (0.498-0.751)
NAG		0.83	0.45	0.61	0.71	1.51	0.38	3.82	0.64	0.28	0.708 (0.590-0.825)
AAP		0.68	0.35	0.55	0.48	1.05	0.91	1.13	0.50	0.03	0.482 (0.347-0.617)
Lyso-Gb3	♀	-	-	-	-	-	-	-	-	-	0.644 (0.398-0.891)
	♂	-	-	-	-	-	-	-	-	-	0.330 (0.040-0.627)

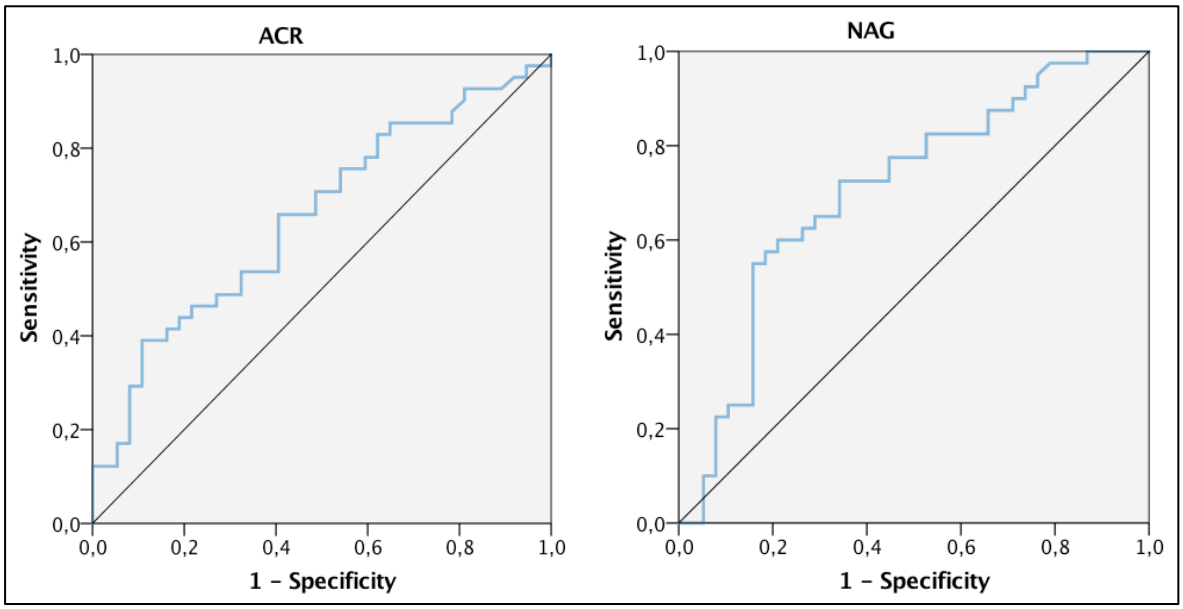


Figure 29: Global diagnostic accuracy to identify CKD stage ≥2. Left image: ACR; right image: NAG. ACR: urinary albumin to creatinine ratio; NAG: urinary N-acetyl-β-D-glucosaminidase excretion.

2.5 Glomerular and tubular damage biomarkers in the longitudinal study

2.5.1 *Compliance and population characteristics*

There was high compliance with longitudinal evaluation (figure 30), with all recruited patients attending their appointments after 12 months. After 24 months, 70 (89.7%) patients attended the appointment; 6 patients re-scheduled their visits, one

patient died during the study (due to haemorrhagic stroke) and another patient changed follow-up to another hospital.

During follow-up, biomarkers were measured in 76.9% of the patients, either after 12 or 24 months. The main reason for no sampling to biomarkers assessment was the re-scheduling of appointments, so the research team was not aware that the patient was present and the samples were not collected; a few samples were also inadvertently wasted.

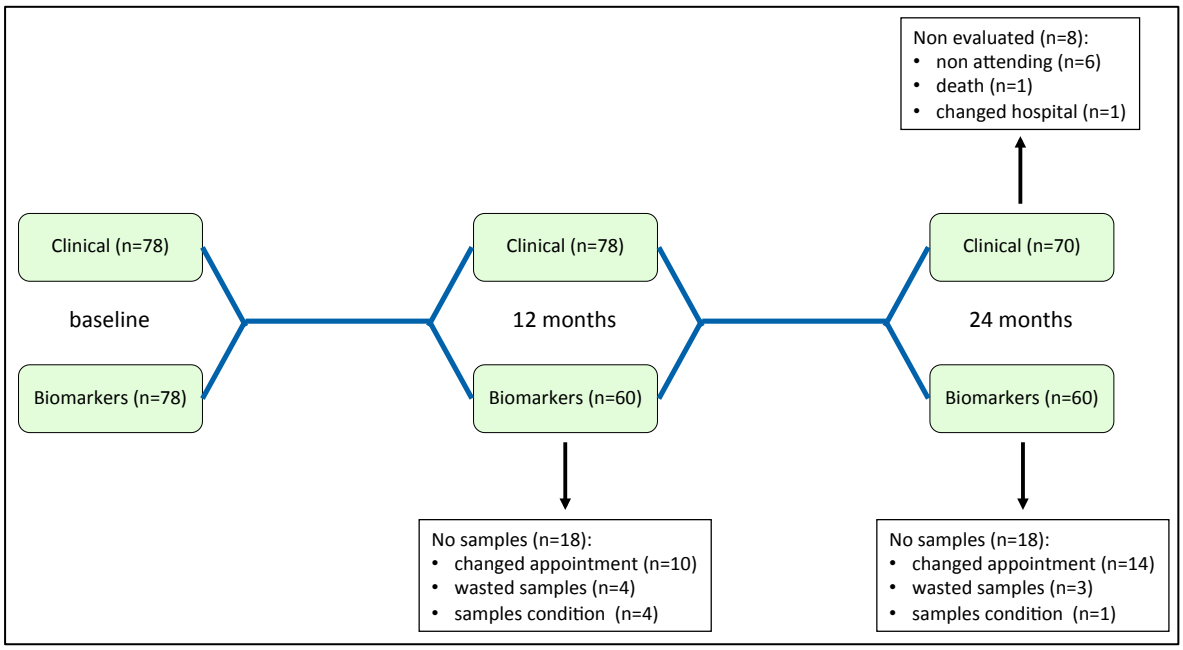


Figure 30: Nephropathy study: flow diagram of longitudinal evaluation.

Glomerular function remained essentially stable during the 2 years follow-up period (table 51), with no significant changes in serum creatinine or estimated GFR; although serum creatinine and estimated GFR significantly increased and decreased, respectively after 12 months, there was a recovery to near baseline values for both variables after 24 months. Furthermore, the two severity indexes (MSSI and FIPI) did not change significantly during follow-up, and likewise the percentage of patients treated with ACEi or ARB and β-blockers. Six patients (7.7%) started ERT during the study.

Analysing glomerular function by FD subgroups or CKD stage, estimated GFR did not change significantly between baseline and 24 months follow-up in any subgroup of FD patients (figure 31); similar results were also found for the control group, with no change in estimated GFR after 12 months.

Table 51: Nephropathy study: longitudinal variation of kidney function, severity and treatment.

ACR: urinary albumin to creatinine ratio; eGFR: estimated glomerular filtration rate; MSSI: Mainz Severity Score Index; FIPI: Fabry International Prognostic Index; ERT: enzyme replacement therapy; ACEi: angiotensin converting enzyme inhibitors; ARB: angiotensin II receptor blockers.

	baseline (n=78)	**12 months** (n=78)	**24 months** (n=70)	p value		
				0 versus 12	12 versus 24	0 versus 24
Kidney function (median/IQR)						
Creatinine (mg/dL)	0.85 (0.60)	0.86 (0.64)	0.83 (0.59)	0.005	<0.001	0.488
eGFR (ml/min/1.73m^2)	92.5 (49.0)	86.0 (52.0)	91.0 (51.0)	0.002	0.004	0.675
Severity (median/IQR)						
MSSI	21.0 (20.0)	21.0 (20.0)	20.0 (20.0)	0.277	0.089	0.075
FIPI	2.0 (3.0)	2.0 (3.0)	2.0 (3.0)	0.903	0.003	0.078
Treatment (n/%)						
ERT (yes)	54 (69.2)	58 (74.4)	54 (77.1)	0.125	0.500	0.031
ACEi / ARB (yes)	38 (48.7)	42 (53.8)	39 (55.7)	0.219	1.000	0.375
β-blockers (yes)	16 (20.5)	16 (20.5)	13 (18.6)	1.000	1.000	1.000

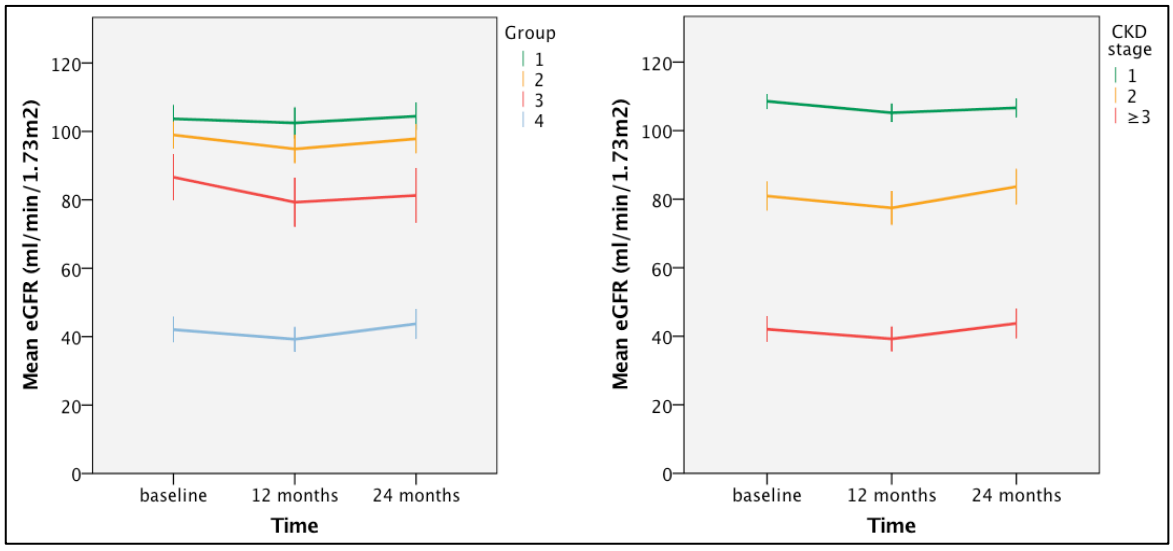


Figure 31: Nephropathy study: longitudinal variation of kidney function by subgroups and CKD stage in FD patients. Left image: FD subgroups; right image: CKD stage. Errors bars: ±standard error; eGFR: estimated glomerular filtration rate; CKD: chronic kidney disease; FD Fabry disease.

According to the definition of ACR progressors (increase ≥1.5 times of ACR between baseline and 24 months, for at least ≥30mg/g or as a change between albuminuria A1 and A2 or A2 and A3) or GFR progessors (decrease ≥5ml/min/1.73m^2 in GFR between baseline and 24 months, for at least a GFR <90ml/min/1.73m^2), mentioned above in the methods section, 20.8% and 28.2% of the FD patients presented ACR and GFR progression, respectively (table 52). There was no significant difference in the proportion of ACR progressors across the FD subgroups; however, there were significantly more GFR progressors in the FD subgroups in the most severe subgroups.

Table 52: Patients progressing according to ACR and GFR criteria in the entire FD cohort and by subgroups. ACR and GFR progressors were defined in the methods sections. ACR: urinary albumin to creatinine ratio; GFR: estimated glomerular filtration rate; FD: Fabry disease.

	ACR		GFR	
	non-progressors	progressors	non-progressors	progressors
FD cohort	61 (79.2)	16 (20.8)	56 (71.8)	22 (28.2)
Subgroup 1	22 (88.0)	3 (12.0)	23 (92.0)	2 (8.0)
Subgroup 2	18 (81.8)	4 (18.2)	17 (77.3)	5 (22.7)
Subgroup 3	8 (80.0)	2 (20.0)	4 (40.0)	6 (60.0)
Subgroup 4	13 (61.9)	7 (33.3)	12 (57.1)	9 (42.9)

2.5.2 *Variation in controls and Fabry disease patients*

There was no significant change in any biomarker, including ACR, in the control group between baseline and 12 months (table 53).

Table 53: Nephropathy study: longitudinal variation of biomarkers in control group. Expressed as median (IQR); ACR: urinary albumin to creatinine ratio; UTE: urinary transferrin excretion; ColIV: urinary collagen type IV excretion; A1MG: urinary α1-microglobulin excretion; NAG: urinary N-acetyl-β-D-glucosaminidase excretion; AAP: urinary alanine aminopeptidase excretion.

	baseline (n=25)	**12 months** (n=25)	**p value**
ACR (mg/g)	3.9 (4.4)	3.3 (4.8)	0.626
UTE (µg/g)	0.81 (0.62)	0.60 (0.30)	0.317
ColIV (mg/g)	5.09 (5.54)	5.30 (2.51)	0.909
A1MG (mg/g)	298.3 (260.9)	250.7 (206.4)	0.689
NAG (U/g)	8.72 (5.66)	10.10 (8.06)	0.167
AAP (U/g)	4.98 (7.13)	6.79 (5.07)	0.693

I did not identify a significant change in biomarker urinary concentration between baseline and 24 months (including ACR), except for urinary NAG excretion, which increased significantly during that period (table 54). Furthermore, there was a significant increase in urinary ColIV excretion after 12 months, which returned to near baseline values after 24 months.

Table 54: Nephropathy study: longitudinal variation of biomarkers in the entire FD cohort. Expressed as median (IQR); FD: Fabry disease; ACR: urinary albumin to creatinine ratio; UTE: urinary transferrin excretion; ColIV: urinary collagen type IV excretion; A1MG: urinary α1-microglobulin excretion; NAG: urinary N-acetyl-β-D-glucosaminidase excretion; AAP: urinary alanine aminopeptidase excretion.

	baseline (n=78)	**12 months** (n=60)	**24 months** (n=60)	**p value**		
				0 versus 12	12 versus 24	0 versus 24
ACR (mg/g)	59.2 (269.2)	94.5 (257.4)	30.4 (210.0)	0.074	0.055	0.474
UTE (µg/g)	1.08 (1.38)	1.19 (1.44)	1.14 (1.39)	0.480	0.158	0.834
ColIV (mg/g)	8.61 (11.53)	13.08 (17.36)	10.12 (12.71)	<0.001	0.007	0.519
A1MG (mg/g)	530.3 (638.3)	563.0 (799.9)	574.3 (633.2)	0.216	0.095	0.900
NAG (U/g)	14.68 (18.83)	26.23 (49.06)	22.41 (33.17)	<0.001	0.492	0.001
AAP (U/g)	13.21 (13.30)	13.74 (16.62)	12.63 (15.88)	0.126	0.037	0.206

Accordingly, even when analysing all biomarkers by FD subgroups, there was no significant change after the follow-up period in any subgroup of patients, except for a significant increase in UTE in subgroup 3, after 24 months of follow-up (figure 32). Furthermore, as previously mentioned, at baseline only ACR and urinary NAG excretion differed significantly across the FD subgroups of increasing severity. This finding remained during follow-up, with an enlargement in the difference of urinary NAG excretion between subgroups, driven by an almost significant increase in subgroups 3 (p=0.091) and 4 (p=0.056).

Analysing biomarker variations according to baseline CKD stage, I identified similar findings, with a significant increase only in urinary NAG excretion, in patients with CKD stage 1 at baseline (figure 33). Furthermore, as noted above only NAG excretion differed significantly between CKD stages at baseline and this difference widened during the follow-up period.

2.5.3 *Baseline concentration and risk of progression*

To assess the effect of baseline concentration of each biomarker on the ACR and GFR variation during follow-up, I divided the biomarkers into quartiles and compared the patients in quartiles 1 and 4.

According to baseline concentration, none of the biomarkers, including ACR, significantly predicted the ACR change during follow-up, without a significant difference between quartiles 1 and 4 for any of them (table 55).

Contrariwise, GFR variation during the follow-up period was significantly distinct between quartiles 1 and 4 of urinary NAG excretion (table 56), with a mean difference of 6.8ml/min/1.73m^2 (95% CI: 0.7-12.9ml/min/1.73m^2; p=0.028). For the remaining biomarkers, including ACR, there was no significant difference between quartiles 1 and 4; for example, for ACR the mean difference was 2.0ml/min/1.73m^2 (95% CI: -4.6-8.6ml/min/1.73m^2; p=0.540).

Furthermore, comparing the GFR change according to baseline quartiles, NAG evidenced a progressive negative variation of GFR across the increasing quartiles (figure 34); for ACR, quartiles 1, 2 and 3 showed similar declines in GFR, only somewhat different in quartile 4.

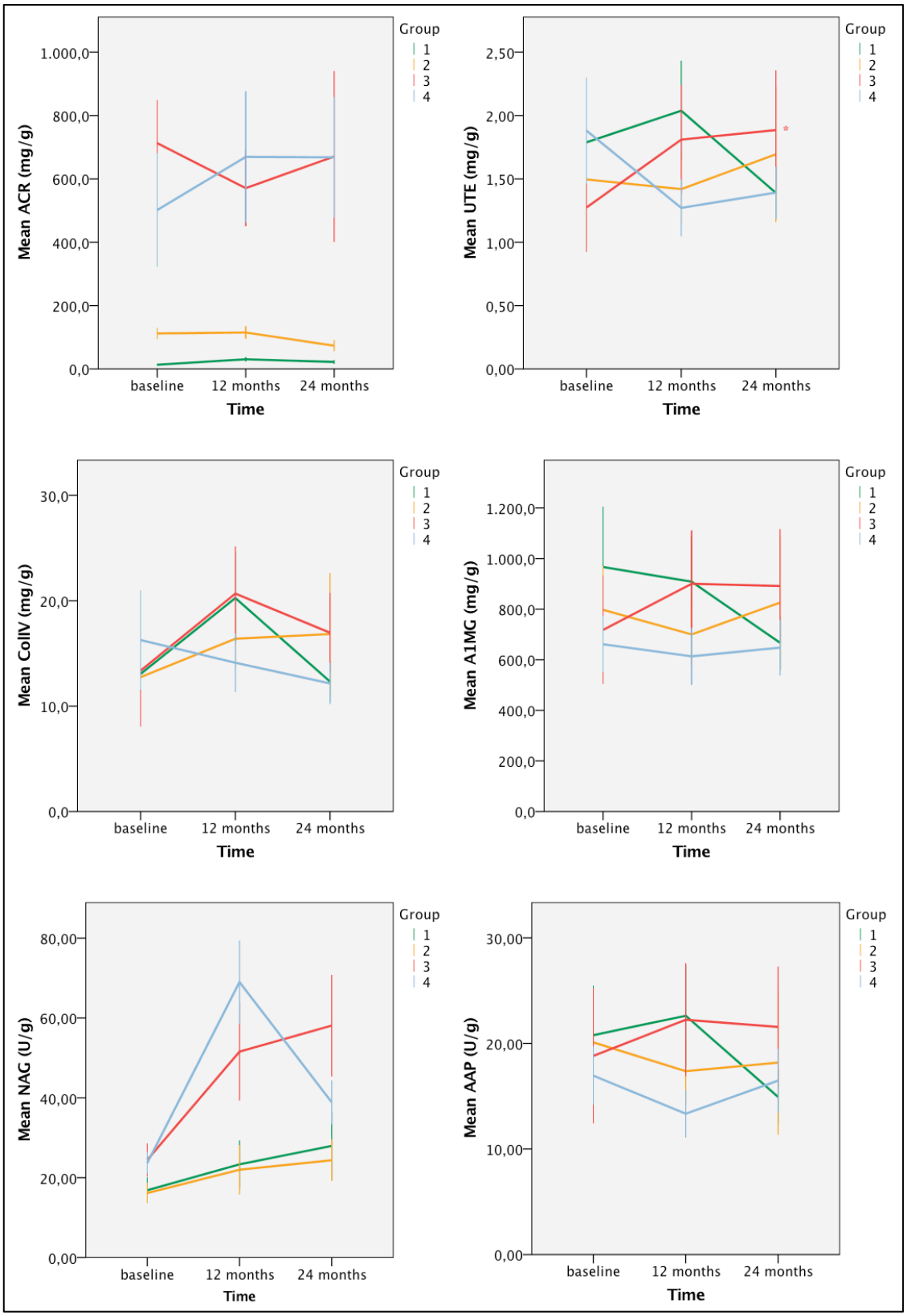


Figure 32: Nephropathy study: longitudinal variation of biomarkers by subgroups of FD patients.

Errors bars: ±standard error; * p<0.05 for variation between baseline and 24 months; ACR: urinary albumin to creatinine ratio; UTE: urinary transferrin excretion; ColIV: urinary collagen type IV excretion; A1MG: urinary α1-microglobulin excretion; NAG: urinary N-acetyl-β-D-glucosaminidase excretion; AAP: urinary alanine aminopeptidase excretion.

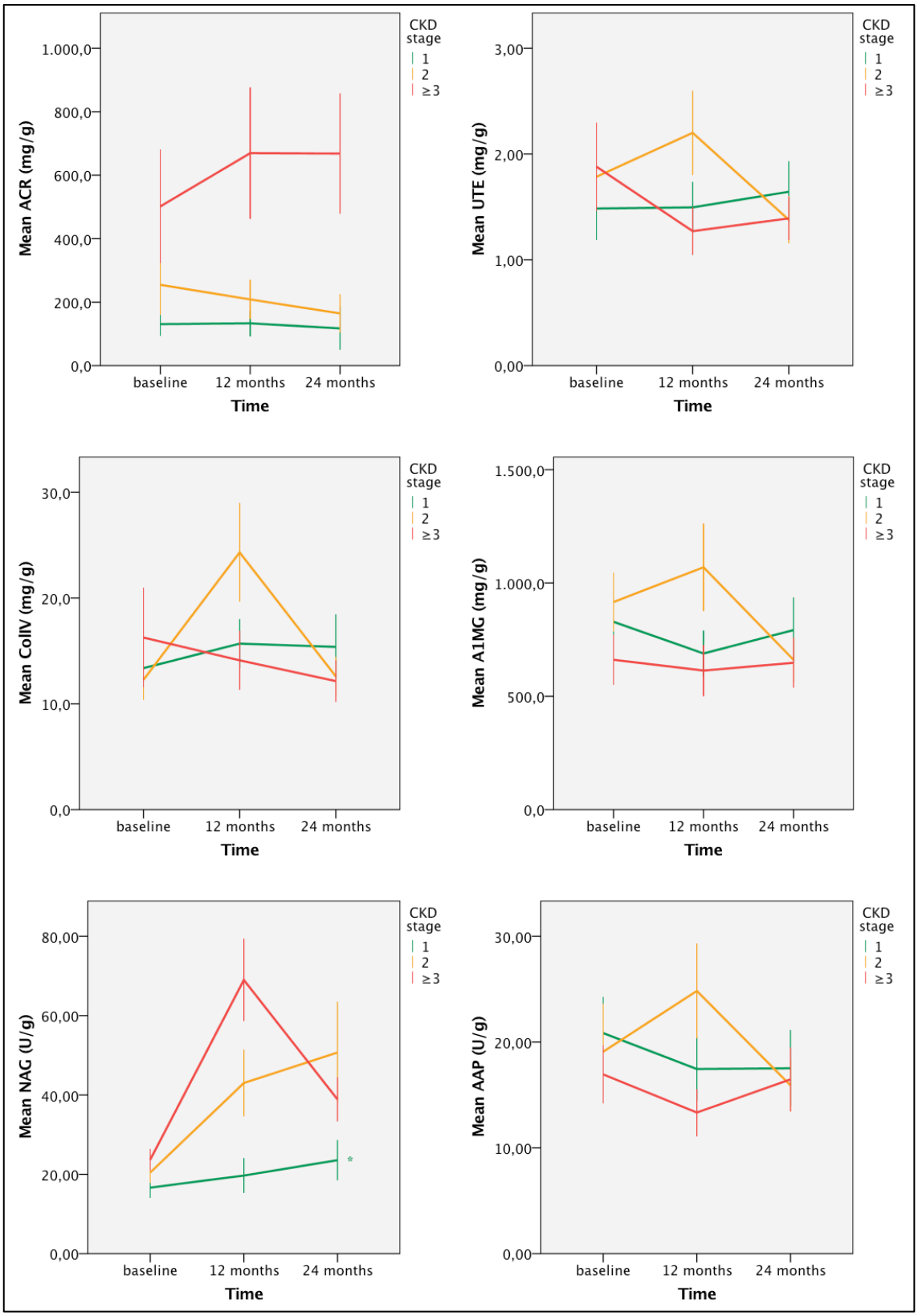


Figure 33: Nephropathy study: longitudinal variation of biomarkers according to CKD stage at baseline. Errors bars: ±standard error; * p<0.05 for variation between baseline and 24 months; ACR: urinary albumin to creatinine ratio; UTE: urinary transferrin excretion; ColIV: urinary collagen type IV excretion; A1MG: urinary α1-microglobulin excretion; NAG: urinary N-acetyl-β-D-glucosaminidase excretion; AAP: urinary alanine aminopeptidase excretion; CKD: chronic kidney disease.

Table 55: ACR variation according to quartiles of the baseline concentration of the biomarkers.

Expressed as median (IQR); * represents the comparison between Q4 and Q1; ACR: urinary albumin to creatinine ratio; Q: quartile; UTE: urinary transferrin excretion; ColIV: urinary collagen type IV excretion; A1MG: urinary α1-microglobulin excretion; NAG: urinary N-acetyl-β-D-glucosaminidase excretion; AAP: urinary alanine aminopeptidase excretion.

	ACR change (mg/g)				**p value***
	Q1	**Q2**	**Q3**	**Q4**	
ACR	3.4 (12.9)	-6.1 (24.6)	-31.4 (118.5)	64.8 (1037.2)	0.233
UTE	-0.1 (45.8)	-1.7 (132.0)	-8.9 (155.5)	2.0 (82.1)	0.624
ColIV	0.9 (37.8)	-3.3 (61.6)	-2.0 (220.4)	2.0 (501.6)	0.518
A1MG	-1.0 (99.2)	-0.1 (49.1)	-2.1 (334.7)	0.1 (52.6)	0.599
NAG	1.9 (34.6)	-13.2 (407.5)	-4.7 (126.6)	6.0 (421.8)	0.433
AAP	-3.5 (112.5)	4.8 (69.2)	-5.4 (85.1)	2.0 (154.3)	0.308

I also assessed the risk of each patient being an ACR or GFR progressor according to baseline quartiles of the biomarkers.

Table 56: GFR variation according to quartiles of the baseline concentration of the biomarkers.

Expressed as median (IQR); * represents the comparison between Q4 and Q1; GFR: glomerular filtration rate; Q: quartile; ACR: urinary albumin to creatinine ratio; UTE: urinary transferrin excretion; ColIV: urinary collagen type IV excretion; A1MG: urinary α1-microglobulin excretion; NAG: urinary N-acetyl-β-D-glucosaminidase excretion; AAP: urinary alanine aminopeptidase excretion.

	GFR change (ml/min/1.73m^2)				**p value***
	Q1	**Q2**	**Q3**	**Q4**	
ACR	0.0 (14.0)	1.0 (12.0)	0.0 (8.0)	-5.0 (15.0)	0.540
UTE	1.0 (14.0)	-5.0 (18.0)	-5.0 (9.0)	-2.0 (13.0)	0.534
ColIV	0.0 (14.0)	-6.0 (12.0)	-3.5 (11.0)	1.0 (10.0)	0.718
A1MG	2.0 (14.0)	-5.0 (12.0)	-5.0 (11.0)	-2.0 (13.0)	0.471
NAG	3.0 (12.0)	-2.0 (11.0)	-5.0 (15.0)	-5.0 (12.0)	0.028
AAP	2.0 (15.0)	-2.0 (7.0)	-5.0 (16.0)	-1.5 (2.0)	0.706

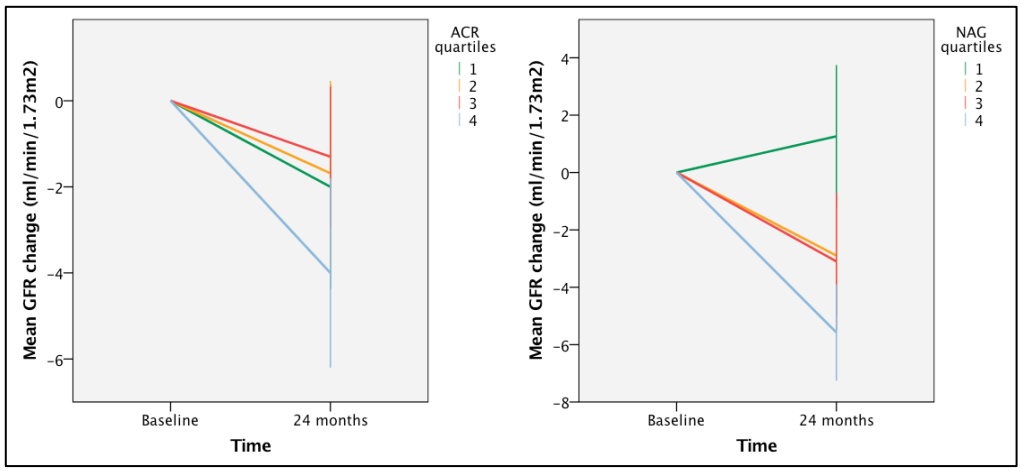


Figure 34: Longitudinal variation of GFR according to ACR (left image) or NAG (right image) quartiles at baseline. Errors bars: ±standard error; GFR: glomerular filtration rate; ACR: urinary albumin to creatinine ratio; NAG: urinary N-acetyl-β-D-glucosaminidase excretion.

There was no significant difference in the distribution of ACR or GFR non-progressors and progressor between the quartiles for all studied biomarkers (table 57); however, there was a trend of a great proportion of progressor in the higher quartiles.

For ACR progression, one patient in the fourth quartile of ACR concentration at baseline has OR of 10.5 (95% CI: 1.14-96.58) to be progressor, in comparison with a patient in the first quartile; none of the other biomarkers exhibited a significant OR comparing quartile 1 and 4.

ACR and urinary NAG excretion both presented a significant OR to identify a GFR progressor, comparing quartile 1 and 4. A patient in the fourth quartile of ACR or NAG distribution presented an OR of 4.17 (95% CI: 1.00-17-31) or 4.80 (95% CI: 1.04-22.10), respectively to be a progressor, in comparison with a patient in the first quartile. For the other biomarkers the OR was non-significant.

Table 57: Distribution of ACR or GFR progressors according to quartiles of the baseline concentration of the biomarkers. * for the distribution of non-progressors and progressors across the quartiles; ** of being a progressor comparing fourth to first quartile; ACR: urinary albumin to creatinine ratio; GFR: glomerular filtration rate; OR: odds ratio; NP: non-progressor; P: progressor; Q: quartile; UTE: urinary transferrin excretion; ColIV: urinary collagen type IV excretion; A1MG: urinary α1-microglobulin excretion; NAG: urinary N-acetyl-β-D-glucosaminidase excretion; AAP: urinary alanine aminopeptidase excretion.

		ACR		p value*	OR**	GFR		p value*	OR**
		NP (%)	P (%)			NP (%)	P (%)		
ACR	Q1	94.7	5.3	0.122	10.50 (1.14-96.58)	78.9	21.1	0.065	4.17 (1.00-17.31)
	Q2	80	20			75.0	25.0		
	Q3	78.9	21.1			85.0	15.0		
	Q4	63.2	36.8			47.4	52.6		
UTE	Q1	90.0	10.0	0.303	4.15 (0.72-23.95)	80.0	20.0	0.433	1.85 (0.43-7.96)
	Q2	72.2	27.8			68.4	31.6		
	Q3	85.0	15.0			55.0	45.0		
	Q4	68.4	31.6			68.4	31.6		
ColIV	Q1	89.5	10.5	0.153	4.96 (0.87-28.15)	78.9	21.1	0.635	1.00 (0.21-4.76)
	Q2	89.5	10.5			65.0	25.0		
	Q3	75.0	25.0			65.0	25.0		
	Q4	63.2	36.8			78.9	21.1		
A1MG	Q1	89.5	10.5	0.665	3.04 (0.51-18.11)	84.2	15.8	0.084	1.42 (0.27-7.44)
	Q2	78.9	21.1			60.0	40.0		
	Q3	75.0	25.0			50.0	50.0		
	Q4	73.7	26.3			78.9	21.1		
NAG	Q1	83.3	16.7	0.238	2.92 (0.62-13.76)	83.3	16.7	0.149	4.80 (1.04-22.10)
	Q2	90.0	10.0			75.0	25.0		
	Q3	80.0	20.0			60.0	40.0		
	Q4	63.2	36.8			52.6	47.4		
AAP	Q1	83.3	16.7	0.825	1.92 (0.38-9.65)	72.2	27.8	0.197	0.52 (0.10-2.61)
	Q2	77.8	22.2			78.9	21.1		
	Q3	84.2	15.8			52.6	47.4		
	Q4	72.2	27.8			83.3	16.7		

Comparing the baseline concentration of biomarkers between ACR progressors and non-progressors, it was significantly higher for ACR (mean: 320.8 versus 250.3mg/g), urinary ColIV excretion (mean: 22.21 versus 11.81mg/g) and urinary NAG excretion (mean: 24.90 versus 18.22U/g) (table 58).

Concerning the comparison of the baseline biomarker concentration between GFR progressors and non-progressors, it was only significantly higher for ACR (mean: 562.0 versus 144.7mg/g) and urinary NAG excretion (mean: 23.44 versus 17.59U/g).

Table 58: Comparison of the baseline concentration of the biomarkers between ACR or GFR non-progressors and progressors. Expressed as median (IQR); ACR: urinary albumin to creatinine ratio; GFR: glomerular filtration rate; NP: non-progressor; P: progressor; UTE: urinary transferrin excretion; ColIV: urinary collagen type IV excretion; A1MG: urinary α1-microglobulin excretion; NAG: urinary N-acetyl-β-D-glucosaminidase excretion; AAP: urinary alanine aminopeptidase excretion.

	ACR		p value	GFR		p value
	NP	P		NP	P	
ACR (mg/g)	42.6 (239.8)	131.0 (1077.6)	0.032	37.6 (122.0)	252.1 (678.5)	0.019
UTE (µg/g)	1.07 (1.42)	1.20 (2.06)	0.312	1.02 (1.49)	1.18 (0.86)	0.366
ColIV (mg/g)	8.06 (10.26)	13.00 (14.11)	0.037	8.76 (13.43)	8.61 (6.49)	0.890
A1MG (mg/g)	522.9 (623.6)	710.6 (983.7)	0.292	510.5 (870.5)	587.3 (289.5)	0.338
NAG (U/g)	13.99 (15.39)	22.20 (23.0)	0.049	13.80 (14.60)	22.02 (19.18)	0.029
AAP (U/g)	13.21 (13.50)	16.68 (17.25)	0.571	12.86 (17.44)	13.78 (9.31)	0.843

Moreover, there was a progressive increase in the ACR and urinary NAG excretion between non-progressing patients, patients with only ACR progression, patients with only GFR progression and patients presenting ACR and GFR progression (figure 35).

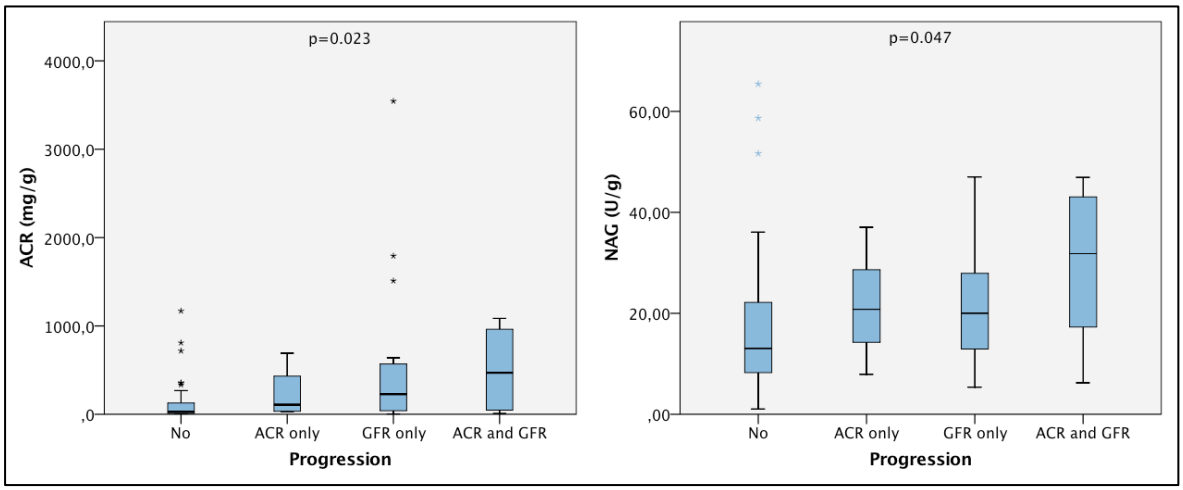


Figure 35: Comparison of ACR (left image) or NAG (right image) baseline concentration between subgroups of progression. From left to right: non-progressors, only ACR progressor patients, only GFR progressor patients and patients presenting ACR and GFR progression. ACR: urinary albumin to creatinine ratio; GFR: glomerular filtration rate; NAG: urinary N-acetyl-β-D-glucosaminidase excretion.

2.5.4 *Correlation between variations of biomarkers and glomerular filtration rate*

To evaluate the correlation between the variation of the biomarkers and the variation of ACR or estimated GFR, I evaluated the variation of the different variables between baseline and 12 months and between 12 and 24 months and considered each variation in correlation analysis.

ACR variation only significantly correlated with urinary A1MG excretion (ρ=0.226; p=0.019) (table 59). The unique biomarker whose variation has significant inverse correlation with GFR variation was NAG (ρ=-0.361; p<0.001); remarkably, this inverse correlation was non-significant between ACR and GFR variations (ρ=-0.127; p=0.131) (figure 36).

Table 59: Correlation between the variation of the biomarkers and ACR or GFR variation. ACR: urinary albumin to creatinine ratio; GFR: glomerular filtration rate; Δ: variation; UTE: urinary transferrin excretion; ColIV: urinary collagen type IV excretion; A1MG: urinary α1-microglobulin excretion; NAG: urinary N-acetyl-β-D-glucosaminidase excretion; AAP: urinary alanine aminopeptidase excretion.

	ACR variation		GFR variation	
	ρ	p value	ρ	p value
Δ ACR	-	-	-0.127	0.131
Δ UTE	0.133	0.175	0.118	0.227
Δ ColIV	0.148	0.125	0.020	0.837
Δ A1MG	0.226	0.019	0.094	0.332
Δ NAG	0.135	0.161	-0.361	<0.001
Δ AAP	0.148	0.131	0.228	0.019

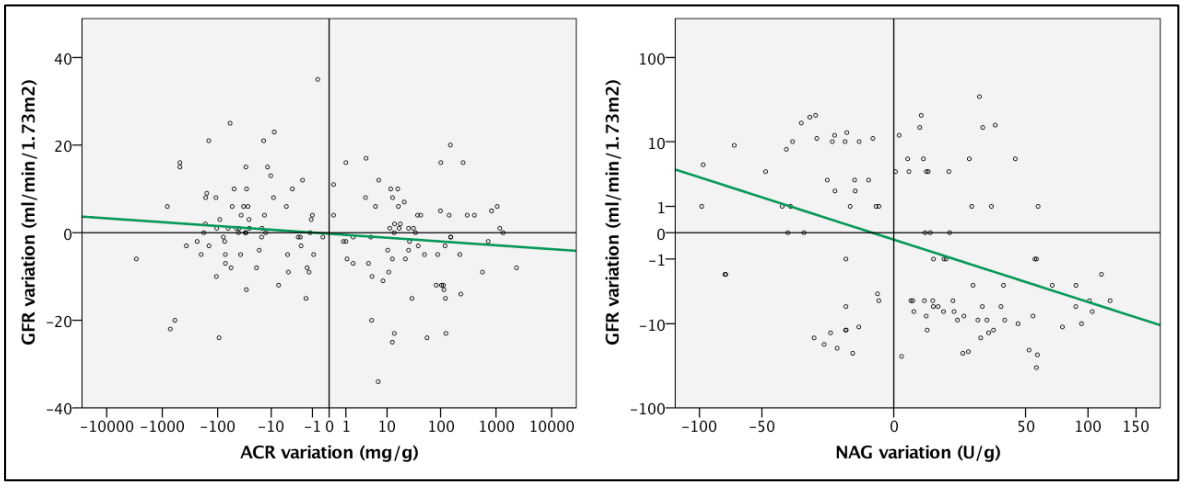


Figure 36: Correlation between GFR variation and ACR (left image) or NAG (right image) variation. GFR: glomerular filtration rate; ACR: urinary albumin to creatinine ratio; NAG: urinary N-acetyl-β-D-glucosaminidase excretion.

2.5.5 *Longitudinal variation and risk of progression*

Comparing the ACR or GFR non-progressors and progressors, the variation of the biomarkers during follow-up was only significantly higher in progressors for urinary NAG excretion, both for ACR and GFR progressors (table 60). For example, there was a mean difference of 31.74U/g (p<0.001) for urinary NAG excretion, comparing GFR progressors and non-progressors; whereas for ACR the mean difference was 34.1mg/g (p=0.792).

Table 60: Comparison of the variation in the concentration of the biomarkers between ACR or GFR non-progressors and progressors. Expressed as median (IQR); ACR: urinary albumin to creatinine ratio; GFR: glomerular filtration rate; NP: non-progressor; P: progressor; Δ: variation; UTE: urinary transferrin excretion; ColIV: urinary collagen type IV excretion; A1MG: urinary α1-microglobulin excretion; NAG: urinary N-acetyl-β-D-glucosaminidase excretion; AAP: urinary alanine aminopeptidase excretion.

	ACR		p value	GFR		p value
	NP	P		NP	P	
Δ ACR (mg/g)	-	-	-	-0.05 (42.7)	-0.1 (261.2)	0.792
Δ UTE (µg/g)	0.09 (1.30)	0.17 (1.57)	0.792	0.09 (1.89)	0.13 (0.78)	0.919
Δ ColIV (mg/g)	2.39 (10.59)	-3.21 (16.74)	0.060	1.33 (16.27)	2.43 (8.60)	0.780
Δ A1MG (mg/g)	-17.06 (525.33)	78.62 (632.49)	0.983	69.79 (648.69)	-39.03 (406.16)	0.591
Δ NAG (U/g)	4.92 (27.84)	23.22 (22.76)	0.027	1.49 (19.77)	27.55 (48.95)	<0.001
Δ AAP (U/g)	-2.56 (13.63)	2.37 (33.25)	0.396	-2.71 (15.88)	-1.51 (12.05)	0.554

There was no significant difference in the distribution of ACR non-progressors and progressors between the quartiles for all studied biomarkers (table 61), although there was a trend of a great proportion of progressors in the higher quartile of variation of urinary NAG excretion. However, the OR of a patient in the fourth quartile to be an ACR progressor, in comparison with a patient in the first quartile was non-significant: OR 3.08 (95% CI: 0.51-18.54).

Furthermore, the distribution of urinary NAG excretion variation between GFR non-progressors and progressors was also significantly different (p<0.001), with an OR of 13.00 (95% CI: 2.59-65.20) to be a progressor comparing quartiles 4 and 1; 72.2% of the patients in the fourth quartile were GFR progressors, in comparison with only 16.7% in the first quartile.

Contrariwise, ACR variation during follow-up did not significantly predict the probability of an individual patient being a progressor, with an OR of 1.25 (95% CI: 0.34-4.59) between quartiles 4 and 1. The distribution of the variation of the remaining biomarkers was also non-statistically significant.

Table 61: Distribution of ACR or GFR progressors according to quartiles of the variation in the concentration of the biomarkers. * for the distribution of non-progressors and progressors across the quartiles; ** of being a progressor comparing fourth to first quartile; ACR: urinary albumin to creatinine ratio; GFR: glomerular filtration rate; OR: odds ratio; NP: non-progressor; P: progressor; Δ: variation; Q: quartile; UTE: urinary transferrin excretion; ColIV: urinary collagen type IV excretion; A1MG: urinary α1-microglobulin excretion; NAG: urinary N-acetyl-β-D-glucosaminidase excretion; AAP: urinary alanine aminopeptidase excretion.

		ACR		p value*	OR**	GFR		p value*	OR**
		NP (%)	P (%)			NP (%)	P (%)		
Δ ACR	Q1	-	-	-	-	63.2	36.8	0.266	1.25 (0.34-4.59)
	Q2	-	-			82.4	17.6		
	Q3	-	-			80.9	19.1		
	Q4	-	-			57.9	42.1		
Δ UTE	Q1	77.8	22.2	0.480	0.44 (0.07-2.76)	77.8	22.2	0.067	0.70 (0.13-3.70)
	Q2	83.3	16.7			61.1	38.9		
	Q3	66.7	33.3			44.4	55.6		
	Q4	88.9	11.1			83.3	16.7		
Δ ColIV	Q1	61.1	38.9	0.138	0.20 (0.03-1.13)	77.8	22.2	0.447	1.00 (0.21-4.81)
	Q2	77.8	22.2			72.2	27.8		
	Q3	88.9	11.1			55.6	44.4		
	Q4	88.9	11.1			77.8	22.2		
Δ A1MG	Q1	77.8	22.2	0.919	0.70 (0.13-3.70)	77.8	22.2	0.067	0.70 (0.13-3.70)
	Q2	83.3	16.7			44.4	55.6		
	Q3	72.2	27.8			61.1	38.9		
	Q4	83.3	16.7			83.3	16.7		
Δ NAG	Q1	88.9	11.1	0.291	3.08 (0.51-18.54)	83.3	16.7	<0.001	13.00 (2.59-65.20)
	Q2	88.9	11.1			100	0		
	Q3	66.7	33.3			55.6	44.4		
	Q4	72.2	27.8			27.8	72.2		
Δ AAP	Q1	82.4	17.6	0.750	1.44 (0.27-7.68)	76.5	23.5	0.344	1.35 (0.29-6.26)
	Q2	88.2	11.8			82.4	17.6		
	Q3	70.6	29.4			52.9	47.1		
	Q4	76.5	23.5			70.6	29.4		

Only the higher quartile of variation of two biomarkers, ACR and urinary NAG excretion, was associated with a significantly higher risk of an individual patient being a progressor (ACR and/or GFR) (table 62). However, for ACR it was a predictor (OR: 9.14 [95% CI: 1.95-42.90]) due to the definition of an ACR progressor (patients certainly in the higher quartiles for ACR variation), given that there was no difference between non-progressors and GFR progressors (figure 37), only between ACR progressors or GFR and AACR progressors and the remaining subgroups.

On the other hand, the higher quartile of urinary NAG excretion variation not only gives an OR of 28.00 (95% CI: 4.44-176.78) to be a progressor (in comparison with the first quartile), but urinary NAG variation increased progressively between non-progressing patients, patients with only ACR progression, patients with only GFR progression and patients presenting both ACR and GFR progression ($p<0.001$).

Table 62: Distribution of progressors (ACR and/or GFR) and non-progressors in the first and fourth quartile of the biomarkers' variation. * for the distribution of non-progressors and progressors between quartiles 1 and 4; ** of being a progressor comparing fourth to first quartile; OR: odds ratio; NP: non-progressor; P: progressor; Q: quartile; Δ: variation; ACR: urinary albumin to creatinine ratio; UTE: urinary transferrin excretion; ColIV: urinary collagen type IV excretion; A1MG: urinary α1-microglobulin excretion; NAG: urinary N-acetyl-β-D-glucosaminidase excretion; AAP: urinary alanine aminopeptidase excretion.

	Q1		Q4		p value*	OR**
	NP (%)	P (%)	NP (%)	P (%)		
Δ ACR	63.2	36.8	15.8	84.2	0.007	9.14 (1.95-42.90)
Δ UTE	61.1	38.9	77.8	22.2	0.471	0.45 (0.10-1.93)
Δ ColIV	55.6	44.4	72.2	27.8	0.489	0.48 (0.12-1.93)
Δ A1MG	61.1	38.9	72.2	27.8	0.725	0.60 (0.15-2.45)
Δ NAG	77.8	22.2	11.1	88.9	<0.001	28.00 (4.44-176.78)
Δ AAP	64.7	35.3	58.5	41.5	1.000	1.28 (0.32-5.13)

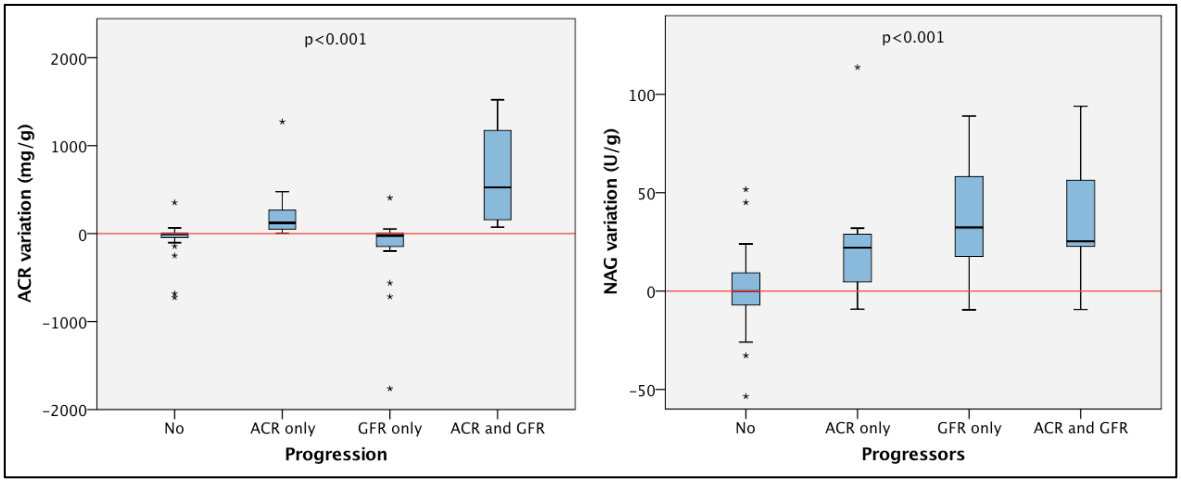


Figure 37: Comparison of ACR (left image) or NAG (right image) variation in concentration between subgroups of progression. From left to right: non-progressors, only ACR progressor patients, only GFR progressor patients and patients presenting ACR and GFR progression. ACR: urinary albumin to creatinine ratio; GFR: glomerular filtration rate; NAG: urinary N-acetyl-β-D-glucosaminidase excretion.

2.5.6 *Longitudinal variation: influence of gender and treatment*

Male gender was a significant risk factor to be an ACR progressor (OR 5.71 [95% CI: 1.64-19.92), but not a GFR progressor (OR: 1.54 [95% CI: 0.53-3.87]) (table 63).

Table 63: Progressing and non-progressing Fabry disease patients: analysis by gender. ACR: urinary albumin to creatinine ratio; GFR: estimated glomerular filtration rate; NP: non-progressors; P: progressors; OR: odds ratio.

	ACR			GFR		
	NP (%)	P (%)	OR	NP (%)	P (%)	OR
Females	90.9	9.1	5.71	75.0	25.0	1.44
Males	63.6	36.4	(1.64-19.92)	67.6	32.4	(0.53-3.87)

However, this exploratory analysis by gender was hampered by the small sample of patients classified as progressors, mainly in the female gender. Therefore, the significant difference in baseline concentration of ACR and urinary NAG excretion, between ACR or GFR non-progressors and progressors, was no longer significant in the female patient subgroup (table 64). Furthermore, the same was also true for the difference of ACR concentration between ACR non-progressors and progressor in male patients. Nonetheless, the difference in baseline urinary A1MG concentration, between ACR or GFR non-progressors and progressors, emerged as significant in the male patient subgroup.

Table 64: Comparison of the baseline concentration of the biomarkers between ACR or GFR non-progressors and progressors: stratification by gender. Expressed as median (IQR); ACR: urinary albumin to creatinine ratio; GFR: glomerular filtration rate; NP: non-progressor; P: progressor; UTE: urinary transferrin excretion; ColIV: urinary collagen type IV excretion; A1MG: urinary α1-microglobulin excretion; NAG: urinary N-acetyl-β-D-glucosaminidase excretion; AAP: urinary alanine aminopeptidase excretion.

		ACR			GFR		
		NP	P	p value	NP	P	p value
ACR (mg/g)	♀	28.7 (116.1)	106.8 (522.4)	0.351	26.1 (111.1)	43.4 (1506.2)	0.650
	♂	206.2 (320.6)	242.8 (513.2)	0.258	74.2 (311.5)	350.5 (433.5)	0.013
UTE (μg/g)	♀	1.09 (1.33)	1.36 (3.17)	0.953	1.18 (1.73)	1.00 (0.74)	0.612
	♂	1.01 (1.26)	1.20 (1.95)	0.200	0.70 (1.20)	1.31 (1.13)	0.060
ColIV (mg/g)	♀	8.93 (13.38)	11.42 (26.81)	0.768	11.18 (15.37)	8.60 (5.51)	0.321
	♂	6.58 (7.23)	14.79 (14.11)	0.006	7.71 (11.25)	8.62 (10.12)	0.403
A1MG (mg/g)	♀	600.61 (818.1)	569.35 (1116.3)	0.738	683.5 (912.1)	537.6 (343.1)	0.594
	♂	464.1 (365.7)	710.6 (977.7)	0.030	342.7 (365.7)	628.8 (301.6)	0.022
NAG (U/g)	♀	14.64 (17.40)	18.88 (24.07)	0.798	15.34 (16.04)	17.94 (20.93)	0.928
	♂	13.45 (9.80)	25.95 (25.99)	0.016	9.67 (8.02)	22.02 (20.57)	0.001
AAP (U/g)	♀	16.02 (16.19)	7.82 (23.63)	0.293	17.56 (20.78)	11.78 (12.89)	0.238
	♂	11.70 (8.34)	16.95 (31.12)	0.035	11.70 (13.69)	14.47 (4.29)	0.145

The significant difference in longitudinal variation of urinary NAG concentration between ACR non-progressors and progressors was no longer significant in the sub-analysis by gender (table 65). However, the significant difference in longitudinal variation between GFR non-progressors and progressors remained significant in both female and male patients. The variation of all other biomarkers was no different between non-progressors and progressors.

This study was not designed to access the effect of ERT or anti-proteinuric agents (ACEi or ARB) on the biomarkers. However, I approached this issue as an exploratory endpoint. All comparative analysis has been adjusted for age, ACR, estimated GFR and MSSI, as treated patients usually have a more severe disease.

Table 65: Comparison of the variation in the concentration of the biomarkers between ACR or GFR non-progressors and progressors: stratification by gender. Expressed as median (IQR); ACR: urinary albumin to creatinine ratio; GFR: glomerular filtration rate; NP: non-progressor; P: progressor; Δ: variation; UTE: urinary transferrin excretion; ColIV: urinary collagen type IV excretion; A1MG: urinary α1-microglobulin excretion; NAG: urinary N-acetyl-β-D-glucosaminidase excretion; AAP: urinary alanine aminopeptidase excretion.

		ACR			GFR		
		NP	P	p value	NP	P	p value
Δ ACR (mg/g)	♀	-	-	-	-3.0 (54.1)	-0.1 (103.7)	0.612
	♂	-	-	-	3.4 (79.2)	-0.1 (821.3)	0.925
Δ UTE (µg/g)	♀	0.13 (1.73)	0.25 (.)	0.723	0.07 (2.30)	0.18 (0.37)	0.591
	♂	-0.01 (1.30)	0.13 (1.40)	0.675	0.10 (1.71)	-0.15 (1.09)	0.441
Δ ColIV (mg/g)	♀	2.44 (14.38)	-1.47 (.)	0.345	1.41 (22.27)	3.73 (7.08)	0.393
	♂	1.06 (7.03)	-3.55 (16.00)	0.164	1.20 (7.42)	-3.10 (9.14)	0.502
Δ A1MG (mg/g)	♀	4.98 (744.68)	78.62 (.)	0.869	15.78 (1073.24)	45.90 (290.08)	0.896
	♂	-17.06 (416.72)	2.50 (597.17)	0.965	72.94 (454.13)	-146.26 (372.01)	0.401
Δ NAG (U/g)	♀	1.47 (24.61)	0.17 (.)	0.759	-1.18 (15.67)	27.54 (66.63)	0.007
	♂	11.99 (37.05)	24.26 (8.60)	0.269	9.62 (23.30)	34.92 (41.29)	0.007
Δ AAP (U/g)	♀	-2.43 (13.88)	7.00 (8.41)	0.122	-2.27 (18.15)	2.18 (11.55)	0.466
	♂	-2.68 (13.62)	-2.39 (16.44)	0.857	-3.41 (16.07)	-1.98 (11.30)	0.859

After adjustment for disease severity all biomarkers, except urinary AAP excretion, evidenced a trend of a decreased variation in patients under ERT, but this difference was not significant for any biomarker (table 66). The effect of anti-proteinuric treatment in the variation of the biomarkers concentration was less evident, without any consistent decreased variation in treated patients.

Table 66: Nephropathy study: comparison of the variation in the concentration of the biomarkers between patients under or not under ERT or ACEi / ARB. Expressed as mean (standard error); ERT: enzyme replacement therapy; ACEi: angiotensin converting enzyme inhibitors; ARB: angiotensin receptor blockers; Δ: variation; ACR: urinary albumin to creatinine ratio; UTE: urinary transferrin excretion; ColIV: urinary collagen type IV excretion; A1MG: urinary α1-microglobulin excretion; NAG: urinary N-acetyl-β-D-glucosaminidase excretion; AAP: urinary alanine aminopeptidase excretion.

	ERT		p value	ACEi / ARB		p value
	No	Yes		No	Yes	
Δ ACR (mg/g)	30.4 (90.4)	17.3 (55.1)	0.910	-33.5 (63.2)	79.0 (65.1)	0.245
Δ UTE (µg/g)	-0.09 (0.45)	-0.19 (0.29)	0.865	-0.29 (0.34)	-0.02 (0.33)	0.590
Δ ColIV (mg/g)	1.91 (4.21)	-0.18 (2.68)	0.700	1.02 (3.14)	-0.04 (3.14)	0.823
Δ A1MG (mg/g)	-181.2 (201.2)	-40.6 (129.0)	0.586	-261.0 (152.9)	80.5 (148.1)	0.139
Δ NAG (U/g)	17.53 (7.38)	12.60 (4.99)	0.609	13.84 (6.60)	14.60 (5.70)	0.938
Δ AAP (U/g)	-4.11 (4.17)	-0.18 (2.65)	0.471	-1.36 (2.98)	-1.54 (3.08)	0.968

Due to the reduced number of patients starting ERT or anti-proteinuric during the study, the comparison between patients starting these treatment, or not, was clearly underpowered. However, there was a non-significant trend of a decreased variation in all biomarkers in patients staring ERT or ACEi / ARB (table 67).

Table 67: Nephropathy study: comparison of the variation in the concentration of the biomarkers between patients starting ERT or ACEi / ARB, or not, during the study. Expressed as mean (standard error); ERT: enzyme replacement therapy; ACEi: angiotensin converting enzyme inhibitors; ARB: angiotensin receptor blockers; Δ: variation; ACR: urinary albumin to creatinine ratio; UTE: urinary transferrin excretion; ColIV: urinary collagen type IV excretion; A1MG: urinary α1-microglobulin excretion; NAG: urinary N-acetyl-β-D-glucosaminidase excretion; AAP: urinary alanine aminopeptidase excretion.

	Starting ERT		p value	Starting ACEi / ARB		p value
	No	Yes		No	Yes	
Δ ACR (mg/g)	36.0 (44.4)	-150.3 (153.0)	0.247	29.5 (44.4)	-95.8 (172.6)	0.486
Δ UTE (μg/g)	-0.10 (0.23)	-0.67 (0.77)	0.483	-0.16 (0.23)	-0.21 (0.88)	0.949
Δ ColIV (mg/g)	0.96 (2.15)	-4.64 (7.19)	0.459	0.71 (2.14)	-2.42 (8.10)	0.711
Δ A1MG (mg/g)	-46.9 (103.1)	-510.4 (345.2)	0.204	-81.8 (105.3)	-135.4 (396.5)	0.897
Δ NAG (U/g)	16.61 (3.35)	9.87 (11.21)	0.567	16.74 (3.37)	5.69 (12.69)	0.406
Δ AAP (U/g)	-1.31 (2.06)	-3.73 (8.43)	0.782	-1.41 (2.06)	-2.07 (8.43)	0.940

2.6 Discussion

In this study my main objective was the identification of biomarkers of incipient and progressive nephropathy in FD and not biomarkers of FD or FD nephropathy (not elevated in other forms of nephropathy). I also defined the effect of therapeutic interventions on biomarkers as an exploratory endpoint.

The results suggest that the studied biomarkers are more sensitive than ACR in detecting incipient FD nephropathy, with a significant increase for all of them in the early stages of FD nephropathy (subgroup of patients with albuminuria A1 and estimated GFR ≥ 60ml/min/1.73m^2). Around 50% of the patients with albuminuria A1 presented increased urinary excretion of every novel biomarker. These findings were further supported by the highest sensitivity of urinary NAG excretion in the diagnosis of patients with FD disease, showing that it identifies more patients with nephropathy than ACR.

Moreover, urinary NAG excretion outperformed albuminuria in the sensitivity and diagnostic accuracy for CKD stage ≥ 2. The relatively modest DOR of the various biomarkers was mainly related to their ability to identify patients with GFR ≥ 90ml/min/1.73m^2, but certainly with kidney involvement from FD, thereby diminishing their specificity for CKD stage ≥ 2. Nonetheless, the different biomarkers are complementary, given that for a few patients only some of them are increased (including some patients with ACR elevation only) and may be used as a whole in the diagnosis of incipient FD nephropathy, reflecting the aforementioned several pathophysiological pathways contributing to the development of FD nephropathy (figure 2).

Concerning the prognostic value of the biomarkers, although a significant progressive increase of the biomarkers along the FD subgroups of increasing severity or CKD stages was only found for urinary NAG excretion, the correlation with estimated GFR was strongest for urinary NAG excretion, but also significant for ACR, UTE and urinary ColIV excretion. Moreover, in the earlier stage of FD nephropathy, all but one (urinary AAP excretion) of the biomarkers showed better correlations with GFR than albuminuria and in multivariate regression analysis urinary NAG excretion was the only remaining in the model of estimated GFR prediction, after adjustment for age.

The prognostic value of urinary NAG excretion was further confirmed in longitudinal evaluation, showing that urinary NAG was not only the sole biomarker significantly increasing its urinary concentration during follow-up, but also that the difference in urinary NAG excretion between CKD stages widened in the same period. Moreover, urinary NAG concentration at baseline was the only one, compared with the other biomarkers (including ACR), accurately predicting the subsequent slope in GFR variation. Urinary NAG variation was also the only one that had a significant inverse correlation with GFR variation in the same period, suggesting a possible pathophysiological link.

The importance of the prognostic value of a biomarker is clearly depicted in this study, since, considering the FD cohort as a whole, kidney function remained essentially stable, even in patients within CKD stages ≥3 or in the most severe subgroups; however, according to the definition in the study, one fifth or more than a quarter of the patients were ACR or GFR progressors, respectively.

This is in accordance with several studies in treated patients and natural history studies, showing that the progression of FD nephropathy is heterogeneous and that a proportion of patients progress, even under ERT.[149, 194, 206, 207] Several risk factors for progression in treated and untreated patients were identified, namely male gender, proteinuria, CKD stage at baseline, higher degree of glomerulosclerosis, longer time between symptom onset and ERT beginning and residual enzymatic activity.[82, 141, 149, 152, 194, 206, 207, 212] Actually, as noted above, proteinuria seems to have an important role in FD nephropathy progression, but the magnitude of this influence is not well established.[15, 83, 141] These findings were confirmed by the present study, showing that the prognostic value of ACR to predict subsequent GFR decline, though significant, is limited.

Nonetheless, both ACR and urinary NAG excretion were significantly higher in ACR or GFR progressors, in comparison with non-progressors and the proportion of progressors was also higher in the highest quartiles of both ACR and urinary NAG distributions. Though, the variation of urinary NAG concentration during follow-up was clearly superior to the variation of ACR in the identification of progressing patients, with a very high OR to be a progressor in patients within the fourth quartile of urinary NAG variation.

Thus, not only do the studied biomarkers seem to have an important added value in the identification of incipient FD nephropathy, but also urinary NAG excretion appears to outperform ACR as a prognostic biomarker, clearly identifying patients at increased risk of progressive nephropathy.

The correlations with variables related to demographics, diagnosis, severity and treatment were not significant, which argues for these biomarkers to be markers of FD nephropathy and not of overall disease burden. One exception was the correlation between ACR and urinary NAG excretion with severity indexes (MSSI and FIPI), but both indexes comprise a renal component / involvement[229, 693] and nephropathy is a well-known determinant of overall disease prognosis and burden.[10, 86, 87] The correlation between urinary NAG excretion and age seems related to the decrease in kidney function associated with ageing, as evidenced by the maintenance of the significant inverse correlation between NAG and estimated GFR after adjustment for age.

The absence of difference in the various biomarkers according to gender is understandable, given the balanced gender distribution within the subgroups of increasing FD nephropathy. However, when cross-sectional and longitudinal correlations and comparisons were stratified by gender, the correlations were weaker and comparisons of smaller magnitude in female patients. This finding may be related to the small sample for subgroup analysis; however, as in female patients disease onset occurs latter and the phenotype tends to be milder and nephropathy progression slower,[10, 11, 82, 141] accurate prognostic biomarkers are crucial and an appropriate study design to establish the prognostic value of biomarkers in both genders is mandatory.

The study was also not designed to access the effect of ERT on the biomarkers, but given the greater proportion of patients on ERT in the more severe subgroups, it is probably due to this reason that the concentration of biomarkers was similar between treated and untreated patients. Actually, in an exploratory analysis, adjusted for age and

nephropathy and overall disease severity, I was able to identify that in patients under / starting ERT, the biomarkers increased less or even decreased during follow-up, although the difference to the remaining patients was non-significant.

The effects of ERT on nonspecific chronic histological lesions of FD nephropathy, like glomerulosclerosis, vascular hyalinosis and tubulointerstitial fibrosis in not known. A few studies confirmed that ERT clears glycosphingolipid inclusions from several renal cell types, including endothelial, tubular, interstitial and mesangial cells (with an inconsistent and only partial clearance of inclusions in glomerular podocytes),[79, 166, 178] but only one study showed that ERT decreased mesangial widening.[165] Thus, the influence of ERT on these biomarkers that seems to be related to histological findings, like mesangial widening (urinary ColIV excretion) and tubular atrophy and dysfunction (urinary NAG excretion), deserves detailed study and may be of paramount interest in clinical practice.

2.6.1 *Glomerular damage biomarkers*

As previously mentioned, transferrin is a molecule slightly larger than albumin (molecular radius 4.0nm versus 3.6nm), but less anionic, thus expected to be more readily filtered through the glomeruli and useful as a surrogate marker of glomerular damage.[480, 481] Moreover, UTE is also a marker of proximal tubular dysfunction, because transferrin resorption depends on the integrity of late endosomal / lysosomal trafficking for intracellular processing in the tubular proximal cells.[485]

Therefore, as both GBM and lysosomal system in proximal tubular cells are damaged in FD, there is a good rational for the use of transferrin as a biomarker in FD nephropathy. Actually, an increase in UTE was found in a small cohort of females with FD, which tended to decrease in patients treated with ERT;[307] I was able to confirm these results, showing an increase in UTE in FD patients in comparison with healthy controls.

Moreover, as reported in diabetic nephropathy (in up to 61% of the patients with albuminuria A1),[488, 498] UTE may be a more sensitive marker of glomerular damage being raised in the subgroup of FD patients and albuminuria A1. However, distinct to diabetic nephropathy, I did not find a progressive increase in UTE with increasing albuminuria and an almost universal increase in UTE in patients presenting albuminuria A2 and A3 (I found some patients in subgroups 2 and 3 with UTE in the range of the control group). This difference may be explained by methodological aspects (studies evaluating UTE in 24

hour urine and the laboratorial methods used) and the heterogeneity of FD phenotype and physiopathological pathways involved.[488, 498] Nonetheless, as in diabetic nephropathy, I was able to show a significant correlation with ACR, documenting a similar glomerular leak and tubule handling for these two proteins, as well as a significant inverse correlation with estimated GFR.[488, 490]

UTE was identified as a good prognostic marker in diabetes, because it identified patients at higher risk of developing albuminuria A2 in two studies with follow-up periods of 24 and 60 months and may contribute to tubular damage due to release of reactive iron and oxidative stress in the tubular epithelium;[480, 490, 509] in diabetic nephropathy model there are no reports evaluating the prognostic significance of UTE in terms of GFR decline. In this study, the prognostic value of UTE was, at least, limited, since UTE just increased in the subgroup of patients with albuminuria A3 and neither its baseline values, nor its variation during follow-up predicted the progression in terms of an increase in ACR or a decline in GFR. However, this study is limited by the sample dimension (in the setting of a rare disorder), with only 25 normoalbuminuric patients recruited (a very small proportion progressing to albuminuria A2) and by the short follow-up period of 24 months. In a slowly progressive disorder in the early phases of nephropathy and with heterogeneous phenotypes, kidney function remained stable during the follow-up period (considering the whole cohort), thus I think that a prognostic value of UTE should not be ruled out without further research.

The effect of ERT on UTE was approached as an exploratory endpoint and there was a trend of a greater decrease in UTE in treated patients during the follow-up period, in comparison with untreated patients. A similar effect was identified in diabetic nephropathy after correction of the metabolic defect with hypoglycaemic agents, with a significant decrease in the percentage of patients with increased UTE.[508]

As previously mentioned, in males ERT does not seem to decrease total proteinuria and ACR in FD patients. As UTE depends, as ACR, on increased permeability of glomerular membrane to plasma proteins, the response to ERT may be limited. However, UTE is also a biomarker of proximal tubular dysfunction and, although Gb3 storage in proximal tubules seems of lesser magnitude than in distal tubules,[57, 77, 79] UTE may decrease in response to ERT if tubular proximal function ameliorates. Gb3 storage in proximal tubular cells may decrease in patients under ERT,[79, 178] but the evidence

supporting the response of tubulointerstitial fibrosis to ERT is scant, with two studies showing no increase in tubulointerstitial fibrosis in treated patients.[79, 165] Nevertheless, experiments in animal models and cell lines, not only showed that recombinant α-galactosidase A reaches the proximal tubular cells mainly via the glomerular filtrate (and to a lesser extent by uptake by mannose-6 phosphate receptors in the basolateral membrane) and is uptake via the megalin receptor, but also that ERT inhibits the epithelial-mesenchymal transition induced by Gb3 and lyso-Gb3 in tubular proximal cells, which is one of the pathways involved in tubulointerstitial fibrosis.[702, 715, 716]

Concluding, transferrin and albumin seems to be handled by the glomerulus and proximal tubule in a similar manner, but UTE appears to have higher sensitivity than ACR to detect incipient FD nephropathy, although its prognostic value seems limited in comparison with ACR.

Due to its high molecular weight, ColIV is not filtered through the glomerulus and its urinary excretion seems only marginally affected by the serum levels; thus, since the source of urinary ColIV seems to be GBM and mesangial matrix, it appears to be a surrogate marker of glomerular basement membrane synthesis and mesangial matrix remodelling.[537] Histological studies corroborated urinary ColIV excretion as a biomarker of mesangial expansion and GBM thickening.[545, 546]

In FD, podocytes are among the earliest cells to be (heavily) loaded with glycosphingolipids deposits, which may activate pathophysiological cascades culminating in podocyte detachment, GBM thickening and mesangial expansion.[58, 77] Lyso-Gb3 activates various deleterious pathways in podocytes: expression of CD80, activation of Notch1 pathways and expression of the cytokine receptor CD74, increased expression of the fibrogenic cytokine TGF-β1 and subsequent increasing in extracellular matrix synthesis (fibronectin and ColIV).[18, 70, 71]

Urinary ColIV excretion has never been studied in FD nephropathy, but, due to the aforementioned reasons, its concentration would be increased in FD patients. In this study, I was able to confirm this hypothesis and also showed an increase of ColIV excretion in early stages of FD nephropathy (in FD patients with albuminuria A1), consistent with the pathological findings of thickening or duplication of glomerular basement membrane and increased mesangial extracellular in incipient FD nephropathy.[57, 77] The better sensitivity than albuminuria to detect initial glomerular

damage has also been documented in diabetic nephropathy, with up to 49% of T2DM patients with albuminuria A1 presenting increased urinary ColIV excretion.[536, 547, 552] However, as also reported in diabetic nephropathy, some patients with increased albuminuria did not show an increase in urinary ColIV excretion and there was no progressive increase in urinary ColIV excretion across CKD stages or subgroups of increasing severity.[469, 536, 555] As in diabetic nephropathy, I found a significant correlation between urinary ColIV excretion and ACR;[536, 555] furthermore, ACR progressing patients presented significantly higher excretion of ColIV at baseline. Thus, ACR and urinary ColIV excretion does not seem to reflect the same pathophysiological pathway, but rather complementary processes, certainly linked and contributing to each other, given that mesangial and GBM pathology is associated with altered permeability of glomerular filtration barrier to plasma proteins.

The significant inverse correlation, in both genders, between urinary ColIV excretion and estimated GFR would suggest that ColIV excretion is a prognostic factor in FD nephropathy. This is in accordance with several findings in diabetic nephropathy: patients with greater thickening of glomerular basement membrane and mesangial fractional volumes were at greater risk of decline in GFR;[471] a significant inverse correlation between ColIV and the reciprocal of serum creatinine;[558] greater risk of progression from normoalbuminuria to albuminuria A2 in patients with increased ColIV excretion;[536, 556] baseline values of urinary ColIV were strong predictors of GFR changes in follow-up studies in patients with T1DM and T2DM.[557, 559, 560] Even in FD, degenerative glomerular / tubulointerstitial lesions or arterial sclerosis scores are the histological alterations with best correlation with age or GFR and, under light microscopy, segmental and/or global glomerulosclerosis were found to be the only pathologic correlates of proteinuria at early stages of Fabry nephropathy.[83, 84, 717]

However, in the longitudinal analysis, baseline urinary ColIV excretion was related, as noted above, to ACR progression, but not to GFR progression. Moreover, although urinary ColIV significantly increased in the first twelve months and decreased thereafter, in a pattern similar to the changes observed in estimated GFR, there was no correlation between the two variables and variation of urinary ColIV excretion did not predict the GFR progression status. These findings may be related to the small sample and short time of follow-up, as explained above for UTE.

I also found a non-significant trend of decreased urinary ColIV excretion in patients treated with ERT or ACEi / ARB, whereas an increasing trend was identified in untreated patients. Actually, ColIV may be a good biomarker of response to ERT, given that the mesangial widening was showed to decrease in patients under ERT.[165] Similarly, in T2DM, glycaemic control with agonist of glucagon-like peptide-1 and thiazolidinediones significantly decreased urinary ColIV excretion.[562-564] The effect of anti-proteinuric treatment in ColIV excretion, in patients with T2DM is less consistent, with studies showing conflicting results.[565-567]

Concluding, in strict correlation with histological findings, showing mesangial expansion as an early event in FD nephropathy, urinary ColIV excretion appears to increase in incipient FD nephropathy and seems a good predictor in terms of ACR progression. However, its prognostic significance in terms of GFR progression and value in assessing response to ERT deserves further research, due to the strong rational supporting its role as a biomarker in these two settings.

2.6.2 *Tubular damage biomarkers*

As stated previously, in FD glycosphingolipids storage occurs also in tubular cells, where they may have a direct toxic effect, even in the early phases of the disease, which are clinically silent in terms of the standard renal tests.[57, 77] Moreover, the main source of urinary Gb3 seems to be the tubular cells and the urinary collecting system, with an 80% reduction in urinary Gb3 after nephrectomy or transplantation.[162, 718] Other contributors to tubular injury in FD may be the hemodynamic damage (as result of Gb3 accumulation in the renal microvasculature) and persistent proteinuria.[74, 75, 494]

Tubular injury preceding glomerular involvement was also observed in other renal diseases, such as diabetic nephropathy, where an increase in several tubular proteins and enzymes become apparent before albuminuria A2.[642, 649] In diabetic nephropathy, the concentration of tubular injury biomarkers has been correlated with the severity of nephropathy, so it has been hypothesized that the long-term outcome in diabetic nephropathy was more related to the degree of the tubulointerstitial impairment than to the severity of glomerular lesions.[719] In this study I also demonstrated that tubular injury biomarkers increase in FD nephropathy before albumin excretion and one of them (NAG)

seems to have better prognostic value in terms of GFR decline. Previous smaller studies in FD have shown an increase in tubular injury biomarkers.[307, 308]

A1MG is a small protein that is freely filtered through the glomerular membrane and reabsorbed by the proximal tubules. Hence, a decrease of creatinine clearance will increase its plasma levels and any proximal tubular cell dysfunction results in increased quantities of A1MG in the urine.[594, 720]

As in diabetic nephropathy (where up to 33.6% of the normoalbuminuric patients presents increased A1MG excretion), I was able to demonstrate that urinary A1MG excretion increases in patients with albuminuria A1 and was more sensitive than albuminuria to detect FD and CKD stage ≥2.[579, 607] Thus, it seems a good marker of incipient FD nephropathy, which is in accordance with the findings of severe tubular inclusions (mainly in the distal tubules) and tubule-interstitial fibrosis in patients with clinically silent FD nephropathy.[57, 77] However, urinary A1MG excretion is a biomarker mainly related to proximal tubular dysfunction, demonstrating that despite the relatively smaller amounts of inclusions in the proximal nephron, significant dysfunction may occur without clinical signs of glomerular damage. This is supported by the finding of highest α-galactosidase A in proximal tubular cells of normal human kidneys, in comparison with other renal cells, so these cells, with high turnover rate, may be particularly susceptible in the setting of α-galactosidase A deficiency. Furthermore, in the early stages of FD nephropathy, increased filtration of albumin and other proteins, that will be reabsorbed by proximal tubular cells (resulting in no increase in net excretion of these proteins), will compete for megalin receptors, also responsible for A1MG resorption in the proximal tubules and may result in an increased excretion of this protein.[715]

Nonetheless, as in diabetic nephropathy, some patients with albuminuria A2 and A3 may present A1MG within in the normal range, resulting in no increase of urinary A1MG excretion across the subgroup of patients with increasing severity and absence of a significant correlation with ACR.[469, 607] This finding may indicate that increased A1MG excretion and ACR represent different pathophysiological pathway involvement and that A1MG excretion may complement ACR rather than outperform it.

I found no significant inverse correlation between urinary A1MG excretion and GFR, as well a significant increase of A1MG excretion across CKD stages. Conflicting results regarding the correlation between A1MG and GFR were also found in diabetic

nephropathy.[579, 614] A possible explanation is that if plasma concentration of A1MG is influenced by the GFR, in the setting of proximal tubular dysfunction, urinary A1MG excretion may be influenced by its filtration, justifying decreased A1MG excretion in patients with lower GFR.[594] This hypothesis is further supported by the absence of prognostic value of A1MG variation in terms of prediction of ACR or GFR progression.

The prognostic value of urinary A1MG excretion has never been studied in diabetic nephropathy, but in FD, despite no significant OR to predict ACR and GFR progression in patients with higher urinary A1MG concentration at baseline, in sub-analysis by gender, male patients showed a significantly higher A1MG excretion at baseline in both ACR and GFR progressors, in comparison with non-progressors. The lack of predictive value in female patients may be related to the small cohort and short follow-up period (with minimal number of progressing female patients), but also to the X-linked nature of FD.

As in diabetic nephropathy, where metabolic control with hypoglycaemic agents significantly decreased A1MG excretion,[501, 618, 622] in this study I found a clear trend of a decrease in urinary A1MG excretion in the small subgroup of patients that started ERT during the study. This finding is also in accordance with a previous small previous study in FD (with 13 women) reporting a significant decrease in urinary A1MG excretion with ERT,[307] and could mean a surrogate of an amelioration of proximal tubular dysfunction or an improvement in glomerular filtration barrier, leading to decreased competition of the proteins in the glomerular filtrate for megalin receptors.

Concluding, urinary A1MG excretion increases in incipient FD nephropathy, supporting proximal tubular injury as an early event in FD, but it does not appears to correlate with GFR; nonetheless, A1MG excretion seems to be of prognostic value in male patients and needs to be further studied as predictor in females.

NAG is an urinary enzyme that is distributed along the whole nephron, but with highest activity in the straight portion of the proximal tubule.[637] Due to its high molecular weight, it cannot be filtered through the glomeruli and its presence in the urine results exclusively from tubular secretion.[633] Moreover, increased urinary NAG excretion may not only occur in tubular damage, but also in increased tubular lysosomal turnover.[640]

I was able to confirm previous data showing an increase in urinary NAG excretion in FD,[641] but I also showed that this biomarker rises before and significantly correlates

with albuminuria. Moreover, among the studied biomarkers, confirming their added value in detecting incipient FD nephropathy, urinary NAG excretion had the highest sensitivity to detect FD patients and CKD stage ≥2. These results are in accordance with histological findings previously described for A1MG excretion.[57, 77] Comparable results were found in diabetic nephropathy, reporting urinary NAG excretion as the most sensitive marker for early detection of tubular damage or for prediction of albuminuria A2.[642, 649, 721] Moreover, this greater sensitivity than albuminuria to detect early renal malfunction may be related to an increase in urinary NAG excretion at a stage where tubular reabsorption capacity may still be sufficient to balance increased glomerular filtration of albumin.[481, 651]

As reported in diabetic nephropathy, the prognostic value of urinary NAG excretion has been clearly depicted in this study, overcoming albuminuria as a predictor of estimated GFR (urinary NAG excretion was the only significant predictor in multivariate regression analysis) and progressively increasing across CKD stages.[642, 643] Furthermore, urinary NAG excretion at baseline and its variation during follow-up had the best value to predict GFR progression: not only did patients in the higher quartiles at baseline present more pronounced decrease of GFR, but also patients for whom urinary NAG excretion increased more during follow-up had a worse prognosis in terms of GFR decline. Urinary NAG excretion has also established prognostic value in diabetic nephropathy.[658, 666, 667] As noted above, degenerative glomerular, tubular, interstitial and arterial lesions are the histological alterations best correlated with age or GFR;[83, 84, 717] however, longitudinal studies are required to establish the predictive value of these degenerative changes for the ultimate progression of Fabry renal disease. Nonetheless, in the present study, for the first time, I present evidence supporting that a biomarker related to degenerative changes in the tubulointerstitial compartment has better prognostic value than the biomarkers related to glomerular barrier (ACR). Notwithstanding, this evidence needs to be further confirmed and correlated with histological findings.

As for the other biomarkers, there was a trend of a smaller increase in urinary NAG excretion in patients treated with ERT or starting ERT during the study. This is in accordance with finding in diabetic nephropathy showing a decrease in NAG excretion after amelioration of the metabolic defect by glycaemic control.[646, 647, 668] As previously

mentioned, these results may be an evidence supporting improvement of tubular dysfunction during ERT.

The better diagnostic and prognostic performance of NAG in comparison with other tubular injury biomarker may be related to various aspects: biomarker proper value (in terms of measuring tubular damage); more accurate laboratorial technique, with a colorimetric assay, in comparison with ELISA methodology applied for the other biomarkers' measurement; widespread distribution of NAG along the nephron (not a specific marker of proximal or distal tubular dysfunction); NAG is not only a biomarker of tubular injury, but also of increased lysosomal turnover in tubular cells.

Concluding, performance of urinary NAG excretion as a biomarker may substantiate several concepts: tubular injury in FD nephropathy occurs early; long-term outcome of FD nephropathy may be more related to the extent of the tubulointerstitial impairment than to the severity of glomerular lesions; proteinuria is a contributor to tubular injury.

AAP is another tubular enzyme present in the brush border membrane (mainly in the proximal tubules). I was able to show that, as in diabetic nephropathy, urinary AAP excretion increases early in the course of FD nephropathy, even in patients with albuminuria A1.[645, 684, 685] However, as also shown in diabetic nephropathy, the prognostic value of urinary AAP excretion and its response to ERT appears limited.[646, 689]

The restricted clinical utility of urinary AAP excretion as a biomarker in FD nephropathy may be related to its: infradian rhythm,[677] inverse correlation with age[678] and reduced stability after urine collection.[682]

2.6.3 *Difficulties and limitations*

I faced several minor difficulties during the study, most of them related to the epidemiology of FD and longitudinal and multicenter design of the study. As a rare disease, it is difficult to recruit a cohort large enough to allow sub-analysis and to detect small differences, which may have clinical importance. While it was possible to recruit a cohort with the initially planned size, the recruitment period was larger than planned (not affecting the longitudinal follow-up period). Ideally, a longer follow-up period would be desirable for a more accurate analysis of the prognostic value of the biomarkers, but due to constrains of the timeline in the research and doctoral program and in the setting of a

long recruitment period, a 24 months longitudinal evaluation was the possible timeline. The multicenter design of the study generated some difficulties, due to the organization of patients collecting samples at various centres at the same time, which caused some loss of data during follow-up, but did not hamper accurate statistical analysis. I did not experience difficulties in the laboratorial tasks and interpretation of the results.

This study has, *per se*, a few limitations, namely: absence of histological correlation, which would be very useful in biomarker validation, for example, of ColIV as a marker of mesangial expansion and NAG as a marker of tubular injury; cohort dimension and follow-up period, hindering the prognostic value of these biomarkers and allowing only an exploratory analysis of the influence of ERT and ACEi / ARB. It would be important to standardize the assay of biomarkers to allow more widespread use in clinical practice.

Final discussion and future perspectives

Only in 1967, almost 70 years after the first description, in 1898, of FD by Johannes Fabry and William Anderson, was the genetic defect causing this multisystem disorder identified.[1, 2] However, the last 20 years have provided an impressive increase in the understanding of the disease and knowledge about the pathophysiological pathways involved. Notwithstanding, this emerging comprehension about FD has raised many more unanswered questions, than those that we can, actually, undoubtedly answer. Moreover, when research in FD is considered, various challenges arise, including: advantages and limitations of cell and animal models, disease epidemiology, diagnostic difficulties, therapeutic objectives, new treatment approaches, biomarkers and many others.

The introduction, in 2001, of a disease-specific ERT, with two commercially available preparations,[165, 166] both based on the infusion of recombinant enzymes fully changed the scenario of FD in the scientific community: from an orphan disease causing significant morbidity and premature death[3, 4] to a treatable disease with multiple and intriguing genetic issues, pathological pathways and organ systems involved.

This increasing knowledge in FD completely changed its epidemiology and clinical picture from the classical concept of a rare disorder with reported incidences ranging from 1:117.000 to 1:833.000,[19-21] to a much higher prevalence revealed by the screening of high-risk populations and newborns.[22, 23] However, up to 90% of the mutations / genetic variants identified in these screenings are associated with attenuated phenotypes or late-onset forms and some of them are genetic variants of unknown significance. The genetic variants of unknown significance have posed new diagnostic challenges and dilemmas in FD.[222]

Therefore, the full-blown classical phenotype described in male patients, with a relatively well-described natural history constitutes no longer the largest cohort of patients that are currently under follow-up across the FD outpatient clinics and reference centres across the entire world.[82, 83] Instead, the FD cohorts comprise mainly patients with late-onset forms, with an unpredictable and fully heterogeneous course of the disease, for whom the scientific evidence supporting the utilization of disease-specific treatment is, at least, scarce. The disease clinical heterogeneity is expanded by its X-linked inheritance pattern, with females usually presenting a milder and time-delayed

phenotype.[10, 15, 82] Skewed X-chromosome inactivation may explain part of the clinical heterogeneity in female patients, but many other unknown factors are certainly involved.[14]

However, in the new ERT and chaperones therapy era, diagnostic dilemmas and clinical heterogeneity are not the only unsolved questions. Currently, after more than 15 years of treatment there is increasing evidence supporting its efficacy, but there is still little evidence supporting that ERT can actually prevent major clinical events and a proportion of patients, namely those with severe LV hypertrophy or cardiac fibrosis, advanced CKD and heavy proteinuria, are still progressing despite standard treatment with ERT.[187, 190, 194, 199, 202] Moreover, a bulk of increasing evidence suggest that an early treatment strategy may be more effective to prevent disease progression and major clinical events, given that patients experiencing irreversible organ changes may not fully respond to ERT. Nevertheless, the ideal moment for disease-specific treatment initiation, as well as the treatment's ability to reverse the earliest pathophysiological pathways and pathological findings involved in FD are still motives of debate in the scientific community.

Therefore, the greatest challenges and most urgent needs in FD are the identification of very early signs of cardiac and kidney involvement enabling a timely institution of disease-specific treatment (permitting each patient to get the maximal benefit from it) and the accurate definition of each patient prognosis, understanding which untreated and treated patients are at higher risk for disease progression. Reliable and validated biomarkers, ideally measured by a non-invasive technique may aid filling these gaps of unanswered questions of routine clinical practice.

Actually, biomarkers may be useful in various clinical settings: diagnosis (distinguishing pathogenic from non-pathogenic mutations), identification of incipient organ, risk prediction (patients at greater risk of disease progression), response to treatment and design of clinical trials (surrogate biomarkers of the long term-response to a treatment). Currently, even with the gold-standard biomarkers in the identification of early heart (advanced echocardiographic techniques) or kidney (albuminuria) involvement, there is a long clinically silent period, characterized by histological (often irreversible) and transcriptional profile changes, before overt evidence of cardiac or renal involvement.[57, 77, 104, 195]

Due to the all the aforesaid reasons, I decided to proceed with the search for biomarkers to detect incipient cardiac and renal involvement and to predict the risk of disease progression in these two organs. Searching for new biomarkers is a very challenging and laborious task, for which I can use a one by one approach or "omics" medicine tools. In these research projects I tried to identify biomarkers, using a one by one approach, with the aforementioned goals in mind, but also strictly related to possible pathophysiological pathways involved in FD cardiomyopathy and nephropathy, in order to deepen our understanding about FD and its mechanisms.

As noted above, myocardial fibrosis is one of the histological hallmarks of FD cardiomyopathy, appearing to be predominantly interstitial diffuse (with only focal areas of replacement fibrosis).[28, 29, 53, 54] Moreover, some serum biomarkers related to collagen metabolism seem to be closely related to the histological evidence of myocardial fibrosis in patients with hypertensive heart disease and there is evidence that their serum concentration may increase in very early phases of cardiac diseases (prior to an overt clinical or imaging phenotype).[378, 397]

Thus, in a longitudinal study, recruiting a relatively large cohort of 60 FD patients, I measured the serum concentration of various biomarkers related to collagen type I, in order to assess not only their value as biomarkers of incipient cardiomyopathy and assess their predictive value of progressive cardiac involvement, but also the natural history of fibrogenesis in FD cardiomyopathy.

In this study, I was able to demonstrate that collagen type I synthesis is increased even before any echocardiographic evidence of FD cardiomyopathy and seems to directly contribute or is correlated to the development of LV hypertrophy in patients with FD. Moreover, I showed that an inhibition of collagen type I degradation, with suppression of MMPs activity is also involved in the progression of FD cardiomyopathy, not only in terms of LV hypertrophy, but also of diastolic dysfunction. These findings are supported by an accumulating bulk of rationale and evidence: accumulating substrates in FD can induce activation of pathophysiological pathways directly involved in fibrogenesis.[18, 70, 702, 716] As these substrates accumulate since the antenatal period, an increase in collagen synthesis certainly occurs early in the disease course, as suggested by the study results.[722]

I was also able to show that biomarkers related to collagen metabolism are more sensitive than LGE in the detection of myocardial fibrosis, and appear to have an excellent

performance in ruling out focal replacement fibrosis. These findings are also in clear agreement with the limited resolution of LGE and its inability to detect diffuse interstitial fibrosis.[272-274]

The prognostic value of the biomarker of collagen type I synthesis was also evidenced in this study. Not only is the serum PICP concentration in a defined time point a good predictor of LV mass, but with greater significance, its longitudinal variation clearly correlates with the probability of a progressive disease and may identify patients at higher risk for a worse prognosis. Serum MMPs measurement also appears to be valuable in terms of prognosis, because it may identify patients at high risk of progression in terms of diastolic dysfunction in subsequent years.

In an exploratory way, I raised the possibility that ERT may mitigate fibrogenesis in FD cardiomyopathy, decreasing collagen type I synthesis and increasing its degradation. These findings are supported by the experimental evidence showing that ERT may suppress TGF-β1 activation induced by Gb3 in a cell line.[702] The prognostic value of the biomarkers related to collagen metabolism and their possible response to ERT clearly suggest that activation of profibrotic pathways is linked to disease progression and that measurements of the studied biomarkers may be clinically meaningful in the follow-up and risk stratification of FD patients.

Concluding, this study of biomarkers related to collagen type I provided new relevant data to understand the natural history of fibrogenesis in FD cardiomyopathy and showed, for the first time, that serum biomarkers of collagen type I metabolism identify ongoing fibrosis in the early pre-clinical stages of FD cardiomyopathy and possibly predict the development of LV hypertrophy, heralding the importance of developing therapies to mitigate fibrosis and change the natural history of FD cardiomyopathy. Moreover, they may be useful biomarkers in the evaluation of the short-term response to the treatments that are under development and evaluation in clinical trials, namely substrate reduction therapy and the modified forms of plant derived ERT.

FD nephropathy is also characterized by involvement of both glomerular and tubule-interstitial compartments, with patients without alteration in the standard renal tests already presenting heavy glycosphingolipids storage, as well as glomerular sclerosis, tubular atrophy and interstitial fibrosis.[57, 77, 80] Thus, I designed a longitudinal study to identify the early diagnostic and prognosis value of various biomarkers of mesangial

expansion and tubular dysfunction, which have previously demonstrated their added value in diabetic nephropathy.[723, 724]

I was able to demonstrate that biomarkers either related to mesangial expansion / altered permeability of glomerular barrier or to tubular dysfunction / injury identified patients with incipient FD nephropathy with better S than ACR. This is in accordance with previous findings showing that accumulating glycosphingolipids may induce activation of pro-fibrotic and pro-inflammatory pathways in podocytes and mesangial or proximal tubular cells; they may also promote epithelial-mesenchymal transition in proximal tubular cells (a known critical step in the development of interstitial fibrosis).[18, 62, 70, 71, 702] Thus, in contrast to albuminuria, that increases as consequence of glomerular damage, the identified biomarkers seems to be directly related to and support the current knowledge about FD nephropathy.

Moreover, one of the studied biomarkers related to tubular injury, urinary NAG excretion, evidenced a clearly better prognosis value than ACR: not only did its excretion at a specific time point predict the subsequent variation of GFR, but, with an expressive significance, its longitudinal clearly correlated with the changes in GFR. These findings support the concept that tubular injury is, at least, as important as glomerular pathology in FD nephropathy and may be critical for subsequent progressive decline of GFR. This is further reinforced by the reports showing that chronic kidney lesions have better prognostic significance, than the severity of kidney cell inclusions.[717]

Furthermore, ERT seems to have a beneficial effect on urinary excretion biomarkers, which is supported by the fact that ERT may actually ameliorate mesangial widening and allowing my hypothesis that it may also probably improve tubule-interstitial pathology.[165] However, this hypothesis needs to be further studied and accurately correlated with histological findings.

Concluding, in this study of kidney biomarkers I showed that albuminuria limitations as a sensitive marker of early renal dysfunction in FD and as a marker for risk of progression of CKD, may be overcome with the identification of these newly identified biomarkers, all increased in normoalbuminuric patients and one of them (NAG) with better prognostic value than albuminuria. Apart from improving the management of FD nephropathy (with potential implications for ERT initiation criteria), these biomarkers may also define novel early stages of FD nephropathy characterized by mesangial

expansion (ColIV) and/or tubular damage (NAG). They may also be useful in the evaluation of new treatment strategies under development, assessing treatment ability to reverse these initial changes related to FD nephropathy.

Although assuming that all these results needs to be further confirmed in other cohorts of patients, in this final chapter of my thesis, I think that it is appropriate to reflect on the work I have done so far, highlight the questions it raised and considerer how they are being or may be pursued in the future.

With this research project I not only constructed a relatively large database, including more than 80 patients with FD diagnosis, with clinical and research meaningful information, but also started and pursued important investigational collaborations, of paramount significance in the development of new research projects not only in the field of biomarkers in FD, but also in other research topics in FD.

This research project answered some of the questions that I had in my mind when I started this work. However, I now clearly realise that these first results are only the beginning of a path in the demanding search for new biomarkers, because my work has generated many more questions than answers.

I clearly showed that cardiac fibrosis, mesangial expansion and tubular injury are early events in FD and that biomarkers translating these processes may be of good value in the identification of incipient FD cardiomyopathy and nephropathy. However, this value in the identification of incipient organ involvement needs to be confirmed in other cohorts of patients together with histological evaluation.

Histological validation of the serum biomarkers related to collagen metabolism is hampered by the potential harm caused by endomiocardial biopsies. However, this correlation between peripheral blood PICP concentration and the myocardial collagen type I content has already been established in hypertensive heart disease and there is no rational to suppose a different and specific behaviour in FD.[326, 378]

Nonetheless, advanced cardiac imaging techniques, like strain and strain rate echocardiography and T1 mapping in cardiac MRI may present better sensitivity to detect incipient FD cardiomyopathy than the TDI abnormalities that I used in this study. Moreover, T1 mapping may detect lipid deposition in the myocardium, so it is directly related with the disease pathophysiology, characterized by glycosphingolipids storage.[278-280] In this setting, I have already started a research project in order to validate the results

of the biomarkers related to collagen metabolism in incipient FD cardiomyopathy in a large cohort of 120 FD patients and also to correlate the results with strain, strain rate and advanced cardiac MRI techniques (T1 mapping, T2 mapping and feature tracking). This study will certainly provide new insights in FD pathophysiology, evaluating the correlation between glycosphingolipids deposits and stimulus for collagen type I synthesis / suppression of collagen type I degradation.

Furthermore, this study of correlation with cardiac imaging has a longitudinal design, with a follow-up period of 60 months (evaluations at baseline, after 24 months and after 60 months). Therefore, I hope to confirm the prognostic value of the biomarkers related to collagen metabolism in a larger cohort and for a longer follow-up period. I will also evaluate the exploratory trend of a beneficial effect of ERT in cardiac fibrogenesis and further explore the effect of renin-angiotensin-aldosterone antagonists.

Histological correlation of the urinary biomarkers is paramount for their validation. Both urinary ColIV and NAG excretion has been correlated, respectively with mesangial widening and scores of tubule-interstitial fibrosis in patients with diabetic nephropathy or primary glomerulopathies.[545, 546, 671, 672] I am currently developing a protocol and establishing the research collaboration for evaluation of histological correlations of urinary ColIV and NAG excretion, in a small cohort of up to 20 FD patients.

If the results of the abovementioned studies of correlation with cardiac imaging or kidney biopsy findings confirm the results of the present research, I will design studies to confirm the prognostic value of these biomarkers and specifically to evaluate their behaviour in patients starting ERT.

So far, my results clearly support the importance of fibrosis in FD cardiomyopathy and nephropathy, but these biomarkers do not explain the heterogeneity of FD and the reasons behind the prognosis in each individual patient, because they are the “effectors” at the end of the pathophysiological cascades.

In an attempt to identify accurate biomarkers implied in the regulation of fibrotic and other pathophysiological pathways, I have already started a research project for evaluation of microRNAs in FD. MicroRNAs are short non-coding RNAs, able to regulate gene expression at the post-transcriptional level. The available evidence shows that microRNAs are key players in heart development and myocardial conditions, as well as in organ fibrosis with different origins.[338, 725] They have also been implicated in the up and

down-regulation of the TGF-β/Smad signalling pathway and the epithelial-mesenchymal transition (mediated by E-cadherin expression), both involved in mesangial expansion and tubulointerstitial fibrosis.[726, 727] Given the promising results of the entire microRNAnome evaluation in a small cohort of 12 FD patients with a severe phenotype, I am presently finishing the evaluation of the 31 differentially expressed microRNAs in a large cohort of 100 FD patients; depending on these results, research of microRNAs biomarkers may proceed with a longitudinal study.

Finally, as an inflammatory background also characterizes FD and infusion of recombinant proteins (ERT) may cause an immunological response, not only humoral, but also cellular or innate, I am currently starting a research project aimed at evaluating ERT immunogenicity, where I will also try to evaluate, as an exploratory endpoint, the effects of immunological response in these studied biomarkers.

To conclude, my overall research goal would be to establish a reliable and accurate panel of biomarkers to define early cardiac and renal involvement and to delineate a prognosis model in untreated and treated patients. If achieved, both these tools will certainly help in clinical decisions, timely initiation of disease-specific treatment and an individualized approach to the patient treatment, according to the risk profile of each patient.

Scientific output

The overall work developed during this PhD thesis originated the following publications, as well as participation in several scientific meetings.

1. Publications

Currently, I already published the results of the baseline evaluation of the nephropathy and cardiomyopathy studies:

- Aguiar P, Azevedo O, Pinto R, Marino J, Baker R, Cardoso C, Ducla Soares JL, Hughes D. New biomarkers defining a novel early stage of Fabry nephropathy: A diagnostic test study. *Mol Genet Metab*. 2017; 121 (2): 162-169. (appendix A.1)

- Aguiar P, Azevedo O, Pinto R, Marina J, Cardoso C, Sousa N, Cunha D, Hughes D, Ducla Soares JL. Biomarkers of myocardial fibrosis: revealing the natural history of fibrogenesis in Fabry disease cardiomyopathy. *J Am Heart Assoc*. 2018; 7 (6): pii: e007124. (appendix A.2)

Two more publications are now under preparation, with the results of the longitudinal evaluation of cardiomyopathy and nephropathy studies.

The interim results of these research projects have been published as abstracts in scientific proceedings or special issues of scientific journals:

- Aguiar P, Azevedo O, Pinto R, Marino J, Ducla Soares JL, Hughes D. Collagen turnover biomarkers in Fabry disease cardiomyopathy. *J Inherit Metab Dis*. 2014; 37 (Suppl 1): S142 (appendix B.1);

- Aguiar P, Azevedo O, Pinto R, Marino J, Hughes D, Ducla Soares JL. Glomerular and tubular damage biomarkers in Fabry disease. *J Inherit Metab Dis*. 2014; 37 (Suppl 1): S142 (appendix B.2);

- Aguiar P, Pinto R, Azevedo O, Marino J, Baker R, Cardoso C, Ducla Soares JL, Hughes D. Urinary type IV collagen: Better than albuminuria to identify incipient Fabry nephropathy. *Mol Genet Metab*. 2016; 117 (2): S16 (appendix B.3);

- Aguiar P, Azevedo O, Pinto R, Marino J, Cardoso C, Ducla Soares JL, Hughes D. Increased synthesis of collagen type I: an early event in Fabry cardiomyopathy. New biomarkers?. *Mol Genet Metab*. 2017; 120 (1-2): S17-S18 (appendix B.4);

- Aguiar P, Azevedo O, Pinto R, Marino J, Cardoso C, Sousa N, Cunha D, Hughes D, Ducla Soares JL. Collagen type I synthesis biomarkers predict the progression of Fabry disease cardiomyopathy. *Mol Genet Metab*. 2018; 123 (2): S16 (appendix B.5);

- Aguiar P, Azevedo O, Pinto R, Marino J, Cardoso C, Ducla Soares JL, Hughes D. Tubular dysfunction biomarkers in Fabry disease: better than albuminuria to identify patients at risk of nephropathy progression. *Mol Genet Metab*. 2018; 123 (2): S16-S17 (appendix B.6).

2. Communications in scientific meetings

Presented as oral communications:

- "Urinary type IV collagen: Better than albuminuria to identify incipient Fabry nephropathy": presented as oral communication (as well as poster) at the 12th Annual WORLD Symposium (2016) – San Diego, United States of America;

- "New biomarkers defining a novel early stage of Fabry nephropathy": presented as oral communication (as well as poster) at the Fabry Master Class IX (2017) – Madrid, Spain.

Presented as panel communication (posters):

- "Collagen turnover biomarkers in Fabry disease cardiomyopathy": presented as poster at the Annual Symposium of the Society for the Study of Inborn Errors of Metabolism (2014) – Innsbruck, Austria;

- "Glomerular and tubular damage biomarkers in Fabry disease": presented as poster at the Annual Symposium of the Society for the Study of Inborn Errors of Metabolism (2014) – Innsbruck, Austria;

- "Increased synthesis of collagen type I: an early event in Fabry cardiomyopathy. New biomarkers?": presented as poster at the 13th Annual WORLD Symposium (2017) – San Diego, United States of America.

- "Collagen type I synthesis biomarkers predict the progression of Fabry disease cardiomyopathy": presented as poster at the 14th Annual WORLD Symposium (2018) – San Diego, United States of America.

- "Tubular dysfunction biomarkers in Fabry disease: better than albuminuria to identify patients at risk of nephropathy progression": presented as poster at the 14th Annual WORLD Symposium (2018) – San Diego, United States of America.

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Appendices

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New biomarkers defining a novel early stage of Fabry nephropathy: A diagnostic test study

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ABSTRACT

Background: Renal involvement in Fabry disease is a major determinant of overall disease prognosis and early enzyme replacement therapy seems effective in preventing progression of kidney injury. Gb3 storage, glomerular sclerosis and tubulo-interstitial fibrosis may occur with minimal or no changes on standard renal tests, hence alternative markers of renal dysfunction are crucial. In this study we compared several biomarkers with albuminuria in the identification of incipient Fabry nephropathy and their diagnostic accuracy to identify chronic kidney disease (CKD) stage ≥ 2.

Methods: In this multicentre, prospective, cross-sectional and diagnostic test study, a cohort of 78 Fabry patients and 25 healthy controls was consecutively recruited. Patients were grouped by severity of nephropathy: 1) albuminuria < 30 mg/g; 2) albuminuria 30–299 mg/g; 3) albuminuria > 300 mg/g; 4) glomerular filtration rate (GFR) < 60 mL/min/1.73 m^2. Several index tests, namely biomarkers of glomerular (transferrin and type IV collagen) and tubular (α1-microglobulin, *N*-acetyl-β-glucosaminidase and alanine aminopeptidase) dysfunction were compared with the reference standard (albuminuria).

Results: Significant increase of all tested biomarkers in Fabry patients, even in the subgroup of patients without evidence of nephropathy. We also found inverse significant correlations between estimated GFR and collagen type IV ($\rho = -0.289$; $p = 0.003$) or *N*-acetyl-β-glucosaminidase ($\rho = -0.448$; $p < 0.001$), which were stronger than with albumin ($\rho = -0.274$; $p = 0.019$). There was also better diagnostic accuracy of *N*-acetyl-β-glucosaminidase to predict CKD stage ≥ 2.

Conclusions: These results suggest that studied biomarkers may overcome the limitations of albuminuria as sensitive marker of early renal dysfunction and as marker for CKD progression risk. These biomarkers may also define novel early stages of nephropathy characterized by mesangial expansion and/or tubular damage.

1. Introduction

Anderson-Fabry disease (FD) is an X-linked lysosomal storage disorder caused by mutations in the GLA gene that encodes the enzyme α-galactosidase A. The deficiency of this enzyme leads to the lysosomal accumulation of neutral glycosphingolipids (mainly globotriaosylceramide [Gb3]) and organ failure. Involvement of the heart, kidney and brain causes significant morbidity and premature death [1,2].

In the past, before renal dialysis and transplant became widely available, kidney failure was the main cause of death in male patients with FD [3,4]. More recent studies reported cardiovascular disease as the main cause of death. However, the majority of patients dying from cardiovascular events had previously received renal replacement therapy [4,5].

In FD nephropathy Gb3 storage occurs in all renal cells and the disease progression results in the development of glomerulosclerosis, tubular atrophy and interstitial fibrosis [6]. There is limited knowledge about the renal histology of young patients with incipient nephropathy. However, heavy storage in podocytes and distal tubules, as well as segmental foot process effacement have been shown in children and patients with minimal or no alterations in standard renal tests [7–12]. Moreover, mesangial and endothelial cells inclusions,

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glomerulosclerosis, tubule-interstitial fibrosis and arteriopathy have also been described in this group of patients [7,10]. There is massive storage in the podocytes and early podocytopathy, with increased podocyturia and reduced expression of nephrin in the slit diaphragm in patients without increased albuminuria [13–15].

Albuminuria is usually considered to be the gold standard and a sensitive marker of early renal dysfunction in FD [16–18]. In fact, proteinuria is one of the most important indicators of renal disease progression in adult FD patients [19,20] and there is a significant correlation between urinary protein excretion rates and foot process width and fractional volume of Gb3 inclusions in the podocytes [8]. Nevertheless, significant histological changes may occur without pathological albuminuria and/or proteinuria [7–12], thus the sensitivity of albuminuria and/or proteinuria to identify incipient FD nephropathy is questionable.

Nowadays, enzyme replacement therapy (ERT) is the gold standard for the treatment of FD. ERT has shown to slow or halt the deterioration of renal function in patients with mild to moderate renal impairment [21–26]. These benefits may be limited patients with advanced renal disease [23,24,26–28]. Moreover, early ERT has a good safety profile (even in Paediatric population) [29] and is effective preventing kidney injury progression and reversing early pathological changes previously described [9,10].

Based on the two assumptions that early kidney involvement is clinically silent and early treatment is more likely to prevent progressive kidney injury, alternative markers of kidney dysfunction are mandated. So, the identification of biomarkers correlated to the earliest pathological findings is paramount, as these biomarkers may become a non-invasive, diagnostic method of pre-clinical renal involvement by FD.

In this study, we investigated several markers of glomerular and tubular damage in a large cohort of FD patients (within the entire spectrum of FD nephropathy severity), with an emphasis on the usefulness of these markers in incipient FD nephropathy.

2. Material and methods (Appendix 1 for extended methods)

2.1. Study design and population

In this multicentre, cross-sectional, prospective and diagnostic test study, a cohort of 78 FD patients and 25 healthy controls was consecutively recruited, between February 2013 and June 2014.

For FD patients the sole inclusion criteria were diagnosis of FD and age ≥ 18 years old. The control group included individuals with none of the exclusion criteria hereinafter listed and a normal kidney function (estimated GFR ≥ 60 mL/min/1.73 m^2 and albuminuria <30 mg/g).

The exclusion criteria defined for the control group included diseases with possible kidney involvement: systemic hypertension, diabetes, nephritic or nephrotic syndrome, immunoglobulin A nephropathy, systemic vasculitis, systemic lupus erythematosus, hepatitis C, amyloidosis and multiple myeloma. The exclusion criteria for FD patients were the same, except systemic hypertension, because it may be manifestation of FD nephropathy.

To ensure the entire spectrum of severity of FD nephropathy, the recruitment was made in accordance to subgroups of increasing severity: 1) albuminuria <30 mg/g and GFR ≥ 60 mL/min/1.73 m^2 (objective: 25 patients); 2) albuminuria 30–299 mg/g and GFR ≥ 60 mL/min/1.73 m^2 (objective: 20 patients); 3) albuminuria ≥300 mg/g and GFR ≥ 60 mL/min/1.73 m^2 (objective: 10 patients); 4) GFR < 60 mL/min/1.73 m^2 (objective: 20 patients). The control group was age- and sex-matched with the less severe FD subgroup.

The study protocol was approved by the local or national Ethical Committees of each participating centre and study was conducted in accordance with this protocol and the ethical principles of the Declaration of Helsinki. Written informed consent was obtained from all participants before enrolment.

2.2. Clinical work-up, renal function assessment and outcomes

For each recruited patient, clinical data were collected and renal function was evaluated by the determination of serum creatinine, albuminuria and measurement or estimation of GFR.

Albuminuria (albumin-to-creatinine ratio) was used as the reference test to compare the index tests, as it is considered to be the gold standard and a sensitive marker of early renal dysfunction in FD [16–18]. It was quantified from spot urine, in random urine samples. Increased albuminuria was defined as ≥30 mg/g [30].

In FD patients, serum creatinine was assessed at routine follow-up (collected in the same day of the urine used to determine the albuminuria and the index tests). GFR was estimated by the 2009 CKD-$EPI_{creatinine}$ formula [30]. In a single centre GFR is often measured using Cr-51 EDTA clearance in FD patients. CKD staging by GFR categories [30] was based in estimated GFR, except in patients in which radioisotopic measurement of GFR was available.

Laboratories assessing the renal function were unaware of the measurements of the investigated glomerular and tubular damage biomarkers and clinical data.

The prespecified outcomes were to compare the index tests with albuminuria in the identification of incipient Fabry nephropathy (comparing subgroup 1 patients with controls) and their diagnostic accuracy to identify chronic kidney disease (CKD) stage ≥ 2.

2.3. Novel biomarkers of kidney injury analysis/index tests

Two markers of glomerular damage (transferrin and type IV collagen [ColIV]) and three markers of tubular injury (α1-microglobulin [A1MG], *N*-acetyl-β-D-glicosaminidase [NAG] and alanine aminopeptidase [AAP]) were quantified from spot urine. Each spot urine has been splitted from the sample used to determine albuminuria and immediately stored at −20 °C, transported to the laboratory at the same temperature conditions in dry ice and thawed just prior to the assay.

Transferrin, col IV, A1MG and AAP were evaluated by an ELISA method, according to manufacturer instructions and measured at 450 nm (SunRed®, Shanghai, PRC); NAG was evaluated by a colorimetric assay at 580 nm (Roche® Diagnostics GmBH, Mannheim; Germany) (detailed description in Supplementary methods). Duplicate determinations were made for each individual and mean results are presented. All biomarkers levels were normalized for the urinary creatinine concentration. Laboratory researchers were blinded to clinical data and renal function assessment (reference test).

Given that there are no validated reference values for the tested biomarkers, the upper limit of 95% confidence interval for mean of the control group was assumed to be the upper limit of the reference value.

2.4. Statistical analysis

Statistical analysis was performed with SPSS® (Statistical Package for the Social Sciences, version 21) software. Continuous variables were expressed as medians and interquartile range (IQR) and as number and percentage for categorical variables. Normal distribution of continuous variables was tested using Shapiro-Wilk test.

For continuous variables, comparison of means/medians was performed using Student *t*-test for variables that followed a normal distribution and Mann–Whitney test/related samples Wilcoxon signed rank test for variables who did not.

If the qualitative variable had >2 categories, an ANOVA test (post-hoc analysis with Bonferroni correction) was used for variables with normal distribution, and a Kruskal–Wallis test was used for those without. For categorical variables, the comparison of the variables distribution between groups was done using the Qui-square or Fisher exact tests.

To evaluate the correlation between the several biomarkers and the quantitative variables, Pearson's correlation coefficient was determined

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and presented in dispersion diagrams. Due to the skewed distribution of all the studied biomarkers (index and reference tests), a logarithmic transformation of these variables has been performed for correlation analysis. Normal distribution of log-transformed variables was tested using Shapiro-Wilk test. For easier visualization, a log scale was also used in scatterplots.

We evaluated the diagnostic accuracy of the several biomarkers to diagnose CKD stage ≥ 2 (defined by GFR < 90 mL/min/1.73 m^2) by calculating the diagnostic odds ratio (DOR) and the area under the curve (AUC) from receiver operating characteristic (ROC) curves (we determined the confidence intervals of area under the curve [nonparametric method], whereas values between 1 [perfect test] and 0.5 [useless test]).

For all comparisons and correlations a probability value of <0.05 was considered statistically significant. For comparisons including all groups, with about 25 subjects per group, we expect a power of ~90% to detect differences among groups, if the within group SD is no more than twice the SD between the groups. For correlations, using all the FD patients ($n \approx 75$), we will have at least ~90% power to detect even small correlations ($\rho 2 \sim 0.2$) as significant.

3. Results

3.1. Population characteristics

78 FD patients (subgroups: 1–25 patients; 2–22 patients; 3–10 patients; 4–21 patients) and 25 controls (age- and sex- matched with subgroup 1) were recruited (Fig. 1). Population characteristics are given in Table 1 (detailed information in Supplemental Table 1). Altogether 35 different GLA pathogenic mutations were identified (Supplemental Table 2), with p.N215S and p.F113L, mutations associated with late-onset phenotypes with predominant cardiac involvement, accounting for 34.6% of the patients; the remainder mutations were usually associated with classical phenotype.

3.2. Novel biomarkers of kidney injury (index tests): correlation with albuminuria (reference standard)

The index tests were all increased in FD patients compared with controls (Table 2), even in the less severe subgroup of FD patients.

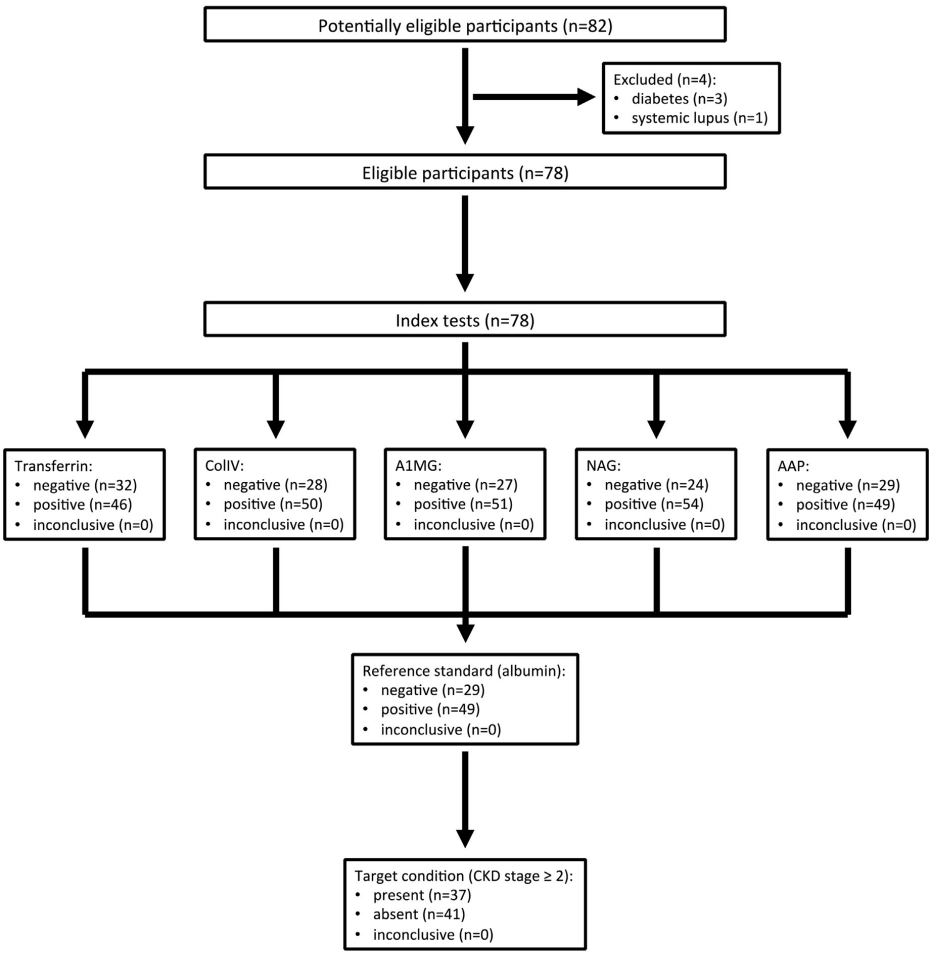

Fig. 1. Study flow diagram. ColIV: collagen type IV; A1MG: α1-microglobulin; NAG: *N*-acetyl-β-D-glicosaminidase; AAP: alanine aminopeptidase; CKD: chronic kidney disease.

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Table 1
Clinical characteristics of study population.

		FD subgroups			
	FD cohort (n = 78)	1 (n = 25)	2 (n = 22)	3 (n = 10)	4 (n = 21)
Categorical variables (n/%)					
Sex (female)	44 (56.4)	18 (72.0)	14 (63.6)	4 (40.0)	8 (38.1)
ERT (yes)[*]	54 (69.2)	15 (60.0)	13 (59.0)	6 (60.0)	20 (95.2)
ACEi/ARB (yes)[Ŧ]	38 (48.7)	8 (32.0)	8 (36.4)	4 (40.0)	18 (85.7)
β-Blockers (yes)	16 (20.5)	2 (8.0)	3 (13.6)	3 (30.0)	8 (38.1)
Continuous variables (median/IQR)					
Age (yrs)[ζ]	50.0 (20.0)	43.0 (21.0)	48.5 (13.0)	59.0 (16.0)	62.0 (24.0)
Age at diagnosis (yrs)	42.0 (24.0)	35.0 (27.0)	42.5 (17.0)	53.5 (32.0)	49.0 (44.0)
Age at ERT initiation (yrs)[§]	48.1 (17.3)	42.9 (25.2)	44.0 (16.2)	50.7 (17.1)	53.5 (25.0)
Time in ERT (yrs)	4.9 (7.6)	3.9 (8.4)	4.8 (5.4)	4.2 (6.7)	8.5 (8.9)
MSSI[˚]	21.0 (20.0)	10.0 (12.0)	20.5 (18.0)	31.0 (11.0)	32.0 (10.0)
FIPI[**]	2.0 (3.0)	1.0 (1.0)	2.0 (2.0)	4.0 (1.5)	5.0 (2.0)
Plasma lyso-Gb3 (nmol/L)	9.58 (15.33)	6.03 (12.88)	9.22 (14.36)	11.79 (71.50)	17.79 (36.60)
Plasma α-gal A ♀ (nmol/h/mL)	4.3 (4.0)	6.6 (5.0)	3.8 (7.2)	4.2 (.)	4.5 (4.7)
Plasma α-gal A ♂ (nmol/h/mL)	0.11 (0.30)	0.15 (0.50)	0.16 (0.80)	0.10 (0.20)	0.20 (0.30)
Albuminuria (mg/g)[ŦŦ]	59.2 (269.2)	9.6 (14.2)	88.2 (108.2)	614.8 (454.7)	206.2 (515.4)
Creatinine (mg/dL)[ξξ]	0.85 (0.60)	0.76 (0.23)	0.76 (0.27)	0.87 (0.21)	1.53 (0.63)
eGFR (mL/min/1.73 m^2)[ξξ]	92.5 (49.0)	105.0 (30.0)	102.0 (22.0)	87.5 (28.0)	39.0 (25.0)

ERT: enzyme replacement therapy; ACEi: angiotensin converting enzyme inhibitors; ARB: angiotensin II receptor blockers; MSSI: Mainz Severity Score Index; FIPI: Fabry International Prognostic Index; eGFR: estimated glomerular filtration rate.
Note: conversion factors for units: creatinine in mg/dL to μmol/L: ×88.4; albuminuria in mg/g to mg/mmol: ×0.113.
[*] $p < 0.05$ for difference in the distribution of categorical variable between subgroups.
[Ŧ] $p < 0.01$ for difference in the distribution of categorical variable between subgroups.
[ζ] $p < 0.01$ for difference between groups 1 and 4; $p < 0.05$ for difference between groups 2 and 4.
[§] $p < 0.05$ for difference between groups 1 and 4.
[˚] $p < 0.01$ for difference between group 1 and all other groups and also between groups 2 and 4.
[**] $p < 0.01$ for difference between group 1 and all other groups and also between groups 2 and 4; $p < 0.05$ for difference between groups 2 and 3.
[ŦŦ] $p < 0.01$ for difference between groups 1 and 3 or 4 and also between groups 2 and 3; $p < 0.05$ for difference between groups 2 and 4.
[ξξ] $p < 0.01$ for difference between group 4 and all other groups.

However, a significant progressive increase between FD subgroups of increasing severity was only found for NAG (Supplemental Fig. 1).

For the entire FD cohort, the correlation between albuminuria and the novel biomarkers (Table 3) was stronger for NAG (Fig. 2), although significant for all but one (A1MG) of the studied biomarkers. Several normoalbuminuric patients had biomarkers concentrations above the assumed upper limit of the reference value (Fig. 2; Supplemental Fig. 2).

3.3. Biomarkers of kidney injury: correlation with GFR and CKD stage (target condition)

For the whole FD cohort, the significant negative correlations between Col IV and estimated GFR ($\rho = -0.289$; $p = 0.003$) and between NAG and estimated GFR ($\rho = -0.448$; $p < 0.001$) were stronger than the correlation between albumin and estimated GFR ($\rho = -0.274$; $p = 0.019$) (Table 4). In subgroup 1, all biomarkers but AAP had better correlation with estimated GFR than albuminuria (Table 4; Fig. 3).

A significant increase of the biomarkers, according to CKD stage, was only found for NAG ($p = 0.016$). Notably, it was not significant even for albuminuria ($p = 0.062$) (Supplemental Fig. 3).

The global diagnostic accuracy of the biomarkers (including albuminuria) for CKD stage ≥ 2 (GFR < 90 mL/min/1.73 m^2) was better for NAG excretion (Fig. 4), with a diagnostic accuracy of 74.6%, compared with the diagnostic accuracy of 65.9% for albuminuria. Moreover, the DOR was also higher for NAG, compared with all the other biomarkers.

3.4. Novel biomarkers of kidney injury: correlation with other variables

For other variables not directly related to kidney function (including demographic, diagnosis, severity and treatment), a significant correlation was sole found between albuminuria or NAG and disease severity (MSSI and FIPI) (Supplemental Table 3). Remarkably, a significant difference between male and female patients was only observed for albuminuria; nonetheless, although slightly weaker in female patients, the correlation between estimated GFR and CollV or NAG was significant for both genders (Supplemental Table 4).

Moreover, there was a significant correlation between NAG and age ($\rho = 0.365$; $p = 0.001$). However, the significant inverse correlation between estimated GFR and NAG ($\rho = -0.355$; $p = 0.002$) remained significant after adjustment for age.

Table 2
Novel biomarkers of kidney injury in controls and FD subgroups (median (IQR)).

	Controls	FD subgroups				p value[a]	p value[Ŧ]
	(n = 25)	1 (n = 25)	2 (n = 22)	3 (n = 10)	4 (n = 21)		
Transferrin (μg/g)	0.81 (0.62)	0.99 (2.10)	1.09 (0.94)	0.95 (0.94)	1.26 (1.16)	0.018	0.45
CollV (mg/g)	5.09 (5.54)	7.92 (14.28)	10.88 (8.31)	7.53 (9.76)	9.75 (11.86)	0.040	0.53
A1MG (mg/g)	298.3 (260.9)	489.2 (1121.9)	570.5 (549.7)	580.7 (767.9)	522.9 (360.1)	0.009	0.91
NAG (U/g)	8.72 (5.66)	10.79 (17.91)	13.44 (8.37)	25.04 (23.04)	22.02 (19.22)	0.037	0.023
AAP (U/g)	4.98 (7.13)	13.07 (20.46)	13.02 (15.98)	16.68 (12.73)	13.37 (9.25)	0.009	0.97

All biomarkers presented a non-normal distribution, so non parametric tests were used.
CollV: collagen type IV; A1MG: α1-microglobulin; NAG: *N*-acetyl-β-D-glicosaminidase; AAP: alanine aminopeptidase.
[a] Controls vs FD disease subgroup 1.
[Ŧ] Between FD subgroups.

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Table 3
Correlation of novel biomarkers of kidney injury with albuminuria for all FD cohort (a logarithmic transformation of the variables has been performed).

	ρ	p value
Transferrin	0.251	0.027
ColIV	0.306	0.007
A1MG	0.179	0.12
NAG	0.468	<0.001
AAP	0.249	0.033

ColIV: collagen type IV; A1MG: α1-microglobulin; NAG: *N*-acetyl-β-D-glicosaminidase; AAP: alanine aminopeptidase.

Table 4
Correlation of biomarkers of kidney damage with estimated GFR for all FD cohort and subgroup 1 (a logarithmic transformation of the biomarkers has been performed).

	All FD cohort		Subgroup 1	
	ρ	p value	ρ	p value
Albumin	−0.274	0.019	−0.381	0.060
Transferrin	−0.230	0.043	−0.551	0.004
ColIV	−0.289	0.003	−0.487	0.014
A1MG	−0.149	0.19	−0.542	0.005
NAG	−0.448	<0.001	−0.623	0.001
AAP	−0.049	0.68	−0.007	0.97

ColIV: collagen type IV; A1MG: α1-microglobulin; NAG: *N*-acetyl-β-D-glicosaminidase; AAP: alanine aminopeptidase.

4. Discussion

In this study, with the objective to identify biomarkers of incipient nephropathy in FD and not biomarkers of FD or FD nephropathy (not elevated in other forms of nephropathy), our results suggest that several of the studied biomarkers are more sensitive than albuminuria in detecting incipient FD nephropathy, with a significant increase for all of them in patients without any evidence of nephropathy.

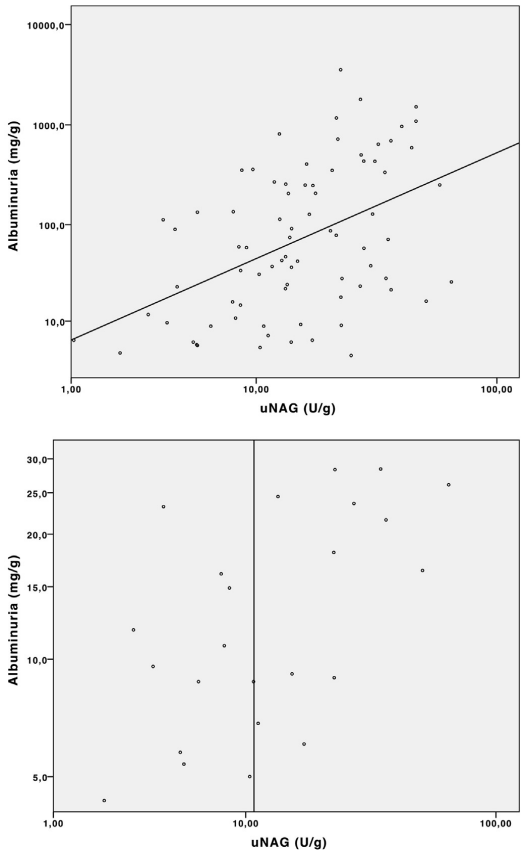

Fig. 2. Concentration of urinary NAG and albuminuria: entire FD cohort (top image) and subgroup 1 (bottom image). Vertical line in right image represents the upper limit of 95% confidence interval for mean of the control group (assumed to be the upper limit of the reference value). For easier visualization, a log scale has been used for the studied biomarkers.

Furthermore, although a significant progressive increase of the biomarkers across the prespecified FD subgroups and the CKD stage (notably non-significant for albuminuria) was sole found for NAG, the correlation with estimated GFR was stronger for ColIV and NAG, than for albuminuria. Thus, the studied biomarkers (NAG and ColIV) seems, at least, as good as albuminuria in the identification of the FD nephropathy severity spectrum (increasing correspondingly).

Additionally, NAG outperformed albuminuria in the diagnostic accuracy for CKD stage ≥ 2. The relatively modest DOR of the several biomarkers were mainly related to their ability to identify patients with GFR ≥ 90 mL/min/1.73 m^2, but certainly with kidney involvement from FD, thereby diminishing their specificity for CKD stage ≥ 2. Thus, NAG seems to have an important added value, not only in the identification of incipient FD nephropathy, but also as a prognostic marker, with significant correlation with estimated GFR and diagnostic accuracy to identify CKD stage ≥ 2.

The correlation with other variables was not significant, which argues for these biomarkers to be markers of FD nephropathy and not of overall disease burden. One exception is the correlations between albuminuria or NAG with severity indexes: both comprise a renal component/involvement [31,32] and nephropathy is a well-known determinant of overall disease prognosis [3–5]. The correlation between NAG and age seems to be related to the decrease in kidney function associated with ageing. The absence of difference in the several biomarkers according to gender and ERT is understandable, given the balanced gender distribution within the subgroups of increasing FD nephropathy and a greater proportion of patients on ERT in the more severe subgroups (may have, by this reason, biomarkers' levels similar to the patients not in ERT). Furthermore, the stratified analysis of correlations with estimated GFR by gender showed that significant correlations remained in male and female patients. Nevertheless, an ongoing longitudinal evaluation of these biomarkers will help to clarify the influence of ERT on them.

4.1. Glomerular damage biomarkers

Transferrin is a molecule slightly larger than albumin, but less anionic, thus expected to be more readily filtered through the glomeruli [33]. To our knowledge, it has never been studied in FD, but as reported in diabetic nephropathy, transferrinuria may be a more sensitive marker of glomerular damage, raised in normoalbuminuric patients [34].

ColIV excretion appears to be a surrogate marker of glomerular basement membrane synthesis and mesangial matrix remodelling [35] and podocytes are among the earliest cells to be loaded with glycosphingolipid deposits [7,8]. The progressive accumulation of lyso-Gb3 in these cells leads to increased expression of the fibrogenic cytokine TGF-β1 and, subsequent, increasing in extracellular matrix synthesis (fibronectin and ColIV) [36]. This study results shows, as in diabetic nephropathy [37], an increase of ColIV excretion in early stages of FD nephropathy, consistent with the pathological findings of thickening or duplication of glomerular basement membrane and increased mesangial extracellular in incipient FD nephropathy [7,11]. The

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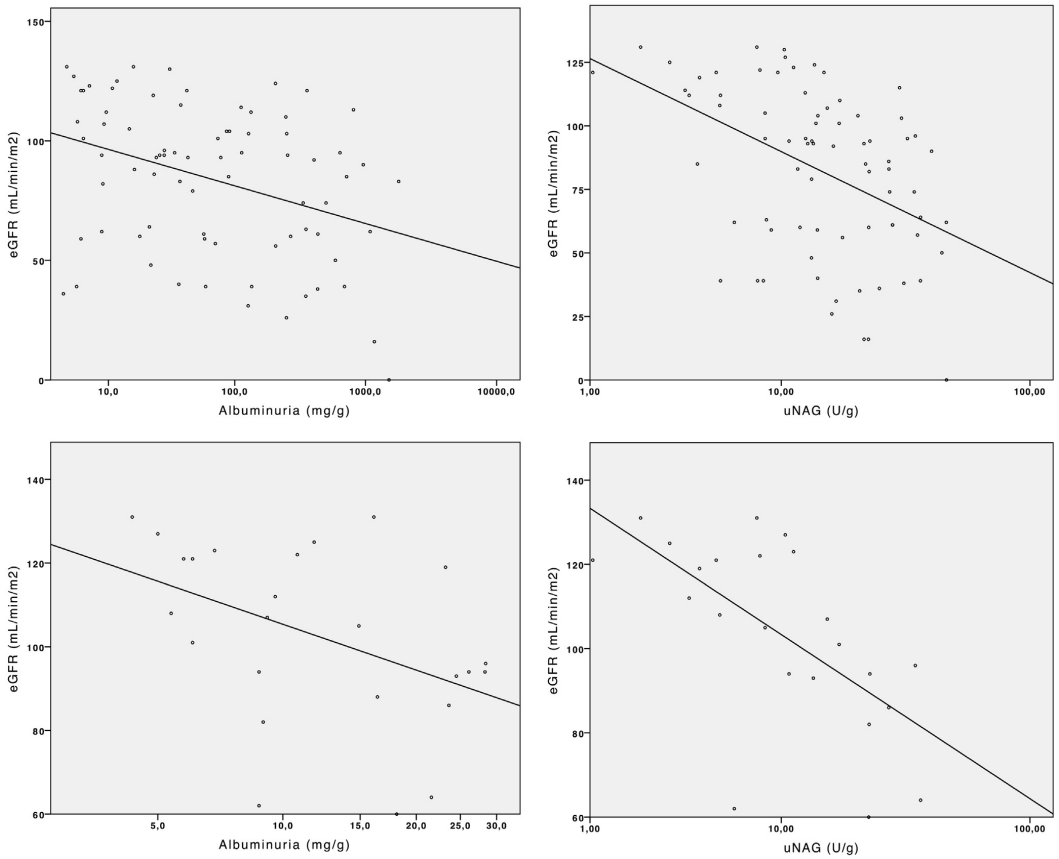

Fig. 3. Correlation of estimated GFR with albuminuria or NAG: entire FD cohort (upper images) and subgroup 1 (bottom images). For easier visualization, a log scale has been used for the studied biomarkers.

significant inverse correlation between ColIV and estimated GFR suggests that ColIV excretion is a prognostic factor in FD nephropathy, as previously demonstrated in diabetic nephropathy [38,39]. Furthermore, ColIV may be a good biomarker of response to ERT, given that the mesangial widening was showed to decrease in patients under ERT [22].

In FD nephropathy, other biomarkers of glomerular dysfunction have been studied, mainly related to podocytopathy. However, in previous studies evaluating podocyturia in FD, it was increased in patients without albuminuria and overt nephropathy, but a significant inverse correlation with estimated GFR was only found in one study (and only in male patients) and the added value against albuminuria, in terms of diagnostic accuracy and nephropathy prognosis, has not been established [13,14,40].

4.2. Tubular injury biomarkers

Sphingolipid storage occurs also in tubular cells [6] and may be present in early phases of the disease, in patients with minimal albuminuria [7]. Other contributors to tubular injury in FD may be the hemodynamic damage and persistent proteinuria [41].

The tubular injury preceding glomerular involvement was also observed in diabetic nephropathy, where, as in our study, an increase in several tubular proteins and enzymes became apparent before albuminuria A2 and has been correlated with the severity of nephropathy [42]. Thus, it has been hypothesized that the long-term outcome in diabetic nephropathy was more related to the degree of the tubulointersticial impairment than to the severity of glomerular lesions [43]. Previous smaller studies in FD have shown an increase in tubular injury biomarkers [44,45].

A1MG is a small protein, whose urinary excretion increases with proximal tubular cell dysfunction [46]. A small previous study in FD (with 13 women) reported a significant decrease in A1MG with ERT [45]. In type 2 diabetes, A1MG has been also correlated with albuminuria [47].

NAG is an urinary enzyme and, due to its high molecular weight, it cannot be filtered through the glomeruli and its presence in the urine results exclusively from tubular secretion [33]. We were able to confirm previous data showing an increase in urinary NAG in FD [48], but also that this biomarker rises before and significantly correlates with albuminuria and outperformed albuminuria as predictor of estimated GFR and CKD stage ≥ 2, like previously reported in type 2 diabetes [44,49]. These findings substantiate several concepts: tubular injury in FD nephropathy occurs early; long-term outcome of FD nephropathy may be more related to the extent of the tubulointersticial impairment than to the severity of glomerular lesions; proteinuria is a contributor to tubular injury. This greater sensitivity than albuminuria to detect

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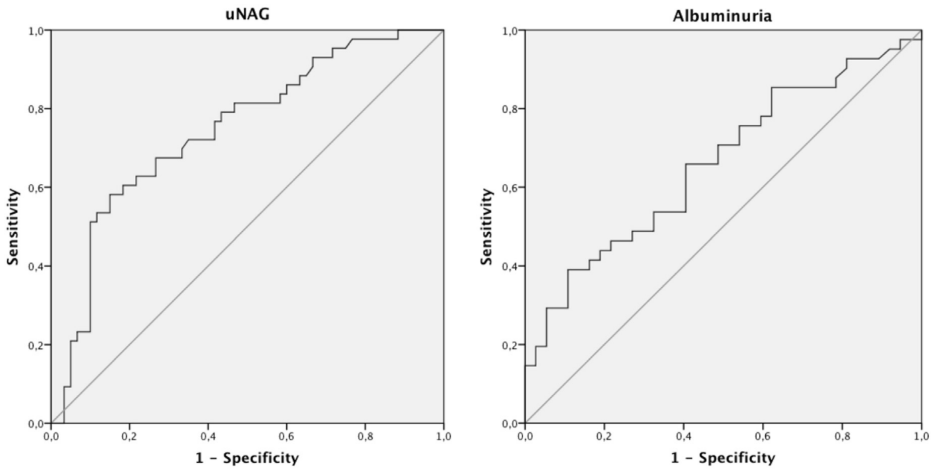

	AUC (95% CI)	DOR
Albumin*	0.659 (0.539 – 0.780)	2.60
Transferrin*	0.653 (0.529 – 0.776)	2.62
ColIV*	0.651 (0.528 – 0.773)	2.31
A1MG	0.625 (0.498 – 0.751)	2.85
NAG*	0.746 (0.650 – 0.843)	4.17
AAP	0.482 (0.347 – 0.617)	1.11

Fig. 4. Diagnostic accuracy of studied biomarkers for CKD stage ≥ 2 (GFR < 90 mL/min/1.73 m^2). 95% confidence interval for AUC was calculated using a nonparametric method. *p < 0.05 for true area = 0.5. AUC: area under the curve; CI: confidence interval; DOR: diagnostic odds ratio; ColIV: collagen type IV; A1MG: α1-microglobulin; NAG: *N*-acetyl-β-D-glicosaminidase; AAP: alanine aminopeptidase.

early renal malfunction, may be related to an increase in urinary NAG at a stage where tubular reabsorption capacity may still be sufficient to balance increased glomerular filtration of albumin [33].

AAP is another tubular enzyme, to our knowledge, never studied before in FD nephropathy. However, as for diabetic nephropathy, although useful to detect tubular dysfunction at an early stage, it has lower sensitivity and prognostic value than NAG excretion [50].

4.3. Limitations

This study has several limitations, namely: absence of histological correlation, which would be very useful in biomarkers validation; the cross-sectional design (an ongoing longitudinal evaluation is in progress), hindering the prognostic value of these biomarkers and the influence of ERT and ACEi/ARB on them. It would be important to standardize the assay of biomarkers to allow more widespread use in clinical practice.

4.4. Conclusion

In this study, we have demonstrated that limitations of albuminuria as a sensitive marker of early renal dysfunction in FD and as a marker of risk of progression of CKD, may be overcome by the newly identified biomarkers, all increased in normoalbuminuric patients and some of them (ColIIV and NAG) with better correlations with estimated GFR than albuminuria. These biomarkers may improve the management of FD nephropathy, inform criteria for starting ERT and may also define novel early stages of FD nephropathy characterized by mesangial expansion (ColIV) and/or tubular damage (NAG).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ymgme.2017.05.007.

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ORIGINAL RESEARCH

Biomarkers of Myocardial Fibrosis: Revealing the Natural History of Fibrogenesis in Fabry Disease Cardiomyopathy

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Background—Cardiomyopathy is a major determinant of overall Fabry disease (FD) prognosis, with the worst outcomes in patients with myocardial fibrosis. Late gadolinium enhancement is currently the gold standard for evaluation of replacement myocardial fibrosis; however, this event is irreversible, thus identification of biomarkers of earlier diffuse fibrosis is paramount.

Methods and Results—Type I collagen synthesis and degradation biomarkers (PICP [carboxyterminal propeptide of procollagen type I], ICTP [carboxyterminal telopeptide of type I collagen], and MMP1 [matrix metalloproteinase 1] and MMP2) and markers of bone synthesis and degradation were evaluated (to adjust type I collagen metabolism to bone turnover) in FD patients and controls. FD patients were grouped by cardiomyopathy severity, according to echocardiogram: (1) normal, (2) tissue Doppler abnormalities, (3) left ventricular hypertrophy. A significant increase in PICP and a significant decrease in matrix metalloproteinases were observed in FD patients; even the group with normal echocardiogram had a significant increase in PICP. We also found a significant correlation between left ventricular mass and PICP (ρ=0.378, P=0.003) and MMP1 (ρ=−0.484, P<0.001). PICP (adjusted for bone turnover) was the better predictor of left ventricular mass in multivariable regression, and its diagnostic accuracy to predict late gadolinium enhancement was also significant.

Conclusions—Collagen type I synthesis is increased in FD cardiomyopathy, even in the earlier stages of the disease, and this profibrotic state has good predictive value for and is likely to be critical to the development of overt left ventricular hypertrophy. Moreover, inhibition of enzymes involved in collagen type I cleavage also seems crucial to myocardial collagen deposition. (***J Am Heart Assoc***. 2018;7:e007124. DOI: 10.1161/JAHA.117.007124.)

Key Words: biomarkers • carboxyterminal propeptide of procollagen type I • cardiac fibrosis • Fabry disease cardiomyopathy • matrix metalloproteinases

Fabry disease (FD) is an X-linked lysosomal storage disorder caused by mutations in the *GLA* (galactosidase α) gene that encodes the enzyme α-galactosidase A, resulting in diffuse lysosomal accumulation of neutral glycosphingolipids (mainly Gb3 [globotriaosylceramide]). Both classical and attenuated phenotypes are associated with significant cardiac involvement. In adulthood, involvement of the heart, kidney, and brain causes significant morbidity and premature death.[1,2] Most recent studies have shown cardiovascular disease as the main cause of death in FD and a major determinant of overall disease prognosis.[3,4] Arrhythmias are the most frequent cardiac event in FD,[4,5] with a recent study reporting the annual increase in cardiac fibrosis as the sole independent predictor of malignant ventricular arrhythmias.[5]

Late gadolinium enhancement (LGE) imaging techniques using cardiac magnetic resonance imaging (MRI) is the gold

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Accompanying Data S1, Table S1, and Figures S1, S2 are available at http://jaha.ahajournals.org/content/7/6/e007124/DC1/embed/inline-supplementary-material-1.pdf

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Clinical Perspective

What Is New?

- Collagen type I synthesis is increased in Fabry disease cardiomyopathy, even in the early preclinical stages.
- Inhibition of enzymes involved in collagen type I degradation also seems crucial for myocardial collagen type I deposition.

What Are the Clinical Implications?

- Serum biomarkers of collagen type I metabolism may identify ongoing fibrosis in the early stages of Fabry disease cardiomyopathy and might predict the development of left ventricular hypertrophy.

standard for noninvasive detection of focal replacement fibrosis in the myocardium. More than 50% of FD patients present with LGE, with characteristic midmyocardial distribution in the inferolateral basal or midbasal segments of the left ventricle wall that seems to be specific of FD cardiomyopathy.[7,8] Nevertheless, LGE has several limitations as an imaging biomarker: (1) It detects only irreversible tissue damage with focal replacement fibrosis and has limited resolution of $\approx 0.2 g$[9]; (2) it may not detect early, potentially reversible, diffuse interstitial fibrosis[10]; (3) there is no universally accepted technique to quantify fibrosis volume.[11] A novel technique, T1 mapping, has been studied for assessment of diffuse interstitial fibrosis.[10] In FD, however, it has been studied more extensively as an imaging biomarker for early detection of cardiac involvement (due to lipid storage, yielding low native T1) than for evaluation of diffuse interstitial fibrosis.[12,13]

Enzyme replacement therapy (ERT) is the standard of care in the treatment of FD; however, the benefits of ERT may be limited in patients with cardiac fibrosis[14,15] and are probably greater when administered early in the course of the disease. A study using tissue Doppler imaging to detect early cardiac involvement reported that ERT prevented the appearance of tissue Doppler abnormalities in FD patients with no left ventricular (LV) hypertrophy or tissue Doppler abnormalities at baseline.[16]

The presence of LGE on cardiac MRI is a late event and predicts a worse prognosis. Consequently, identifying early predictors of overt disease is clinically relevant. Echocardiography is currently the gold standard to assess early signs of cardiomyopathy, which is associated with diastolic dysfunction. Several studies have demonstrated that tissue Doppler abnormalities precede and correlate with LV hypertrophy progression.[16,17] Newer tools like speckle tracking, which allows measurement of myocardial systolic and diastolic strains, seem to be superior to conventional echocardiographic measurements (including tissue Doppler) for the identification of myocardial contraction and relaxation abnormalities.[18,19]

These manifestations may be preceded by histologic changes or deregulated gene expression, as demonstrated previously in hypertrophic cardiomyopathy (HCM) due to sarcomere protein gene mutations.[20,21] These results indicate that a profibrotic milieu, with extracellular matrix expansion and collagen deposition, is present early in the pathogenesis of the disease, even when cardiac function and histology are normal.

The identification of biomarkers of collagen synthesis and degradation could represent an advance in the identification of preclinical involvement of the heart in FD, with possible therapeutic implications. Type I collagen is the main collagen type of the myocardium. During its synthesis from its precursor, procollagen type 1, PICP (carboxyterminal propeptide of procollagen type I) is released into the bloodstream with a stoichiometric ratio of 1:1, and its serum level reliably reflects myocardial type I collagen synthesis.[22,23] Collagen turnover biomarkers have been studied in HCM, hypertension, heart failure, and myocardial infarction.[24–26] In the HCM model, an increase in serum PICP was reported even in mutation carriers without LV hypertrophy or visible fibrosis in cardiac MRI.[26] In FD cardiomyopathy, to our knowledge, there are only 2 publications of a limited evaluation of extracellular matrix turnover, reporting increased levels of MMP9 (matrix metalloproteinase 9), PICP, ICTP (carboxyterminal telopeptide of type I collagen), and PIIINP (procollagen type III aminoterminal propeptide).[4,27]

In this study, we investigated several biomarkers of collagen type I turnover in a large cohort of FD patients (within the entire spectrum of FD cardiomyopathy severity), with an emphasis on the usefulness of these markers in early and prehypertrophic stages of FD cardiomyopathy.

Methods

The data, analytic methods, and study materials will not be made available to other researchers for purposes of reproducing the results or replicating the procedure. See Data S1 for extended methods.

Study Design and Population

In this multicenter, cross-sectional, and prospective study, a cohort of 60 consecutive FD patients was recruited from 3 centers (Centro Hospitalar Lisboa Norte, Lisbon, Portugal; Hospital Senhora da Oliveira, Guimarães, Portugal; Royal Free Hospital, London, United Kingdom) between February 2013 and June 2014. Twenty healthy controls were also recruited.

For FD patients, the only inclusion criteria were diagnosis of FD and age ≥18 years. FD was defined in male patients as

low α-galactosidase A activity and/or identification of a proven pathogenic mutation in the *GLA* gene and in female patients as the presence of a proven pathogenic mutation in the *GLA* gene.

FD patients with conditions, other than the usual manifestations of FD cardiomyopathy, that possibly affected cardiac collagen turnover were excluded, namely, HCM due to sarcomere protein gene mutations or other cardiomyopathies, previous myocardial infarction, moderate or severe valvular heart disease, previous heart surgery, a cardiac device implanted in the previous 6 months, surgery or major trauma within the previous 6 months, inflammatory or fibrotic diseases, and active cancer.

To enroll patients who were representative of the entire spectrum of FD cardiomyopathy severity, recruitment was done in accordance with subgroups of increasing severity of FD cardiomyopathy (aiming at a specific number of patients), defined by echocardiogram. Subgroup 1 had no evidence of cardiac involvement: no LV hypertrophy or tissue Doppler abnormalities (20 patients). Subgroup 2 had tissue Doppler abnormalities (defined as at least 1 of the following: systolic tissue Doppler velocities <6 cm/s; early diastolic tissue Doppler velocities <10, <8, or <6 cm/s at the septal corner of the mitral annulus in patients aged <40, between 41 and 60, and >60 years, respectively; early diastolic tissue Doppler velocities <14, <12, or <6 cm/s at the lateral corner of the mitral annulus in patients aged <40, between 41 and 60, and >60 years, respectively) and no LV hypertrophy (20 patients). Subgroup 3 had LV hypertrophy, defined as diastolic interventricular septum or posterior wall thickness ≥12 mm (20 patients).

The control group included healthy individuals with normal echocardiograms who were age and sex matched with the less severe FD subgroup (subgroup 1) and who did not have conditions influencing cardiac collagen turnover, including not only those previously listed for FD patients but also systemic arterial hypertension (defined as systolic blood pressure ≥140 mm Hg, diastolic blood pressure ≥90 mm Hg, or use of antihypertensive medication), LV hypertrophy from any cause, coronary artery disease, pacemaker placement (regardless of time since implantation), and atrial fibrillation.

The study protocol was approved by the local or national ethics committees of each participating center, and the study was conducted in accordance with this protocol and the ethics principles of the Declaration of Helsinki. Written informed consent was obtained from all participants before enrollment.

Clinical Assessment

For each patient recruited, routine follow-up data were collected, namely sex, age, age at diagnosis, plasma α-galactosidase A activity, *GLA* gene mutation, current medication (angiotensin-converting enzyme inhibitors, angiotensin II receptor blockers, aldosterone antagonists, and β-blockers), data about ERT (product, dose, and duration), clinical manifestations (to calculate the disease severity indexes: Mainz Score Severity Index [MSSI][28] and Fabry International Prognostic Index [FIPI][29]), echocardiographic measurements, presence of LGE on cardiac MRI (if available), and laboratory results (NT-proBNP [amino-terminal fragment of the pro-hormone of brain natriuretic peptide], kidney function tests [glomerular filtration rate, creatinine, and albuminuria] and plasma lyso-Gb3 [globotriasosylsphingosine]).

Cardiomyopathy assessment/reference test and outcomes

Cardiac function and structure were evaluated by echocardiogram and cardiac MRI. Echocardiogram (LV mass and tissue Doppler abnormalities) was used as the reference test for comparison with index tests because it is considered the gold standard for evaluation of early cardiac dysfunction in FD.

Data from the M-mode, 2-dimensional, and Doppler transthoracic echocardiographic study were collected for each patient and control. FD patients' echocardiograms were done using a Vivid 7 (General Electric) ultrasonographic system at all recruitment sites. Evaluation of control participants was performed using a Vivid 7 or Toshiba Xario ultrasonographic systems, with a protocol identical to that for FD patients for imaging acquisition. Echocardiograms were undertaken in routine clinical practice, and there was no core reading of the results.

LV dimensions (interventricular septum, posterior wall, and LV end-diastolic diameter) were assessed from the long-axis view, and ventricular mass was calculated according to the Devereux formula and normalized for height ($g/m^{2.7}$). LV hypertrophy was defined as LV mass ≥50 $g/m^{2.7}$. Left atrium area was obtained in the apical 4-chamber view and expressed in square centimeters.

Mitral inflow pattern (rapid filling [E wave] and atrial contraction [A wave] peak velocities, E-wave decelerating time, A-wave duration, and isovolumic relaxation time) was obtained at the mitral level by Doppler echocardiography in the apical 4-chamber view. Retrograde atrial flow velocity and duration were acquired from the pulmonary veins also by Doppler echocardiography in the same view.

Myocardial velocities during systole and early diastole were collected from tissue Doppler imaging at lateral and septal corner of the mitral annulus in the apical 4-chamber view. Tissue Doppler abnormalities were defined as aforementioned. The ratio between LV rapid filling and average early diastole (septal and lateral) was used to estimate LV end-diastolic pressure.

Echocardiographic studies were performed and reported by people who were blinded to the measurement of collagen turnover biomarkers (index tests) and clinical data.

Data about cardiac MRI were collected if it was performed as part of the follow-up protocol and there was no core reading of the images. Cardiac MRI studies were performed in a 3.0-T system (Philips Intera). LGE is the standard for detection of focal myocardial replacement fibrosis and was defined in the study as 2 SD above the mean signal intensity of the distant myocardium.

The specified outcomes were to compare the index tests with tissue Doppler abnormalities in the identification of incipient Fabry cardiomyopathy (comparing patients in subgroup 1 with controls) and to correlate index tests with LV mass and their diagnostic accuracy to identify LGE in cardiac MRI.

Measurement of Collagen Turnover Biomarkers and Index Tests

To assess type I collagen turnover, we measured levels of peptides released during collagen synthesis and degradation and enzymes involved in collagen degradation. During collagen synthesis, PICP is cleaved from procollagen I and released into blood. Collagen is degraded by matrix metalloproteinases (MMPs) as follows: MMP1 cleaves collagen I and releases ICTP into the blood; further degradation is performed by gelatinases MMP2 and MMP9.

Type I collagen is also a major component of bone; so the measured pro- and telopeptides may reflect bone formation and resorption. To minimize such confounding factors, we also measured markers of bone synthesis (B-AP [bone-specific alkaline phosphatase]) and resorption (TRAP5b [tartrate-resistant acid phosphatase type 5b]) and determined the ratios of PICP:B-AP and ICTP:TRAP5b.

Peripheral venous blood samples were obtained <6 months apart from the echocardiographic study, spun within 120 minutes after phlebotomy (489×g, 10 minutes), immediately stored at −20°C, transported to the laboratory in dry ice, and thawed and mixed thoroughly just before the assay.

TRAP5b was evaluated by an ELISA method, according to manufacturer instructions (Immunodiagnostic Systems), and measured at 405 nm; MMP1 and MMP2 were evaluated by an ELISA method, according to manufacturer instructions (SunRed), and measured at 450 nm; B-AP and PICP were evaluated by an ELISA method, according to manufacturer instructions (Quidel Corp), and measured at 405 nm; ICTP was evaluated by an electrochemiluminescence immunoassay in a COBAS e411 instrument (Roche). Duplicate determinations were made for each individual, and the average result was considered. Laboratory researchers were blinded to clinical data and cardiac assessments (reference test).

Because there are no validated reference values for the tested biomarkers, the upper limit of the 95% confidence interval for the mean of the control group was assumed to be the upper limit of the reference value.

Statistical Analysis

Statistical analysis was performed with SPSS (version 21; IBM Corp) software. Categorical variables were expressed as number and percentage and continuous variables as median and interquartile range.

For categorical variables, the χ^2 or Fisher exact tests were used to compare the variable distributions between the groups. Normal distribution of continuous variables was tested using the Shapiro–Wilk test. For continuous variables, comparison of means or medians was performed using the Student *t* test for variables that followed a normal distribution and the Mann–Whitney test or related-samples Wilcoxon signed rank test for variables that did not. If the qualitative variable had >2 categories, ANOVA (post hoc analysis with Bonferroni correction) was used for variables with normal distribution, and a Kruskal–Wallis test was used for those without.

Because of the skewed distribution of all studied biomarkers (index and reference tests), the Spearman correlation coefficient was determined to evaluate the correlation between the biomarkers and the quantitative variables.

To construct a regression model to quantify LV mass, all potentially related variables were correlated, using the Spearman rank correlation coefficient, with LV mass (in univariate analysis). Those variables showing significant correlation ($P<0.05$) were entered into the multivariable analysis. For multivariable analysis, stepwise regression combining both forward selection and backward elimination was used. A cutoff limit to remain in the model was set at an F-statistic *P* value of <0.05.

We evaluated the diagnostic accuracy of the studied biomarkers to detect cardiac fibrosis (defined by presence of LGE in cardiac MRI) by calculating the sensitivity, specificity, and area under the curve from receiver operating characteristic curves (we determined the confidence intervals of the area under the curve [nonparametric method], with values between 1 [perfect test] and 0.5 [useless test]).

For all comparisons and correlations, $P<0.05$ was considered significant.

Results

Population Characteristics

From February 2013 to June 2014, we recruited 60 FD patients (20 in each subgroup) and 20 controls (age and sex matched

with subgroup 1; Figure 1). Population characteristics are given in Table 1. Only 1 FD patient was taking a mineralocorticoid receptor antagonist (in subgroup 3). Overall, 28 different *GLA* pathogenic mutations were identified (Table S1), with p.N215S and p.F113L—2 mutations associated with attenuated or late-onset phenotypes with predominant cardiac involvement—accounting for 31.7% of the patients; the remaining mutations are usually associated with a classical phenotype. As expected, no adverse events resulted from collecting blood samples to determine index tests.

No significant echocardiographic differences were noted between the control group and FD subgroup 1 (Table 2). A gadolinium-based contrast cardiac MRI result was available for 37 FD patients (60%; 9, 13, and 15 in subgroups 1, 2, and

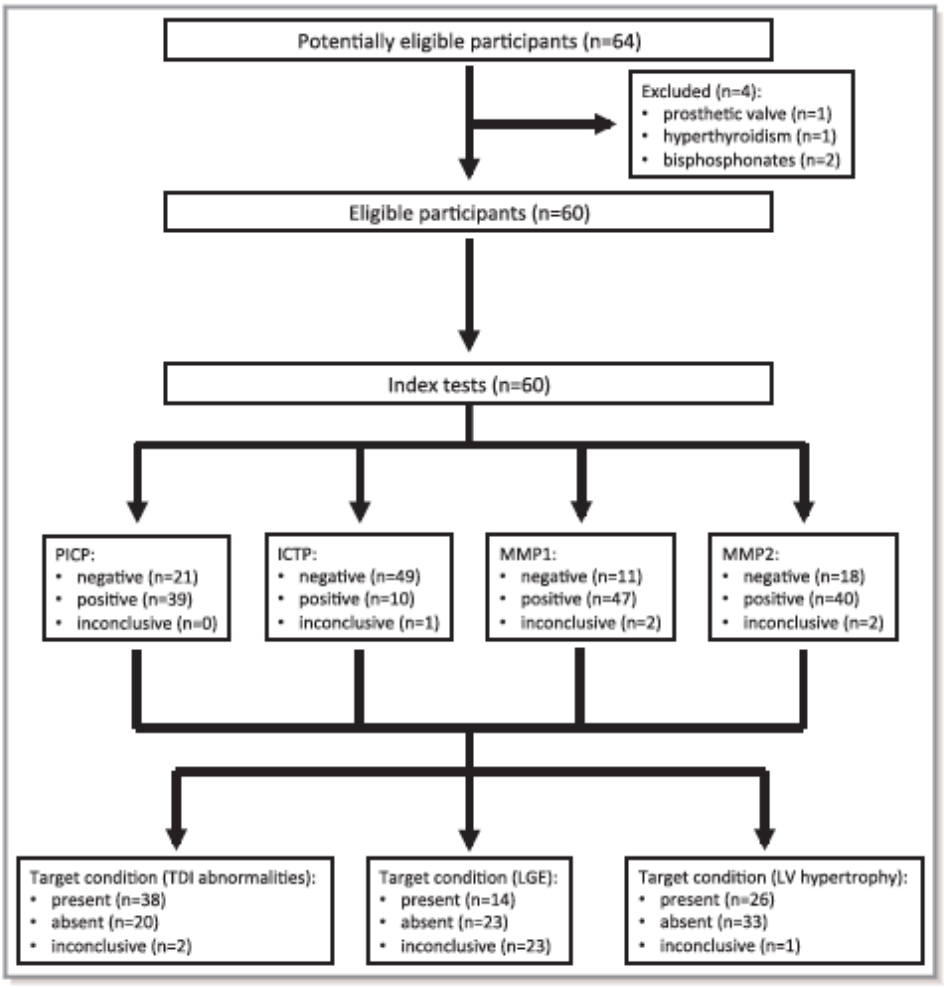


Figure 1. Study flow diagram. ICTP indicates carboxyterminal telopeptide of type I collagen; LGE, late gadolinium enhancement; LV, left ventricular; MMP, matrix metalloproteinase; PICP, carboxyterminal propeptide of procollagen type I; TDI, tissue Doppler imaging.

3, respectively); LGE was present in 0%, 15.4%, and 80.0% of patients in FD subgroups 1, 2, and 3, respectively.

Collagen Type I Turnover Biomarkers (Index Tests)

Collagen type I synthesis (PICP) was increased in FD patients (Table 3). Compared with PICP levels in controls, PICP levels in FD subgroup 1 were significantly elevated, a 61% increase (P=0.006). Comparing FD subgroups, PICP levels were significantly higher in FD subgroup 3 (P=0.001). The significant results comparing FD subgroup 1 and controls and FD subgroups remained with the PICP:B-AP ratio.

Collagen type I degradation (ICTP) and levels of the enzymes involved in its degradation (MMP1 and MMP2) were similar between the control group and FD subgroup 1 (Table 3). The difference between these 2 groups attained statistical significance only after adjustment to bone degradation with the ICTP:TRAP5b ratio (P=0.011). Moreover, there was a significant trend of decreased activity of MMP1 as severity increased.

The PICP:ICTP ratio is considered to reflect the balance between type I collagen synthesis and degradation. PICP:ICTP evaluation reveals a clear trend (significant only after adjustment for bone turnover [P=0.012]) of prevalent type I collagen synthesis over degradation within the subgroups of FD patients with greater severity (Figure S1). Correlation between PICP:ICTP and MMP1 reflects the influence of higher collagenase activity balancing PICP:ICTP toward type I collagen degradation and lesser collagen type I deposition. Accordingly, we identified a significant inverse correlation between PICP:ICTP and MMP1 activity before (ρ=−0.348; P=0.008) and after (ρ=−0.322; P=0.015) adjustment to bone turnover.

Correlation With Cardiac Imaging and LGE (Reference Standards)

For the entire FD cohort, a significant direct correlation between the biomarker of collagen type I synthesis (PICP) and LV wall thickness and mass (Table 4) was found (PICP with LV mass: ρ=0.378, P=0.003). This significant correlation remained after adjustment for bone turnover (PICP:B-AP ratio with LV mass: ρ=0.313, P=0.016). For biomarkers of collagen type I degradation, there was a significant inverse correlation between the enzyme MMP1, involved collagen type I cleavage, and LV thickness and mass parameters (MMP1 with LV mass: ρ=−0.484, P<0.001). A significant direct correlation was found for the MMP1 and echocardiographic parameters of diastolic dysfunction, namely, early diastolic mitral velocities measured at the septal (ρ=0.354, P=0.016) and lateral (ρ=0.280, P=0.042) corners of the mitral annulus.

There was a clear trend for higher values of collagen type I synthesis and decreased activity of the enzymes involved in collagen type I cleavage in patients with LGE in cardiac MRI (Table 4), but the difference between patients with and without LGE was significant only for PICP, even after adjustment for bone turnover (for PICP:B-AP ratio: mean difference 12.5, a 74% increase for LGE positive group; P=0.01).

After adjustment for bone turnover, there was a significant correlation between PICP:ICTP and LV mass and higher values of this ratio in LGE positive patients (Figure S2).

Correlation With ERT, Severity Indexes, and Other Variables

For the rest of the variables (including demographic, diagnostic, and severity- and treatment-related variables), not directly related to cardiac imaging, consistent and significant correlations were found among the biomarker of collagen type I synthesis or MMP1 and the disease severity indexes (MSSI and FIPI) and NT-pro BNP (Table 5). Correlations among other biomarkers and these variables seemed less reliable, except for the correlations between age or estimated glomerular filtration rate and MMPs; however, the significant correlation with estimated glomerular filtration rate did not remain after adjustment for LV mass.

The serum concentration of PICP and PICP adjusted for bone turnover was significantly higher in male patients, and there was a nonsignificant trend of lower serum MMP1 concentration in the same sex. This is in agreement with the unbalanced sex distribution across FD patient subgroups and the significantly higher mean LV mass in male patients (57.6 $g/m^{2.7}$ versus 41.4 $g/m^{2.7}$; P<0.001). Nonetheless, the significant direct correlation between LV mass and PICP and the inverse correlation between LV mass and MMP1 remained significant for both sexes in the subanalysis by sex.

Association Between Predictive Variables and Clinical End Points (LV Mass and LGE)

Variables with significant correlation with LV mass (in univariate analysis) were included in a multivariable regression model (Table 6). Within this model only 2 variables retained statistical significance: PICP:B-AP ratio and age, with the former as the better predictor of LV mass (β=0.919; SE=0.095; P<0.001).

The global diagnostic accuracy of several biomarkers to detect patients with LGE in cardiac MRI was significant for LV mass, PICP, PICP:B-AP ratio, PICP:ICTP ratio adjusted to bone turnover, and NT-proBNP (Figure 2), achieving diagnostic accuracy of 75.9% for the PICP:ICTP adjusted ratio. Moreover, lower estimated glomerular filtration rate, systolic average,

Table 1. Clinical Characteristics of Study Population

	Controls (n=20)	FD Cohort (n=60)	FD Subgroups		
			1 (n=20)	2 (n=20)	3 (n=20)
Categorical variables, n (%)					
Sex (female)*	14 (70.0)	37 (61.7)	14 (70.0)	18 (90.0)	5 (25.0)
HBP (yes)	0 (0.0)	25 (41.7)	8 (40.0)	10 (50.0)	7 (35.0)
HF class (0/I/II)	…	39/6/14	15/3/2	14/2/4	10/2/8
ERT (yes)	…	39 (65.0)	14 (70.0)	8 (40.0)	17 (85.0)
ACEI/ARB (yes)	0 (0.0)	29 (48.3)	9 (45.0)	10 (50.0)	10 (50.0)
β-Blockers (yes)	0 (0.0)	11 (18.3)	3 (15.0)	3 (15.0)	5 (25.0)
Continuous variable, median (IQR)					
Age, y†	41.0 (23.0)	44.0 (23.0)	40.5 (21.0)	48.5 (18.0)	59.5 (13.0)
Age at diagnosis, y‡	…	41.5 (22.0)	34.5 (24.0)	44.0 (15.0)	50.5 (35.0)
Age at ERT initiation, y§	…	49.0 (17.1)	38.1 (25.4)	52.4 (17.0)	49.4 (19.9)
Time in ERT, y	…	6.3 (8.4)	4.8 (8.0)	5.9 (7.6)	8.2 (9.1)
MSSI‖	…	18.5 (21.0)	11.0 (11.0)	12.5 (18.0)	31.0 (16.0)
FIPI¶	…	2.0 (3.0)	1.0 (2.0)	1.0 (3.0)	4.0 (2.0)
Plasma lyso-Gb3, nmol/L¶	…	9.1 (16.6)	3.6 (27.3)	8.3 (9.9)	18.8 (45.1)
Plasma α-gal A (female), nmol/h/mL	…	4.3 (4.1)	5.1 (2.8)	3.5 (4.5)	3.8 (.)
Plasma α-gal A (male), nmol/h/mL	…	0.11 (0.3)	0.14 (0.50)	0.06 (.)	0.20 (0.30)
NT-proBNP, ng/mL†	…	142.0 (334.0)	40.0 (122.0)	129.5 (166.0)	940.7 (1602)
eGFR, mL/min/1.73 m²†	108.5 (20.0)	93.0 (51.0)	99.5 (53.0)	101.0 (91.0)	67.5 (51.0)

ACEI indicates angiotensin-converting enzyme inhibitors; α-gal A, α-galactosidase A; ARB, angiotensin II receptor blockers; eGFR, estimated glomerular filtration rate; ERT, enzyme replacement therapy; FD, Fabry disease; FIPI, Fabry International Prognostic Index; HBP, high blood pressure (arterial hypertension); HF, heart failure; IQR, interquartile range; lyso-Gb3, globotriaosylsphingosine; MSSI, Mainz Severity Score Index; NT-proBNP, N-terminal probrain natriuretic peptide.
*$P<0.01$ for difference in the distribution of categorical variables between subgroups.
†$P<0.01$ for difference between subgroups 1 and 3.
‡$P<0.05$ for difference between subgroups 1 and 2.
§$P<0.05$ for difference between subgroups 1 and 3.
‖$P<0.01$ for difference between group 3 and all other groups.
¶$P<0.05$ for difference between subgroups 2 and 3.

and early diastolic average had significant predictive value for presence of LGE in cardiac MRI.

Using a model of binary logistic regression for the diagnosis of LGE, a model with LV mass and NT-proBNP correctly classified 73.9% of patients regarding LGE status; this percentage increased to 87.0% after addition of the PICP: ICTP adjusted ratio to the model.

Discussion

Increased Collagen Type I Synthesis

The results of our study suggest increased myocardial collagen type I synthesis in FD patients with established LV hypertrophy and, to a lesser extent, in FD patients with normal echocardiograms or with isolated tissue Doppler abnormalities. PICP increase remained after adjustment for bone synthesis (with the ratio to B-AP), confirming the myocardial origin of the excessive collagen type I synthesis (rather than higher bone metabolic activity). This correction for bone activity is important mainly in postmenopausal women because the concentration of collagen type I synthesis and degradation biomarkers is increased in this population.[30]

Serum PICP concentration was slightly, but nonsignificantly, lower in patients with tissue Doppler abnormalities (subgroup 2) compared with patients with normal echocardiograms (subgroup 1). We attributed this to the similar LV masses in these subgroups because there was a significant direct correlation between LV mass and serum PICP.

Elevation of PICP levels in FD patients with normal echocardiograms suggests that it may be used as a serologic marker of risk, detectable before the earlier echocardiographic signs of cardiac dysfunction. The ongoing longitudinal evaluation of these biomarkers will help clarify this issue. Moreover, it hints that the stimulus for myocardial fibrosis is an early event in FD cardiomyopathy (probably directly related to the disease

Table 2. Echocardiographic Characteristics of Study Population

	Controls	FD Subgroups		
		1	2	3
dIVS, mm*	9.0 (2.0)	9.0 (2.0)	9.0 (2.2)	14.5 (4.0)
dLVPW, mm*	9.0 (1.0)	9.0 (3.0)	8.9 (2.8)	12.1 (4.5)
LVMI, g/m$^{2.7}$*	39.3 (10.9)	35.2 (9.7)	39.5 (19.6)	63.3 (10.6)
LA area, cm^2*	—	17.0 (4.6)	18.9 (5.6)	23.3 (10.2)
E/A ratio	—	1.47 (0.85)	1.25 (0.87)	0.98 (0.95)
DT, ms†	—	184.0 (73.0)	230.0 (62.0)	249.5 (25.0)
S'(s), cm/s‡	8.0 (2.0)	8.0 (2.0)	6.0 (2.0)	6.0 (2.0)
S'(l), cm/s‡	9.5 (5.0)	11.0 (3.0)	7.0 (3.0)	6.5 (3.0)
E'(s), cm/s§	11.5 (5.0)	11.5 (3.0)	7.0 (4.0)	5.0 (2.0)
E'(l), cm/s‡	15.5 (6.0)	14.5 (4.0)	9.0 (4.0)	8.0 (4.0)
E/E'‖	6.3 (2.1)	6.0 (2.3)	9.4 (5.0)	11.8 (6.5)

Data are shown as median (interquartile range). dIVS indicates diastolic interventricular septal thickness; dLVPW, diastolic left ventricular posterior wall thickness; DT, E-wave decelerating time; E/A, left ventricular rapid filling (E wave)/atrial contraction (A wave) peak velocities; E/E'(‖), left ventricular rapid filling velocity/average (septal and lateral) early diastolic myocardial velocity measured at the mitral annulus; E'(l), early diastolic myocardial velocity measured at the lateral corner of the mitral annulus; E'(s), early diastolic myocardial velocity measured at the septal corner of the mitral annulus; LA, left atrium; LVMI, left ventricular mass indexed for height; S'(l), systolic myocardial velocity measured at the lateral corner of the mitral annulus; S'(s), systolic myocardial velocity measured at the septal corner of the mitral annulus.
*P<0.01 for difference between subgroup 3 and all other FD groups.
†P<0.01 for difference between subgroups 1 and 3.
‡P<0.01 for difference between subgroup 1 and all other FD groups.
§P<0.01 for difference between all FD groups.

pathophysiology) and not a secondary event caused by mechanical stress or ischemia at microcirculatory level. Similar to our study, in HCM it has been shown that serum levels of PICP are elevated even in mutation carriers without LV hypertrophy, supporting a profibrotic state preceding development of overt cardiomyopathy.[26] Moreover, a histologic study in cats with preclinical HCM showed an increase in myocardial collagen deposition.[31] Furthermore, studies in murine models of HCM have shown upregulation of several extracellular matrix proteins (CTGF [connective tissue growth factor] and POSTN [periostin]) in the very early stage of the disease (prehypertrophic stage, with normal histologic findings).[20,21]

In FD, one of the accumulating substrates is lyso-Gb3, a product of Gb3 deacylation. Lyso-Gb3 promotes proliferation of vascular smooth muscle cells in culture[32] and increases the expression of TGF-β (transforming growth factor β) in human cultured podocytes, with subsequent increase in extracellular matrix synthesis.[33] These findings support our observations that upregulation of cardiac fibrogenesis is an early event and directly related to the disease pathophysiology.

Type I Collagen Degradation: MMP Downregulation

In contrast, we found no increase in collagen type I degradation biomarker (ICTP), even after adjustment for bone turnover. Nonetheless, we found a significant decrease in the activity of MMP1 across the subgroups of increasing severity of FD cardiomyopathy. As an index of degree of coupling between the synthesis and degradation of collagen type I, the PICP:ICTP ratio, after adjustment to bone turnover, was significantly higher in the subgroup of patients with established LV hypertrophy and inversely correlated with MMP1 activity. Thus, as reported in HCM,[26,34,35] we can hypothesize that in incipient FD cardiomyopathy, increased collagen synthesis is balanced by degradation (limiting fibrogenesis), but when collagen type I synthesis exceeds the degradation, there is deposition of collagen in the myocardium and LV hypertrophy; the suppression of MMPs may be another mechanism of myocardial collagen type I buildup.

Table 3. Collagen Type I Turnover Biomarkers in Controls and FD Subgroups

	Controls (n=20)	FD Subgroups			*P* Value*	*P* Value†
		1 (n=20)	2 (n=20)	3 (n=20)		
PICP, ng/mL	107.3 (39.3)	148.5 (129.0)	115.7 (88.8)	219.0 (98.4)	0.006	0.001
PICP/B-AP	10.1 (4.3)	16.2 (10.3)	12.7 (9.1)	19.4 (24.1)	0.003	0.001
ICTP, ng/mL	0.34 (0.20)	0.25 (0.27)	0.20 (0.19)	0.29 (0.23)	0.687	0.236
ICTP/TRAP5b	0.11 (0.07)	0.16 (0.10)	0.11 (0.07)	0.13 (0.06)	0.011	0.186
MMP1, ng/mL	16.8 (26.3)	13.5 (19.3)	14.1 (4.3)	9.6 (4.6)	0.159	0.002
MMP2, ng/mL	440.3 (541.0)	341.8 (849.5)	356.5 (187.0)	318.7 (101.9)	0.872	0.108

Data are shown as median (interquartile range). B-AP indicates bone-specific alkaline phosphatase; FD, Fabry disease; ICTP, carboxyterminal telopeptide of type I collagen; MMP, matrix metalloproteinase; PICP, carboxyterminal propeptide of procollagen type I; TRAP5b, tartrate-resistant acid phosphatase type 5b.
*Controls vs FD disease subgroup 1.
†Between FD subgroups.

Table 4. Correlation Between Type I Collagen Turnover Biomarkers and Cardiac Imaging Parameters

	PICP (ng/mL)	PICP/B-AP	ICTP (ng/mL)	ICTP/TRAP5b	MMP1 (ng/mL)	MMP2 (ng/mL)
Echocardiogram						
dIVS, mm	0.438*	0.376*	0.178	−0.017	−0.519*	−0.300*
dLVPW, mm	0.322*	0.234	0.204	0.043	−0.438*	−0.294*
LVMI, g/m$^{2.7}$	0.378*	0.313*	0.197	0.013	−0.484*	−0.295
LA area, cm^2	0.173	0.066	0.092	0.080	−0.129	−0.239
DT, ms	0.103	0.059	−0.024	−0.187	−0.294*	−0.145
S'(s), cm/s	−0.023	0.071	0.020	−0.046	0.354*	0.188
S'(i), cm/s	−0.029	−0.017	−0.063	0.083	0.230	0.127
E'(s), cm/s	−0.125	−0.113	0.130	0.139	0.354*	0.288
E'(i), cm/s	−0.037	0.000	0.127	0.294	0.280*	0.200
E/E'(a)	0.105	0.005	0.027	−0.082	−0.097	0.087
Cardiac MRI						
LGE (yes)	88.8*	12.5*	0.066	−0.024	−5.27	−239.3

Data are shown as mean difference for categorical variables and as ρ for continuous variables. B-AP indicates bone-specific alkaline phosphatase; dIVS, diastolic interventricular septal thickness; dLVPW, diastolic left ventricular posterior wall thickness; DT, E-wave decelerating time; E/E'(a), left ventricular rapid filling velocity/average (septal and lateral) early diastolic myocardial velocity measured at the mitral annulus; E'(i), early diastolic myocardial velocity measured at the lateral corner of the mitral annulus; E'(s), early diastolic myocardial velocity measured at the septal corner of the mitral annulus; ICTP, carboxyterminal telopeptide of type I collagen; LA, left atrium; LGE, late gadolinium enhancement; LVMI, left ventricular mass indexed for height; MMP, matrix metalloproteinase; MRI, magnetic resonance imaging; PICP, carboxyterminal propeptide of procollagen type I; S'(i), systolic myocardial velocity measured at the lateral corner of the mitral annulus; S'(s), systolic myocardial velocity measured at the septal corner of the mitral annulus; TRAP5b, tartrate-resistant acid phosphatase type 5b.
*P<0.05.

The suppression of MMPs as a pathophysiological mechanism for cardiac fibrosis has been described previously in HCM.[34,35] The multiple pathways involved in MMP suppression are not well understood, but recent studies imply aldosterone-induced expression of TIMP1 (tissue inhibitor of metallopeptidase 1), acting mainly through MMP1 inhibition increasing net cardiac collagen content.[36] Furthermore, microRNA miR-214 (an antifibrotic microRNA) may play an important role in MMP1 regulation, as shown by Dong et al,[37] demonstrating that suppression of MMP1 in cardiac fibroblasts, induced by angiotensin II, could be reversed after administration of pre–miR-214 (this also decreased TIMP1 and TGF-β).

Correlations Between Collagen Metabolism Biomarkers and Cardiac Imaging

The biomarker of type I collagen synthesis (PICP), before and after adjustment for bone turnover, had a significant correlation with LV mass, and after multivariable adjustment, PICP: B-AP ratio remained the better predictor of LV mass. In addition, there was a significant inverse correlation between MMP-1 and LV mass. These 2 findings might indicate that increased collagen synthesis and inhibition of collagenolysis are syntropic in the process of LV hypertrophy.

Moreover, although female patients presented significantly lower serum PICP concentration and LV mass, subanalysis by sex showed that PICP or MMP1 remained predictors of LV mass in both sexes.

Nevertheless, the correlation between biomarkers of collagen type I metabolism and LV thickness and mass is not a universal finding in previous studies in HCM.[26,34,35] Studies in HCM elucidating the relative contribution of fibrosis to the magnitude of hypertrophy and heart weight found clear but weak correlations.[38] In FD, the magnitude of the increase of fibrotic tissue in endomyocardial tissue was not as high as the cardiomyocyte area and glycosphingolipids vacuoles.[39] Despite the possibility of greater contribution of cardiomyocytes hypertrophy than fibrosis to the degree LV hypertrophy, the predictive value of the collagen type I synthesis biomarker to LV mass is evident.

In addition, there was significant correlation between MMP1 and tissue Doppler systolic and early diastolic myocardial velocities, further supporting the role of MMP inhibition in diastolic function impairment, as described previously for HCM.[34]

As expected, PICP was the sole biomarker with significant difference between LGE-positive and -negative FD patients; however, we cannot rule out that these findings reflect only the significant correlation between PICP and LV mass, as LV mass was significantly higher in patients with LGE. Furthermore, PICP was already elevated in FD patients with normal echocardiograms and without LGE. Nonetheless, the diagnostic accuracy of PICP and the PICP:ICTP ratio (reflecting the

Table 5. Influence of Other Variables in Biomarkers of Collagen Type I Turnover

	PICP (ng/mL)	PICP/B-AP	ICTP (ng/mL)	ICTP/TRAP5b	MMP1 (ng/mL)	MMP2 (ng/mL)
Demographic						
Sex (female)	−48.9*	−7.5*	−0.11*	−0.02	5.7	−55.6
Age, y	0.077	0.071	−0.153	−0.175	−0.334*	−0.277*
Diagnosis						
Age at diagnosis, y	−0.059	0.009	−0.142	−0.100	−0.308*	−0.309*
Plasma α-gal A (female), nmol/h/mL	−0.318	−0.444*	0.028	0.029	0.071	−0.017
Plasma α-gal A (male), nmol/h/mL	0.120	0.145	0.012	0.035	−0.121	−0.055
Disease severity						
MSSI	0.394*	0.287*	0.194	0.189	−0.392*	−0.222
RFI	0.258*	0.141	0.158	0.043	−0.374*	−0.211
Plasma lyso-Gb3 (female), nmol/L	0.402*	0.463*	0.225	0.255	−0.123	0.094
Plasma lyso-Gb3 (male), nmol/L	0.240	0.095	0.171	0.308	0.037	0.266
NT-proBNP, ng/mL	0.289	0.489*	−0.163	−0.084	−0.533*	−0.262
eGFR, mL/min/1.73 m^2	−0.122	−0.081	−0.060	−0.059	0.269*	0.262*
Treatment						
ERT (yes)	13.6	3.8	0.03	−0.01	−1.0	−56.3
Age at ERT initiation, y	−0.187	−0.139	−0.217	−0.218	−0.217	−0.220
Time in ERT, y	0.204	0.110	−0.213	−0.288	−0.014	0.146
ACEI/ARB (yes)	−27.0	−1.8	−0.03	−0.04	−8.2	−173.4
β-Blockers (yes)	6.3	0.2	−0.03	−0.00	4.7	67.5

Data are shown as mean difference for categorical variables and as ρ for continuous variables. ACEI indicates angiotensin-converting enzyme inhibitors; α-gal A, α-galactosidase A; ARB, angiotensin II receptor blockers; B-AP, bone-specific alkaline phosphatase; eGFR, estimated glomerular filtration rate; ERT, enzyme replacement therapy; FIPI, Fabry International Prognostic Index; ICTP, carboxyterminal telopeptide of type I collagen; lyso-Gb3, globotriaosylsphingosine; MMP, matrix metalloproteinase; MSSI, Mainz Severity Score Index; NT-proBNP, N-terminal probrain natriuretic peptide; PICP, carboxyterminal propeptide of procollagen type I; TRAP5b, tartrate-resistant acid phosphatase type 5b.
*$P<0.05$.

balance between synthesis and degradation) to detect LGE was high, ≈75% after adjustment for bone metabolism, and significant. Moreover, the addition of the PICP:ICTP ratio to routine clinical predictors (LV mass and NT-proBNP) seems to increase the ability to correctly classify a patient regarding LGE status.

Previous results in HCM found no significant correlation between collagen type I metabolism biomarkers and LGE,[26] and these results are certainly related to the limited resolution of LGE to detect focal replacement fibrosis[9] and the inability to detect early diffuse interstitial fibrosis.[10] Nevertheless, sensitive ELISAs may detect very small amounts of circulating PICP; they provide a more sensitive index of fibrosis and reflect subtle changes in myocardial composition that are not detectable by cardiac MRI.

A novel cardiac MRI technique, T1 mapping (assessing T1 relaxation times), may overcome the inability of LGE to detect diffuse interstitial fibrosis. High T1 relaxation times are observed in diffuse fibrosis, protein deposition, and water in edema. Low T1 values are seen in iron or lipid deposition; however, in FD cardiomyopathy, cardiac lipid storage is a hallmark, and T1 mapping has been studied as an imaging biomarker for early detection of cardiac involvement (with low native T1).[12,13] Consequently, the capacity of T1 mapping to detect early diffuse fibrosis may be impaired because of low native T1 (fibrosis may be detected only in the "pseudonormalization" stage, when the amount of fibrotic tissue is enough to overcome the low native characteristic FD cardiomyopathy) and needs further evaluation.

Collagen Type I Metabolism Biomarkers: Previous Findings in FD Cardiomyopathy

To our knowledge, only 2 studies have evaluated cardiac extracellular matrix turnover in FD. Shah et al[27] reported increased levels of MMP9, with significant correlation with MSSI and inverse correlation with midwall fractional shortening of the left ventricle. No difference was found in levels of TIMP1 and TIMP2 between FD patients and controls. In contrast to the identified decrease in MMP1 and MMP2 with increasing cardiomyopathy severity, MMP9 has been identified in HCM as a profibrotic marker.[35]

Table 6. Predictive Model of LV Mass by Univariate and Multivariable Regression Analysis

	LV Mass (Univariate)		LV Mass (Multivariable)	
	R	*P* Value	β	*P* Value
PICP	0.413	0.001	0.081	0.418
PICP/ß-AP	0.510	<0.001	0.919	<0.001
ICTP	0.170	0.202		
ICTP/TRAP5b	0.016	0.906		
MMP1	−0.339	0.010	−0.066	0.646
MMP2	−0.285	0.032	0.016	0.910
PICP/ICTP	0.217	0.102		
PICP/ICTP adjusted	0.379	0.004	0.035	0.751
Age	0.486	<0.001	0.392	0.010
Age at diagnosis	0.274	0.035	0.066	0.644
Age at ERT initiation	0.310	0.058		
Time in ERT	0.320	0.050	−0.094	0.288
Plasma α-gal A (female)	−0.027	0.906		
Plasma α-gal A (male)	−0.004	0.987		
MSSI	0.631	<0.001	0.041	0.686
RPI	0.658	<0.001	−0.149	0.417
Plasma lyso-Gb3	0.409	0.009	−0.012	0.898
NT-proBNP	0.414	0.040	0.028	0.759
eGFR	−0.388	0.003	−0.057	0.645

α-gal A indicates α-galactosidase A; ß-AP, bone-specific alkaline phosphatase; eGFR, estimated glomerular filtration ratio; FIPI, Fabry International Prognostic Index; ICTP, carboxyterminal telopeptide of type I collagen; lyso-Gb3, globotriaosylsphingosine; LV, left ventricular; MMP, matrix metalloproteinase; MSSI, Mainz Severity Score Index; NT-proBNP, N-terminal pro-brain natriuretic peptide; PICP, carboxyterminal propeptide of procollagen type I; TRAP5b, tartrate-resistant acid phosphatase type 5b.

Kržimer et al[6] reported an increase in 3 biomarkers of collagen metabolism (PICP, ICTP, and PIIINP) compared with healthy historical controls; however, no difference in these markers was observed between patients with and without fibrosis in the cardiac MRI. The authors explained this finding assuming a systemic fibrotic state in FD, involving the heart, the kidneys, and other organs. Nevertheless, a subanalysis by sex and adjustment to bone turnover was not performed and could be crucial; usually female patients have a milder phenotype, but the rise of the collagen markers due to bone turnover can misleadingly overestimate myocardial fibrosis. Moreover, the previously described limitations of LGE techniques to detect cardiac fibrosis certainly influenced the results.

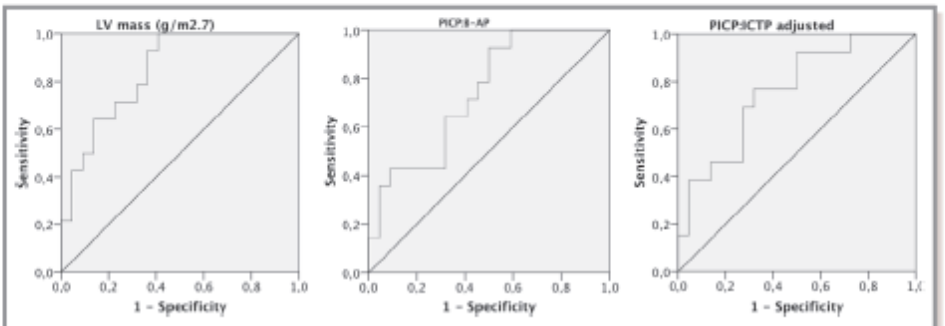
Limitations

This study has several limitations. First, histologic correlation is absent, although it could be very useful for biomarker validation, but endomiocardial biopsy is an invasive and potentially harmful technique. Second, the cross-sectional design hinders the prognostic value of these biomarkers and the influence of ERT and angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers on them (we are currently performing a longitudinal evaluation). Third, laboratory standardization of these biomarker assays is an urgent need to improve their clinical application. Fourth, no core reading of the echocardiograms and cardiac MRIs was performed as part of the standardized research protocol. Fifth, there is no correlation with speckle-tracking analysis of echocardiograms because this was not available in the majority of the cohort, and there is no quantification of the LGE area, although no correlation was identified in previous studies. Sixth, there is no correlation with T1 mapping cardiac MRI techniques.

Moreover, in this study, we recruited a relatively small cohort of FD patients that does not represent the entire spectrum of FD cardiomyopathy phenotypes. Further research is needed to address the value of the studied biomarkers in a larger and more heterogeneous cohort of FD patients.

Conclusion

This study provides new, relevant data to understand the natural history of fibrogenesis in FD cardiomyopathy. It shows, for the first time, that serum biomarkers of collagen



	AUC (95% CI)	Sensitivity (%)	Specificity (%)
LV mass (g/m$^{2.7}$) *	0.844 (0.720 – 0.969)	71.4	65.2
- S' average (cm/s) *	0.732 (0.561 – 0.904)	46.2	73.9
- E' average (cm/s) *	0.822 (0.683 – 0.961)	100	39.1
PICP (ng/mL) *	0.718 (0.552 – 0.883)	100	43.5
PICP/B-AP *	0.740 (0.579 – 0.902)	100	30.4
ICTP (ng/mL)	0.572 (0.372 – 0.771)	23.1	78.3
ICTP/TRAP5b	0.458 (0.262 – 0.654)	38.5	59.1
PICP/ICTP	0.636 (0.450 – 0.823)	69.2	47.8
PICP/ICTP adjusted *	0.759 (0.597 – 0.921)	61.5	73.9
MMP-1 (ng/mL)	0.388 (0.199 – 0.576)	100	18.2
MMP-2 (ng/mL)	0.432 (0.238 – 0.626)	71.4	36.4
NT-pro BNP (ng/mL) *	0.742 (0.513 – 0.970)	-	-
- eGFR (ml/min/1.73m^2) *	0.762 (0.597 – 0.927)	-	-

Figure 2. Diagnostic accuracy of several biomarkers for presence of LGE in cardiac MRI. Minus S' average, E' average, and eGFR have been used to identify the presence of LGE; lower values of these variables are associated with the presence of LGE. *P<0.05 for true area=0.5. B-AP indicates bone-specific alkaline phosphatase; E', early diastolic myocardial velocity measured at the mitral annulus; eGFR, estimated glomerular filtration rate; ICTP, carboxyterminal telopeptide of type I collagen; LV, left ventricular; MMP, matrix metalloproteinase; NT-proBNP, N-terminal probrain natriuretic peptide; PICP, carboxyterminal propeptide of procollagen type I; S', systolic myocardial velocity measured at the mitral annulus; TRAP5b, tartrate-resistant acid phosphatase type 5b.

type I metabolism identify ongoing fibrosis in the early stages of FD cardiomyopathy and possibly predict the development of LV hypertrophy, highlighting the importance of developing therapies to mitigate fibrosis and change the natural history of FD cardiomyopathy.

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Author Contributions

Research idea and study design: Aguiar, Azevedo, Ducla Soares and Hughes; data acquisition and laboratorial measurements: Aguiar, Pinto, Marino, Cardoso; data analysis/interpretation: Aguiar, Pinto; statistical analysis: Aguiar; supervision: Sousa, Cunha, Ducla Soares, Hughes. Each author contributed important intellectual content during article drafting or revision and accepts accountability for the overall work by ensuring that questions pertaining to the accuracy or integrity of any portion of the work are appropriately investigated and resolved. Aguiar takes

responsibility that this study has been reported honestly, accurately and transparently; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned have been explained.

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Supplemental Material

Data S1.
Supplemental Methods

Laboratorial methods

Plasma lyso-Gb3 was quantified by liquid chromatography tandem-mass spectrometry (Agilent, ultra performance liquid chromatography [UPLC] triple quadrupole [QqQ] electrospray ionization [ESI]). The quantification was achieved by multiple reaction monitoring (MRM) of the transitions mass-to-charge ratios (m/z) 786,4→282,3 and, as internal standard 1-β-D-glucosylsphingosine (GSG), 460,5→280,3. The result was extrapolated from the calibration curve obtained from responses of calibrators of known concentrations versus internal standard.

PICP was measured in serum, by an ELISA method (QUIDEL Corporation®, Hannover, Germany; category number 8003), according to manufacturer instructions. The microelisa strip plate is pre-coated with human PICP monoclonal antibody and the kit uses p-nitrophenyl phosphate substrate to measure the immune complex obtained during the reaction; final colorimetric reaction is measured by photometry at 405 nm.

ICTP was measured in serum, by an ECLIA method (Roche® Diagnostics GmBH, Mannheim; Germany; reference 11972308122) in an automated analyser COBAS e411 instrument, according to manufacturer instructions. The detection of the marker in the samples is made using human ICTP monoclonal antibody.

MMP-1 was measured also in serum, in an assay using a double-antibody sandwich ELISA (SunRed® Biotechnology Company; category number 201-12-0917), according to the instructions of the manufacturer. The microelisa strip plate is pre-coated with human MMP-1 monoclonal antibody and the kit uses biotin-streptavidin-HRP technology for measure de immune complex obtained during the reaction; final colorimetric reaction is measured by photometry at 450 nm.

MMP-2 was measured in serum, also by a double-antibody sandwich ELISA assay (SunRed® Biotechnology Company; category number 201-12-0905), according to the instructions of the manufacturer. The microelisa strip plate is pre-coated with human MMP-2 monoclonal antibody and the kit uses biotin-streptavidin-HRP technology for measure de immune complex obtained during the reaction; final colorimetric reaction is measured by photometry at 450 nm.

B-AP was measured in serum, by ELISA methodology (QUIDEL Corporation®, Hannover, Germany; category number 8012), according to the instructions of the manufacturer. The microelisa strip plate is pre-coated with human B-AP monoclonal antibody and thee kit uses p-nitrophenyl

phosphate substrate to measure the immune complex obtained during the reaction; final colorimetric reaction is measured by photometry at 405 nm.

TRAP-5b was measured in serum, by an ELISA assay (ids, Immunodiagnostic Systems®, United Kingdom; category number SB-TR201A), according to the manufacturer instructions. The microelisa strip plate is pre-coated with human TRAP5b monoclonal antibody and the kit uses p-nitrophenyl phosphate substrate to measure the immune complex obtained during the reaction; final colorimetric reaction is measured by photometry at 405 nm.

Table S1. Mutation frequency.

Mutation	n	%
p.N215S	10	16.7
p.F113L	9	15.0
p.G35E	7	11.7
c.700_702del	2	3.3
p.R227X	2	3.3
p.C52G	2	3.3
p.L166P	2	3.3
p.N42V	2	3.3
p.R342Q	2	3.3
unknown	2	3.3
other*	20	33.3

* Mutations presented by only one patient within the study

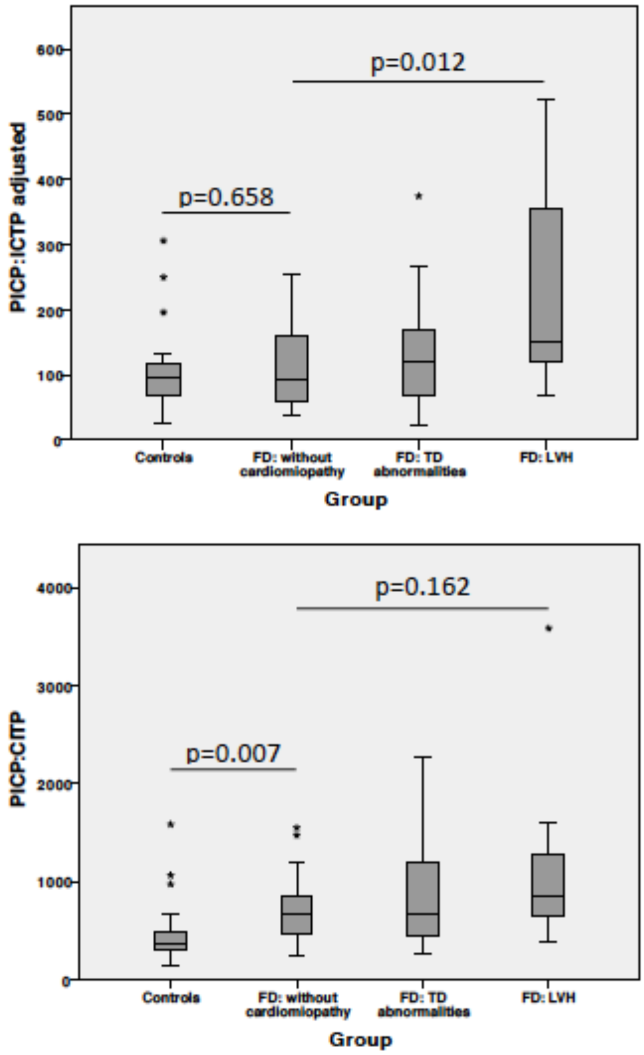

Figure S1. Boxplot of PICP to ICTP ratio (before and after adjustment for bone collagen turnover) in controls and FD subgroups.

PICP: carboxy-terminal propeptide of procollagen type I; ICTP: carboxy-terminal telopeptide of type I collagen.

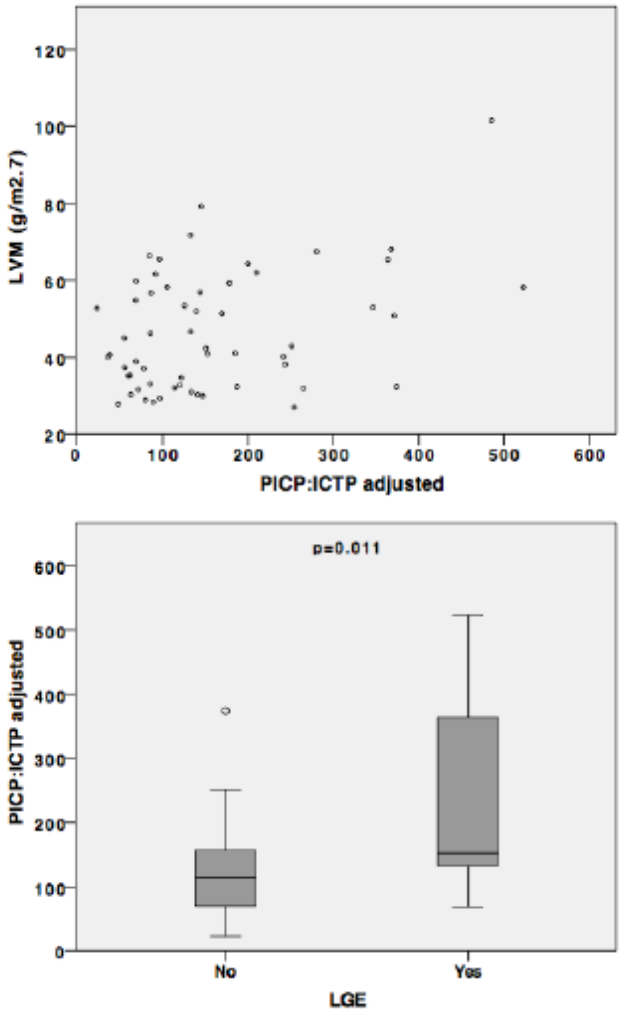

Figure S2. Correlation between PICP to ICTP ratio (after adjustment for bone collagen turnover) and left ventricular mass (upper image); boxplot of PICP to ICTP ratio (after adjustment for bone collagen turnover) in LGE negative and positive patients (lower image).

PICP: carboxy-terminal propeptide of procollagen type I; ICTP: carboxy-terminal telopeptide of type I collagen; LVM: left ventricular mass; LGE: late gadolinium enhancement.

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Biomarkers of Myocardial Fibrosis: Revealing the Natural History of Fibrogenesis in Fabry Disease Cardiomyopathy

Patrício Aguiar, Olga Azevedo, Rui Pinto, Jacira Marino, Carlos Cardoso, Nuno Sousa, Damião Cunha, Derralynn Hughes and José Luís Ducla Soares

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P-376

Collagen turnover biomarkers in Fabry disease cardiomyopathy

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Background and objectives: Cardiomyopathy is the most common cardiac abnormality seen in Fabry disease (FD). Since myocardial fibrosis is one of the histological hallmarks of FD cardiomyopathy the assessment of the collagen turnover biomarkers is paramount.
Methods: FD patients with left ventricle hypertrophy and age- and sex-matched controls were studied. All patients and controls underwent echocardiography and markers of collagen type I and III synthesis and degradation were measured. Since type I collagen is a major component of bone, bone-specific alkaline phosphatase (b-AP) and tartarate resistant acid phosphatase 5b (TRAP5b) were also assessed.
Results: Ten patients and ten controls were evaluated. Levels of propeptide of type I procollagen (PICP) were significantly higher in FD patients ($p=0.015$) as also the PICP:b-AP ratio ($p=0.002$). The PICP to C-terminal telopeptide of type I collagen ratio (balance between collagen synthesis and degradation) was non-significantly increased in FD ($p=0.258$) and the matrix metalloproteinase-2 (MMP-2) was significantly lower in FD ($p=0.028$).
Conclusion: Collagen type I synthesis is increased in FD cardiomyopathy and there is a statistical trend for the synthesis to prevail over degradation, related to the inhibition of the MMP. As in hypertrophic cardiomyopathy this profibrotic state is likely to be critical in FD cardiomyopathy.
Conflict of Interest declared.

P-377

Glomerular and tubular damage biomarkers in Fabry disease

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Background and objectives: In Fabry disease (FD) nephropathy storage occurs in all renal cells (endothelial, glomerular, interstitial and tubular cells). These histologic findings can occur with minimal or no alterations on standard renal tests, so alternative markers of glomerular and tubular dysfunction are crucial.
Methods: FD patients with chronic kidney disease and age- and sex-matched controls were studied. All patients and controls underwent kidney function evaluation (glomerular filtration rate [GFR] and albuminuria) and markers of glomerular (transferrinuria and collagen type IV) and tubular (α1-microglobulin, N-acetyl-glucosaminidase and alanine aminopeptidase) damage were measured.
Results: Ten patients and ten controls were evaluated. All the patients had GFR<60 ml/min/1.73 m^2 and 9 had albuminuria>30 mg/g creatinine. Markers of glomerular damage were significantly higher in FD patients (transferrinuria p=0.001; collagen type IV p=0.001). A similar result was achieved for markers of tubular injury (α1-microglobulin p=0.006; N-acetyl-glucosaminidase p=0.001; alanine aminopeptidase p=0.005).
Conclusion: The kidney damage in FD affects the glomeruli and the tubules. This is the first evaluation of tubular dysfunction markers in FD. These results suggests further study of these markers which have shown promise in the early detection of nephropathy in other diseases such as diabetes, so should also be pursued in FD.
Conflict of Interest declared.

7

Urinary type IV collagen: Better than albuminuria to identify incipient Fabry nephropathy

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Introduction: Renal involvement in Fabry disease (FD) is a major determinant of overall disease prognosis. Accumulating evidence suggest that early enzyme replacement therapy (ERT) is safe and effective in preventing progression of kidney injury. Gb3 and lyso-Gb3 storage in renal cells may occur with minimal changes on standard renal tests, hence alternative markers of glomerular dysfunction are crucial. In podocytes, lyso-Gb3 induced expression of fibronectin and type IV collagen. Thus, urinary type IV collagen (uColIV) may be an early marker of FD nephropathy.

Methods: uColIV, glomerular filtration rate (GFR) and albuminuria were determined in FD patients and controls. FD patients were grouped by nephropathy severity: 1) albuminuria < 30 mg/g and GFR ≥ 60 ml/min/m^2; 2) albuminuria 30-299 mg/g; 3) albuminuria > 300 mg/g; 4) GFR < 60 ml/min/m^2.

Results: 78 FD patients (25, 22, 10 and 21 in the groups 1, 2, 3 and 4, respectively; 43,6% males, mean age 50,1 ± 15,1 years, 69,2% on ERT) and 25 controls (age- and sex-matched with group 1) have been recruited. uColIV was significantly higher in group 1 FD patients compared with controls (130% increase; $p = 0{,}041$). There was no difference in uColIV between the several groups of FD patients ($p = 0{,}898$), as well as no difference/correlation with age ($p = 0{,}957$), sex ($p = 0{,}880$), use of ERT ($p = 0{,}508$) or use of anti-proteinuric agents ($p = 0{,}196$). Regarding group 1 of FD patients, there was a significant correlation between uColIV and albuminuria ($R = 0{,}474$; $p = 0{,}017$) or GFR ($R = 0{,}413$; $p = 0{,}040$); additionally, 52% of the patients within this group had uColIV above the reference range.

Conclusion: Our findings, suggests that uColIV is a better marker of incipient FD nephropathy than albuminuria (we purpose uColIV as new marker, instead of albuminuria, to define the early stage of FD nephropathy), probably reflecting the ongoing alteration of the extracellular matrix turnover induced by lyso-Gb3. Whether increased excretion of uColIV predicts progression to renal failure is being longitudinally evaluated.

3

Increase synthesis of collagen type I: an early event in Fabry cardiomyopathy. New biomarkers?

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Cardiovascular disease is the main cause of death in Fabry disease (FD) and a major determinant of overall disease prognosis, with worst outcomes in patients with myocardial fibrosis. Currently, late gadolinium enhancement (LGE) is the gold standard for evaluation of replacement myocardial fibrosis. However, this is an end stage / irreversible event, thus identification of biomarkers of earlier diffuse fibrosis or increased collagen deposition is paramount. Type I collagen synthesis and degradation biomarkers (carboxy-terminal propeptide of procollagen type I [PICP], carboxy-terminal telopeptide of type I collagen and matrix metalloproteinases [MMP] 1 and 2), as well as markers of bone synthesis and degradation were evaluated (to adjust type I collagen metabolism to bone turnover) in FD patients and controls. FD patients were grouped by cardiomyopathy severity: 1) normal echocardiogram; 2) tissue Doppler abnormalities in echocardiogram; 3) left ventricular (LV) hypertrophy in echocardiogram. 60 FD patients (20 in each group; 38,3% males, mean age 50,2 ± 14,7years, 65,0% on ERT) and 20 controls (age- and sex-matched with group 1) were recruited. A significant increase of PICP and a significant decrease in MMPs was observed in FD patients, even in the group 1 for the former biomarker. There was a significant correlation between LV mass and PICP ($\beta = 0.413$, $p = 0.001$) or MMP-1 ($\beta = -0.339$, $p = 0.01$). PICP (adjusted for bone turnover) was the better predictor of LV mass in a multivariate regression model and its diagnostic accuracy to predict LGE was also significant. Collagen type I synthesis is increased in FD cardiomyopathy (even in the earlier stages of the disease) and this profibrotic state is a good predictor and is likely to be critical to the development of overt LV hypertrophy. Moreover, inhibition of enzymes involved in collagen type I cleavage seems to be a crucial pathophysiological link in myocardial collagen deposition.

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4

Collagen type I synthesis biomarkers predict the progression of Fabry disease cardiomyopathy

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Introduction: Fabry disease (FD) cardiomyopathy is characterized by a hypertrophic phenotype, in the histological setting of cardiomyocyte hypertrophy and myocardial fibrosis; myocardial fibrosis is an irreversible event and affects the prognosis. Thus, the identification of biomarkers to predict the development of myocardial fibrosis and overt left ventricular (LV) hypertrophy is paramount.

Methods: a cohort of 60 FD patients (38,3% males, mean age 50,2 ± 14,7years, 65,0% on ERT) was consecutively recruited and the patients were followed for 24 months. Patients with an increase ≥10g/m$^{2.7}$ in LV mass (LVM) or an increase ≥1.5 in E/E' average, associated with TDI abnormalities upon follow-up were defined as LVM or LV end-diastolic pressure (LVEDP) progressors, respectively. The prognostic value of type I collagen synthesis (carboxy-terminal propeptide of procollagen type I [PICP]) and degradation (matrix metalloproteinase 1 [MMP-1]) biomarkers was evaluated.

Results: during follow-up, both LVM and E/E' ratio remained stable, but 28,3% and 31,3% of the patients presented criteria of LVM and LVEDP progression, respectively; there was no significant change in PICP and MMP-1 during follow-up. MMP-1 serum concentration at baseline was significantly lower in patients presenting with LVEDP progression. Moreover, there was a significant correlation between PICP variation and LVM variation ($\rho = 0.492$; $p = 0.001$) and PICP decreased by a median 7,6ng/mL in LVM non-progressor and increased by a median 20,6ng/mL in LVM progressors ($p = 0{,}001$); furthermore, a patient in the forth quartile of PICP variation presented an OR = 25,0 ($p = 0{,}008$) of being a LVM progressor compared to a patient in the first quartile of PICP variation.

Conclusion: our findings suggest that collagen type I synthesis has good prognostic value for and is likely to be critical to the development of overt LV hypertrophy. Moreover, inhibition of enzymes involved in collagen type I cleavage also seems crucial to myocardial collagen deposition and is related to the risk of progressive diastolic dysfunction.

5

Tubular dysfunction biomarkers in Fabry disease: better than albuminuria to identify patients at risk of nephropathy progression

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Introduction: Fabry disease (FD) nephropathy is clinically heterogeneous in terms rate of progression. Proteinuria/albuminuria influence nephropathy progression, although the magnitude of this influence is not well established, with studies showing conflicting results. Therefore, the identification of prognosis biomarkers is paramount to the identification of patients at increased risk of progressive nephropathy.

Methods: in this multicentre, prospective, longitudinal and diagnostic test study, a cohort of 78 FD patients (43,6% males, mean age 50,1 ± 15,1years, 69,2% on ERT) was consecutively recruited and the patients were followed yearly, for 2 years. The accuracy of a biomarker of tubular dysfunction (urinary N-acetyl-β-glucosaminidase [uNAG]) in the identification of GFR decline was compared with the reference standard (albuminuria).

Results: during 24 months of follow-up, GFR remained stable in the whole cohort (mean decline of -1,5ml/min/1.73m2, p = 0,675), but in 28,2% of the patients GFR declined ≥5ml/min/1.73m^2(defined as progressors); during the same period, albuminuria remained stable and there was a significant increase in uNAG. Both albuminuria and uNAG were significantly higher at baseline, comparing GFR progressors with non-progressors. However, comparing the GFR change according to baseline quartiles of the biomarkers, only uNAG was consent with progressive reduction in GFR across the increasing quartiles; for albuminuria, quartiles 1, 2 and 3 showed similar declines in GFR, only somewhat different in quartile 4. Moreover, only uNAG variation has a significant inverse correlation with GFR variation ($\rho = -0.361$; $p < 0.001$, compared with $\rho = -0.127$; $p = 0.131$ for albuminuria).

Conclusion: our findings, suggests that uNAG appears to outperform albuminuria as a prognostic biomarker, clearly identifying patients at increased risk of progressive nephropathy. These results support the evidence that long-term outcome of FD nephropathy may be more related to the extent of the tubulointerstitial impairment than to the severity of glomerular lesions, which is in accordance with findings in diabetic nephropathy.