Thesis

Endothelial dysfunction in Fabry disease

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Graz, 30.06.2025 Gregor Regenstein m.p.

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Zusammenfassung

Einleitung: Morbus Fabry ist eine seltene lysosomale Speicherkrankheit, die durch pathogene Varianten des *GLA*-Gens verursacht wird. Dieses Gen kodiert für das Enzym alpha-Galactosidase (AGAL), welches für den Abbau von Globotriaosylceramid (Gb3) erforderlich ist. Mangel an AGAL führt zu einer Anhäufung von Gb3 in verschiedenen Zellen und Geweben. In der Regel sind die Endothelzellen betroffen, wobei ihre Beeinträchtigung zu einer endothelialen Dysfunktion (ED) führen kann. Obwohl bei Männern aufgrund der X-chromosomalen Vererbung meist schwerere Fälle auftreten, können auch Frauen schwerwiegende Symptome aufweisen, wenn auch mit einer geringeren Gb3-Akkumulation. Interessanterweise zeigen auch Patient*innen, unter Enzymersatztherapie (ERT) erhalten, ED-assoziierte Symptome. Dies deutet darauf hin, dass die endotheliale Dysfunktion bei Morbus Fabry nicht ausschließlich auf die Gb3-Speicherung zurückzuführen ist, sondern möglicherweise weitere pathophysiologische Mechanismen eine Rolle spielen, die noch erforscht werden müssen.

Methoden: Diese Literaturübersicht gibt einen Überblick über die vorhandene Literatur in der PubMed-Datenbank. Es wird eine systematische Suche mit Hilfe von Schlüsselwörtern durchgeführt, wobei der Schwerpunkt auf Forschungsarbeiten liegt, die sich auf den Menschen oder *in vitro* Modelle beziehen.

Ergebnisse: Die Mehrheit der Patient*innen zeigte klinische Veränderungen, die mit einer ED in Zusammenhang stehen. Gb3-Akkumulation in Endothelzellen wurde am häufigsten berichtet, wobei Frauen weniger betroffen waren. Eine ERT führte zu einer effizienten Gb3 Reduktion, obwohl andere klinische Aspekte nicht verbessert wurden. Zahlreiche Marker der ED waren bei beiden Geschlechtern verändert, und die meisten Patient*innen zeigten nach der ERT keine Verbesserung. Die Genotypen Daten wurden analysiert und mit dem phänotypischen Auftreten der ED in Verbindung gebracht, führten jedoch zu keinen signifikanten Ergebnissen. Der größte Teil der untersuchten Genotypen entfiel auf Missense-Genvarianten. Berichte über diverse *in vitro* Modelle von

Morbus Fabry wurden gefunden, die ED-Merkmale aufweisen. Zudem wurden epigenetische Veränderungen wie DNA-Methylierung oder erhöhte MikroRNA-Spiegel beobachtet. In *in silico* Modellen konnten potenzielle Modifikatorgene und Hochrisikomarker für zelluläre Schäden festgestellt werden. Nach einer ERT wurden häufig Anti-Drug-Antikörper nachgewiesen.

Schlussfolgerung: Die Ergebnisse deuten auf eine ähnliche Entwicklung der ED bei Frauen und Männern hin, unabhängig von der sichtbaren Gb3-Speicherung. Zudem weisen die Daten auf eine geringe Wirksamkeit der ERT bei der Verbesserung der ED hin. Dies könnte auf eine verbleibende Gb3-Akkumulation in anderen Zellentypen oder auf epigenetische Veränderungen in den Endothelzellen zurückzuführen sein, die durch die ERT nicht rückgängig gemacht werden. Eine frühzeitige ERT und Verhinderung der Gb3-Akkumulation wurde mit besseren Ergebnissen in Verbindung gebracht und sollte in Betracht gezogen werden. Es lagen nicht genügend Daten vor, um den Genotyp mit dem Phänotyp der ED zu korrelieren. Zur weiteren Erforschung dieser Hypothesen sowie zur Validierung relevanter Marker, sind sowohl mehrere der vorgeschlagenen FD-*In-vitro*-Krankheitsmodelle sowie *in vivo* Studien mit größeren Kohorten durchgeführt werden.

Schlüsselwörter: Morbus Fabry, Alpha-Galaktosidase A, Globotriaosylceramid, endotheliale Dysfunktion, Entzündung, Endothel, Enzymersatztherapie, Biomarker, Genetik

Abstract

Introduction: Fabry disease (FD) is a rare lysosomal storage disorder caused by pathogenic variants in the *GLA*-gene, which encodes the enzyme alpha-Galactosidase (AGAL), required for degradation of globotriaosylceramid (Gb3). Deficiency of AGAL results in Gb3 accumulation in various cells and tissues. Endothelial cells are commonly affected, and their impairment leads to endothelial dysfunction (ED). Although men usually present with more severe symptoms sue to, X-linked inheritance, women can also present with serious manifestations, despite showing less Gb3 accumulation. Patients under enzyme replacement therapy (ERT) may still develop ED-related symptoms. Therefore, ED in FD might not solely be related to Gb3 storage, and other influences need to be elucidated.

Methods: This literature review provides an overview of existing studies available in the PubMed database. A systematic search using relevant keywords was conducted, with a focus on research involving human subjects or *in vitro* models.

Results: The majority of patients showed ED-related clinical changes. Gb3 accumulation in endothelial cells was the most common, while females were generally less affected. ERT led to efficient clearance, although other clinical aspects showed limited improvement. Numerous markers of ED were altered in both sexes, with most patients showing no improvement after ERT. There was not enough data to perform a correlation analysis between patients' genotype and ED phenotype. Missense gene variants accounted for the largest portion of reported genotypes.
Multiple *in vitro* models of FD were reported, which show ED characteristics. Epigenetic changes like DNA-methylation or elevated micro-RNA levels were observed as well. *In silicio* models identified multiple potential modifier genes and high-risk markers of cellular damage. Anti-drug antibodies were frequently reported following ERT.

Conclusion: The results suggest a similar development of ED in both females and males, regardless of Gb3 storage. They also indicate limited efficacy of ERT in reversing ED, which may be due to remaining Gb3 accumulation in other cell types

or epigenetic changes not addressed by ERT. Early initiation of ERT and prevention of Gb3 accumulation have been associated with better outcomes and should be considered. Not enough data was available to correlate genotype to ED phenotype. To further investigate these hypothesis and validate potential markers, multiple FD *in vitro* models, as well as *in vivo* studies with larger cohorts are necessary.

Key Words: fabry disease, alpha-galactosidase A, globotriaosylceramid, endothelial dysfunction, inflammation, endothelium, enzyme replacement therapy, biomarkers, genetics

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Glossary and Abbreviations

3-NT = 3-Nitrotyrosine

ACTB = Beta-actin

ADAMTS-13 = A disintegrin and metalloproteinase with thrombospondin motifs

ADMA = Asymmetric dimethylarginine

AGAL = Alpha-galactosidase A

AI = Augmentation index

AMPK = Adenosine monophosphate-activated protein kinase

Ang II = Angiotensin II

ANG2 = Angiopoietin-2

ASAH1 = N-acylsphingosine amidohydrolase 1

ATP = Adenosine triphosphate

BNP = Brain natriuretic peptide

CAC = Circulating angiogenic cells

C4B = Complement component 4B

COL4A1/2 = Collagen type IV alpha 1 and 2 chains

COX = Cyclooxygenase

CRISPR = Clustered Regularly Interspaced Short Palindromic Repeats

CRP = C-reactive protein

E-selectin = Endothelial selectin

EC = Endothelial cell

ED = Endothelial dysfunction

EDN1 = Endothelin-1

EMP = Endothelial microparticles

eNOS = Endothelial nitric oxide synthase

EPC = Endothelial progenitor cells

ERT = Enzyme replacement therapy

ESM1 = Endothelial cell specific molecule 1

FD = Fabry disease

FGF2 = Fibroblast growth factor 2

FMD = Flow-mediated dilation

Gb2 = Globotriaosylceramide 2

Gb3 / GL3 = Globotriaosylceramide

Gb4 = Globoside (globotetraosylceramide)
GDF-15 = Growth differentiation factor 15
GLA = Galactosidase alpha (gene for AGAL)
HDL-C/ = High-density lipoprotein
HNRNPH2 = Heterogeneous nuclear ribonucleoprotein H2
HPSE = Heparanase
HUVEC = Human umbilical vein endothelial cells
hArg = L-homoarginine
ICAM-1 = Intercellular adhesion molecule 1
iC3b = Inactivated complement component 3b
IL = Interleukin
IMFE1 = Intermediate filament elongation protein 1
IMT = Intima-media thickness
iNOS = Inducible nitric oxide synthase
iPSC = Induced pluripotent stem cell
KDR = Kinase insert domain receptor (VEGF receptor 2)
LacCer = Lactosylceramide
Let 7 = Let-7 microRNA family
LSD = Lysosomal storage disorder
Lyso-Gb3 = Globotriaosylsphingosine
MCP-1 = Monocyte chemoattractant protein-1
miRNA = MicroRNAs
MIP-1beta = Macrophage inflammatory protein 1 beta
MMP = Matrix metalloproteinase
MPO = Myeloperoxidase
MR-pro ANP = Mid-regional pro-Atrial natriuretic peptide
NADH = Nicotinamide adenine dinucleotide (reduced form)
NFC = Nailfold capillaroscopy
NF-kB = Nuclear factor kappa B
NO = Nitric oxide
P-SMAD2 = Phosphorylated SMAD family member 2
P-selectin = Platelet selectin
PF4 = Platelet factor 4

PFN1 = Profilin-1
Rab = Ras-related proteins in brain
RAG-1 = Recombination activating gene 1
REA = Residual enzyme activity
RHI = Reactive hyperemia index
RIP = Receptor-interacting protein
ROS = Reactive oxygen species
SAM = S-adenosyl methionine
SD = Standard deviation
SDMA = Symmetric dimethylarginine
siRNA = Small interfering RNA
sICAM-1 = Soluble intercellular adhesion molecule-1
SNP = Single nucleotide polymorphism
SOD2 = Superoxide dismutase 2
sTF = Soluble tissue factor
suPAR = Soluble urokinase plasminogen activator receptor
SRT = Substrate reduction therapy
TAT III = Thrombin-antithrombin III complex
TGF-beta = Transforming growth factor beta
Tie-2 = Tyrosine kinase with immunoglobulin-like and EGF-like domains 2
TLC = Thin-layer chromatography
TM = Thrombomodulin
TNF-α = Tumor necrosis factor alpha
TNFR = Tumor necrosis factor receptor
TSP-1 = Thrombospondin-1
VCAM-1 = Vascular cell adhesion molecule 1
VE-PTP = Vascular endothelial-protein tyrosine phosphatase
VEC = Vascular endothelial cells
VECAD = Vascular endothelial cadherin
VEGF = Vascular endothelial growth factor
vWF = von Willebrand factor
VUS = Variant of uncertain significance
WT = Wild type

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1 Introduction

1.1 Lysosomal storage diseases

More than 70 distinct genetic disorders of metabolism, known as lysosomal storage diseases, result from a pathogenic gene variant and a subsequent lysosomal dysfunction. [1]

Lysosomes are ubiquitous cellular organelles, which consist of a single lipid bilayer membrane that contains an acidic lumen. Segregated from the basic cytoplasma the pH-level of the lysosomal lumen is approximately 5 and enables the function of soluble hydrolytic enzymes, the hydrolases. [2] Other than that, they also contain activators called sphingolipid activator proteins or saposins, which allow binding of substrates to the enzymes. [3] While there are a handful of known diseases originating in activator protein deficiency or in lysosomal membrane transport, most lysosomal storage diseases are caused by deficient enzyme activity. Categorization of LSD depends on the specific enzyme deficit and the particular type of substance that accumulates in the lysosomes. This leads to classifications like sphingolipidoses, mucopolysaccharidoses, mucolipidoses, and oligosaccharidoses, amongst others. Notable examples include Fabry disease (FD), Gaucher disease, Niemann-Pick disease, Pompe disease, the GM2-gangliosidoses (encompassing both Sandhoff disease and Tay-Sachs syndrome), and I-cell disease.

Although most LSD lead to multisystemic symptoms, alongside a progressive disease course, the severity and the timeframe over which symptoms unfold are highly variable and, influenced by the specific disease subtype.

The wide range of symptoms and the uncommon nature of these diseases often complicate the diagnostic process. Most lysosomal storage disorders follow an autosomal recessive inheritance pattern, although there are exceptions, like FD, MPS II and Danon disease which are inherited in an X-linked pattern. [1,4]

1.2 Sphingolipidoses

One of the largest groups of enzyme-deficiencies causing LSD, are the sphingolipidoses. These diseases are defined through accumulation of various forms of sphingolipids in different types of cells. Sphingolipids consist of

sphingosine, a long-chain amino alcohol, which is combined with a fatty acid to form ceramides, which are complex lipids. There are several subtypes, depending on additional groups to the ceramides. Globosides are such a subtype and are generated through the addition of polysaccharides. The most basic globoside is globotriaosylceramide, also known as Gb3, GL-3 or ceramide trihexoside and consists of ceramide-glucose-galactose-galactose. This molecule is physiologically cleaved by the exoglycohydrolase alpha-galactosidase A (AGAL) and can then be further degraded and recycled. [5]

1.3 Fabry disease

Definition

Fabry disease (FD) is a rare, inherited, X-linked LSD which belongs to the group of sphingolipidoses. The *GLA*-gene carries a pathogenic variant, which leads to a significantly reduced or non-existent enzyme activity of AGAL. [6]

Genetic aspects

Reduced or absent activity of AGAL is caused by pathogenic variants of the *GLA*-gene in the chromosomal location Xq22. Up to date, over a thousand gene variants have been described in the Human Gene Mutation Database (HGMD) [7] in association with FD. Not all of the genetic variants are pathogenic, with some being benign and some being variants of uncertain significance (VUS). Since FD is X-linked inherited, the hemizygous males are generally severely affected. Due to lyonization (i.e. X-chromosome inactivation), the phenotype in heterozygous females can be extremely heterogeneous and is not predictable by its genotype. [8]

Epidemiology

The incidence ranges from 1 in 476,000 [9] to 1 in 117,000 [10]. It is pan-ethnic and its incidence is most likely massively underestimated as confirmed with recent newborn screening studies reporting incidences of up to 1:3,100.[11]

Clinical presentation

Usually, patients show no symptoms during their early years or can even remain symptom free well into adulthood. Nevertheless, clinical manifestations that affect

child's health and activities begin most commonly within the 3-to-10-year age range, while being a bit delayed in females. [12] The quality of life deteriorates considerably for males and females beginning in their thirties and risk of major organ involvement is considerably increased with age for both, although later in females. [13]

Most diagnosed men display a "classic" symptom profile, which is associated with the most severe progression. The late-onset or atypical FD with milder manifestations is further differentiated into "cardiac variant" and "renal variant" and is used to classify patients who develop mainly heart, or mainly kidney-related problems, respectively. [14,15]

This clinical spectrum reaches from the severe "classic" presentation typically seen in males (hemizygous), to a disease course that appears asymptomatic, as sometimes observed in females. Therefore, a multitude of clinical phenotypes are possible even in siblings and relatives carrying same genetic variant of the *GLA*-gene. Most women carrying one copy of the faulty gene (heterozygotes) develop symptoms, although the underlying reasons remain unclear at present. In a rare homozygous female with FD on the other hand a similar phenotype to an affected male with same gene variant p.Q279R was observed. [16]

Therapeutic options

The most common form of therapy is the enzyme replacement therapy (ERT) in form of biweekly intravenous injections of the recombinant AGAL enzyme to replace the enzymatic function and stop Gb3 accumulation. ERT was first approved in this indication in 2001 with agalsidase alfa (Replagal) and agalsidase beta (Fabrayzme)[17] and since 2023 pegunigalsidase alfa (Elfabrio) was approved as well.[18] An issue in this approach is the development of anti-drug-antibodies (ADA), resulting in increased tolerance to infusions and reduced therapy efficacy.[17] A novel ERT with the enzyme α-N-Acetylgalactosaminidase (NAGA) might solve this problem in the future, since this NAGA is modificated to cleave Gb3, but is known to the immune system and will not cause ADA development.[19]

A very effective therapy option is the use of the chaperone Migalastat, which can be used in patients carrying genetic variants leading to impaired protein-folding. Chaperone binds to the endogenously produced, but impaired enzyme and

enables its transport to the lysosome. This restores its function resulting in a normalized enzyme activity. [20]

A potential therapeutic option which is not yet approved and still under research is substrate reduction therapy (SRT). The idea is to reduce metabolites, which are substrates in the production of Gb3, like glucosylceramide. One of these is GZ-161 which is an analogue of Venglustat and is currently in clinical trials for FD [21], another is Eliglustat which has been reported to reduce Gb3 accumulation[22], but is mostly used in Gaucher disease type 1.[23]

Gene therapy is also a possible approach, which could heal FD with a single injection of the genetic code for the *GLA*-gene via RNA. At the moment, no gene therapy has been approved, but there is currently an ongoing phase 1/2 trial study.[24]

Pathophysiology

The reduced or absent enzyme activity leads to progressive accumulation of mainly Gb3, but also other similar glycosphingolipids like galabiosylceramide (Ga2), in the lysosomes of various cell types. [25] Another relevant metabolite is the hydrophilic and soluble lyso-Gb3, which is synthesized from Gb3 by ASAH1, an acid ceramidase. [26]

Mostly affected are renal cells like podocytes, tubular, mesangial, interstitial and glomerular endothelial cells, cardiac cells like cardiomyocytes and fibroblasts, nerve cells and capillary endothelial cells. The severity of the disease is determined by the remaining enzyme activity (REA) of AGAL, with lower levels indicating earlier disease manifestation and worse outcomes. The pathophysiological process of metabolite accumulation starts at birth or sometimes even in the fetal-development. [27]

In FD specifically, the accumulation of material in lysosomes, alongside resulting cellular malfunction, is considered to start a cascade of detrimental effects. This includes cell death, interruptions in energy metabolism [28], improper function of K(Ca)3.1 channels within endothelial cells [29], increased oxidative stress[30], damage to small blood vessels [31], abnormal formation of autophagosomes [32] and the establishment of irreversible scarring caused by insufficient oxygen delivery to

tissues in the heart resulting in Fabry cardiomyopathy (FCM) [33] and kidneys leading to chronic kidney disease. [34].

Since the specific focus of this work is on endothelial dysfunction (ED) stemming from FD, the subsequent chapter will elaborate on the most important features of ED in the context of FD.

1.4 Endothelial dysfunction

The endothelium is the layer located between the vascular walls and the blood circulating within them. This layer behaves as a sensor, responding to both mechanical stimuli, such as the pressure applied by blood flow and the shear stress from friction, as well as chemical stimuli including hormonal influences from factors that impact blood vessel tone. Upon receiving these inputs, the endothelium subsequently releases diverse substances. These secreted compounds then influence blood vessel dilation, trigger inflammatory processes, and play a role in coagulation.

Other functions of the endothelium are angiogenesis, vascular permeability, homeostasis and mitogenesis. [35]

Endothelial dysfunction (ED) therefore includes reduced vasodilatory capabilities as well as a proinflammatory and prothrombic state leading to an impairment of the endothelium. [36]

ED plays a crucial role in the development of various cardiovascular diseases (CVD) via atherosclerotic processes.[37,38] These encompass, among others, hypertension, coronary artery disease, chronic heart failure, peripheral artery disease, diabetes mellitus, and chronic renal failure.

Not only can ED lead to increased disease risk, but it is also often a direct consequence of diseases. In case of FD, ED is considered to start by Gb3 accumulation among other processes.

But it is also caused by a myriad of other diseases or conditions, associated with increased cardiovascular risk. These include metabolic syndrome, high blood pressure, tobacco use, sedentary lifestyle, hyperhomocysteinemia and diabetes mellitus.[39,40] Patients with these comorbidities are therefore usually excluded from the following studies.

Clinical assessment of ED

The earliest documented ED symptoms in FD focus mostly on the dermal manifestation in form of angiokeratoma corporis diffusum, a benign lesion affecting the upper dermis and exhibiting ectatic vessels. This is caused by an excessive build-up of Gb3 within the EC found in the dermis, which results in distension and impaired function of the vascular wall. [41]

The accumulation of Gb3 in EC can be observed in microscopic studies and is an indirect assessment of ED, since it leads to impairment of EC. [42]

A useful metric for ED is endothelium-dependent vasodilation. This is often analyzed using angiography and Doppler flow measurements within coronary arteries. This approach measures how endothelium-dependent agonists, like acetylcholine, impact the vessels. [43]

The performance of the endothelium can also be determined by measuring the forearm blood flow by strain-gauge plethysmography and then evaluation of the resistance of arteries.[44]

Another method utilizes reactive hyperemia, since subjecting EC to elevated shear stress triggers them to release NO. This allows evaluation of flow-mediated dilation (FMD) of the brachial artery, employing ultrasound technology.[37]

The intima-media thickness (IMT), is a parameter for vascular stiffness and therefore represents an indicator of endothelial function. [45]

After measurement with a peripheral arterial tonometry method based on a plethysmographic device[46], the reactive hyperemia index (RHI) can be calculated. This is the ratio between the mean finger arterial pulse wave amplitude during reactive hyperemia after the occlusion of the vessel compared with values before the occlusion and can indicate ED. Arterial stiffness can also be measured as ED surrogate, by using the augmentation index (AI) which is the difference between the first and second peaks of the central arterial waveform.[47]

Examination of capillaries by using nailfold capillaroscopy is most commonly associated with rheumatic diseases [48], but provides information about the endothelial function in non-rheumatic diseases as well. [49] The impairment of capillaries can also be measured by optical coherence tomography (OCT) and corneal confocal microscopy (CCM), these are not typically used in FD but are useful to evaluate corneal and retinal endothelial disease. [50]

Evaluation of cardiac manifestations, hypertension and thrombosis which can lead to severe clinical events e.g. cardiac events, stroke and death is another assessment of ED. [51]

Markers of ED

Typical markers for ED are the soluble ICAM-1, VCAM-1 and E-selectin,[52] as well as markers like ischemia modified albumin, pentraxin-3, angiopoietin (Ang2), endothelial cell specific molecule 1 (ESM1), asymmetrical dimethylarginine (ADMA), von Willebrand factor (vWF), endothelial microparticles (EMP) and endothelial progenitor cells (EPC).[53]

While these are commonly used to assess ED, the following different processes of ED, each have several other markers which might be useful in assessing and monitoring ED as well.

Angiogenesis

Angiogenesis is the process of remodeling of tube-like structures formed from endothelial cell precursors. This allows vessels to grow as they form branches and sprout new vessels. [54]

Among the markers for angiogenesis are vascular endothelial growth factor A (VEGF-A)[55], VEGF-C[56], kinase insert domain receptor (KDR/VEGFR-2)[57], matrix metallproteinase-2 (MMP-2)[58], fibroblast growth factor 2 (FGF2)[59], angiopoietins and Tie2 [60] and circulating angiogenic cells (CAC). [47]

Circulating angiogenic cells (CAC) are relevant for ED, since they are a subtype of circulating endothelial progenitor cells (EPC), which correlate inversely with the degree of ED in humans at various degrees of cardiovascular risk.[61]

Some markers for inhibition of angiogenesis are thrombospondin-1 (TSP-1)[62], angiostatin[63] and transforming growth factor beta (TGF beta).[64]

TSP-1 binds to free- and or cell-associated VEGF [65] and scavenges EC membrane-associated FGF2. [66] Thus, TSP-1 regulates the bioavailability of VEGF and FGF2 in each cell's micro-environment. [67]

Vasodilation and constriction

Appropriate reaction to changes in blood pressure of the endothelium is necessary to ensure adequate perfusion and is often impaired in capillaries due to ED. Agents that induce vasodilation, which is the enlargement of blood vessels, involve nitric oxide (NO), prostacyclins, various endothelium-derived hyperpolarizing factors and C-type natriuretic peptide.

Vasoconstrictors on the other hand, cause blood vessels to constrict and include endothelin-1 (ET-1), angiotensin II (Ang II), thromboxane A2,[68] and reactive oxygen species (ROS).[69]

Angiotensin II (Ang II) which is an important vasoconstrictor, also plays a part in inflammation[70] and the activation of NAD(P)H oxidase and consequently increases ROS levels, which leads to ED.[70]

Inflammation

The inflammatory processes in ED cause a vicious cycle, which causes further damage to the endothelium and consequently potentiates inflammation.

Markers for inflammation include nitric oxide (NO), intercellular adhesion molecule-1 (ICAM-1), soluble ICAM-1 (sICAM-1), vascular adhesion molecule-1 (VCAM-1), endothelial selectin (E-selectin), nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB), platelet selectin (P-selectin), interleukin-6 (IL-6), IL-7, tumor necrosis factor alpha (TNF-alpha), TNF-receptor 1 (TNFR1), TNF-receptor 2 (TNFR2), matrix metalloproteinase (MMP-9), high-sensitive C-reactive protein (hs-CRP), complement component 4B (C4B), inactivated complement component 3b (iC3b), monocyte chemoattractant protein-1 (MCP-1),[71] myeloperoxidase (MPO), [72] growth differentiation factor 15 (GDF-15), [73] macrophage inflammatory protein 1beta (MIP-1beta), [74] soluble urokinase plasminogen activator receptor (suPAR), [75] heparanase (HPSE), [76] IL-12p70[77] and galectin-3.[78]

Some relevant anti-inflammatory markers are IL-10 [71] and galectin-1. [79]

Activation of NF-kB, is also measured as an inflammation indicator. NF-kB is an essential regulator of the NO availability, and thus leads to reduced NO levels upon activation, which consequently elevates VCAM-1 expression in EC.[80]

Elevation of MPO in the serum indicates significant increase in leukocyte priming, which is known to be associated with inflammation and increased production of

ROS [81] Numerous lines of evidence implicate this inflammatory protein as a participant in vascular injury, and increased levels of MPO have been associated with increased risk of atherosclerosis and incident major adverse cardiac events in patients presenting with chest pain. [82–84]

Oxidative stress

Oxidative stress with increased ROS leads to ED, since the endothelium-dependent vasodilation can be restored to normal levels after anti-oxidative treatment. [85] Moreover, there is a link between dysfunctional endothelium-dependent relaxation, the severity of clinical manifestations and increased oxidative stress levels. [86] The markers of ED are correlating with the degree of endothelial function, regardless of kidney function[87] and diabetes diagnosis. [88]
Another not completely elucidated function of ROS in the response to endothelial damage, is anoikis, which is a type of programmed cell death similar to apoptosis. It leads to a separation of EC from extracellular matrix and ROS levels are increased intracellularly.[89]
Markers of oxidative stress include radical oxygen species (ROS), 3-Nitrotyrosin (3-NT), [90] asymmetric dimethylarginine (ADMA), L-homoarginine/ADMA ratio (hArg/ADMA ratio),[91] symmetric dimethylarginine (SDMA), hArg/SDMA ratio, [92] thrombomodulin (TM), [93] superoxide dismutase 2 (SOD2) [94] and catalase. [95]

Nitric oxide (NO) and endothelial NO synthase (eNOS)

Nitric oxide (NO) is a vasodilator of the endothelium, it also plays part in inhibition of inflammation, growth, as well as thrombocyte aggregation. The NO reduction in association with ED is a well-documented occurrence. One of the reasons of NO reduction, is diminished endothelial NO synthase (eNOS) activity. This might be due to endogenous or exogenous inhibitors or caused by a reduction in the availability of its substrate, L-arginine. Another cause is decreased NO bioavailability. The radical oxygen species (ROS), superoxide ($O2\cdot-$) can cause NO inactivation by transformation to peroxynitrite ($ONOO\cdot-$). [96]
This is a highly reactive oxidant, which can impair protein functionality subsequent to protein nitration. It also plays a key role in the oxidation process affecting low-

density lipoproteins, thus highlighting its contribution to the development of atherosclerosis. [97]

Peroxynitrite also instigates the breakdown of tetrahydrobiopterin (BH4), a cofactor vital for eNOS function, which causes “uncoupling” of eNOS. [98]

A surplus of oxidants additionally prompts BH4 to diminish, causing a rise in dihydrobiopterin (BH2) levels. This shift disrupts the formation of eNOS's active dimer, which is vital for oxygenase activity and subsequent NO production. Essentially, eNOS becomes "uncoupled."

Consequently, eNOS's reductase activity is upregulated, leading to elevated ROS production. Effectively, the enzyme transitions from generating NO (via its oxygenase role) to generating ROS (via its reductase role). This further intensifies the oxidant imbalance and has a detrimental impact on both endothelial and vascular functionality. [99]

Surplus oxidation is associated with the vessel wall exhibiting a pro-inflammatory condition. The ROS increase the expression of adhesion molecules e.g. VCAM-1 and ICAM-1, as well as molecules responsible for chemotaxis, such as macrophage chemoattractant peptide-1 (MCP-1). [97]

Reduced bioavailability of NO is linked to inflammation. For example, studies have demonstrated that the inflammation associated C-reactive protein (CRP) inhibits the function of endothelial nitric oxide synthase (eNOS). [100]

Associated is also prolonged adenosine monophosphate-activated protein kinase (AMPK) activation, which causes vascular endothelial impairment. This might be caused by reduced availability of eNOS due to faster breakdown or decreased eNOS expression. [101]

A normal plasma nitrate level on the other hand suggests normal systemic production of NO. Nitrate is produced by the reaction of NO with oxyhemoglobin (HbFeIIO2), resulting in methemoglobin (HbFeIII) and nitrate (NO3−) formation. This pathway is considered the major inactivation pathway of vascular NO. [102]

Asymmetric dimethylarginine (ADMA)

Asymmetric dimethylarginine (ADMA), which is a metabolite of L-arginine is involved in another important pathway causing NO decrease. This molecule acts as an intrinsic competitive inhibitor of eNOS and has been implicated in ED.[103]

Furthermore, ADMA concentrations displayed an inverse relationship with endothelium-dependent vasodilation [104] in patients with hypercholesterolemia. The administration of L-arginine, which is both the substrate for eNOS and a competitor of ADMA, successfully restored normal endothelial function. The build-up of this internal eNOS inhibitor is thought to contribute to diminished effective plasma flow, as well as elevations in vascular resistance and blood pressure (BP). [105] Intravenous administration of a low dose of ADMA led to reduced heart rate and cardiac output while raising mean BP. [106]

In individuals experiencing renal failure, elevated levels of ADMA are associated with increased cardiovascular risk factors. These include, but aren't limited to, C-reactive protein (CRP)[107], the thickness of the carotid intima-media[108], concentric left ventricular hypertrophy, and impaired left ventricular function. [109] It also has prognostic value of acute coronary events[110] and mortality rates in critically ill patients.[111] Symmetric dimethylarginine (SDMA) is similar but is a more specific marker for kidney function and used as a control parameter for ADMA values. [112]

Cardiovascular disease risk markers

Cardiovascular events are caused by ED and patients diagnosed with FD often experience cardiac complications. This encompasses dyspnea, arrythmia, left ventricular hypertrophy, angina pectoris and myocardial infarction.[113]

Several markers for cardiovascular damage might be elevated due to ED and its long-term consequences. Among them are brain natriuretic peptide (BNP), mid-regional pro-atrial natriuretic peptide (MR-pro ANP), [114] L-homoarginine (hArg), [115] high density lipoprotein-cholesterol (HDL-C)/Total cholesterol ratio, [116] vascular endothelial cadherin (VECAD) [117] and syndecan-1. [118]

Hypercholesterolemia is a well-recognized risk factor for atherosclerosis and is intertwined with endothelial dysfunction. [119]

Endothelin-1 is implicated in multiple cardiovascular diseases[120], but the plasma and tissue levels can differ significantly, which can lead to false negative values measured in plasma, while tissue levels are increased.[121]

Prothrombotic state

The prothrombotic state observed in ED not only further increases inflammation, but also leads to clinical manifestations like embolisms, strokes and other thrombotic events, which are observed in FD patients. [113]

Markers for prothrombotic processes include plasminogen activator, tissue factor inhibitor, von Willebrand factor (vWF), NO, prostacyclin, thromboxane A2, plasminogen-activator inhibitor-1, fibrinogen, soluble tissue factor (sTF), beta thromboglobulin (beta TG), anti-platelet factor 4 (PF4) [122] and thrombin-antithrombin III complex (TAT III). [123]

An important prothrombotic marker is vWF, a glycoprotein with adhesive properties and critical importance in the initial binding of platelets and the subsequent development of a thrombus. [124] Elevated levels are indicative of a heightened likelihood for developing thrombotic cardiovascular conditions, such as strokes and coronary heart disease. [125]

A relevant antithrombotic marker is A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS-13). [126]

Other markers of ED

Several other changes were observed in ED, which are involved in distinct aspects of ED.

One of these is the thickness of the glycocalyx layer, which is on the uppermost portion of the endothelial cells and extends into the vessels. It is of utmost importance for sustaining the structural integrity of blood vessels and maintains cardiovascular homeostasis. The partial degradation of the endothelial glycocalyx by the release of heparanases is one of the first responses to an inflammatory stimulus within the endothelium. Persistent degradation results in a chronic inflammatory response, leading to a severe damage of the vascular endothelium. [127] with impaired vascular permeability. [128,129]

Other potential markers are microRNAs (miRNA), which are RNA molecules lacking protein-coding capabilities, but are critically involved in regulation of gene expression.[130] They are involved in the pathogenesis of atherosclerosis among other diseases. [131]

Endothelial cell membrane derived microparticles (EMP) are vesicular formations released by endothelial cells undergoing activation or programmed cell death. They are integral in inflammation, thrombus formation, angiogenesis and vascular homeostasis.[132] EMP have been described as useful predictive factors for ED and are potentially part of a vicious cycle with ED increasing EMP levels and EMP further damaging the endothelial function. [133]

Beta-actin (ACTB) plays a crucial role in a multitude of cellular functions. It is heavily implicated in cellular movements, structural maintenance, and ensuring the overall stability of cells. But since there is an interaction between ACTB and profilin-1 (PFN1), with the endothelial nitric oxide synthase (eNOS), it could also function as a possible indicator of ED.[134,135]

The protein caveolin-1 is thought to be involved in the control of how quickly eNOS is broken down. This, in turn, influences the amount of nitric oxide (NO) released when blood vessels experience shear stress.[136].

1.6 Knowledge and research gap

A wide variety of clinical phenotypes in relation to ED and endothelial damage are possible in FD. Available literature addressing both, clinical manifestations and cellular changes due to substrate accumulation and in relation to remaining enzyme activity or patients´ genotype is sparse. As ED is a prominent manifestation of FD it is essential to establish which phenotypes occur predominantly in patients and if they are reversible upon therapy.

1.7 Reasoning behind the research objective

Since ED in FD can lead to systemic damage and consequently to organ failure and death, it is important to recognize its pathophysiological processes, clinical results, potential prognostic markers, which might lead to novel intervention options.

A better understanding of ED and the underlying mechanisms in FD would be important for both, men and women, since female, heterozygous FD patients can develop equally severe symptoms as hemizygous men.

1.8 Aims and objectives

The specific aim of the thesis is to provide a comprehensive overview of all measured clinical phenotypes and markers of ED in FD patients and to assess their responsiveness to Fabry-specific therapy based on available data. This will be achieved by following three objectives:

1) To generate an overview of all clinically assessed parameters pertaining to ED for FD patients before therapy administration;

2) To generate an overview of all measured markers of ED in FD patients before therapy vs healthy controls; and

3) To compare clinical phenotypes and respective ED marker in FD patients before and after therapy.

A sub-aim of the study is to establish an association between therapy responsiveness and patients' genotype. For this purpose, patients' genotypes will be extracted and associated with clinical data and markers of ED.

Another sub-aim is to assess functionality and markers of ED in *in vitro* models as well as documenting other ED specific epigenetic or proteomic changes in FD.

2 Methodology

For this thesis, a systematic review of the existing literature on 'Endothelial dysfunction in Fabry disease' was conducted. Only primary sources of literature listed in the PubMed database regarding the respective topic were included. Beforehand, appropriate MESH terms were identified and used in PubMed.
The search used typical database search techniques such as quotation marks, apostrophe, parentheses, asterisk and Boolean operators 'AND', 'OR' and 'NOT' to specify the search target and cover related synonymous and compatible terms.
First search term: ((fabry) AND ((endothelium) OR (endothelial cells) OR (endothelial dysfunction))
A first approach to search for scientific publications on this topic yielded a large number of results, with 438 in total. Therefore, the search term needed to be adapted to reduce the number of results. Following additional criteria for inclusion were defined:

- the publication date cannot be before 1988, since earlier articles contained no relevant information or were not accessible
- cannot be a review
- can be human or cell-model study

With this, a new search term was created:
(fabry) AND ((endotheli*) OR (endothelial cell*) OR (endothelial dysfunction*)) NOT ("review"[Publication Type]) AND (("1988"[Date - Publication] : "2024/09/25"[Date – Publication]))
This term led to 363 results (25.9.24). After manually scanning through the abstracts and accessible articles, 110 articles were included. Excluded articles did not contain relevant information regarding the endothelium or FD (157), were false positive due to authors named Fabry (52) or were describing studies using animal models (26).
A spreadsheet containing the most important data from all papers was created. Based on different types of studies and different aspects of ED the articles and the results in this thesis were accordingly divided in different categories.

3 Results

A total of 110 articles consisting of case studies, clinical trials and original research articles including cellular models were identified as relevant to the outcome and included in the systematic overview. The review of relevant articles showed that while some articles had information on multiple aspects, most of the literature focused on specific aspects of endothelial function.
Based on the thesis objectives the wide variety of different types of studies and analyzed endothelial characteristics, the research articles and results were categorized into following sections:

- Part 1) Clinical aspects of ED in FD patients and response to therapy
- Part 2) Systemic markers of ED in FD patients before and after ERT
- Part 3) Gene variants reported across publications
- Part 4) Functional assessment of ED and markers of ED in cellular models
- Part 5) Epigenetic and proteomic aspects of ED in FD

Part 1 describes clinical changes regarding ED in analyzed patients and therapy responses. In this part 73 articles were included.
In Part 2 the findings of 37 publications addressing systemic markers for ED in FD patients were collected and compared.
In Part 3 different gene variants of *GLA* gene, which were described in 110 publications, are listed and sorted into categories.
Part 4 discusses changes and markers in cellular models for FD, 23 articles were included in this part.
Part 5 includes the remaining 8 research articles, which look at aspects including microRNA changes, DNA-methylation, modifier genes and *in silico* models for FD.

3.1 Part 1: Clinical aspects of ED in FD patients and response to therapy

There is a plethora of possible clinical phenotypes regarding ED in FD patients. Overall, in the included research papers clinical phenotypes of 648 males and 180 females are described.

3.1.1 Angiokeratoma corporis diffusum

Angiokeratoma were reported in 57.4% of 134 FD patients, which were examined in 12 publications. [137–148]

After therapy

Therapy effect was analyzed in only one publication, which reports that after ERT with Fabrazyme for 5 years angiokeratoma were still present in 31 males. [149]

3.1.2 Histological aspects

The Gb3/GL3 accumulation in vascular EC is a well documented clinical aspect and was described in 42 publications.

It was observed in 82.5% of 258 examined FD-patients. [143,145,146,150–162] and in 82.8% of 821 total examined EC.

The superficial skin capillary EC (SSEC) were affected in 56.5% of the 205 total examined patients,[137,139,141,143,144,146,148,151,153,160,163–166] in female participants only 4.4% of 67 [137,139,141,151,153] and in males 81.8% of 138 were affected. Deep vessel EC showed Gb3 accumulation in 87.6% of 81 examined patients [143,151], kidney capillary EC in 95.7% of 118 patients [32,143,150,154,163,167–171], glomerular capillary EC in 89% of 91 patients [152,157,159,161,165,167,171–175], peritubular capillary EC in 98.4% of 64 patients [143,152], hearth capillary EC in 86.8% of 160 patients [150,155,156,158,159,162], non-capillary EC in 88.8% of 27 patients [143,171], arterial/arteriolar EC in 95.3% of 43 patients [152], muscle EC in all of 12 patients [156], interstitial EC in all of 13 patients [161,167], sural nerve/epineurial EC in one case [138], HUVEC in one case [176] and in not specified EC in two cases. [145,177]

In one case the sweat gland surrounding vessel EC were affected [178], suggesting that this might be involved in hypohydrosis, one with bronchiolar/arteriolar EC [179], pulmonary clinical manifestations with reduced total lung capacity and one with splenic sinusoidal EC [180], with splenomegaly and hypersplenism due to compromised splenic bloodflow.

Typical observed Gb3 caused manifestations were myelin like depostis, lamellated inclusions and vacuolation..[137–139,141,144,148,154,159,163–165,167–169,172–174,176–179]
Other observations in microscopic studies of EC are “sunbursts” villus like structures [166], zebra bodies, arteriolar hyalinization and occlusion, numerous osmiophilic inclusions in EC of glomeruli [174] and toluidine blue staining in EC. [170]
Another publication showed EC degeneration and death with subsequently transformation to depocytes. [181]
In only two publications, no patient showed accumulation of Gb3 in the endothelium. These publications investigated the cardiac late onset IVS4+919G>A variant [158] and VUS p.A143T, p.D313Y and p.S126G. [160]

After therapy

Changes in Gb3-accumulation and histological aspects after ERT were reported in 20 publications. For changes after chaperone therapy insufficient data was found with the used searchterm, this might be due to longer availability of ERT in comparison. They showed complete clearance in the EC of FD patients in 84% of 315 FD patients treated with ERT [32,146,149–154,157,162,171,175,182–187] and in 89% of 714 total examined EC.
SSEC showed total clearance in 92.1% of 191 examined patients [146,149–151,153,182,184], deep vessel EC in 79.7% of 64 [149,151], kidney capillary EC in 92.8% of 125 [32,150,154,171,182,183,186], glomerular capillary EC in 97.4% of 79 [152,157,171,175,183], peritubular capillary EC in 96% of 49 [152], hearth capillary EC in 76.2% of 139 [150,155,158,162,182,184,187], arterial/arteriolar EC in 91% of 44 [152], saphenous vein tunica intima and adventitia EC in 3 cases [184,185] and in non-capillary EC in 20 cases.[171]
Total clearance was reported in most of the FD patients after 5 to 6 months of therapy[149,153,162] and it correlated positively with duration of ERT [150] and start of therapy at earlier age. [187] Interestingly, in a study by Ramaswami et al. after 5 years of ERT there was no improvement in the endothelial alterations, angiokeratomas, arteriopathy, retinal artery dilation and tortuosity, despite effective Gb3 clearance. [149] In the biopsies of a 20 year old male with p.T412Sfs38 genotype Gb3 persisted.[154] No significant differences between genotypes and sexes could be found in this population.

3.1.3 Intima media thickness (IMT) and flow mediated dilation (FMD)

The IMT and FMD were measured in 5 publications. The IMT was increased in different vessels like carotid, brachial and aortic arteries in 87% of 79 and the FMD was reduced in 88% of 85 studied patients compared to controls.[188–192] In the carotid arteries of 33 FD patients the IMT was increased and FMD decreased, but less atherosclerotic plaques were found compared to controls. [191]

After nitroglycerin application the normal endothelium independent vasodilation was observed, while the endothelium dependent vasodilation as response to reactive hyperemia was impaired. [192]

In 10 cases no significant differences in cerebral hemodynamic, IMT or FMD were described compared to controls. [188]

After therapy

Only two studies evaluated the influence of ERT on IMT and FMD.

The study by Rombach et al. analyzed 67 FD patients who received ERT. Compared to controls, only 22 male patients with classic FD (n=22), showed increased IMT and reduced FMD. In 35 females with classic FD, 5 females and 5 males with atypical FD no changes were observed. However atypical male FD patients demonstrated increased AGEs values. In this population, elevated lysoGb3 levels (>7 nmol/L) contributed to a 2.9% lower FMD, independent of age and sex (p=0.02). [193]

In 4 FD patients with genotype p.G373S the FMD was improved by ERT for one year with agalsidase beta. [194]

3.1.4 Reactive hyperemia index (RHI), augmentation index (AI) and circulating angiogenic cells (CAC)

In a publication by Lorenzen et al., for 26 FD patients RHI values indicating ED were measured. Arterial stiffness was also measured, using AI. Arterial stiffness showed tendency towards increased values in FD patients, but remained not significant. Regarding left ventricular hypertrophy (LVH), the only significant correlation was with AI, which is age-dependent. Therefore, the onset of cardiac hypertrophy was determined to be likely age-dependent as well.

The same publication describes elevated numbers of circulating angiogenic cells (CAC) and their impaired angiogenic functions possibly due to Gb3 accumulation.

Treatment with ERT for one year was started for 16 patients, which attenuated CAC dysfunction and reduced numbers to normal levels.
Treatment with ERT for one year normalized RHI values. [47]
In contrast to Lorenzen et al., which found no changes in AI, Stamerra et al, report 4 FD patients with genotype p.G373S and an increased AI, which significantly improved after one year of ERT with agalsidase beta. [194]

3.1.5 Nailfold capillaroscopy (NFC)

In a study by Faro et al., 11 males and 14 females were examined with nailfold capillaroscopy (NFC). In classic FD patients reduced capillary length, loop tortuosity below 20%, ectasia of the subpapillary plexus, angiotectonic disorder and neovascularization was visible. In the VUS p.A143T, p.R220X, and p.S126G the loop tortuosity was between 20 and 50%, angiotectonic disorder and neovascularization were observed. In late onset patients the loop tortuosity was between 20 and 50%, but no other changes were visible. Overall no avascular areas, giant capillaries, microhemorrhagia or thrombosed loops were found.[195]
Another publication using NFC in FD patients was published by Costanzo et al., where they report atypical capillaries in 52.6% compared to none in controls and irregular nailfold capillaroscopy architecture in 78.9% vs 36.8% in controls. [189]

3.1.6 Capillary impairment

In 13 cases with p.N34H, in 3 with p.L54P and in one unknown genotype, corneal confocal microscopy (CCM) was performed and no significant differences to healthy controls were found in corneal EC density, cell area, cell perimeter, pleomorphism and polymegathism. [196]
Optical coherence tomography (OCT) was performed in 11 male and 14 female FD patients. Lower vascular density in superficial and deep capillary plexuses, larger foveal avascular zone and equal density of radial peripapillary capillaries was observed compared to controls indicating retinal microvascular alterations related to FD. [197]
The glomerular capillary endothelial coverage was significantly less fenestrated in FD-patients compared to controls, it was even less in the mesangial than in the peripheral zone. [198]

Reduced endothelial fenestration was also observed in 14 children, indicating increased impermeability which could lead to reduced filtration rate. [140]

3.1.7 Cardiac manifestations

Angina pectoralis was described as symptom of ED by Chimenti et al. [155] In two p.E341X females with cardiac manifestation and two p.R227P females, it was shown that ED was involved in cardiomyopathy. [199] Using intravascular ultrasound of coronary arteries on 9 FD patients, diffuse plaques, hypoechogenicity and more lipid cores were observed by Kovarnik et al. [200]

After therapy

In 13 FD patients with angina pectoralis, hypertrophy, proliferation and engulfment by Gb3 was observed in EC. They also demonstrated perfusion defects, slow coronary flow, and luminal narrowing of intramural arteries associated with replacement fibrosis of the myocardium. They had a mean REA of 8 mol/L/h, 5 were female, the mean age was 48.5±12.1 years. The genotypes represented in the study population were p.Q279K, p.C946delG, p.R227Q, p.Y216C, p.N215S, p.C378Y and p.G328R. No reduction in the accumulation of Gb3 in EC was observed in the 5 reevaluated patients after one year of ERT. Also, no improvement of coronary slow flow and intramural coronary artery narrowing was observed. [155]

In a study by Hsu et al. 17 male and 5 female FD patients aged 47 to 75 years with the *GLA* variant IVS4+919G>A, were examined. They had no Gb3 in capillary EC, but in cardiomyocytes. They all had increased LVH and 12 had hypertension. In 5 patients without ERT and GLA-activity between 1.15 and 2.75nmol/hr/ml, the lysoGb3 levels were at 3.21-7.47nM. In 17 patients with ERT with AGAL for 8 to 51 months the lysoGb3 levels were at 2.12-14.54nM. This was interpreted as a cardiac phenotype with late onset and the ERT showed no significant improvement. [158]

In a study by Gregório et al. with 37 female and 15 male FD patients, ERT was given for 62 ±SD 27.5 months. The patients were divided by being below or under the age of 40 at ERT initiation. The gene variants were listed with p.Y365X in 36, p.W47X in 9, and 7 cases with p.R118C, p.G11Afs*110 or p.W204X. However, the clinical data was not directly linked to the different genotypes. The interventricular

septal thickness (IVST) was significantly higher in patients who initiated ERT later. [201]

3.1.8 Hypertension

Elevated blood pressure was observed in 41.4% of 123 examined patients in 11 publications. [139,158,167,168,170,173,190,194,202–204] One case with p.A37P variant without early symptoms showed renal failure at 25 years and a blood pressure at 200 over 120 mmHg. [202] One 40 year old male FD patient exhibited hypertension and end stage renal disease (ESRD). [173] In a chinese population of 9 patients, no hypertension related to FD was observed. [170] In 4 FD patients with p.G373S genotype only the males showed hypertension. [194]

After therapy

In 40 patients treated with agalsidase beta, patients in their first 3 years of ERT had significantly reduced systolic and diastolic blood pressures. Patients who already had 3 years of ERT, showed significantly decreased systolic blood pressure after 4 to 7 years of therapy. [205]

In 4 FD patients with genotype p.G373S the blood pressure values were maintained in the normal range by ERT for one year with agalsidase beta.[194]

In a study by Hsu et al. in 58.8% of 17 FD patients increased BP was measured after ERT. [158]

3.1.9 Prothrombotic state

Massive thrombosis and stroke were reported in one male patient with p.R227X variant [206] and recurrent thrombosis in a 57 year old female with p.R342Q variant. [145]

3.1.10 Severe clinical events

Severe clinical events e.g. renal failure, cardiac events, stroke and death, as well as Gb3 clearance were recorded in a cohort of 1044 FD patients.

Treatment with agalsidase beta (Fabrazyme) 1 mg/kg/2 weeks, cleared EC of Gb3 after 6 months. In the first 6 months of therapy severe clinical events were 111 per 1000 person-years (95% CI 84 to 145), after 6 months there were 40–58 events per 1000 patient-years, which showed a decrease in their frequency after ERT. [207]

3.2 Part 2: Systemic markers of ED in FD patients before and after ERT

A plethora of markers related to ED were analyzed in FD and reported in 19 publications. To facilitate the presentation of the results, markers of ED were divided into categories according to their respective role in: pro- and anti-inflammatory, oxidative stress, cardiovascular processes, angiogenesis, antiproliferative, as well as pro- and antithrombotic markers.

Data was collected and summarized separately for therapy naive populations and populations under therapy. In the population under therapy (tFD), the significance levels were inconsistently determined either by comparison to healthy controls (C) or by comparing them to therapy naive, not-treated FD-patients (nFD). The compared groups for each value are shown in the tables 1 to 9.

If available, information about patient gender was included.

3.2.1 Pro-inflammatory markers

Altered levels of 20 inflammatory markers were reported in 12 publications (Table 1). [134,188,191,199,201,208–214] Most of the studied pro-inflammatory markers were significantly elevated in the majority of studied patients, when compared to controls. Among them were sICAM-1, ICAM-1, sVCAM, P-selectin, IL-7, TNFR-1, TNFR-2, MMP-9, IL-6, TNF-alpha, hsCRP, IC3b, C4B, MPO and MCP-1. E-selectin levels on the other hand were reduced in 36% of studied patients.

No significant changes were observed in GDF-15, IL-12p70, suPar and MIP-1beta.

There were differences between sexes in ICAM-1 levels with 88% of males and only 55% of females showing elevation and in the MPO levels with 92% of males and none of the females exhibiting increased levels.

The other markers showed no significant differences between males and females.

After therapy, normalized or improved levels were observed in TNF-alpha, IC3b, C4B, MIP-1beta, MPO and MCP-1. The GDF-15 levels were elevated in 27% of the treated population, but not in the non-treated patients.

No significant changes after therapy were reported for ICAM-1, sVCAM, IL-7, IL-12p70, TNFR 1, TNFR 2, MMP-9, IL-6, hsCRP, suPAR and E-selectin.

Only for TNF-alpha considerable differences in changes after therapy were observed between sexes with males remaining at elevated levels in 76% and

females in 52%. For sICAM-1 and P-selectin no information for changes after therapy was available.

Table 1. Pro-inflammatory markers in FD.

Marker	Elevated in nFD vs C (n)	males (n)	females (n)	tFD vs nFD/C (n)	males (n)	females (n)
sICAM-1	**100% (25)** (25/25 ***) [208]					
ICAM-1	**70% (40)** (29/40 *) [209]	**88% (18)** (16/18 *) [209]	**55% (22)** (13/22 *) [209]	**Unchanged vs. nFD (14)** (14/14N.S.) [209]		
sVCAM	**100% (43)** (14/14 *) [191] (25/25 ***) [208] (4/4 *) [188]		**100% (4)** (4/4 *) [188]	**100%vsC (25)** (19/19 *) [191] (6/6 *) [188]	**100% (4)** (4/4 *) [188]	**100% (2)** (2/2 *) [188]
P-selectin	**100% (25)** (25/25 ***) [208]					
IL-7	**100% (23)** (23/23 S.)[210]	**100% (5)** (5/5 S.) [210]	**100% (18)** (18/18 S.) [210]	**Unchanged vs nFD (46)** (46N.S.) [210]	**100% (29)** (29N.S.) [210]	**100% (17)** (17N.S.) [210]
IL-12p70	**0% (6)** (0/6 N.S.) [211]	**0% (1)** (0/1 N.S.) [211]	**0% (5)** (0/5 N.S.) [211]	**5.5% vsC (18)** (1/18N.S.)[211]	**0% (8)** (0/8 N.S.) [211]	**10% (10)** (1/10N.S.) [211]
TNFR 1	**100% (31)** (31/31 **) [212]	**100% (8)** (8/8 **) [212]	**100% (23)** (23/23 **) [212]	**100%vsC (37)** (37/37 **) [212]	**100% (26)** (26/26 **) [212]	**100% (11)** (11/11 **) [212]
TNFR 2	**100% (31)** (31/31***) [212]	**100% (8)** (8/8***) [212]	**100% (23)** (23/23***) [212]	**100%vsC (37)** (37/37***) [212]	**100% (26)** (26/26***) [212]	**100% (11)** (11/11***)[212]
MMP-9	**100% (41)** (10/10 *)[199] (31/31***)[212]	**100% (8)** (8/8 ***) [212]	**100% (23)** (23/23***) [212]	**100%vsC (49)** (12/12*) [199] (37/37***)[212]	**100% (26)** (26/26***)[212]	**100% (11)** (11/11***)[212]
IL-6	**72% (65)** (14/14**)[191] (31/31*)[212] (2/16N.S.)[213] (0/4 N.S.)[188]	**73% (11)** (8/8 *)[212] (0/3 N.S.)[213]	**78% (50)** (14/14 **)[191] (23/23*)[212] (2/13 **)[213]	**74%vsC(76)** (19/19**)[191] (37/37*)[212] (0/20N.S.)[213]	**74% (53)** (13/13**)[191] (26/26*)[212] (0/14N.S.)[213]	**74% (23)** (6/6**)[191] (11/11*)[212] (0/6N.S.)[213]
TNF-alpha	**67% (55)** (0/14N.S.)[191] (0/4N.S.)[188] (31/31**)[212] (6/6*)[211]	**100% (25)** (8/8**)[212] (1/1*)[211]	**100% (42)** (23/23**)[212] (5/5*)[211]	**76% vs nFD reduced & vs C elevated (74)** (0/19N.S.)[191] (37/37**)[212] (18/18***)[211]	**76% high (34)** (26/26**)[212] (8/8***)[211]	**52% high (21)** (11/11**)[212] (10/10***)[211]
hsCRP	**89% (18)** (14/14**)[191] (0/4 N.S.)[188]			**100%vsC (19)** (19/19**)[191]		

iC3b	**100% (8)** (8/8*)[134]	**100% (8)** (8/8*)[134]		**100%vs nFD Improved (8)** (8/8***)[134]	**100% improved vs nFD (8)** (8/8***)[134]	
C4B	**100% (8)** (8/8**)[134]	**100% (8)** (8/8**)[134]		**100%vs nFD improved (8)** (8/8*)[134]	**100% improved vs nFD (8)** (8/8*)[134]	
suPAR	**0% (10)** (0/10 N.S.)[199]			**0%vsC (12)** (0/12N.S.)[199]		
GDF-15	**0% (6)** (0/6 N.S.)[211]	**0% (1)** (0/1N.S.)[211]	**0% (5)** (0/5N.S.)[211]	**27%vsC (70)** (0/18N.S.)[211] (19/52*)[201]	**0% (8)** (0/8N.S.)[211]	**0% (10)** (0/10N.S.)[211]
MIP-1beta	**0% (6)** (0/6N.S.)[211]	**0% (1)** (0/1N.S.)[211]	**0% (5)** (0/5N.S.)[211]	**100%vs nFD Reduced (18)** (18/18*)[211]	**100% reduced (8)** (8/8*)[211]	**100% reduced (10**) (10/10*)[211]
MPO	**66% (18)** (12/12****)[214] (0/6N.S.)[211]	**92% (13)** (12/12****)[214] (0/1 N.S.)[211]	**0% (5)** (0/5N.S.)[211]	**0%vsC (24)** (0/6N.S.)[214] (0/18N.S.)[211]	**0% (14)** (0/6N.S.)[214] (0/8N.S.)[211]	**0% (10)** (0/10N.S.)[211]
MCP-1	**100% (6)** (6/6*)[211]	**100% (1)** (1/1*)[211]	**100% (5)** (5/5*)[211]	**100%vs nFD reduced (18)** (18/18**)[211]	**100% reduced (8)** (8/8**)[211]	**100% reduced (10**) (10/10**)[211]
	Reduced					
E-selectin	**36% (39)** (14/14**)[191] (0/25N.S.)[208]			**100%vsC (19)** (19/19**)[191]		

Data in bold represent the percentage of FD patients showing altered levels of respective marker in relation to the total number of analyzed patients (in parentheses) in a total of 12 publications. Comparison vs. C refers to elevation, except for E-selectin. The significance level is indicated for tFD vs. C or for tFD vs. nFD. Data listed in parentheses describe the numbers of patients in individual sources.
Abbr: n=number of studied FD patients, C=healthy controls, nFD=non-treated FD patients, tFD=treated FD patients; ICAM-1 = intercellular adhesion molecule-1, sICAM-1 = soluble ICAM-1, sVCAM = soluble vascular cell adhesion molecule-1, IL = Interleukin, TNFR = Tumor necrosis factor receptor, MMP-9 = Matrix Metalloproteinase 9, TNF-alpha = Tumor necrosis factor alpha, hsCRP = high sensitive C-reactive protein, iC3b = inactivated Complement component 3b, C4B = Complement component 4, suPAR = soluble urokinase plasminogen activator receptor, GDF-15 = Growth Differentiation Factor 15, MIP-1beta = Macrophage infammatory protein 1beta, MPO = Myeloperoxidase, MCP-1 = Monocyte chemoattractant protein-1.
*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, N.S.=not significant

3.2.2 Anti-inflammatory markers

Altered levels of two anti-inflammatory markers were reported in two publications (Table 2). [211,212] Galectin-1 was significantly elevated in the all studied patients compared to controls, while the levels of IL-10 were unchanged.

No significant differences between sexes and treated or non-treated patients were observed.

Table 2. Anti-inflammatory markers in FD.

Marker	Elevated in nFD vs C (n)	males (n)	females (n)	Elevated in tFD vs C (n)	males (n)	females (n)
IL-10	**0% (6)** (0/6 N.S.)[211]	**0% (1)** (0/1 N.S.)[211]	**0% (5)** (0/5 N.S.)[211]	**5.5% (18)** (1/18N.S.)[211]	**12.5% (8)** (1/8 N.S.)[211]	**0% (10)** (0/10N.S.)[211]
galectin-1	**100% (31)** (31/31***)[212]	**100% (8)** (8/8***)[212]	**100% (23)** (23/23***)[212]	**100% (37)** (37/37***)[212]	**100% (26)** (26/26***)[212]	**100% (11)** (11/11***)[212]

Data in bold represent the percentage of FD patients showing altered levels of respective marker in relation to the total number of analyzed patients (in parentheses) in a total of 2 publications. Data listed in parentheses describe the numbers of patients in individual sources. n=number of studied FD patients, C=healthy controls, nFD=non-treated FD patients, tFD=treated FD patients;
***p<0.001, N.S.=not significant

3.2.3 Oxidative stress and cardiovascular markers

Altered levels in 11 markers of oxidative stress or cardiovascular damage were reported in 8 publications (Table 3). [134,199,201,208,209,212,215,216] Of the studied markers 3-NT, ACTB, BNP and MR-pro ANP were significantly elevated in all studied patients, compared to controls. Significantly increased levels were also observed for SDMA in 40%, for HDL-C/Total cholesterol ratio in 32% and for ADMA in 28.5% of patients. The hArg/SDMA ratio and TM levels on the other hand were reduced in 40% and 100% of studied patients respectively. No significant changes were observed in hArg and the hArg/ADMA ratio. There were differences between sexes in HDL-C/Total cholesterol ratio levels with 73% of the males and none of the females showing elevation. The other markers showed no significant differences between males and females. After therapy normalized or improved levels were observed in 3-NT and ACTB, while ADMA levels were only slightly reduced to 12.5% when compared to controls. The hArg/SDMA ratio was reduced in 92% of the treated population, but only in 40% of the non-treated patients and

the SDMA levels were accordingly elevated in the same population, compared to controls. SDMA levels also were only elevated in FD patients with cardiomyopathy (CM).

No significant changes after therapy were reported in hArg, hArg/ADMA ratio, BNP and MR-pro ANP concentrations. For HDL-C/Total cholesterol ratio and TM no information for changes after therapy was available.

Table 3. Oxidative stress and cardiovascular markers in FD

Marker	nFD vs C (n)	males (n)	females (n)	tFD vs nFD/C (n)	males (n)	females (n)
3-NT	**100% (13)** (13/13**)[215]	**100% (13)** (13/13**)[215]		**0%vs C (52)** (52 N.S.)[201]	**0% (15)** (15 N.S.)[201]	**0% (37)** (37 N.S.)[201]
ADMA	**28.5% (14)** (4/4*)[216] (0/10N.S.)[199]	**100% (2)** (2/2*)[216]	**100% (2)** (2/2*)[216]	**13%vsC(16)** (2/4*)[216] (0/12N.S.)[199]	**50% (2)** (1/2*)[216]	**50% (2)** (1/2*)[216]
SDMA	**40% (10)** (4/10**)[199]			**92%vsC(12)** (11/12**)[199]		
hArg	**0% (10)** (0/10N.S.)[199]			**0%vsC (12)** (0/12N.S.)[199]		
hArg/ADMA ratio	**0% (10)** (0/10N.S.)[199]			**0%vsC (12)** (0/12N.S.)[199]		
ACTB Beta-actin	**100% (8)** (8/8*)[134]	**100% (8)** (8/8*)[134]		**100%vsnFD Improved (8)** (8/8**)[134]	**100% Improved (8)** (8/8**)[134]	
HDL-C/Total cholesterol ratio	**32% (67)** (22/67*)[209]	**73% (30)** (22/30*)[209]	**0% (37)** (0/37N.S.)[209]			
BNP	**100% (31)** (31/31**)[212]	**100% (8)** (8/8**)[212]	**100% (23)** (23/23**)[212]	**100%vsC (37)** (37/37**)[212]	**100% (26)** (26/26**)[212]	**100% (11)** (11/11**)[212]
MR-pro ANP	**100% (31)** (31/31#)[212]	**100% (8)** (8/8#)[212]	**100% (23)** (23/23#)[212]	**100%vsC (37)** (37/37#)[212]	**100% (26)** (26/26#)[212]	**100% (11)** (11/11#)[212]
	Reduced					
hArg/SDMA ratio	**40% (10)** (4/10**)[199]			**92% (12)** (11/12**)[199]		
TM	**100% (25)** (25/25*)[208]					

Data in bold represent the percentage of FD patients showing altered levels of respective marker in relation to the total number of analyzed patients (in parentheses) in a total of 8 publications. Comparison vs. C refers to elevation, except in hArg/SDMA ratio and TM. The significance level is indicated for tFD vs. C or for tFD vs. nFD. Data listed in

parentheses describe the numbers of patients in individual sources. n=number of studied FD patients, C=healthy controls, nFD=non-treated FD patients, tFD=treated FD patients; 3NT = 3-Nitrotyrosine, ADMA = Asymmetric dimethylarginine, SDMA = symmetric dimethylarginine, hArg = L-homoarginine, ACTB = Beta-actin, HDL-C = High density lipoprotein-cholesterin, BNP = Brain natriuretic peptide, MR-pro ANP = Mid-regional pro-Atrial natriuretic peptide, TM = thrombomodulin
*p<0.05, **p<0.01, #p=0.013, N.S.=not significant

3.2.4 Angiogenesis markers

Eight published studies revealed altered levels of four angiogenesis indicators (Table 4). [199,203,204,209–212,217] In the majority of the reports, these were notably higher in all examined subjects, with the exception of VEGF-A, which showed an increase in 65% of cases when compared to the control group.

When considering VEGF-A levels, treated patients presented a more frequent elevation – at 90% – than those patients that didn't receive any treatment, compared to controls. It was documented that MMP-2 and VEGF-C showed no significant shifts after treatment. FGF2 contrarily showed normalized or improved levels with only 58% of males and a total of 76% being elevated, while the levels in females remained unchanged. The other markers showed no differences between sexes.

Table 4. Angiogenesis markers in FD

Marker	nFD vs C (n)	males (n)	females (n)	tFD vs nFD/C (n)	males (n)	females (n)
VEGF-A	**67% (115)** (24/40?)[209] (23/23***)[210] (18/18*)[204] (9/16**)[203] (0/10 N.S.)[199] (2/2*)[217] (1/6*)[211]	**78.5% (28)** (12/18?)[209] (5/5***)[210] (4/4*)[204] (1/1*)[211]	**75% (46/61)** (12/22?)[209] (18/18***)[210] (14/14*)[204] (2/2*)[217] (0/5 N.S.)[211]	**90% vs C (164/182)** (14/14?)[209] (46/46 N.S. vs nFD)[210] (30/30*)[204] (13/19*)[203] (0/12N.S.)[199] (43/43*)[217] (18/18*)[211]	**100% (78)** (29/29***)[210] (20/20*)[204] (21/21*)[217] (8/8*)[211]	**100% (59)** (17/17***)[210] (10/10*)[204] (22/22*)[217] (10/10*)[211]
VEGF-C	**100% (23)** (23/23*)[210]	**100% (5)** (5/5*)[210]	**100% (18)** (18/18*)[210]	**Unchanged vs nFD (46)** (0/46 N.S.)[210]	**Unchanged (29)** (0/29N.S.)[210]	**Unchanged (17)** (0/17N.S.)[210]
MMP-2	**100% (31)** (31/31*)[212]	**100% (8)** (8/8*)[212]	**100% (23)** (23/23*)[212]	**100% vs C (37)** (37/37*)[212]	**100% (26)** (26/26*)[212]	**100% (11)** (11/11*)[212]
FGF2	**100% (25)** (23/23***)[210] (2/2**)[217]	**100% (5)** (5/5***)[210]	**100% (20)** (18/18***)[210] (2/2**)[217]	**76% vs C (89)** (46/46***)[210] (22/43**)[217]	**58% (50)** (29/29***)[210] (0/21N.S.)[217]	**100% (39)** (17/17***)[210] (22/22*)[217]

Data in bold represent the percentage of FD patients showing altered levels of respective marker in relation to the total number of analyzed patients (in parentheses) in a total of 8 publications. Comparison vs. C refers to elevation. The significance level is indicated for tFD vs. C or for tFD vs. nFD. Data listed in parentheses describe the numbers of patients in individual sources. n=number of studied FD patients, C=healthy controls, nFD=non-treated FD patients, tFD=treated FD patients; VEGF-A/C = Vascular endothelial growth factor-A/C, MMP-2 = Matrix metalloproteinase-2, FGF2 = Fibroblast Growth Factor 2; *p<0.05, **p<0.01, ***p<0.001, N.S.=not significant

3.2.5 Antiproliferative markers

In three studies changes in the concentrations of three antiproliferative markers were reported (Table 5). [199,204,217] Findings showed significant increase in TGF beta and active TGF beta across all examined patients. Angiostatin was also increased, though this was observed in only 36% of the patients relative to control subjects. There were no significant differences based on sexes in non-treated patients. Following treatment, active TGF beta levels in males showed no significant alteration compared to controls, but the levels in females remained elevated. Conversely, TGF beta remained unchanged by the treatment in both, male and female patients. No significant changes after therapy were reported in angiostatin with similar elevated levels to non-treated patients.

Table 5. Antiproliferative markers in FD

Marker	Elevated in nFD vs C (n)	males (n)	females (n)	Elevated in tFD vs C (n)	males (n)	females (n)
Angiostatin	**36% (28)** (10/10*)[199] (0/18N.S.)[204]	**0% (4)** (0/4N.S.)[204]	**0% (14)** (0/14N.S.)[204]	**28.5% (42)** (12/12*)[199] (0/30N.S.)[204]	**0% (20)** (0/20N.S.)[204]	**0% (10)** (0/10N.S.)[204]
TGF beta	**100% (2)** (2/2*)[217]		**100% (2)** (2/2*)[217]	**100% (43)** (43/43*)[217]	**100% (21)** (21/21*)[217]	**100% (22)** (22/22*)[217]
Active TGF beta	**100% (2)** (2/2*)[217]		**100% (2)** (2/2*)[217]	**51% (43)** (22/43*)[217]	**0% (21)** (0/21N.S.)[217]	**100% (22)** (22/22*)[217]

Data in bold represent the percentage of FD patients showing altered levels of respective marker in relation to the total number of analyzed patients (in parentheses) in a total of 3 publications. Data listed in parentheses describe the numbers of patients in individual sources. n=number of studied FD patients, C=healthy controls, nFD=non-treated FD patients, tFD=treated FD patients; TGF beta=Transforming growth factor beta *p<0.05, N.S.=not significant

3.2.6 Prothrombotic markers

Two studies documented changes in 5 prothrombotic marker concentrations (Table 6). [208,213] The majority of the investigated markers were notably higher in the majority of the patients, when compared to the control group. These included PAI-1, beta-TG, TAT, and PF4. When sex was not taken into account, no significant alterations were seen in sTF. However, considering sex, sTF concentrations did show some variation, as all males showed increased levels, while no females did. Conversely, with both TAT and PF4, increased levels were found exclusively in the females and in none of the males. After therapy, TAT and PF4 levels demonstrated normalization or improvement, with only 30% of patients showing increased concentrations versus 81% in untreated patients. Concerning beta TG, there were no significant differences between men and women, and no data was available regarding PAI-1.

Table 6. Prothrombotic markers in FD

Marker	Elevated in nFD vs C (n)	males (n)	females (n)	Elevated in tFD vs C (n)	males (n)	females (n)
PAI-1	**100% (25)** (25/25*)[208]					
sTF	**19% (16)** (3/16N.S.)[213]	**100% (3)** (3/3*)[213]	**0% (13)** (0/13N.S.)[213]	**70%vsC (20)** (14/20*)[213]	**100% (14)** (14/14*)[213]	**0% (6)** (0/6N.S.)[213]
beta TG	**100% (16)** (16/16*)[213]	**100% (3)** (3/3*)[213]	**100% (13)** (13/13**)[213]	**100%vsC (20)** (20/20*)[213]	**100% (14)** (14/14*)[213]	**100% (6)** (6/6**)[213]
TAT	**81% (16)** (13/16*)[213]	**0% (3)** (0/3 N.S.)[213]	**100% (13)** (13/13*)[213]	**30%vsC (20)** (6/20*)[213]	**0% (14)** (0/14N.S.)[213]	**100% (6)** (6/6*)[213]
PF4	**81% (16)** (13/16*)[213]	**0% (3)** (0/3N.S.)[213]	**100% (13)** (13/13*)[213]	**30%vsC (20)** (6/20*)[213]	**0% (14)** (0/14 N.S.)[213]	**100% (6)** (6/6*)[213]

Data in bold represent the percentage of FD patients showing altered levels of respective marker in relation to the total number of analyzed patients (in parentheses) in a total of 2 publications. Data listed in parentheses describe the numbers of patients in individual sources. n=number of studied FD patients, C=healthy controls, nFD=non-treated FD patients, tFD=treated FD patients;
PAI-1=plasminogen activator inhibitor, sTF=soluble tissue factor, beta TG=thromboglobulin, TAT=thrombin-antithrombin III complex, PF4=anti-platelet factor 4
Not included: vWF=von Willebrand factor and tPA=tissue plasminogen activator were N.S. changed in 25 nFD vs C.[208]
*p<0.05, **p<0.01, N.S.=not significant

3.2.7 Antithrombotic markers

A single study presented findings on altered levels of an antithrombotic marker (Table 7). [211] Specifically, in the patient group under examination, no significant changes were detected in ADAMTS-13 when compared to the control group. Post treatment a highly significant decline in ADAMTS-13 levels was observed, when compared to the non-treated patients.

Table 7. Antithrombotic markers in FD

Marker	Elevated in nFD vs C (n)	males (n)	females (n)	tFD vs nFD (n)	males (n)	females (n)
ADAMTS-13	**0% (6)** (0/6N.S.)[211]	**0% (1)** (0/1N.S.)[211]	**0% (5)** (0/5N.S.)[211]	**100%vsnFD reduced (18)** (18/18***)[211]	**100% reduced (8)** (8/8***)[211]	**100% reduced(10)** (10/10***)[211]

Data in bold represent the percentage of FD patients showing altered levels of respective marker in relation to the total number of analyzed patients (in parentheses) in the analyzed publication by Alonso-Núñez et al. Data listed in parentheses describe the numbers of patients in individual sources. n=number of studied FD patients, C=healthy controls, nFD=non-treated FD patients, tFD=treated FD patients;
ADAMTS-13=A disintegrin and metalloproteinase with thrombospondin motifs
***p<0.001, N.S.=not significant

3.2.8 Direct comparisons of changes in different markers

Various markers were studied in peritubular renal capillaries of 11 patients and compared to control subjects (Table 8). The expression of eNOS, ANG2 and KDR was significantly reduced, while TSP-1 showed increased expression. [218]

Table 8. Expression of distinct markers of ED in peritubular capillaries of 11 kidney biopsies

Function	Marker	Elevated in FD vs controls
EC junctions	VECAD	Normal (N.S.)
ED	eNOS	Reduced (p<0.001)
Angiogenesis	ANG2	Reduced (p<0.05)
Angiogenesis	KDR	Reduced (p<0.01)
Antiproliferative	TSP-1	Elevated (p<0.001)

VECAD = Vascular Endothelial Cadherin, eNOS = endothelial NO synthase, ANG2 = Angiopoetin-2, KDR= Kinase insert Domain Receptor (VEGFR-2), TSP-1 = Thrombospondin-1; Kidney biopsies from one study by Do et al. [218], without information about treatment or patients`gender.

3.2.9 Circulating angiogenic cells (CAC)

In 26 FD patients increased numbers of circulating CD34+/KDR+ cells and impairment of their function were observed ($p<0.01$), whereas in CD133+ and CD34+/CD133+/KDR+ cells no significant differences were reported. After therapy with ERT the numbers of CD34+/KDR+ cells were not significantly reduced. A role of CAC in ED was suggested by Lorenzen et al. [47]

Table 9. Numbers of CAC in 26 FD patients

Function	Marker	nFD vs C (n)	males (n)	females (n)	tFD vs nFD (n)	males (n)	females (n)
CAC	**CD34+/ KDR+ (VEGFR 2)**	**100% elevated** (26/26**)	**100% elevated** (12/12**)	**100% elevated** (14/14**)	**Reduced still elevated** (16/16N.S.)	**Reduced still elevated** (10/10N.S.)	**Reduced still elevated** (6/6 N.S.)

Data present the percentage of FD patients showing altered levels of respective marker in relation to the total number of analyzed patients in the analyzed publication by Lorenzen et al. [47] n=number of studied FD patients, C=healthy controls, nFD=non-treated FD patients, tFD=treated FD patients;
CD34+=hematopoietic progenitor cell antigen, KDR+/VEGFR2=kinase insert domain receptor/vascular endothelial growth factor receptor 2
**p<0.01, N.S.=not significant

3.3 Part 3: Type of gene variants reported across publications

In 114 publications, a total of 2,957 FD patients were mentioned. For 1,177 no data regarding genotype was available. For additional 976 FD patients no exact genotype was available, however authors report on particular variant type as follows: 529 were classified as missense, 187 as nonsense, 107 as frameshift, 57 as ”classic mutations”, 46 as splice site, 21 as small deletion, 10 as “atypical”, 6 as “other” mutations, 4 as intronic, 3 as initiator codon, 3 as large deletions, 2 as small insertion and 1 as large insertion (Figure 1.).

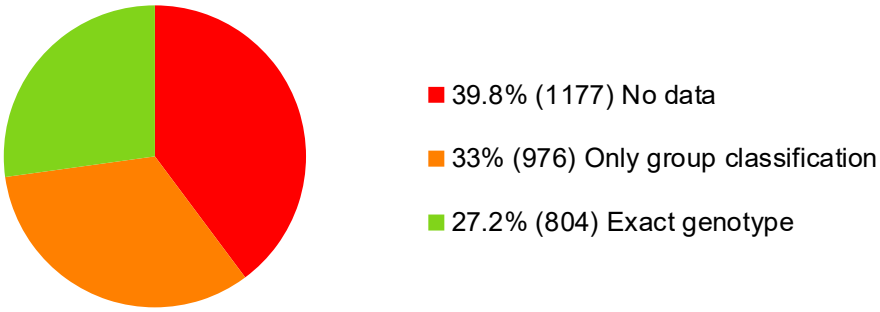

Figure 1. Overview of reported genotype across all selected publications. Data are presented as percentages in relation to the total number of patients enrolled in all selected publications. Numbers in brackets refer to the exact number of patients reported for the respective group.

For the remaining 804 FD patients the exact genotype was mentioned and only these were included in the overview depicted in the Figure 2.

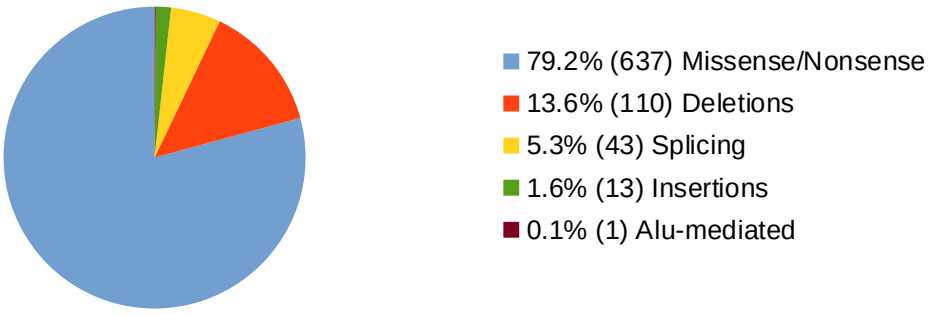

Figure 2. Type of the reported gene variants in analyzed publications.
All included patients were examined for an aspect of endothelial change. Only patients where the exact phenotype was mentioned were included in the chart. Data are presented as percentages in relation to the total number of patients enrolled in all selected publications. Numbers in brackets refer to the exact number of patients reported for the respective group. Detailed information about the reported variants were extracted from the HGMD database accessed on 18.04.25.

The most common variant type was missense/nonsense with 637 (79.2%), followed by deletions with 110 (13.6%), splicing site with 43 (5.3%), insertions with 13 (1.6%) and one case with alu-mediated gene variant (0.1%).

Similar to the documented variant types in the HGMD database, the most reported variant type was missense/nonsense, followed by deletions, splicing and insertions (Figure 2.).
There were no complex, regulatory or small indel variants reported.
Only one Alu-meditated variant is listed in the HGMD, [219] whereas a different Alu-mediated variant was found in this population with a resulting null allele. [137]

Out of the 1189 known *GLA* gene variants listed in the HGMD, 803 are missense/nonsense, 176 are small deletions, 61 small insertions, 56 splicing, 45 gross deletions, 21 small indels, 10 complex, 9 gross insertions, 8 regulatory, one known alu-mediated deletion and 67 are non-coding. The alu-mediated deletion was not separately listed in HGMD, but included in the other deletions.

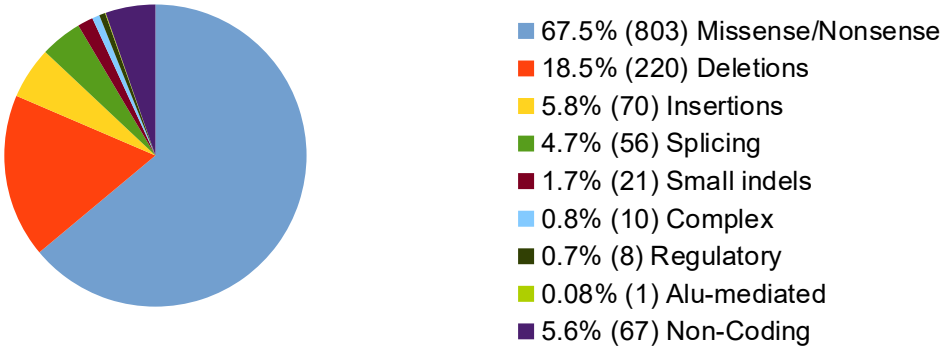

Figure 3. Types of *GLA* gene variants listed in HGMD.
Data are presented as percentages in relation to the total number of patients enrolled in all selected publications. Numbers in brackets refer to the exact number of patients reported for the respective group. Data collected from HGMD database accessed on 18.04.25.

In the included literature, 96 patients presented with 68 genotypes, which were not yet included in the HGMD database (18.04.25).

3.4 Part 4: Functional assessment of ED and markers of ED in cellular models

3.4.1 Immortalized Fabry Endothelial Cell line 1 (IMFE1):

The cell line, IMFE-1 with an eight-fold extended lifespan was created by introducing human telomerase reverse transcriptase gene (hTERT) to hemizygote FD-EC with the genotype R112H. In these cells the expression of Willebrand factor, CD31, CD34 and endothelial nitric oxide synthase (eNOS) were increased, which are markers of ED. They retained functional characteristics such as uptake of acetylated low-density lipoprotein, responsiveness to angiogenic growth factors, up-regulation of eNOS production upon extracellular stimuli and formation of tube-like structures on Matrigel basement membrane matrix. They also showed the FD characteristics of reduced activity of AGAL to 16% in comparison to controls and the accumulation of Gb3 in lysosomes via immunostaining. Treatment with 0.4 IU/ml recombinant AGAL enzyme for 48 h significantly reduced the Gb3 accumulation. [220]

In a further study this cell line was used to show that Gb3 loading led to increased Gb3 uptake in lysosomes, as well as dose-dependent increase in ROS, ICAM-1, VCAM-1 and E-selectin. Addition of the antioxidant vitamin C (100 µmol/L) significantly decreased the intracellular ROS level. Similar to Gb3 loading, FD-patient plasma also led to more ROS generation in IMFE1-EC. Treatment with 1µmol/L of the GSL synthase inhibitor EtDO-P4, as well as with 0.08 IU/ml AGAL for 4 days significantly reduced Gb3, which led to a down-regulation of ICAM-1 and VCAM-1 expression. [30]

A study by Shen et al. examined IMFE-1-EC with loci-specific pyrosequencing and genome-wide DNA-methylation arrays. Dysregulation of DNA methylation homeostasis was revealed. This was linked to an altered methionin metabolism, with consequentially increased SAM (S-Adenosyl methionine) and increased expression of COL4A1/2 (Collagen type IV alpha 1 and 2 chains) due to their decreased methylation level, which led to vasculopathy. [221]

3.4.2 Human macro- (HmaVEC) and microvascular cardiac endothelial cells (HmiVEC):

To exhibit that intracellular Gb3 accumulation and not GLA deficiency causes dysregulation of endothelial pathways, in a study by Namdar et al. HmaVEC and HmiVEC were incubated with Gb3. Both cell types showed downregulation of eNOS and iNOS (cytotoxic) and upregulation of COX-1 and COX-2 (vasoconstrictive). The HmiVEC were more affected. Silencing of *GLA* via siRNA Gla-knockout on the other hand had no similar effects. [42]

3.4.3 Circulating Angiogenic Cells (CAC) and markers of oxidative stress

In a study by Lorenzen et al. silencing of the *GLA*-gene in CAC of 26 FD patients and applying the boyden chamber assay with HUVEC, showed impaired angiogenesis, reduced migratory capacity of HUVEC and CAC and elevated CAC numbers. The adhesion of *GLA*-knockdown-CAC to TNF-α pre-stimulated HUVEC hindered tube formation. After treatment with recombinant AGAL (100 µg/mL) for 24h the migratory capacity of CAC was significantly improved ($p<0.01$), however their numbers were not significantly reduced. On the other hand treatment with the NOS inhibitor L-NAME, led to significantly impaired migratory capacity of CAC ($p < 0.001$). Furthermore in isolated CAC of 4 untreated and of 3 ERT treated FD patients, the expression of SOD2 ($p<0.01$) and catalase ($p=0.03$) was reduced in untreated compared to ERT treated FD patients. The expression of eNOS was not significantly changed in both ($p=0.2$) (Table 10). There was an association of these changes to the reactive hyperemia index and the augmentation index for arterial stiffness. [47]

Table 10. Expression of oxidative markers in isolated CAC

Function	Marker	nFD vs C (n)	tFD vs nFD (n)
Oxidative stress, cardiac	**SOD2**	**100% reduced** (4/4**)	**100% improved** (3/3**)
Oxidative stress	**Catalase**	**100% reduced** (4/4x)	**100% improved** (3/3x)
Oxidative stress	**eNOS**	**Unchanged** (4 N.S.)	**Unchanged** (3 N.S.)

Data in bold represent the percentage of FD patients showing altered levels of respective marker in relation to the total number of analyzed patients (in parentheses) in the analyzed publication by Lorenzen et al.[47] n=number of studied FD patients, C=healthy controls, nFD=non-treated FD patients, tFD=treated FD patients;

SOD2=superoxide dismutase, eNOS=endothelial nitric oxide synthase
x:p=0.03, **p<0.01, N.S.=not significant

3.4.4 Human Umbilical Vein Endothelial Cells (HUVEC)

Several studies investigated the effect of Gb3 loading in HUVEC.

In a study by Choi et al. HUVEC were loaded with Gb3 and downregulated levels of KCa3.1 and especially plasma membrane localized KCa3.1 were observed due to lysosomal degradation. [222]

After the same treatment, Tseng et al. reported downregulation of SOD2 and enhanced AMPK activity. [223]

Maier et al. described increased E-selectin, ICAM and VCAM levels in seven different donors after Gb3 stimulation. It also increased the activity of NF-kB, which led to reduced miRNA levels of let-7a and let-7d. [224]

HUVEC of a hemizygous Fabry fetus showed lamellar Gb3 accumulation in the lysosomes. After treatment with high-uptake AGAL, the results indicated that the EC did not internalize AGAL via the Mannose- 6-P receptor. Immunofluorescence studies showed very few or no Mannose-6-P receptors on their surface. Morphological studies did not show changes, but cell-associated AGAL activity was found, when the enzyme was added to cells preincubated with Concanavalin A (ConA). Since ConA is a lectin it induced ultrastructural changes in the cytoplasm itself, which could obscured the effect of the enzyme. [176]

3.4.5 Retinal pigment epithelial cell line (ARPE-19) and HUVEC

In a study by Hwang et al. HUVEC were treated with conditioned media (CM) of ARPE19 (Retinal pigment epithelial cell line) which were previously treated with lyso-Gb3. This induced necroptosis, inflammation, and senescence in HUVEC, as they showed increased inflammation markers Lc3B, Beclin1, RIP1, RIP3, MLKL, VCAM1 and ICAM1. Further, they showed that autophagy inhibitor (3-MA) and necroptosis inhibitors (necrostatin and GSK-872) reduced these markers of ED, which led to the conclusion that ED is caused by an autophagy dependent necroptosis pathway. Inhibition of RIP1 and RIP3 led to better outcomes and they could be possible markers for FD. Hwang et al. suggested that intervention via necroptosis inhibition might be a possible therapeutic approach. [225]

3.4.6 Immortalized endothelial cell line - EA.hy926

The cell line EA.hy926 was designed by fusion of HUVEC with human lung carcinoma cell line A549 and possesses an indefinite capacity for cell division. [226] A FD model of EA.hy926 was established on one hand by silencing the *GLA*-gene by siRNA and on the other by constructing a *GLA*-gene KO by gene-editing with CRSIPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats). Silencing of the *GLA*-gene led to increased Gb3 levels. [161]

In the CRISPR/Cas9 KO cells, Gb4 was more prevalent than Gb3 [227], they presented lysosomal vacuoles, increased expression of ASAH1 and Ras-related protein Rab-11B. They also exhibited higher cell viability and increased proliferation rates, leading to higher metabolic rates and mitochondrial stress due to an increased NADH and ATP turnover. [26] The glycocalyx thickness was reduced, monocyte adhesion was increased and the expression of angiopoietin-2, heparanase and NF-κB was elevated. Comparable result were found in wild-type EC which were incubated with lyso-Gb3. Functional rescue was attempted with recombinant AGAL, heparin, anti-inflammatory, antioxidant drugs and razuprotafib, which specifically inhibits the binding of VE-PTP to angiopoietin-1 receptor (Tie2) and consequential release of heparanase. All interventions showed improved glycocalyx structure and endothelial function *in vitro*.[127]

In both methods for FD models, higher lyso-Gb3 levels and decreased eNOS activity were observed.[161] Similarly, a three to five-fold increase in vWF secretion and decreased eNOS activity compared to controls were observed by Kang and others. After pharmacological increase of NO bioavailability and ROS decrease, normalized vWF secretion was observed, while ERT reduced vWF levels only slightly and eliglustat increased them. [228]

3.4.7 FD-induced pluripotent Stem Cell (FD-iPSC)

FD-iPSC from peripheral blood mononuclear cells (PBMC)

Only one study reported use of peripheral blood mononuclear cells (PBMC) from a patient with the *GLA*-gene variant IVS4+919G>A, which is associated with late-onset cardiac phenotype, and their transformation to induced pluripotent stem cells (iPSCs; FD-iPSCs). These were then differentiated to VEC which expressed

typical EC traits such as expression of CD31, VE-cadherin, vWF and also GLA reduction. Using them for further research as a VEC phenotype of FD was consequently proposed. They showed intracellular Gb3 accumulation, ROS increase, downregulation of the antioxidant-related SOD2 expression and enhanced AMPK activity most likely in response to increased ROS production. [223]

FD-iPSC from fibroblasts

An other study reports an iPSC model by using skin fibroblasts with the genotype GLA IVS4+919G>A. They were then differentiated to EC and showed Gb3 accumulation, increased ROS and autophagic flux impairment.
The levels of autophagosome markers LC3-II and p62 were measured in FD and in by CRISPR/Cas9 corrected FD cells, respectively, after application of autophagy inhibitor chloroquine and activator rapamycin. Chloroquine led to elevated LC3II/LC3I ratio and p62 levels in both, but rapamycin treatment increased only the LC3II/LC3I ratio in the FD-EC. This was interpreted as rescued autophagosomal function in the by CRISPR/Cas9 corrected FD-EC. They also showed normalization of pro-inflammatory genes, NF-kB reduction and MAPK downregulation. [229]
To examine the pathogenicity of different VUS, iPSCs of 13 hemizygote FD patients were created from dermal fibroblasts and differentiated into EC. They showed a reduction of GLA activity in p.A143T, normal activity in p.D313Y and p.S126G, and no Gb3 accumulation. The three VUS were therefore considered to likely have a benign phenotype. [160]

Another study examined the efficacy of CRISPR/Cas9-mediated *A4GALT* suppression in rescuing ED in FD. The enzyme Lactosylceramide 4-alpha-galactosyltransferase is encoded by the *A4GALT* gene, it is responsible for the synthesis of Gb3 from Lactosylcermide (LacCer) and its inhibition might reduce Gb3 accumulation. EC-lines from iPSCs (WT) as controls, *GLA*-gene variant iPSCs (GLA-KO) and CRISPR/Cas9-mediated *A4GALT*-KO iPSCs (*GLA*/*A4GALT*-KO) were differentiated.
The *GLA*-KO-iPSCs showed lower expression of EC markers, AGAL expression and increased Gb3 deposit. Values for MAPK and AKT phosphorylation levels

were decreased, for SOD and catalase increased. Changes in the transcriptome regarding angiogenesis, cell death, and cellular response to oxidative stress were observed. In the CRISPR/Cas9-mediated *A4GALT*-KO iPSCs higher EC marker expression, normal cell migration and tube formation were observed. Values for MAPK, AKT, SOD and catalase were also normal. The transcriptome appeared normal as well. Therefore it was concluded, that the FD phenotype and the ED were rescued by *A4GALT* suppression. [230]

3.4.8 Development of kidney organoids

In a further study iPSC with *GLA*-gene variant created by CRISPR/Cas9 usage were differentiated into kidney organoids via decellularized extracellular matrix (dECM). The dECM contributed to the recruitment of EC from the host mouse kidney, the vascular network, the vascular integrity and to maturation. The model exhibited limited vascularization compared to the WT and reduced mRNA expression of eNOS, ANG2 and SOD2. After ERT, the model formed a microvasculature that fenestrated into podocytes and surrounded the tubular structures, recovering the structural changes in the podocytes and tubular cells. This vascularized kidney organoid was considered to be useful for disease modeling for vasculopathy in FD. [231]

3.5 Part 5: Epigenetic and proteomic aspects of ED in FD

3.5.1 MicroRNA

In a study by Lo Curto et al. the levels of miR-126-3p, a senescence-associated microRNA were increased in FD patients small extracellular vesicles (sEV) in comparison to healthy controls. In HUVEC used as control the miR-126-3p in sEV was increased, depending on the cumulative population doubling. [232]

In a publication by Cammarata et al., FD patients with ERT showed lower levels than untreated patients for both miR-199a-5p and miR-126-3p, which are suspected to be involved in ED. The miR-423-5 levels were higher after ERT and for miR-451a no significant change was observed. [233]

In a previously mentioned *in vitro* model of FD-EC by Maier et al., the accumulation of Gb3 induced the activation of NF-κB, which directly reduced let-7a and let-7d miRNA expression. In the same study the levels of let-7a and let-7d were also significantly increased in three FD patients after ERT. Both were therefore considered as possible markers for inflammation and low enzyme activity in FD. [224]

3.5.2 DNA-Methylation and gene expression

In a study by Al-Obaide et al., HEK293 (Human embryonic kidney 293 cells) were used to look into the connection between the heterogeneous nuclear ribonucleoprotein H2- (*HNRNPH2*) and *GLA*-gene. Defect in *HNRNPH2* expression is potentially leading to impaired DNA-splicing, which influences the *GLA*-expression. It was revealed that they share a bidirectional promoter, which showed varying methylation patterns in different cell lines and might explain varying phenoptypes of FD. [234]

In a study using the IMFE-1 cell line it was revealed that the ten-eleven translocation 1 (TET1) enzyme, was reduced to 40%. This enzyme is part of the DNA-demethylation process and its downregulation might have an effect on the altered DNA-methylation homeostasis in FD. Global gene expression and DNA-methylation analysis showed changes under SRT with GZ161 and under gene

therapy in the expression of numerous genes and in their methylation status. Shen et al. suggested these approaches to screen for potential therapy efficacy biomarkers. [221]

3.5.3 Modifier genes

A study which used proprietary software SNP clinic v1.0 found seven regulatory SNP (rSNP) in the genes *IL10*, *TGFB1* and *EDN1*. The two rSNP in *IL10*, could explain regulatory mechanisms of active B cells that influence fibrosis. The three rSNP in *TGFB1*, could act in apoptosis-autophagy regulation. The two rSNP in *EDN1*, are possibly involved in chronic inflammation. The three genes were considered as minor genes in FD since they could modulate the FD phenotype. [235]

3.5.4 Proteomic *in silico* modeling

A research approach is the use of *in silico* models, which are computational simulations of different biological processes. They are especially useful in research of rare diseases, which is usually limited by small study populations.[236] An *in silico* model for FD based on machine learning was created by Gervas-Arrguga et al., in which they used a combined systems biology and machine learning approach. It includes a computational model of vascular and nervous system disease. The markers calcium/calmodulin dependent protein kinase II (CAMK2A), integrin-linked kinase (ILK), lamin A (LMNA) and KH-type splicing regulatory protein (KHSRP) had high classification capabilities and are markers for high risk for tissue and cellular damage. An *in vivo* validation of the results was suggested by the authors. [237]

3.6 ERT induced anti-drug-antibodies (ADA)

The development of ADA is a common phenomenon following ERT administration in FD and was reported in 5 publications. In 77% of 283 studied patients showed seroconversion and differing titer levels. [142,150,211,238,239]

After 3 months of ERT 90% of patients developed ADA as reported by Germain et al., [150] but in a study by Wilcox et al. after 30-36 months of therapy the amount of seroconversions stayed similar.[238] In a study by Bénichou et al. seroconversions were associated with less clearance of dermal capillary EC and therefore less therapy efficacy. Furthermore, there were differences between the sexes with 85% of 122 males showing seroconversion and only 50% of 12 females. [239]

Regarding genotype one male patient with genotype p.E358K developed ADA with a titre of 6400 and another whose genotype was not determined a titre of 200, while nine other patients in this study developed no antibodies. [142]

Another limiting factor of ERT is the post-mitotic status of storage cells e.g. cardiomyocytes preventing their replacement by enzyme supplied precursors. Further modification of the lysosomal system by longstanding storage and possibly a relative lack of saposin 1 might be limitations. [184]

4 Discussion

FD is a rare lysosomal storage disease. In pathogenic variants of the *GLA*-gene, the function of the encoded enzyme AGAL is reduced or nonexistent. The resulting accumulation of Gb3 and other metabolites in the lysosomes of different cells and especially EC leads to systemic damage and multiple processes which result in ED and later organ damage. This thesis represents a systemic review and summarizes the available literature on ED in FD, extracted from the PubMed database. This allows conclusions to be drawn regarding the mechanisms by which FD causes ED, potential contributing factors, differences among patient groups, potential disease markers, information gained from *in vitro* models, and how therapy affects the course of the disease.

4.1 Answers to the research

To assess clinical parameters pertaining to ED in FD the data of 648 male and 180 female FD patients was included from overall 110 publications. The angiokeratoma corporis diffusum was described in 57.4%. Gb3 accumulation in various types of EC with different histological presentations was described in 82.5% of patients, with various EC types being differently affected. In SSEC only 4.4% of examined females and 81.8% of males were affected. In IVS4+919G>A variant and in various VUS no accumulation was found. The RHI and FMD was reduced in patients. The IMT was increased in some but not in other studies. NFC showed pathological signs with more severe presentation in classical cases and less severe in late onset cases. The corneal EC showed pathological signs as well and examination with OCT revealed vascular damage. Endothelial fenestration was reduced especially in the mesangial zone of the glomerular capillary. Angina pectoris was described as an ED caused symptom and HCM and LVH was observed also, especially in the cardiac type. The coronary arteries showed diffuse plaques, hypoechogenicity and more lipid cores in intravascular ultrasound examinations. Increased blood pressure was observed in 41.4% of examined patients. Patients with a prothrombotic state with massive recurrent thrombosis and stroke were described. Hypersplenomegaly as a result of compromised blood flow was reported.

An overview of all measured markers of ED in FD patients was created. Of the established ED markers like ESM1, pentraxin-3 and ischemia modified albumin were not evaluated in patients and others like vWF showed no changes. ICAM-1, VCAM-1, EMP, ADMA, CAC were elevated and E-selectin as well as ANG2 reduced. Other ED-related markers with elevated values compared to controls were TSP-1, PAI-1, sTF, beta TG, TAT, PF4, TGF beta, angiostatin, VEGF-A, VEGF-C, MMP-2, FGF2, 3-NT, SDMA, ACTB, HDL-C/Cholesterol ratio, BNP, MR-pro ANP, galectin-1, MCP-1, MPO, iC3b. C4B, hsCRP, TNF-alpha, IL-6, MMP-9, TNFR 1, TNFR 2, IL-7 and P-selectin. Reduced compared to controls were eNOS, ANG2, KDR, TM, hArg/SDMA ratio, and E-selectin levels. Males showed elevation of MPO, HDL-C, sTF and females of TAT and PF4, no other significant differences between sexes were found.

The clinical phenotypes and respective ED marker in FD patients were compared before and after therapy. The choice of ERT between agalsidase alfa, beta and pegunigalsidase alfa showed comparable outcomes. After ERT the EC showed overall a total clearance in 84% of patients. The heart capillary EC and deep vessel EC showed the least clearance with 76.2% and 79.7%, while the other EC types showed clearance in 91 to 100%. While most were cleared after 5 months, early start and longer duration of ERT were positively correlated with total clearance.

Reduction of blood pressure, of severe clinical events, RHI and AI were observed after ERT, although other clinical aspects are not significantly improved. Most of the ED markers were not affected by ERT and remained unchanged as well, only IC3b, C4B, MPO, 3-NT, ACTB and ADAMTS-13 were significantly improved after ERT.

The sub-aim of the study to establish an association between therapy responsiveness and genotype could not be sufficiently fulfilled. The exact genotype was included for only 27.2% of 2,957 FD patients. Most of them were missense/nonsense, while deletions, splicing, insertion and an alu-mediated gene variant was present. In most publications the phenotype data was not directly linked to the genotype and the available data was not enough to show significant differences.

Moreover multiple *in vitro* models of FD were reported. IMFE1, Hma/HmiVEC, CAC, HUVEC, EA.hy926, FD-iPSC and kidney organoids from FD-iPSC were used in models of FD. Models of FD-EC were created either by Gb3 loading of cells, silencing of *GLA* via siRNA, by gene-editing via CRISPR/Cas9 or by differentiating from FD-iPSC. They exhibited various traits of ED in FD and might be useful for further investigation of ED in FD.
Epigenetic changes were reported in multiple microRNA. In comparison to healthy controls miR-126-3p was elevated, while let-7a and let-7d miRNA was reduced. After ERT the levels of miR-126-3p and miR-199a-5p were reduced, miR-423-5, let-7a and let-7d miRNA levels were increased and for miR-451a no significant change was observed compared to untreated FD-patients. The TET1 enzyme activity was reduced to 40% in FD patients and might lead to altered DNA-methylation. Therapy with SRT with GZ161 and gene therapy changed the expression and DNA-methylation of multiple genes.
The *HNRNPH2*-gene which is involved in *GLA*-gene expression, showed varying DNA-methylation patterns as well.
Further *IL10*, *TGFB1* and *EDN1* were proposed as modifier genes in FD. Also an *in silicio* model found CAMK2A, ILK, LMNA and KHSRP as potential markers for high risk for tissue and cellular damage in FD.
Another important observation was the development of ADA which was described in up to 90% after 3 months of ERT and led to deceased therapy efficacy.

Since Gb3 clearance was observed frequently, but clinical aspects and ED markers remained unchanged, two following hypothesis were created:
The first is that other cell types are not cleared of Gb3 and might contribute to ED via inflammation and other pathomechanisms. This hypothesis is supported by the observation of cases with total Gb3 clearance of EC after ERT, which showed persistent storage in cardiomyocytes, smooth muscle cells, fibroblasts, sweat glands, skeletal muscle, neurons, pericytes, glomerular epithelial cells, tubular epithelial cells, podocytes, as well as in non-vascular cells. [183,184]
The other hypothesis is that Gb3 induces permanent changes in the EC-phenotype via epigenetic modifications which cannot be restored with therapy.

This is supported by persistent elevation of ED related markers after ERT and by increased levels of DNA-methylation and micro-RNA.

4.2 Comparative explanations

The results of this systematic review contradict the suggestions from previous studies, that the occurrence of ED in FD is only related to the Gb3-accumulation in EC. [42] While Gb3 accumulation initiates ED, it is influenced by multiple factors and can occur even with low metabolite levels after ERT. Some of these factors are potentially Gb3 accumulation in other cell types and epigenetic changes.
In a publication by Di Risi et al. altered DNA-methylation was also observed in FD and further research was suggested. [240] A publication by Carnicer-Cáceres et al. [241] also highlighted the prognostic value of markers in FD, although they focused on markers for other aspects than ED. They suggested further research and implementation of markers into clinical practice to enable detection in the early disease stages. Like previously mentioned by Oliveira and Ferreira [242] links between phenotype and genotype have not been well established, but could provide information about pathophysiologic mechanisms. Especially the ED phenotype has not been considered and in this work not enough data was found to link genotype to ED phenotype as well.

4.3 Conclusion

The results suggest that most FD patients exhibit ED related clinical aspects. Even though Gb3 accumulation in various EC types is frequently observed in both sexes, females were less affected. While ERT leads to Gb3 clearance it does not improve most clinical aspects. Most ED related markers showed significant changes compared to controls and only a few differed in female compared to male patients. After ERT most of them showed no improvement. This suggests similar development of ED in females and males regardless of visible Gb3 storage. It also indicates a low efficacy of ERT in the improvement of ED. This might be due to remaining Gb3 accumulation in other cells or caused by epigenetic changes like DNA-methylation or microRNA which are not reversed by ERT. Early ERT and prevention of Gb3 accumulation was associated with better outcomes and should be considered. Not enough data was available to correlate genotype to ED phenotype was available. To test these hypothesis and validate the markers the

reported FD *in vitro* models might be useful. Larger populations for *in vivo* studies are necessary to validate the results and investigate genotype-phenotype correlations.

4.4 Critical reflection/restrictions on content and method

In this literature review, the scope of the presented data is restricted to articles stemming from the content available within the PubMed metadata repository.
A limiting factor is the rarity of FD and consequently, studies examining the ED in this already rare disease are often unable to encompass a sufficiently large patient cohort to yield statistically robust results. Most of the publications were not focused on ED, but rather on multiple or different FD aspects, this led to biased data collection for their hypothesis and complicated the comparison of results.
Moreover, the publications often lack data to compare the individual genotypes and phenotypes to draw conclusions.
Another limitation is the fact that more severe cases are more likely to be treated and therefore the data regarding changed marker levels before and after therapy might be biased. This might have been the case in SDMA and sTF measured patients, which were only altered in treated patients.
Therapy with chaperone or SRT were reported sparsely in the included literature. ERT is much longer available and more established, which might have caused this discrepancy. ED is a condition, that can be influenced by a plethora of causes like lifestyle, comorbidities and others which do not relate to FD and were not always properly assessed. Some interesting aspects were beyond the scope of this work. There is evidence of polymorphisms of *eNOS*-gene variants being more prevalent in FD patients than in control groups. These influence the ED progression in FD, as well as development of symptoms e.g. left posterior wall thickness and might be another pathophysiological pathway of ED in FD. [218,243–245]
ED with metabolite accumulation in EC plays a part in various other clinical manifestations like nephropathy and cardiomyopathy. They are however not strictly caused by ED, since Gb3 also accumulates in various other cell types leading to their development.[246,247]

4.5 Implications for theory and practice

An understanding and recognition of ED involvement in FD with different expression of markers and severity of symptoms, depending on Gb3 accumulation in EC is important for both clinical care for patients as well as for researchers and could be useful in the development of better diagnostic options and treatments.
To clinically assess ED in pediatric patients, RHI can be useful, since it can detect impaired vascular endothelial function in early stages and in absence of other symptoms. [248] Affected patients should be considered for early ERT to prevent Gb3 accumulation and ED development. The AI on the other hand seems less useful in children, since it increases age-dependently. Seroconversion with increased ADA titer should be considered more frequently as a limiting factor of ERT. Various markers attributed to different ED-related mechanisms are changed in FD patients and could be used for prognosis or therapy monitoring. Since the decrease of glycocalyx was observed *in vitro* as an ED contributing pathomechanism, Using protective Tie2 treatment of the glycocalyx as a potential therapy option was suggested.[127]

4.6 Outlook and suggestions for further work

Some important markers for ED were only measured in few publications e.g. ADMA, while others were not measured at all e.g. ESM1. Moreover the other reported ED related markers should be validated in larger study populations. Further research pertaining genotype-phenotype correlations is necessary, especially regarding ED. The influence of chaperone therapy and SRT on ED also needs to be investigated. *IL10, TGFB1, EDN1* as modifier genes in FD as well as CAMK2A, ILK, LMNA and KHSRP as potential markers for high risk for tissue and cellular damage in FD need further *in vivo* validation. The *in vitro* models of FD can be useful for further ED investigation. Novel therapy options e.g. NAGA should be studied, since they could circumvent ADA related issues,
The results of this literature review can be used in future studies to better understand the causes of ED in FD and thus encourage a more detailed investigation of the pathophysiology of related manifestations. These studies should also focus on clinical aspects, changes in markers and epigenetic alterations. This might improve early detection, monitoring of therapy efficacy and prognosis.

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