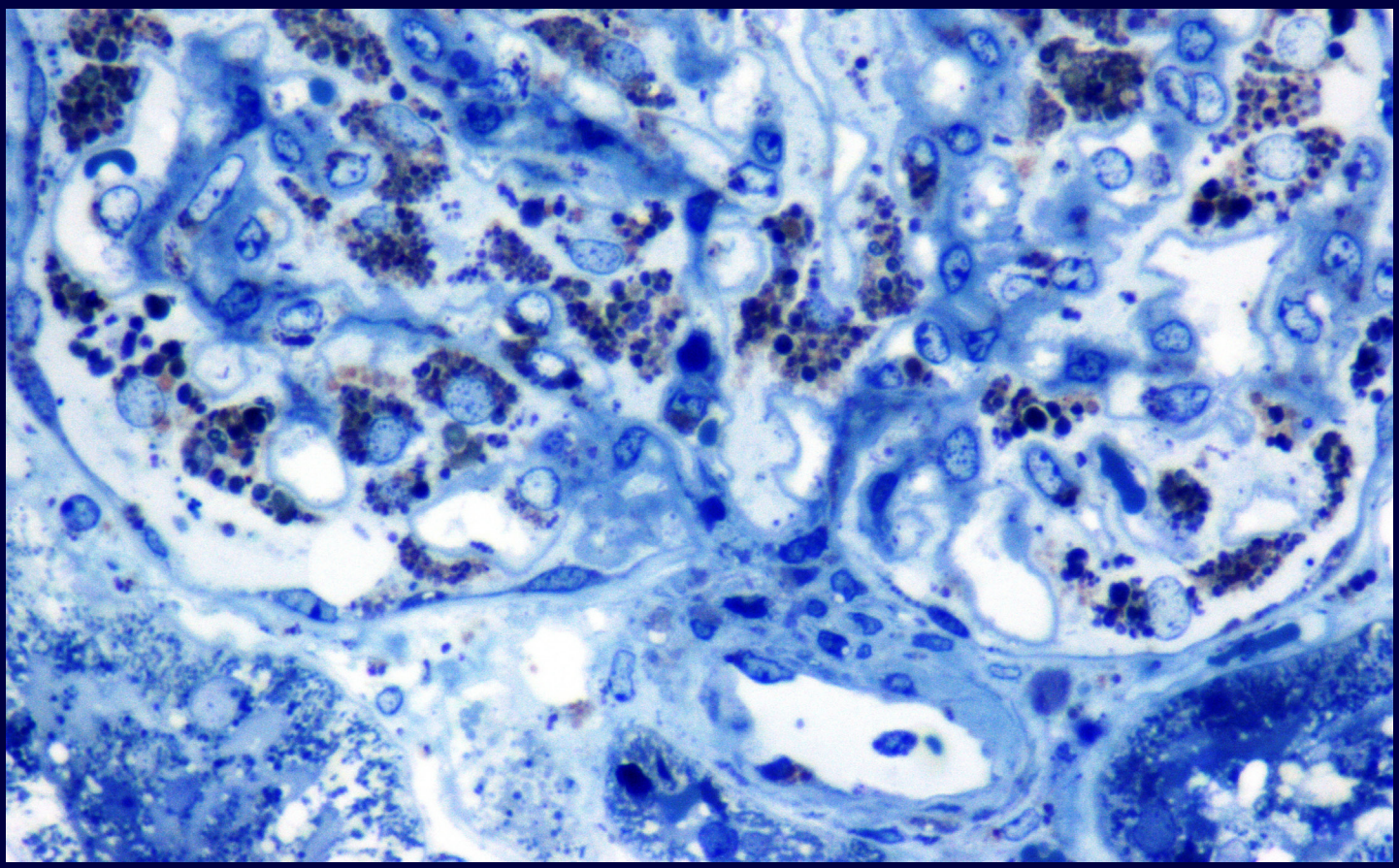
FABRY DISEASE: PATHOGENESIS AND HISTOPATHOLOGY

DOENÇA DE FABRY: PATOGENIA E HISTOPATOLOGIA

Carmen Ruiz de Valbuena Bueno



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- Oliveira JP, **Valbuena C**, Moreira A, Fonseca E, Soares C, Leão Teles E and Waldek S. *Splenomegaly, hypersplenism and peripheral blood cytopaenias in patients with classical Anderson-Fabry disease.* Virchows Arch 2008; **453**: 291-300.

- Ferreira S, **Valbuena C**, Carvalho F and Oliveira JP. *Novel human pathological mutations. Gene symbol: GLA. Disease: Fabry disease.* Hum Genet 2009; **126**: 352.

- Fogo AB, Bostad L, Svarstad E, Cook W, Moll S, Barbey F, Geldenhuys L, West M, Ferluga D, Vujkovac B, Howie AJ, Burns A, Reeve R, Waldek S, Noel LH, Grunfeld JP, **Valbuena C**, Paulo Oliveira JP, Muller J, Breunig F, Zhang X, Warnock D and all members of the International Study Group of Fabry Nephropathy (ISGFN). *Scoring system for renal pathology in Fabry disease: report of the international study group of Fabry nephropathy (ISGFN).* Nephrol Dial Transplant 2010; **25**: 2168-2177.

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- **Valbuena C**, Oliveira JP, Carneiro F, Relvas S, Ganhão M, Sá-Miranda MC and Rodrigues LG. *Kidney histologic alterations in α-Galactosidase deficient mice.* Virchows Arch 2010; **458**: 477-486.

- **Valbuena C**, Leitão D, Carneiro F and Oliveira JP. U*se of anti-Gb3 antibody in paraffin-embedded tissue: an accessible method to diagnose Fabry nephropathy and localize Gb3 deposits.* Virchows Archiv 2011b, Dec 29 [Epub ahead of print].

Em cumprimento do disposto no referido Decreto-Lei, declara que participou activamente na recolha e estudo do material incluído em todos os trabalhos, assim como na análise dos resultados e redação dos artigos, em colaboração com os outros autores. As cópias de todos os artigos foram incluídas com autorização editorial das revistas correspondentes.

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A mi familia

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INTRODUCTION

OVERVIEW OF FABRY DISEASE: historical perspective and recent developments

Fabry disease was originally described in 1898 by two dermatologists, William Anderson in England [*Anderson 1898*] and Johannes Fabry in Germany [*Fabry 1898*], and for many years was considered as a pure dermatological condition until the first autopsy studies [*Pompen et al. 1947*] unequivocally demonstrated its systemic nature, with major involvement of the blood vessels, heart and kidneys. The prominent vacuolation of the affected cells, as observed by routine light microscopy, suggested that Fabry disease might be a metabolic storage disorder and that the accumulated substances were possibly lost in the processing of tissue sections for light microscopy evaluation [*Pompen et al. 1947*]. Accumulation of birefringent material within the affected cells, showing a Maltese cross configuration under polarised light, was actually demonstrated soon thereafter, in frozen tissue autopsy specimens [*Scriba 1950*]. Such deposits, probably of a lipidic substance, were present in many different types of cells, including podocytes and glomerular parietal cells, tubular and interstitial cells in the kidneys, smooth muscle cells of the aorta, cardiomyocytes, cells of the adrenal gland, spleen and lymph nodes, ganglion cells of the brain and cells of the gastric and intestinal myenteric plexuses. The birefringence of the stored material allowed the first *in vivo* histological diagnosis of Fabry disease, which was made on a skin biopsy [*Hornbostel & Scriba 1953*]. End-stage renal disease was a major cause of death of patients with Fabry disease and although all the reported cases were males, the kidney pathology observed by light microscopy was quite similar in clinically affected individuals, irrespective of their gender [*Colley et al. 1958*]. The characteristic ultrastructural features of the intracellular deposits of Fabry disease, with alternating light- and dark-staining bands in a concentric or lamellar pattern were originally described in a percutaneous renal biopsy specimen [*Henry & Rally 1963*]. These established the value of electron microscopy study of kidney biopsies as a ancillary diagnostic tool for Fabry disease. The isolation of two novel neutral glycosphingolipids, particularly a ceramide trihexoside (galactosyl-galactosyl--glucosyl-ceramide) and minor amounts of a ceramide dihexoside (galactosyl-galactosyl--ceramide), from preserved kidney tissue of a young adult male with Fabry disease who had died of renal failure [*Sweeley & Klionsky 1963*], elucidated the biochemical nature of the stored material, and allowed the classification of Fabry disease as a glycosphingolipidosis

(later on, the ceramide trihexoside was eventually classified as globotriaosylceramide (Gb3) and the ceramide dihexoside as galabiosylceramide). Deficiency of a ceramide trihexosidase catalyzing the hydrolysis of the terminal glucose residue of ceramide trihexoside was initially demonstrated in small intestinal biopsies of patients with Fabry disease [*Brady et al 1967*]. The anomeric specificity of the enzyme defect was analysed with synthetic substrates [*Kint 1970*]. Those studies finally proved that Fabry disease was caused by the deficiency of a lysosomal α-galactosyl hydrolase (i.e. α-galactosidase), which blocked the catabolic processing of neutral glycosphingolipids containing terminal α-galactosyl residues. The biosynthesis and the homodimeric structure of the mature human α-galactosidase enzyme were elucidated in 1978 [*Bishop & Sweeley 1978, Kusiak et al. 1978*]. It became apparent that the human α-galactosidase had multiple isoforms and that plasma enzyme had significantly more sialic acid residues than the isoforms extracted from different solid tissues.

A simple and sensitive fluorometric assay to specifically measure α-galactosidase activity in human biological samples was originally presented in 1981 [*Mayes et al. 1981*] and became the gold standard for the enzymatic diagnosis of Fabry disease. The method employs a synthetic substrate for α-galactosidase (4-methylumbelliferyl-α-D-galactopyranoside), and N-acetylgalactosamine to inhibit the activity of α-N-acetylgalactosaminidase, an enzyme which has partial activity against α-galactosidase substrates.

Although previously suspected from clinical studies of extended families [Wise 1962], the X-linked inheritance of Fabry disease was formally demonstrated by linkage analysis in 1965 [*Opitz et al. 1965*]. The localization of the α-galactosidase gene locus (*GLA*) on the X chromosome was confirmed using human-hamster cell hybrids [*Rebourcet et al. 1974*] and later assigned to the Xq22.1 region by a variety of methods [*Fox et al 1984, MacDermot et al. 1987*]. A cDNA clone isolated from a human liver cDNA library contained an open reading frame of 398 residues, with a molecular mass of 45.4 kD, encoding the α-galactosidase protein [*Bishop et al. 1986*]. RNA transfer hybridization analysis detected a 1.45-kb transcript. The complete nucleotide sequence of the *GLA* gene was elucidated in 1989 [*Kornreich et al. 1989*]. The identification of mutations of the *GLA* gene in patients with Fabry disease was first reported in 1989 [*Bernstein et al. 1989*] and since then more than 600 pathogenic *GLA* mutations have been reported to the Human Gene Mutation Database (*HGMD®) [http://www.hgmd.cf.ac.uk/ac/gene.php?gene=GLA, accessed on December 1, 2011*].

Initial efforts to replace the defective enzyme in classically affected males involved the administration of single doses of either normal plasma containing known amounts of α-galactosidase [*Mapes et al. 1970*] or of partially purified enzyme from the human placenta [*Brady et al. 1973*]. These pilot trials demonstrated that the intravenously administered α-galactosidase was metabolically active in the recipient patients. Additionally, *in vitro*

studies provided evidence that cultured fibroblasts from patients with Fabry disease could use an exogenous α-galactosidase preparation and catabolize the glycosphingolipids accumulated intracellularly [*Dawson et al. 1973*]. A subsequent pilot trial compared the effectiveness and safety of the splenic and plasma isoforms of human α-galactosidase in two brothers with Fabry disease [*Desnick et al. 1979*]. The administered enzyme was rapidly cleared from circulation and could be demonstrated in the liver shortly after infusion, but the more sialylated plasma isoform was retained in the circulation much longer than the splenic isoform [*Desnick et al. 1979*]. In cultured fibroblasts from patients with Fabry disease, α-galactosidase uptake was strongly inhibited by addition of mannose-6-phosphate to the culture medium and the pre-treatment with acid phosphatase reduced the efficiency of the endocytosis of high-uptake placental enzyme isoforms [*Mayes et al. 1982*]. Furthermore, in the absence of mannose 6-phosphate residues (as in I-cell fibroblasts), newly synthesized α-galactosidase was secreted to the culture medium [*Lemansky et al. 1987*]. These observations demonstrated that the intracellular transport to the lysosomes and the cellular uptake of α-galactosidase from the culture medium were dependent on mannose-6-phosphate specific receptors. Taken together, these were seminal findings to provide an *in vivo* proof of concept and an *in vitro* biochemical model for enzyme replacement therapy (ERT) of Fabry disease. Yet, for many years thereafter, the inability to produce sufficient amounts of purified active enzyme and the absence of an animal model to evaluate the pharmacokinetics, biodistribution, and pharmacodynamics of the therapeutic enzyme remained as major impediments to the design of human clinical trials of ERT for Fabry disease. These obstacles were ultimately overcome by genetic engineering techniques which allowed the industrial production of α-galactosidase, either through the overexpression of the human *GLA* cDNA in Chinese hamster ovary cells [*Ioannou et al. 1992*] or the activation of the promoter of the *GLA* gene in cultured human skin fibroblasts, and by the generation of a *GLA* knockout mouse model of Fabry disease [*Ohshima et al. 1997*].

Two main clinical presentations of Fabry disease have been recognised in males, with differing clinical severity, rate of progression, and pattern of organ involvement [*Desnick et al. 2001, Desnick et al. 2001a, Desnick et al. 2003*]. The severe classical phenotype characteristically develops in patients with less than 1-5% of residual α-galactosidase activity. The later-onset atypical variants, which are associated with levels of residual activity α-galactosidase above 5-10%, have more restricted organ involvement, limited to kidney and/or the heart, and lack the cutaneous (angiokeratoma, hypohidrosis), neuropathic (acroparesthesias) and ocular (cornea verticillata) that are typically observed in classically affected males, early in the natural history of the disease. The pathophysiology and molecular mechanisms linking glycosphingolipid accumulation to the clinical manifestations of Fabry disease are still not clearly understood. The main pathogenic theory of the classical phenotype attributes its complications predominantly to ischaemic tissue damage resulting from microvascular endothelial disease and/or necrosis of vascular

smooth-muscle cells and/or pericyte injury [*Desnick et al. 2001*]. For this reason, the clearance of endothelial Gb3 deposits has been proposed as a major therapeutic target. However, patients with the cardiac variant have significant Gb3 storage almost confined to the cardiomyocytes.

Fabry disease has been reported in all ethnic groups. The prevalence of the classical phenotype has been estimated between 1:40,000-60,000 male live births [*Desnick et al. 2001*], which makes of Fabry disease a 'rare' or 'orphan' disease, according to the epidemiological criteria defined by the Office of Rare Diseases of the National Institutes of Health, in the United States of America, or by the European Commission in the European Union [*EURORDIS: European Organisation for Rare Diseases 2005*]. However, the accurate estimation of the prevalence of Fabry disease is plagued with difficulties because it is not unusual for diagnosis of affected individuals to be delayed for years, or even decades, due to the phenotypic diversity or failure to recognise the constellation of early signs and symptoms. The later-onset phenotypes may have a population prevalence manifold higher than the classical phenotype [*Spada et al. 2006*].

The differential diagnosis of Fabry disease is determined by the various signs and symptoms of the disease [*Desnick et al. 2001*]. The angiokeratomas may be diagnosed as petechiae. The acroparesthesias are not infrequently associated with low-grade fever and elevated erythrocyte sedimentation rate, a clinical presentation that has been confounded with rheumatic fever. Patients with classic Fabry disease have also been misdiagnosed with "growing pains," "neurosis", juvenile arthritis, rheumatoid arthritis, systemic lupus erythematosus, Raynaud syndrome, erythromelalgia, and multiple sclerosis. The later-onset atypical variants of Fabry disease may be unrecognised in cardiac clinics, misdiagnosed as hypertrophic cardiomyopathy, and in nephrology clinics, misdiagnosed as chronic glomerulonephritis or end-stage renal disease (ESRD) of undetermined aetiology.

The inheritance of Fabry disease follows a typical X-linked pattern: all the daughters of an affected man will be heterozygous for the *GLA* mutation carried by her father but none of the sons of a male patient will inherit the disease from the father; contrastingly, the risk of inheriting a *GLA* mutation from a heterozygous mother is 50%, irrespective of the gender of the offspring. As a clinical consequence of X-linkage, all hemizygous males develop the disease but the heterozygous females have more variable clinical phenotypes, ranging from no or mild clinical manifestations to relatively rare cases that progress as severely as affected males. The attenuated clinical expression of Fabry disease in the heterozygous females is, at least in part, modulated by the random inactivation of one of the X chromosomes that occurs in the female embryo [*Lyon 1961*], probably at blastocyst stage.

In affected males, either with the classic or variant phenotypes, Fabry disease is readily diagnosed by the demonstration of absent or deficient α-galactosidase enzyme activity in plasma, leukocytes, or cultured cells, or alternatively by the identification of a pathogenic *GLA* mutation [*Desnick et al. 2001, Desnick et al. 2003*]. Nearly 100% of the males with Fabry disease will have an identifiable *GLA* mutation. Although the demonstration of significantly decreased α-galactosidase activity confirms the diagnosis of Fabry disease also in females, many heterozygotes will have enzyme activity in the normal range, which renders the enzyme assays unreliable to diagnose Fabry disease in females [*Desnick et al. 2001, Desnick et al. 2003*]. For this reason, the *GLA* mutational analysis is the reference method to confirm the diagnosis of Fabry disease in heterozygous females.

Until recently, recognition of Fabry disease did not affect the patient's prognosis, since no specific treatment was available. However, the recent availability of ERT with bioengineered human α-galactosidase (agalsidase) offers the promise of altering the natural history of the disease [*Eng et al. 2001, Schiffmann et al. 2001*]. This greatly increased the responsibility of making its diagnosis before the occurrence of irreversible complications.

THE BIOLOGICAL BACKGROUND

Lysosomes: Structure and Physiology

Initially discovered by Christian de Duve in 1955 [*de Duve 1959*], lysosomes are acidic, hydrolase-rich organelles that are capable of degrading most biological macromolecules. Lysosomes form part of a highly dynamic endocytotic system. Two types of mannose-6--phosphate receptors were identified because of their ability to bind mannose-6-phosphate containing soluble acid hydrolases in the Golgi apparatus and transport them to the endosomal/lysosomal system [*Ghosh et al. 2003*]. The acidification of endosomes, lysosomes and lysosome-related organelles facilitates the dissociation of the mannose-6-phosphate--receptor-ligand complexes, and the proteolytic processing required for the enzymatic activation of several hydrolases. Alterations in the proteolytic machinery of the lysosomes or in the vesicular transport of the endosomal/lysosomal system, as well as deficiencies of lysosomal hydrolases, their activators or transporters, can occur as a consequence of mutations in the corresponding genes. These genetic defects are the cause of the lysosomal storage diseases, in which there is accumulation of specific substrates in the lysosomes [*Saftig 2006*].

Lysosomes are surrounded by a limiting membrane. The lysosomal membrane has multiple functions including acidification of the lysosomal lumen, sequestration of lysosomal enzymes, mediation of fusion events and transport of degradation products to the cytoplasm. Lysosomal membrane proteins are usually highly glycosylated proteins decorating the luminal surface of lysosomal membranes. Apart from such general central cellular functions, lysosomes are also involved in specific processes outside the cell, exemplified by the bone-degrading capacity of osteoclasts or the secretion of lysosomal hydrolases by granulocytes to act as a first line of defense against bacterial pathogens [*Saftig 2006*].

Glycosphingolipids: classification, biosynthesis, physiological role and catabolism

Glycosphingolipids are a large and heterogeneous family of substances constituted by a saccharide chain glycosidosically linked to ceramide [*Sandhoff & Kolter 2003*]. All glycosphingolipids are glycosides of N-acylsphingosine (ceramide) and variation in their fatty acid, sphingosine, and carbohydrate moieties results in a large number of distinct molecular species [*Ullman & McCluer 1989*]. Ceramide, the hydrophobic portion of glycosphingolipids, is composed of a sphingoid (long-chain) base in amide linkage to a fatty acid. The saccharide linked to ceramide may be a monosaccharide or a polysaccharide chain of up to approximately 40 carbohydrate residues. Major structural and functional classifications of glycosphingolipids have traditionally been based on the glycans. The four principal classes of glycosphingolipids are the cerebrosides, sulfatides, globosides and gangliosides [*Schnaar et al. 2009*].

- **Cerebrosides** have a single sugar group linked to ceramide, most commonly galactose (galactocerebrosides). Glucocerebrosides are less abundant and found at elevated levels only in Gaucher disease, which is caused by mutations in the enzyme β-glucocerebrosidase, resulting in the accumulation of glucosylceramide, particularly in the liver and spleen;

- **Sulfatides** are the sulfuric acid esters of galactocerebrosides. Excess accumulation of sulfatides is observed in metachromatic leukodystrophy;

- **Globosides** are cerebrosides that contain additional carbohydrates, predominantly galactose, glucose or N-acetyl-galactose. Gb3 is a ceramide trihexoside containing two galactose residues linked to glucosylceramide;

- **Gangliosides** are very similar to globosides except that they also contain n-acetylneuraminic acid in varying amounts.

Glycosphingolipids may be further subclassified as neutral (no charged sugars or ionic groups directly connected to a ceramide moiety), acidic (having one or more sialic acid residues) or sulfated.

Glycosphingolipids are normal constituents of the plasma membrane and of some intracellular membranes, including those of the Golgi apparatus and lysosomes, serving essential structural and functional roles. Glycosphingolipids are primarily expressed in the outer leaflet of the limiting plasma membrane of cells, with their glycans facing the external milieu. Thus, glycosphingolipids may act as intermediaries in the flow of information from the outside to the inside of cells. Their functions fall into two major

categories: mediating cell-cell interactions via binding to complementary molecules on apposing plasma membranes (i.e. interaction of leukocytes with the blood vessel wall during the process of inflammation) and modulating activities of proteins in the same plasma membrane (i.e. interactions between gangliosides and members of the receptor tyrosine-kinase family) [*Schnaar et al. 2009*].

Glycosphingolipid biosynthesis occurs in the endoplasmic reticulum and Golgi compartment in a stepwise fashion, with an individual sugar added first to ceramide and then subsequent sugars transferred by glycosyltransferases from nucleotide sugar donors. Ceramide is synthesized on the cytoplasmic face of the endoplasmic reticulum; it subsequently equilibrates to the luminal face and trafficks to the Golgi compartment. Glucosylceramide is synthesized on the cytoplasmic face of the endoplasmic reticulum and early Golgi apparatus; it then flips into the Golgi lumen, where it is typically elongated by a series of glycosyltransferases. In contrast, galactosylceramide is synthesized on the luminal face of the endoplasmic reticulum and then trafficks through the Golgi compartment, where it may be sulfated to form sulfatide. In both cases, the final orientation of glycosphingolipids is consistent with their nearly exclusive appearance on the outer leaflet of the plasma membrane, facing the extracellular milieu. Although ceramide resides on intracellular organelles such as mitochondria, glycosphingolipids of complexity higher than glucosylceramide are not known to exist on membranes facing the cytoplasm.

The breakdown of glycosphingolipids occurs stepwise by the action of lysosomal hydrolases. Glycosphingolipids on the outer surface of the plasma membrane are internalized, along with other membrane components, in invaginated vesicles that then fuse with endosomes, resulting in the glycosphingolipid glycan facing the endosomal lumen. Glycosphingolipid-enriched areas of the endosomal membrane may then invaginate once again to form multivesicular bodies within the endosome. When endosomes fuse with primary lysosomes, glycosphingolipids become exposed to lysosomal hydrolases. As glycosphingolipids are successively cleaved to smaller structures, the remaining "core" monosaccharides become inaccessible to the water-soluble lysosomal hydrolases and require assistance from activator proteins. These include GM2-activator protein (GM2 AP) and four structurally related saposins (SAP-A, SAP-B, SAP-C and SAP-D) [*Schnaar et al. 2009*], each involved in the degradation of different glycosphingolipids. Saposins are thought to bind to their glycosphingolipid substrate, disrupt its interaction with the local membrane environment, and facilitate the access of the glycans to hydrolytic enzymes. Glycosphingolipids are eventually broken down to their individual components, which are then available for reuse.

Alpha-Galactosidase: biosynthesis, structure and function

Alpha-Galactosidase is a typical lysosomal acid hydrolase with an optimal activity towards natural and synthetic substrates at pH 3.8-4.6 [*Dean & Sweeley 1979*]. The physiologic role of α-galactosidase is the hydrolysis of terminal, non-reducing alpha-D-galactose residues in alpha-D-galactosides from neutral glycosphingolipids, with anomeric specificity for the α-1,4 glycosidic bond. The active enzyme is a relatively heat-labile, homodimeric glycoprotein of approximately 101 kDa that requires SAP-B to act on its natural substrates *in vivo* [*Kase et al. 1996*]. SAP-B is a glycoprotein that binds to sulfatide, GM1 ganglioside, and Gb3, rendering these glycosphingolipids available for hydrolysis by their respective lysosomal enzymes [*Desnick et al. 2001*].

Alpha-Galactosidase is synthesized as a glycosylated precursor of 52 kDa in the rough endoplasmic reticulum, which is then phosphorylated and processed to the mature lysosomal form of 49 kDa following removal of a signal peptide during its transport to the lysosome via mannose-6-phosphate pathway [*Lemansky et al. 1987, Desnick et al. 2001, Desnick & Schuchman 2002*]. A proportion of the phosphorylated enzyme is secreted from the cell and is taken up by receptor-mediated endocytosis through mannose-6-phosphate receptors in the plasma membrane. The secretion and reuptake of alpha-galactosidase provides the rationale for ERT. Mutations in the *GLA* gene produce abnormal forms of α-galactosidase that are unable to effectively break down their metabolic substrates [*Guce & Garman 2010*].

The Alpha-Galactosidase (*GLA*) Gene and Heredity

The *GLA* gene locates to the region q22.1 of the X-chromosome and is composed by seven exons, ranging in size from 92 to 291 nucleotide base pairs (bp) [*Desnick et al. 2001, Gal et al 2006*]. The *GLA* cDNA consists of 1290 bp, coding for a polypeptide of 429 amino acids, including a 31-amino acid signal sequence, which is removed in the endoplasmic reticulum [*Gal et al 2006*].

More than 600 pathogenic mutations of the *GLA* gene have already been compiled at the Human Gene Mutation Database® [*http://www.hgmd.cf.ac.uk/ac/gene.php?gene=GLA; last accessed on the 1/12/2011*]. About 75% of those are point mutations, including missense (around 56%), nonsense and splicing mutations [*Pastores & Lien 2002*], about 21% are short length deletions and/or duplications and the remainder are gross deletions and/or duplications and complex rearrangements. In a small minority of patients with biochemically confirmed Fabry disease, no *GLA* mutation can be found [*Schäfer et al. 2005*].

There are no obvious mutational hot spots in the *GLA* gene. Exon 5 has the highest relative frequency of point mutations, followed by exon 6 and exon 3; in total, these three exons which comprise 41.7% of the coding sequence harbour about 53% of the point mutations. Of the 14 CpG dinucleotides in the *GLA* coding sequence, point mutations have been described in ten, reflecting that CpG dinucleotides are prone to point mutations. Haplotype analysis of mutant alleles that occurred in two or more unrelated families reveals that those with rare alleles are probably related, whereas those with mutations involving CpG dinucleotide are not [*Ashton-Prolla et al. 2000*]. About one-third of the small rearrangements occur in exon 7, which accounts for about 22% of the coding region, suggesting that exon 7 is susceptible to rearrangements [*Gal et al 2006*].

The frequency of *de novo* mutations in Fabry disease is unknown, but estimated to occur in 3-10% of all cases [*Gal et al 2006, Schäfer et al. 2005*].

The mutations in the *GLA* gene may affect the synthesis, processing and stability of the α-galactosidase protein and can be grouped into three broad categories. First, there are mutations which compromise the active site of the enzyme, leading to loss or decrease of enzyme activity. Second, perturbations in the hydrophobic core of the protein lead to folding defects in the enzyme. This class comprises the largest group of mutations in Fabry disease, indicating that Fabry disease is usually a protein folding disease. The rest of the mutations are grouped into a third category, which includes mutations that lead to broken disulphide bonds or to the loss of N-linked glycosylation sites, and mutations that affect enzyme activity by as yet unknown mechanisms [*Guce & Garman 2010*].

The Storage Products in Fabry Disease

Globotriaosylceramide (Gb3; also abbreviated as GL-3 or CTH) [Gal (α1→4) Gal (β1→4) Glc (β1→1′) Cer], is the predominant storage product in Fabry disease. Another major natural substrate of α-galactosidase is galabiosylceramide (Ga2) [Gal (α1→4) Gal (β1→1′) Cer], which also accumulates to significant amounts in patients Fabry disease [*Desnick et al. 2001, Mills et al. 2005*]. The blood group B and B1 antigens are additional examples of glycosphingolipids that are not adequately degraded when α-galactosidase activity is deficient [*Asfaw et al. 2002, Wherret & Hakomori 1973*]. Because Gb3 and related glycosphingolipids cannot be catabolised in the plasma, the total amount accumulated in a given tissue depends on time, the level of residual α-galactosidase activity, the individual's ABO blood group, the rate of accumulation from intracellular and circulatory sources, the possibilities for excretion and the half-life of each relevant cell type [*Desnick et al. 2001*].

- **Globotriaosylceramide:** Gb3 is synthesised by α1,4-galactosyltransferase by addition of an α-galactosyl moiety to lactosylceramide [*Taga et al. 1995, Desnick et al. 2001*]. In red blood cells, Gb3 has been identified as the P^k antigen of the P blood group system [*Marcus et al. 1981*]. In leukocytes, Gb3 is the cell surface antigen clustered as CD77 (cluster of differentiation) [*Knapp et al. 1989*]. Monoclonal antibodies against Gb3/CD77 are used as a marker for Burkitt's B-cell lymphoma and are able to initiate apoptosis. Gb3/CD77 has also been localised to a subset of tonsillar B-cells in the germinal center, platelets and uroepithelial cells. The B-subunit of Shiga toxins interacts specifically with Gb3 on the cell surface; thus Gb3 plays a direct role in toxin entry into the cell. Recently, Gb3 has been implicated in the entry of HIV-1 into cells [*Keusch et al. 2000*].

  In normal individuals, the highest concentrations of Gb3 are found in the kidney, followed by aorta, spleen and liver. Kidney, liver, lung and erythrocytes contribute much of the normal Gb3 load. A major source of Gb3 is the catabolic processing of globotetraosylceramide (globoside, Gb4) by removal of its terminal N-acetylgalactoamine residue.

  In males with classical Fabry disease, increased concentrations of Gb3 are found in all analysed sources except erythrocytes, meaning that most tissues are involved in its catabolism. The magnitude of accumulation of Gb3 in Fabry hemizygotes was thirty - to more than three hundredfold higher than normal levels, with the highest concentration found in kidney, lymph nodes, heart, prostate, striated muscle, and autonomic ganglia [*Desnick et al. 2001*].

- **Galabiosylceramide:** The storage of Ga2 in patients with Fabry disease is tissue-specific, being detected only in the kidneys, pancreas, right heart, lung, urinary sediment and spinal and sympathetic ganglia. Ga2 is synthesised by β1,1 galactosylcerebroside-α1, 4galactosyltransferase from galactosylcerebroside, which is found in the brain and kidney. This galactosyltransferase is present in the kidney, but not in the brain, spleen or liver, consistent with the tissue distribution of Ga2 observed in Fabry patients [*Desnick et al. 2001*].

- **Blood group B glycosphingolipids:** The blood group B and B1 erythrocyte antigens are fucose-containing neutral glycosphingolipids with terminal α-galactosyl residues. In general, glandular epithelial tissues such as stomach, pancreas, and intestine are rich sources of these substances, whereas parenchymatous organs and erythrocytes contain lower quantities. Thus, patients with Fabry disease who have blood group B and AB accumulate four glycosphingolipids substrates - Gb3, Ga2, and B and B1 -, whereas patients with A or O blood group accumulate only Gb3 and Ga2 [*Desnick et al. 2001*].

- **Blood group P glycosphingolipids:** The P^k and P antigens of the P blood group system are, respectively, Gb3 and Gb4. Although these antigens are relatively rare, the P1 antigen, which also has a terminal α-galactosyl residue, is present in about 75-80% of the Caucasian population. To date, however, the accumulation of P1 substance in Fabry disease has not been documented, nor has the possibility of increased disease severity due to the accumulation of this blood group substance been assessed [*Desnick et al. 2001*].

The Inheritance of Fabry Disease

As typical in X-linked inherited disorders, Fabry disease is usually more severe in the hemizygous males than in the heterozygous females. Although a substantial proportion of affected women may develop significant clinical complications [*Wilcox et al. 2008*], most females remain asymptomatic or only mildly symptomatic [*Desnick et al. 2001*]. The expression of Fabry disease in females is modulated by the effect of X chromosome inactivation and the resulting proportions of normal and enzyme-deficient cells in individual organs [*Lyon 1961*].

The following general rules are useful for genealogical analysis and genetic counselling of patients and families with Fabry disease [*Germain 2006*]:

A | Parents of an affected male proband:

- The father of an affected male is not affected;
- In a family with more than one affected individual, the mother of an affected male individual is an obligate carrier;
- If only one individual in the family is affected, the mother of the affected male is likely a carrier. Rarely, the affected male may have a *de novo* gene mutation.

B | Siblings of an affected male proband:

- The risk to siblings depends on the carrier status of the mother;
- If the mother of the proband has a disease-causing mutation, the chance of transmitting it in each pregnancy is 50%. Male siblings who inherit the mutation will be affected; female siblings who inherit the mutation will be carriers;
- Female carriers may be symptomatic;
- If the disease-causing mutation cannot be detected in the DNA of the mother of the only affected male in the family, the risk to siblings is low but greater than that of the general population because of the possibility of germline mosaicism. Although maternal germline mosaicism has not been demonstrated in this condition, it remains a possibility.

C | Siblings of a (symptomatic or asymptomatic) heterozygous female:

- If the mutation was inherited from her affected father, all of her sisters will be carriers and none of her brothers will be affected;
- If the mutation was inherited from her mother, there is a 50% chance of transmitting the mutation in each pregnancy. Male siblings who inherit the mutation will be affected; female siblings who inherit the mutation will be carriers;
- Paternal germline mosaicism has been demonstrated in this condition [*Dobrovolny et al. 2005*]. Thus, even if the disease-causing mutation has not been identified in paternal DNA, female siblings of a female carrier may still be at increased risk of inheriting the disease-causing mutation.

D | Offspring of an affected male:

- All daughters of an affected male are obligate carriers and may be symptomatic;
- All sons of an affected male are unaffected.

E | Offspring of a (symptomatic or asymptomatic) heterozygous female:

- The risk of transmitting the mutation in each pregnancy is 50%;
- Male offspring who inherit the mutation will be affected;
- Female offspring who inherit the mutation will be carriers and may be affected.

CLINICAL MANIFESTATIONS IN FABRY DISEASE

The clinical manifestations of Fabry disease result predominantly from the progressive multisystemic deposition of glycosphingolipids in the different tissues and body fluids, with predominance in skin, kidney, heart, blood vessels, peripheral nerves and brain [*Desnick et al. 2001*]. This accumulation of glycosphingolipids initially causes cellular dysfunction and sub-clinical disease. Clinical onset usually occurs during childhood or adolescence, but it may be delayed until the second or third decade. Physical stigmata, such as angiokeratomas in skin and mucous membranes and a characteristic benign corneal dystrophy facilitate the diagnosis of Fabry disease [*Brady & Schiffmann 2000*]. Early symptoms reflect disease progression in the peripheral and autonomic nervous systems and include neuropathic pain, gastrointestinal symptoms and hypohidrosis. The disease becomes progressively more severe and affects multiple organ systems over time. Clinical manifestations of disease progression in the kidney, heart and cerebrovascular system usually occur later in a Fabry patient's life, although in severely affected patients, these may occur in the second and third decades of life. These late complications are the major causes of the morbidity and mortality associated with Fabry disease.

Like most lysosomal storage disorders, Fabry disease encompasses a spectrum of disease severity, with the classic form representing the most severe phenotype. Less severe renal and cardiac variants have been recognised, both of which are characterised by the presence of residual α-Gal activity, onset in adulthood and clinical manifestations confined primarily to the kidney and/or myocardium [*von Scheidt et al. 1991, Nakao et al. 1995, Nakao et al. 2003*].

Early Symptoms in Pediatric Patients with Classic Variant of Fabry Disease

Paediatric Fabry patients have a significantly decreased quality of life than their peers. Unlike many lysosomal storage disorders, Fabry disease is not associated with mental retardation or obvious physical abnormalities, and the onset in male and female paediatric patients varies between 6 and 8.1 years old, respectively [*Ries et al. 2005, Wilcox et al. 2007*]. Table 1 summarises the clinical manifestations in paediatric patients.

Table 1 | Clinical manifestations of classic Fabry disease in pediatric patients.

age 4-16 years	· Intermittent paresthesia and acroparesthesia consisting of chronic, burning, tingling pain in the hands and/or feet, usually beginning in early childhood. Can occur daily and can last minutes to days;
	· Episodic Fabry crises of severe, incapacitating pain, lasting from days to weeks. Often precipitated by stress, illness, physical exertion, or temperature changes and accompanied by fever and an elevated erythrocyte sedimentation rate. Very rare in females;
	· Angiokeratomas that appear in adolescence and worsen in adulthood;
	· Whorled corneal opacity (also frequently observed in females with or without disease manifestations);
	· Gastrointestinal problems, including diarrhea, abdominal discomfort, vomiting, nausea;
	· Hypohidrosis or anhidrosis;
	· Heat, cold, and/or exercise intolerance;
	· Mild proteinuria and urinary sediment containing globotriaosylceramide.
late adolescence to adulthood	· Renal dysfunction that leads to uremia and hypertension and progresses to end-stage renal disease;
	· Cardiovascular dysfunction, including myocardial infarction, valvular abnormalities, arrhythmias, left ventricular hypertrophy;
	· Cerebrovascular complications, such as early stroke, hemiplegia, hemianesthesia, transient ischemic attacks;
	· Pulmonary complications, such as airflow obstruction, dyspnea.

(Desnick 2004)

- **The pain.** Two types of pain have been described: episodic crises and constant discomfort (acroparesthesia - chronic burning or tingling pain in the hands and feet). The Fabry crises are episodes of agonizing neuropathic pain that typically begin in the hands and feet and may radiate proximally. Pain episodes can last from minutes to weeks and are often accompanied by fever and an elevated erythrocyte sedimentation rate. Most boys with classic Fabry disease historically have Fabry crises beginning as early at the age of 4 years (mean age of onset 6.4 years old). Pain is often triggered by stress, heat, fatigue or exercise. Hypohidrosis also leads to heat sensitivity and exercise intolerance, both of which worsen with age. With time, pain intensity increase, and attacks occur more frequently, during sports sessions and also without overheating or pyrexia. At this time, children are often admitted to hospital and require morphine for acute attacks and anticonvulsants for chronic pain relief. Attacks of abdominal or flank pain may simulate appendicitis or renal colic. As the patient ages, the periodic crises usually decrease in frequency and severity; however, in some patients, they may occur more frequently, and the pain could be so excruciating that the patient may contemplate suicide [*Desnick et al. 2001, MacDermot et al 2001, Wilcox et al. 2007*].

Acroparesthesias may occur daily, usually during late afternoon, and may represent an attenuated form of the Fabry crises. Heterozygous females may also have acroparesthesias, usually tingling in nature and beginning at a mean age of 7.9 years old. The acroparesthesias in children with undiagnosed Fabry disease are often dismissed as malingering, or growing pains. Physical examination may not provide clues about the diagnosis, particularly if the cutaneous involvement is subtle. Electromyography and nerve conduction studies usually fail to detect abnormalities because the neuropathology primarily involves small nerve fibers. Pain has also led to misdiagnoses, including rheumatic fever or joint pain *[Desnick et al. 2001, MacDermot et al. 2001a, Desnick & Brady 2004, Wilcox et al. 2007*].

- **Gastrointestinal manifestations.** Beginning in childhood, boys with Fabry disease may have mild-to-severe gastrointestinal disturbances, including diarrhoea, early satiety, abdominal discomfort, nausea and vomiting. Females may also have gastrointestinal symptoms, usually beginning in adolescence [*Sheth et al. 1981, Argoff et al. 1998*].

- **Dermatologic manifestations.** Virtually all male patients with classic Fabry disease develop angiokeratomas, usually beginning in childhood or adolescence and becoming larger and more numerous with age. These small, slightly raised, purplish red, nonblanching telangiectases are most often found between the umbilicus and knees but can occur virtually anywhere, including the oral mucosa and conjunctiva. The hips, back, thighs, buttocks, penis and scrotum are most commonly involved, but there is a wide variation in the pattern of distribution and density of the lesions. Approximately 10% to 35% of females also develop angiokeratomas, generally beginning in adolescence. In female patients, the lesions are usually isolated and consist of small macules on the breasts, groin or flanks. The presence of cutaneous vascular lesions correlates with the severity of the systemic manifestations of the disease, as assessed by a modification of the Mainz severity scoring index [*Brady & Schiffmann 2000, Desnick et al. 2001, Desnick & Brady 2004, Whybra et al. 2004*]. In addition to these vascular lesions, anhidrosis or hypohidrosis are early and almost constant events [*Desnick et al. 2001, Desnick & Brady 2004*].

- **Ocular manifestations.** Most boys and >70% of girls with Fabry disease have a characteristic whorled corneal opacity, known as "cornea verticillata", observed by slit-lamp microscopy, but which does not affect vision. Under slit-lamp examination these appear as creamy-white whorls radiating from the corneal centre to the periphery [*Desnick et al. 2001, Desnick & Brady 2004*].

- **Kidney involvement.** Mild proteinuria, isosthenuria or urinary sediment examination reveals casts, erythrocytes and cells containing the accumulated glycosphingolipids,

which appears as Maltese cross particles under polarisation microscopy. Vasopressin-resistant (nephrogenic) diabetes insipidus has been observed in children. Before the age of 18 years, microalbuminuria or overt proteinuria are present in a minority of patients of both genders, even in children with residual α-galactosidase activity <1% normal. Only a minority of Fabry patients have decreased glomerular filtration rates before the age of 18 years [*Ries et al. 2003, Desnick & Brady 2004, Ries et al. 2005, Ramaswami et al. 2006, Wornell et al. 2006, Hopkin et al. 2008*].

- **Cardiac involvement.** Mitral insufficiency is the most frequent valvular lesion and is typically present in childhood or adolescence. A variety of electrocardiographic abnormalities and morphologic alterations of the myocardium, heart valves and vessels, such as as left ventricular hypertrophy, mitral murmurs or arrhythmias can be found [*Desnick et al. 2001, Desnick & Brady 2004, Ries et al. 2005, Hopkin et al. 2008*].

- **Skeletal and growth abnormalities.** Children with Fabry disease do not have the skeletal abnormalities characteristic of other lysosomal disorders such as Gaucher disease or several of the mucopolysaccharidoses, but many children have subtle evidence of musculoskeletal involvement. Retarded growth, delayed puberty and sparse, fine facial and body hair are common. Many affected male patients have difficulty gaining weight. A characteristic facial dysmorphism is found in about half of male patients and may be recognisable in early adolescence [*Nelis & Jacobs 1989, Desnick et al. 2001, MacDermot et al. 2001*].

- **Cerebrovascular involvement.** Stroke has been reported in 14- and 16-year-old boys, and cerebrovascular abnormalities have been found in boys as young as 8 years old [*Grewal 1994, Cabrera-Salazar et al. 2005, Ries et al. 2006*].

- **Other symptoms.** Tinnitus, vertigo, fatigue and anaemia associated with reticulocyte levels in the low-normal range have been described in paediatric patients. Subclinical hypothyroidism has also been described [*Ries et al. 2005, Ramaswani et al. 2006*].

Clinical Manifestations in adult patients with Classical Variant of Fabry Disease

With increasing age, continuing multisystemic glycosphingolipid deposition in classically affected Fabry patients ultimately leads to ischaemic complications involving predominantly the kidneys, heart and brain and organ failure [*Desnick et al. 2001*].

- **Renal manifestations.** The majority of Fabry patients have kidney involvement, with significant proteinuria and progressive loss of kidney function leading to end-stage renal disease. Chronic kidney disease, progressing to end-stage renal disease, has been a major cause of morbidity and early mortality in adult males with the classical Fabry phenotype. Heterozygous females generally have a more variable, less severe and later-onset clinical course than male Fabry patients [*Desnick et al. 2001, Branton et al. 2002*]. However, a number of heterozygous females may develop severe renal complications, including chronic kidney disease and end-stage renal disease [*Mehta et al. 2004, Deegan et al. 2006, Kobayashi et al. 2008, Ortiz et al. 2008, Wilcox et al. 2008, Schiffmann et al. 2009, Oqvist et al. 2009*].

  An inability to maximally concentrate the urine, and even isosthenuria, has been the most commonly reported tubular function abnormality in Fabry disease, and was demonstrated even in patients with normal glomerular filtration rate [*Colley et al. 1958, Henry & Rally 1963*]. The urinary concentration defect may be the earliest functional manifestation of Fabry renal disease, leading to polyuria, nocturia and polydipsia [*Branton et al. 2002*]. Red blood cells, white blood cells and hyaline and granular casts can be found in the urine sediment [*Desnick et al. 2001*]. Birefringent lipid particles, with the characteristic Maltese cross appearance, may also be noted with polarised light microscopy. These particles are present either free in the urine or within desquamated uroepithelial cells. Lipid-laden renal tubular cells account for about 75% of the exfoliated cells in the urine sediment of classically affected Fabry patients [*Chatterjee et al. 1984*]. Vasopressin-resistant (nephrogenic) diabetes insipidus has been shown in adult males with Fabry disease [*Colley et al. 1958*].

  Most patients with classic Fabry disease develop proteinuria, although the severity of the proteinuria is more marked in males than females [*Desnick et al. 2001, Ortiz et al. 2008, Schiffmann et al. 2009*]. By the age of 35 years, about 50% of the affected males are estimated to have proteinuria, and all patients who survive into the 6th decade of life eventually develop proteinuria [*Branton et al. 2002*]. In almost all cases, proteinuria is of glomerular origin, containing ≥50% albumin. Nephrotic range proteinuria develops in less than 20% of male Fabry patients with chronic kidney disease [*Branton et al. 2002, Ortiz et al. 2008, Schiffmann et al. 2009*]. However, the full clinical and laboratory presentation of nephrotic syndrome is not frequent: hypoalbuminaemia and hyperlipidaemia were respectively seen in only 26 and 21% of patients with nephrotic-range proteinuria [*Branton et al. 2002, Branton et al. 2002a*].

  Systemic hypertension, probably related to vascular involvement of renal parenchymal vessels, is not uncommon, but hypertension is less prevalent in chronic kidney disease Fabry patients than in the general chronic kidney disease population, at comparable levels of glomerular filtration rate [*Desnick et al. 2001, Ortiz et al. 2008*].

Progressive pathological changes in the kidney result in renal failure in midlife in most males affected with classic Fabry disease [*Sessa et al. 2001*]. Without dialysis or kidney transplantation, the average age of death was 41-42 years [*Wise 1962, Colombi et al. 1967*]. More recently the median cumulative survival was 50 years in Fabry hemizygote patients receiving renal dialysis or transplantation [*MacDermot et al. 2001*].

- **Cardiac manifestations.** The deposition of glycosphingolipid in the cardiac myocytes of the heart, valves and cells of the bundle of His and its branches results in left ventricular hypertrophy and electrocardiographic abnormalities. Cardiac disease occurs in most hemizygous males and heterozygous females. The most frequent valvular lesion is mitral insufficiency. Involvement of the myocardium and the conduction system results in electrocardiographic abnormalities that may show left ventricular hypertrophy, ST-segment changes and T-wave inversion. Other alterations such as arrhythmias, intermittent supraventricular tachycardias and a short PR interval are common findings in Fabry patients. Patients with complete atrioventricular block and/or sinus node dysfunction have been reported. Echocardiographic studies demonstrate an increased incidence of mitral valve prolapse, an increased thickness of the interventricular septum and the left ventricular posterior wall, and an enlarged aortic root diameter. Hypertrophic obstructive cardiomyopathy secondary to glycosphingolipid deposition in the interventricular septum has been reported. Late manifestations may include angina pectoris, myocardial ischaemia and infarction, congestive heart failure and severe mitral valve regurgitation [*Colucci et al. 1982, Goldman et al. 1986, Sakuraba et al. 1986, Desnick et al. 2001*].

- **Central nervous system and neurological manifestations.** Multifocal brain and ear small-vessel involvement with glycosphingolipid deposits lead to a broad spectrum of clinical manifestations including headache, vertigo, tinnitus, hearing loss and deafness [*Germain et al. 2002*]. The most frequent cerebrovascular manifestations in descending order are hemiparesis, vertigo and/or dizziness, diplopia, dysarthria, nystagmus, nausea/vomiting, head pain, hemiataxia and gait ataxia, in the hemizygous males and memory loss, dizziness, ataxia, hemiparesis, loss of consciousness and hemisensory symptoms, in the heterozygous females [*Mitsias & Levine 1996, Desnick et al. 2001*]. These manifestations are predominantly due to pathological dilatation of the vertebrobasilar vessels, and the average age of onset of cerebrovascular complications is 33.8 years in men and 40.3 years in women [*Mitsias & Levine 1996*]. Personality changes and psychotic behaviour may become manifest with increasing age [*Liston et al. 1973, Mendez et al. 1997*]. Severe neurological signs may be present without evidence of major thrombosis or hypertension and are presumably due to small-vessel occlusive disease [*Desnick et al. 2001*]. All patients older than 54 years have cerebrovascular involvement, with the typical distribution of a small-vessel disease [Crutchfield et al. 1998]. Abnormalities of cutaneous thermal

sensation are commonly found in patients with Fabry disease, even in asymptomatic heterozygotes [*Morgan et al. 1990*]. Disturbances in the autonomic nervous system are typically seen in Fabry disease, and these include abnormalities of tear and saliva formation, cerebrovascular reactivity, cardiac rhythm, gastrointestinal motility and pain perception [*Kolodny & Pastores 2002*].

- **Ocular manifestations.** Cornea verticillata is seen in almost all patients, irrespective of gender; cream-coloured anterior capsular deposits are seen in one third of hemizygotes and in none of heterozygotes, and a translucent posterior capsular opacity with a branching radial pattern ("Fabry cataract") is observed in 37% of the hemizygotes and in 14% of the heterozygotes [*Sher et al. 1979*]. The corneal and lenticular opacities do not interfere with visual acuity. Aneurysmal dilatation and tortuosity of conjuctival and retinal vessels also occur, and are both more frequent and severe in the hemizygotes than in the heterozygous females [*Sodi et al. 2007*].

- **Lung manifestations.** Although pulmonary involvement has been claimed to have little clinical or functional impact on patients with Fabry disease, several affected individuals have had pulmonary involvement, clinically manifesting as chronic bronchitis, wheezing, dyspnoea or obstructive airway disease [*Bartimmo et al. 1972, Kariman et al. 1978*].

- **Vascular manifestations.** Pitting oedema of the lower extremities may be present in adulthood in the absence of hypoproteinemia, varices or other clinically significant vascular disease. Varicosities, haemorrhoids, and priapism have also been reported [*Mehta et al. 2008*]. Lymphoedema of the lower limbs has been described as a [*Gemignani et al. 1979*].

- **Psychological manifestations.** Depression is not rare, and its diagnosis is frequently missed. Depresion, anxiety, severe fatigue and other psychosocial manifestations lead to decreased quality of life in many affected individuals [*Mehta et al. 2008, Hoffmann 2009*].

- **Other symptoms.** Mild anaemia is probably due to decreased red blood cells survival [Kleinert et al. 2005].

The Atypical Clinical Phenotypes of Fabry Disease

Phenotypic variants predominantly affecting the kidneys and the heart, of later clinical expression than the classic phenotype, have been recognised in patients with residual α-galactosidase activity, usually over 1% of the normal average [*Desnick et al. 2001, Desnick et al. 2001a*].

- **The cardiac variant phenotype.** This atypical variant phenotype of Fabry disease with late-onset manifestations confined to the heart was identified in the early nineties [*Elleder et al. 1990, Ogawa et al. 1990, Nagao et al. 1991, von Scheidt et al. 1991*]. It is characterised by late-onset cardiac involvement and proteinuria but there is usually no renal failure [*Desnick et al. 2001, Desnick et al. 2001a, Beer et al. 2002, Meehan et al. 2004*]. Patients with the cardiac variant of Fabry disease show significantly higher residual α-galactosidase activities (up to 30% of the normal average) than patients with the classical phenotype. Extensive glycosphingolipid deposition in the kidneys is limited to podocytes. These individuals are essentially asymptomatic during most of their lives and do not experience the early classic manifestations. Most of them have cardiomegaly, typically involving the left ventricular wall and interventricular septum, and electrocardiographic abnormalities consistent with cardiomyopathy. Others have hypertrophic cardiomyopathy and/or myocardial infarctions. Most are diagnosed after the onset of cardiac manifestations and most are found to have mild proteinuria, usually of mild to moderate degree, which may be detected as early as the third decade of life, but they maintain normal renal function at ages well beyond those at which classically affected males develop end-stage renal disease [*Desnick et al. 2001*].

- **The renal variant phenotype.** The renal variant of Fabry disease was originally identified in Japanese patients on chronic haemodialysis in whom end-stage renal disease had been misdiagnosed as chronic glomerulonephritis. Fabry disease has also been diagnosed by demonstration of deficient α-galactosidase enzyme activity in patients with long-standing history of proteinuria and advanced chronic kidney disease, who had none or only a few of the typical early signs and symptoms of the disease. The majority of these patients reached end-stage renal disease during their fifth or sixth decade of life, and had moderate to severe left ventricular hypertrophy. These observations indicate that the renal variant may be underdiagnosed due to the absence of the early symptoms of Fabry disease. Therefore, it is appropriate to test for Fabry disease among individuals on renal dialysis and/or undergoing renal transplantation without a primary or biopsy diagnosis of the chronic kidney disease [*Desnick et al. 2001a, Germain 2001, Nakao et al. 2003*]. Patients with the renal variant have lower residual α-galactosidase activities than patients with the cardiac variant [*Desnick et al. 2001, Desnick et al. 2001a*].

- **The cerebrovascular variant phenotype.** Previously unrecognised Fabry disease has been diagnosed by screening Caucasian adults suffering acute cryptogenic stroke up to the age of 55 years. Although a substantial number of these patients of both genders had proteinuria, and some had cardiac abnormalities, many patients had none or only a few of the classic early signs and/or symptoms of Fabry disease [*Rolfs et al. 2005*].

THE PATHOLOGY OF FABRY DISEASE

Histopathological examination of the affected organs in Fabry disease has been critical for the understanding of this disorder, and the autopsy report of two Fabry patients in 1947 rose for the first time the possibility of it being a systemic and congenital metabolic storage disturbance affecting the entire vascular system [*Pompen et al. 1947*].

General Considerations on the Histological Examination of Glycosphingolipid Deposits

Light Microscopy

Morphologically, Fabry disease is characterised by tissue deposits of crystalline glycosphingolipids, which show birefringence with characteristic Maltese cross configuration under polarized microscope [*Desnick et al. 2001*].

Faraggiana et al. described the different histological aspects of Fabry disease as caused by the different ways of fixation and embedding tissues and the different histochemical methods of staining. They found a strong reaction to two peroxidase-labelled lectins from *Ricinus communis* and *Bandeiraea simplicifolia*, PAS and lipid-soluble dyes with the storage material in unfixed-frozen sections [*Faraggiana et al. 1981*].

- **Paraffin-embedded sections:** The most characteristic histological finding on routine light microscopy of tissue biopsies of Fabry patients stained with haematoxylin and eosin (HE) is vacuolation of the cytoplasm of affected cells, which may have a swollen appearance [Figure 1]. This aspect is characteristic of Fabry disease, but it is not pathognomonic and can be found in many other pathological situations, such as metabolic storage disorders in general. Glycosphingolipids have a carbohydrate hydrophilic moiety and a ceramide hydrophobic and lipophilic tail, and from a histochemical point of view, glycosphingolipids display the staining characteristics of both lipids and carbohydrates. The identification of this stored material by histochemical techniques is thus plagued with

difficulties. The ceramide is soluble in non-polar solvents being removed by the process of embedding in paraffin, but the carbohydrate may remain within the vacuoles during this procedure [*Faraggiana et al. 1981, Desnick et al. 2001*]. Residual carbohydrates within these vacuoles can be non-specifically identified in periodic acid-Schiff and Jones methenamine silver in formalin-fixed, paraffin-embedded tissues [Figure 2] [*Desnick et al. 2001, Fischer et al. 2006, Oliveira et al. 2008*].

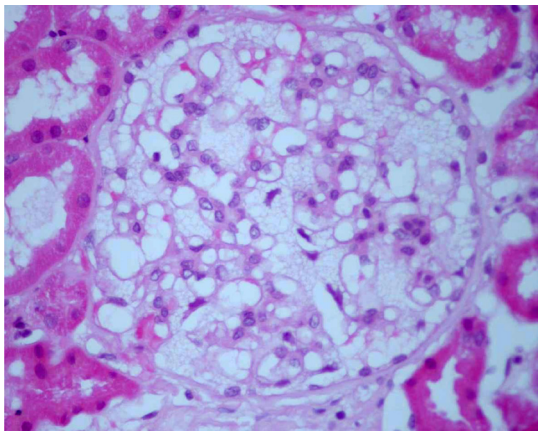

Figure 1 · Extensive vacuolation of epithelial glomerular cells (Hematoxilin and eosin, x400).

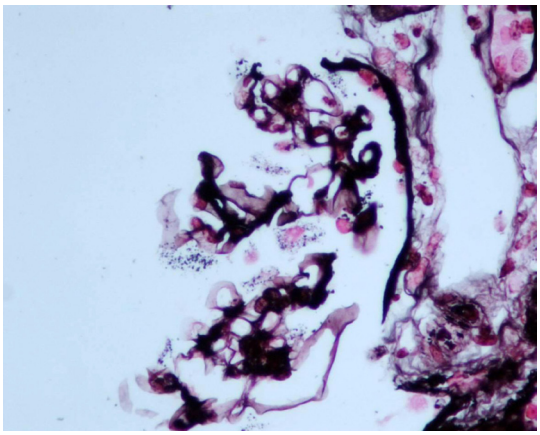

Figure 2 · Formalin fixed, paraffin embedded kidney showing glomeruli with granular and argirophilic inclusions in the podocytes (Jones methe-namine silver, x200).

- **Methacrylate-embedded sections:** Embedding biopsies from patients with suspected Fabry disease in methacrylate retains some of glycosphingolipids inclusions and allows staining with lipid-soluble dyes [*Faraggiana et al. 1981*]. However, this is not a method routinely used in diagnostic pathology.

- **Frozen sections:** Frozen sections of formalin-fixed or unfixed material from Fabry patients allow preservation and histochemical identification of glycosphingolipids but with the consequent loss of fine cellular details [*Faraggiana et al. 1981*].

- **Epon-embedded sections:** Biopsies fixed in formalin or glutaraldehyde, post-fixed in osmium and embedded in epon for electron microscopy purposes, retain the entire glycosphingolipids molecules and allow the identification of this deposit material in semi-thin sections stained with toluidine blue or methylene blue under light microscopy vision. Glycosphingolipids are seen as dark-grey or dark-blue round or spiral inclusions in the cytoplasm of affected cells or even floating in biologic fluids such as urine [Figure 3]. Glycosphingolipids have birefringence, with typical Maltese cross configurations and autofluorescence under polarised light microscopy [Figure 4] [*Faraggiana et al. 1981*].

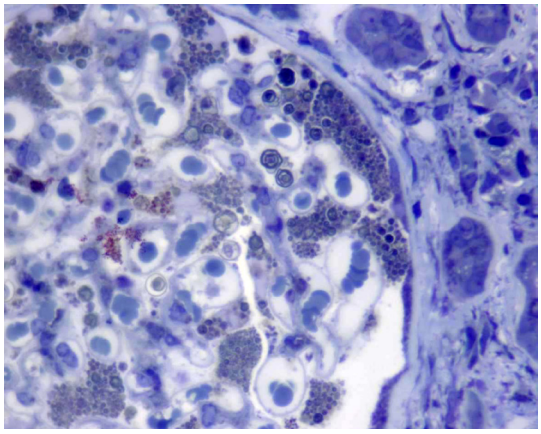

Figure 3 · Glomerulus with numerous dark-grey or dark-blue osmiophilic inclusions, round or spiral shaped, in the podocytes, and small and punctiform in parietal cells. Interstitial cells and peritubular capillary cells, on the right, have also small osmiophilic inclusions (Semi-thin section, methylene blue-stained, x600).

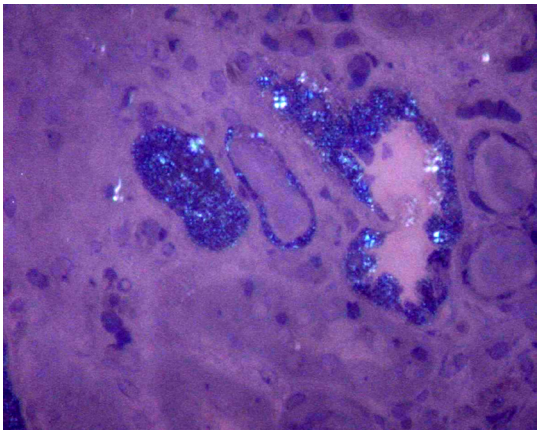

Figure 4 · Polarized light microscopy appearance of a tubulo-interstitial area of the kidney parenchyma, showing three tubuli with their epithelial cells filled with autofluorescent deposits, some of them birefringents and with typical Maltesse cross appearance (Semi-thin section, methylene blue-stained, x600).

Immunofluorescence Microscopy

Antibodies for IgG, IgM, IgA, C3, C1q do not react with the glycosphingolipids storage material in biopsies of Fabry patients, and therefore the routine immunofluorescence studies should be negative [*Tisher & Brenner (ed) 1989*].

Electron Microscopy

Electron microscopy is critical for the diagnosis of Fabry disease, because osmium tetroxide, used in the processing material for electron microscopy, is an excellent fixative for lipids in general, and preserves the glycosphingolipids that otherwise would be extracted by solvents used for processing of tissue (alcohol, xylene) [*Faraggiana et al. 1981*]. The most accurate descriptions of glycosphingolipids deposits seen in electron microscopy were made in renal biopsies from Fabry patients, and the first report of their typical ultrastructure was published in the early sixties, in the morphological study of a kidney needle biopsy specimen [*Henry & Rally 1963*].

Most glycosphingolipids inclusions have a coarsely lamellated appearance, and may be round with a concentric onion-skin like structure, the so called "myelin figures", or ovoid, with a parallel arrangement of dense layers, known as "zebra bodies" [Figure 5 and 6]. Some glycosphingolipids inclusions may appear as dark electrondense and amorphous bodies, without lamellation, and these are more frequently found in endothelial and mesangial cells [Figures 5] [*Gubler et al 1978, Desnick et al. 2001, Sessa et al. 2002, Fischer et al. 2006*].

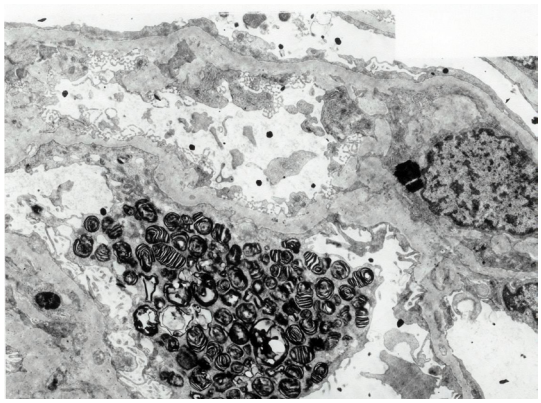

Figure 5 · Electron microscopy image from a kidney biopsy showing part of a glomerulus with "Zebra bodies" and "myelin figures" in a podocyte. "amorphous bodies" are also seen in mesangial cells (Electron microscopy, 2,700x3).

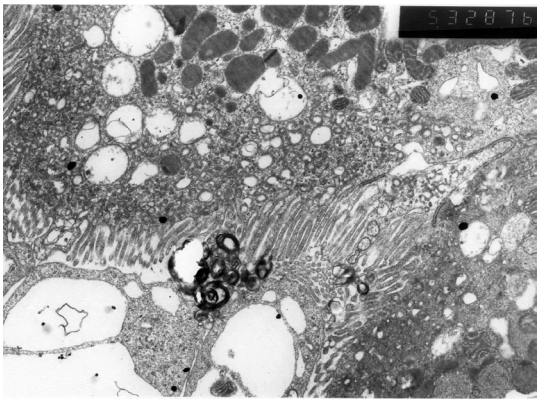

Figure 6 · Electron microscopy image of a proximal tubuli showing osmiophilic myelin figures floating in the tubular lumen, in intimal relation with microvilli of the epithelial cell (Electron microscopy, 5,300x3).

Immunohistochemical Location

The first description of immunohistochemical staining of Gb3 in urinary renal tubular cells, using a fluorescein-labeled antibody specific for Gb3, was reported in 1984 [*Chatterjee et al. 1984*].

A murine, highly specific monoclonal antibody against Gb3, working on frozen sections and paraffin-embedded tissue, was used for immunohistochemical detection of Gb3 in tissue specimens of Fabry patients [*Kotani et al. 1994, Fukushima et al. 1995, Kanekura et al. 2005, Askari et al. 2007*], in human kidneys [*Taguchi et al. 1998, Ergonul et al. 2003*] and in murine tissue [*Fujii et al. 2005*], but its use for the diagnosis of Fabry disease has not yet been validated. Gb3 is intrinsically expressed in the human kidney, with strong intensity in cortical tubular epithelia, and also in podocytes, mesangial cells and endothelium of different renal vessels and microvasculature [*Hughes et al. 1998, Hughes et al. 1998a, Ergonul et al. 2003, Meyers & Kaplan 2000, Taguchi et al. 1998*]. However, the immunohistochemical detection of Gb3 in normal human glomerular cells depends on the technical conditions, with a loss of almost 90% of positive immunoreactions if the immunohistochemistry process is performed at room temperature [*Ergonul et al. 2003*].

The possibility to use this anti-Gb3 antibody as a tool for the histological diagnosis of Fabry disease in paraffin-embedded tissue specimens would be invaluable, given the limited availability of electron microscopy in diagnostic pathology departments [Figure 7] [*Furness 1996*].

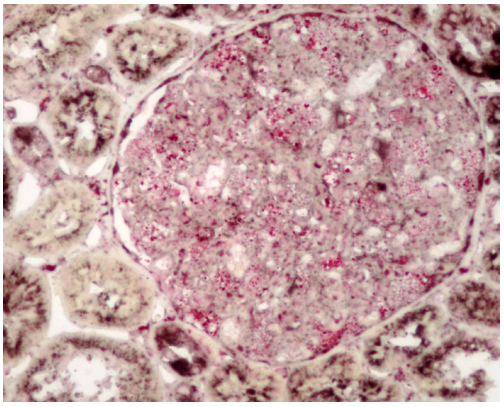

Figure 7 · Immunohistochemical reaction against Gb3 in glomerular, interstitial and peritubular capillary cells is remarkable in this renal biopsy from a hemizygous patient with Fabry nephropathy [Gb3 (bright red precipitate)/LAMP2 (crisp brown precipitate) double immunohistochemistry, x200].

Special Issues in the Pathology of the Different Organs

The Skin: Angiokeratoma Corporis Diffusum

Angiokeratomas are often one of the earliest manifestations of Fabry disease. The skin lesions are telangiectasias or small superficial angiomas. The cutaneous eruption in most instances consists of innumerable small angiomas, which are usually only 1 to 2 mm in diameter. They appear as dark red macules or papules ranging in size from micrometers to millimetres, which do not blanch with pressure. Slight hyperkeratosis is notable in larger lesions. The greater number of lesions are localised in the beltline, abdomen, buttocks, hips, genitalia and upper thighs areas, and tends to be bilaterally symmetric. The oral mucosa, conjunctiva and other mucosal areas are commonly involved with telangiectasias. Less characteristic changes include palmar erythema or splinter haemorrhages. In occasional instances cutaneous lesions are absent [*Clarke et al. 1971*].

In routine microscopic sections, there is a slight hyperkeratoris and a somewhat hyperplastic epidermis. The subepidermal capillaries are dilated and may be enclosed by the hyperplastic epidermis [Figure 8].

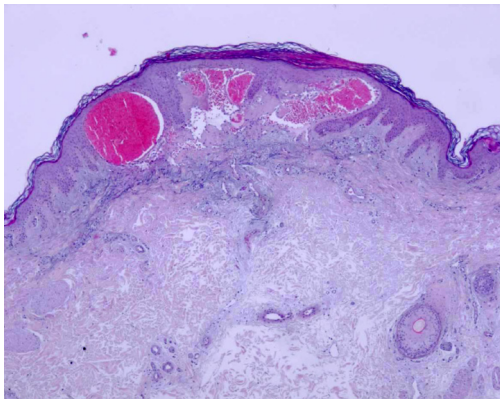

Figure 8 · Angiokeratoma in the skin. There is a slight hyperkeratoris and some acanthosis and papillomatosis of the epidermis. The subepidermal capillaries are dilated and are enclosed by the hyperplastic epidermis (Hematoxilin and eosin, x100).

There is often a moderate dilatation of the deep vessels of the skin. Occasionally, within dilated capillaries there are partially organised fibrinous thrombi [*Nakamura et al. 1981*]. Atrophic or scarce sweat or sebaceous glands have been reported [*Desnick et al. 2001*]. Sometimes cytoplasmic vacuoles representing lipids can be detected in endothelial cells, fibroblasts and pericytes [*Tarnowski & Hashimoto 1969*].

Glycosphingolipids are seen particularly in the endothelial cells, pericytes and smooth muscle of the cutaneous capillaries, venules and arterioles, but also in many fibroblasts and the arrector pilorum muscles, sweat gland epithelium and perineural cells [*Ruiter 1954, Pittelkow et al. 1957, Hashimoto et al. 1965, Tarnowski & Hashimoto 1969*]. The amount of glycosphingolipids in the skin is small and therefore can be detected only using special stains on unfixed or formalin-fixed frozen sections. Staining with Sudan Black B demonstrates the lipids particularly well. The PAS reaction is strongly positive and diastase-resistant, because it stains the carbohydrate component of the glycosphingolipids [*Hashimoto et al. 1965*]. Because the lipid material is birefringent, it can be demonstrated also under polarised microscope of unfixed or formalin-fixed frozen sections. Glycosphingolipid deposits are not restricted to the affected areas but are also seen in normal-appearing skin [*De Groot 1964*]. The earliest pathologic involvement has been observed in the vascular endothelium and perithelium of clinically normal skin from a 1-year-old affected male [*Breathnach et al. 1980*].

On electron microscopy examination of the skin, large electron-dense glycosphingolipids deposits are seen in endothelial cells, pericytes and fibroblasts. They may also be seen in arrector pilorum muscles and in the secretory, ductal and myoepithelial cells of eccrine and sweat glands and in the perineural cells [*Nakamura et al. 1981, Desnick et al. 2001*]. On high magnification, these deposits show a lamellar structure with a periodicity of 5 nm [*Hashimoto et al. 1976*]. Many of the inclusions are surrounded by a membrane, but others are not. Eccrine sweat glands reveal numerous cytoplasmic inclusions in the clear cells of the secretory coil, coiled duct and basal cells of the straight duct. Inclusions are also observed in unmyelinated axons innervating the sweat gland and the small blood vessels around the gland, which have marked involvement of the vascular endothelium [*Lao et al. 1998*].

Differential diagnosis of the cutaneous lesions must exclude the angiokeratoma of Fordyce, angiokeratoma of Mibelli and angiokeratoma circumscriptum, none of which has the typical histological or ultrastructural lysosomal storage pathology of the Fabry lesions. The angiokeratoma of Fordyce are similar in appearance to those of Fabry disease but are limited to the scrotum and usually appear after the age of 30. The angiokeratoma of Mibelli are warty lesions on the extensor surfaces of extremities in young adults and are associated with erythematosus subcutaneous swellings. Angiokeratoma circumscriptum or naeviformus can occur anywhere on the body, are clinically and histologically similar to those of Fordyce and are not associated with chilblains (erythematous subcutaneous swellings).

Angiokeratoma, in clinical appearance and distribution reportedly similar to or indistinguishable from the cutaneous lesions seen in individuals with Fabry disease, have been described in individuals with other lysosomal storage diseases, including fucosidosis, sialidosis (α-neuraminidase deficiency with or without β-galactosidase deficiency), adult-type β-galactosidase deficiency, aspartylglucosaminuria, adult-onset α-galactosidase B deficiency, β-mannosidase deficiency and a lysosomal disorder with mental retardation and some features of the mucopolysaccharidoses [*Desnick et al. 2001*].

The Kidney

Gross descriptions of the kidney in Fabry patients are scarce, but the kidney may be enlarged by the accumulation of storage material. Renal cortical and parapelvic cysts have been demonstrated by ultrasound magnetic resonance imaging and computed tomographic imaging in up to 50% of patients, including classically affected hemizygous males, heterozygous females and cardiac variants. The prevalence of the cysts increase with age, but their presence does not correlate with residual enzyme activity, mutation type, proteinuria, or kidney function [*Glass et al. 2004, Ries 2004*]. The nature and pathogenesis of the cysts remain uncertain. Decreased cortical thickness, increased echogenicity and decreased corticomedullary differentiation have also been detected [*Glass et al. 2004*].

Light microscopy alterations are remarkable and can easily elucidate the diagnosis of Fabry disease. Even in children, protein, casts, red cells, and birefringent lipid globules may be observed in the urinary sediment [*Selvarajah et al. 2011*]. Examination of urinary specimens of patients with Fabry disease under phase-contrast microscopy using a polarized light filter revealed the presence of auto-fluorescent, birefringent Maltese cross particles having a lamellated appearance with protrusions, resembling 'mosquito coils' [*Selvarajah et al. 2011*]. Some reports showed that histological evidence of kidney involvement precedes clinical signs in early Fabry nephropathy [*Grünfeld 2003, Fervenza et al. 2008, Tøndel et al. 2008*]. The accumulation of glycosphingolipids in the kidneys starts in prenatal life and rudimentary deposits have been observed in podocytes of an aborted male foetus, at 19 weeks of gestational age [*Elleder et al. 1998*]. The original descriptions of Fabry nephropathy were in males, but females can develop the same kidney histopathology as males [*Gubler et al 1978, Svarstad et al. 2005*].

Glomeruli appear with a streaking white color when a fresh renal biopsy is visualized in stereomicroscopy. This color and appearance is markedly different from the usual reddish-colored glomeruli seen in the other patients with renal diseases or with normal kidneys [*Svarstad et al. 2004*]. Glomerular tuft contains enlarged and vacuolated glomerular cells, especially podocytes. The vacuoles generally are small and uniform and impart a honeycomb appearance to these cells [Figure 1]. Less commonly, parietal epithelial cells are

affected, and only rarely can the vacuoles be identified in endothelial or mesangial cells. Tubules, especially distal tubules and the loop of Henle, are also vacuolated [Figure 9]. Small arteries and arterioles show vacuolation of endothelial cells and finely vacuolated areas in the smooth muscle. Interstitial foam or lipid-laden cells can also be seen [Figure 10]. Lipid-laden distal tubular epithelial cells desquamate and may be detected in the urine sediment, and these cells have been shown to account for about 75% of the urinary cells shed by an affected hemizygote [*Desnick et al. 1971, Chatterjee et al. 1984*]. Non-specific chronic signs of evolution of kidney disease may be seen from early stages of the disease on. These include segmental and global glomerulosclerosis, mesangial expansion, glomerular hyaline, capillary wall thickening, tubular atrophy and interstitial fibrosis, arterial and arteriolar sclerosis and arterial fibrinoid deposits and may result from the necrosis of severely involved cells [Figures 11, 12] [*Gubler et al 1978, Sessa et al. 2001, Alroy et al. 2002, Sessa et al. 2002, Fischer et al. 2006, Tøndel et al. 2008*]. Focal glomerular and tubular epithelial necrosis, as well as thickening of glomerular and tubular basement membranes have also been reported [*Gubler et al 1978, Alroy et al. 2002*]. In severe disease, there is progressive glomerular sclerosis, tubular atrophy and varying amounts of interstitial fibrosis [*Alroy et al. 2002*].

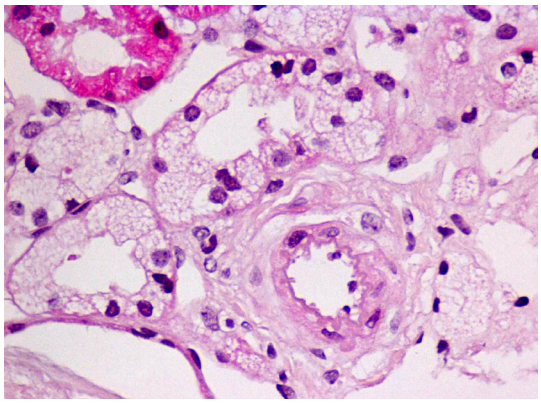

Figure 9 · Tubulo-interstitial area of a renal biopsy showing an extensive vacuolation of epithelial tubular cells with a honey comb appearance (Hematoxilin and eosin, x600).

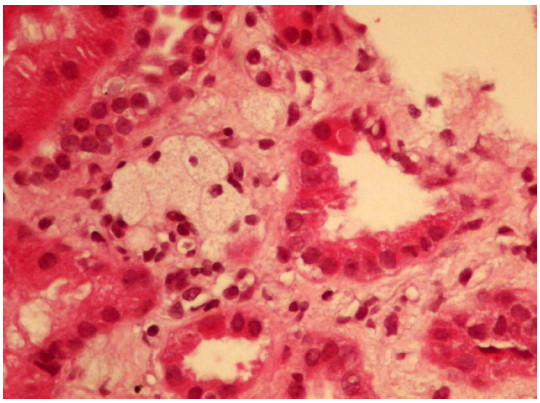

Figure 10 · Tubulo-interstitial area of a renal biopsy showing foam cells in the interstitium (Hematoxilin and eosin, x400).

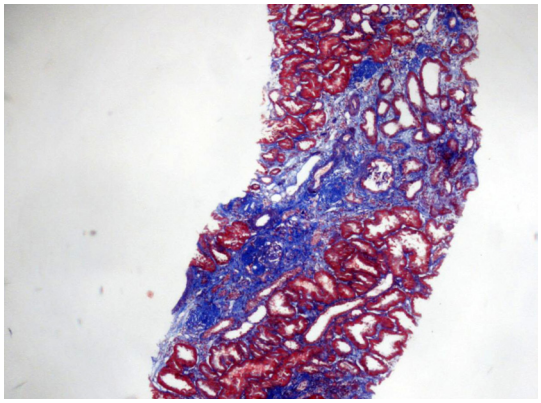

Figure 11 · Renal biopsy showing areas of interstitial fibrosis and tubular atrophy (stained in blue) and also some glomeruli with segmental sclerosis and global sclerosis (Special trichrome, x100).

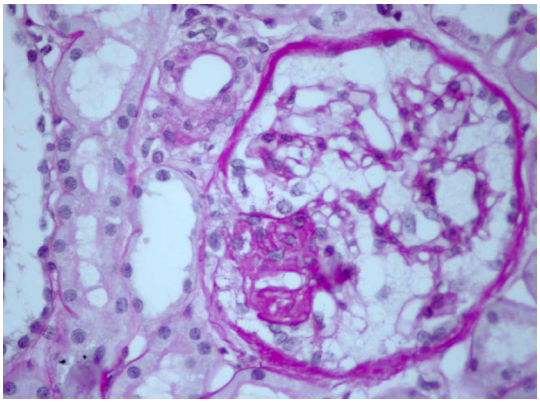

Figure 12 · Glomerulus with segmental sclerosis in a hemizygous patient with early Fabry nephropathy. Enlarged podocytes with fine vacuoles in their cytoplasm are also evident (PAS, x600).

The glycosphingolipids in frozen sections, whether fresh or formalin-fixed, are birefringent, autofluorescent, sudanophilic and positive to oil red O stain, in contrast to their appearance in paraffin embedded tissue [Figures 1 and 4] [*Faraggiana et al. 1981*].

Routine immunofluorescence microscopy is negative or nonspecific except where superimposed immune-mediated glomerulonephritis occurs. In cases with advanced lesions, such as segmental sclerosis, IgM, C3 and C1q may be present in capillary walls and mesangial regions in a segmental distribution and granular pattern [*Tisher & Brenner (ed) 1989*].

In semi-thin sections of plastic-embedded sections of osmium-fixed tissues stained with toluidine blue or methylene blue, glycosphingolipids deposits appear as fine and blue darkly-stained granular or round intracellular inclusions [Figures 3 and 13]. Furthermore, it is also possible to discern that peritubular capillary cells and interstitial cells are affected [Figure 13] [*Thurberg et al. 2002*].

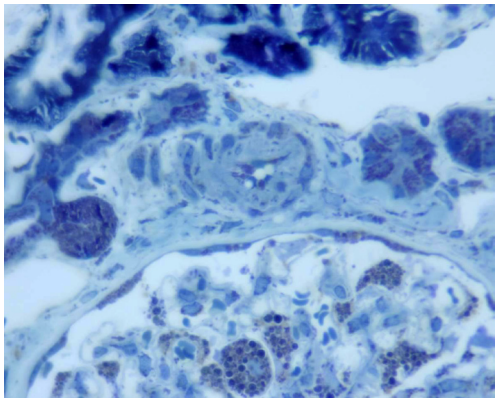

Figure 13 · Part of a glomerulus and tubulo-interstitial area with numerous osmiophilic inclusions within glomerular podocytes and parietal epithelial cells. Endothelial cells and muscle cells in the arteriole located close to the glomerulus (upper center), fusiform interstitial cells, peritubular capillary cells and tubular epithelial cells are filled also with inclusions (Semi-thin section, methylene blue-stained, x600).

Electron microscopy shows enlarged secondary lysosomes filled with osmiophilic granular to lamellated membrane structures (myelin figures and zebra bodies) [Figure 5] [*Schatzki et al. 1979, Gubler et al 1978*]. The periodicity of lamellated structures when measured using routine plastic semi-thin sections is estimated to be 3.5 to 5 nm, but the periodicity of their structures is 14 to 15 nm when studied by freeze-fracture electron microscopy, due to better tissue preservation [*Gubler et al 1978, Simon et al. 1990*]. In lamellar and amorphous bodies, high magnification reveals a regular arrangement of alternatively light- and dark-staining bands with a periodicity that has been reported variably as 35 to 50 Å, 40 to 50 Å, 50 to 60 Å and 60 to 65 Å, or as great as 98 Å. The electron-dense component is 20 to 30 Å in thickness with coarser periods of 150 to 200 Å. Most of the inclusions are contained within lysosomal membranes, but some of them are free in the cytoplasm or even in biological fluids such as urine [Figure 6]. Osmiophilic inclusions measure 0.3 to 10 μ in diameter but their size, shape, abundance, and distribution vary from one type of cell to another. It is speculated that the number and the size of glycosphingolipids deposits are inversely related to the

turnover rate of the renal cell populations: during the 2- to 3-month lifespan of endothelial cells only a modest amount of Gb3 can accumulate before the cell is replaced; in contrast, the largest deposits are found in podocytes, which are terminally differentiated cells and have the slowest turnover rate of all renal cell types [*Thurberg et al. 2002*]. In these latter cells, the size of glycosphingolipids deposits may reach 10 µm in diameter [*Gubler et al 1978, Fischer et al. 2006*], but also epithelial tubular cells, which have mitotic activity, may contain such large inclusions [*Gubler et al 1978*]. The glycosphingolipids inclusions are especially abundant in podocytes, Bowman's capsule epithelium, distal tubular epithelium, vascular endothelial cells and myocytes, peritubular capillary cells and interstitial cells, but may be found in all types of renal cells [*Gubler et al 1978, Desnick et al. 2001, Sessa et al. 2002, Thurberg et al. 2002*]. Involvement of parietal epithelial cells in Bowman's capsule, podocytes, tubular epithelial cells and peritubular capillary cells seem to be early pathologic events in Fabry nephropathy [*Tøndel et al. 2008*].

In **glomeruli** the inclusions are present in every cell type, although the epithelial cells are affected to the greatest degree [Figures 3 and 14]. Podocytes are the most severely affected cells [*Gubler et al 1978, Sessa et al. 2002, Alroy et al. 2002, Sessa et al. 2003a, Fischer et al. 2006*]. Endothelial and mesangial cells contain fewer inclusions, and these are smaller [*Gubler et al 1978, Tisher & Brenner (ed) 1989*]. Effacement of the foot processes of the podocytes could be seen, in particular in cases with heavy proteinuria [Tøndel et al. 2008, Tisher & Brenner (ed) 1989]. Membranofibrillary and non-immunogenic deposits in subendothelial location of the glomerulus are also described in Fabry patients [Figure 15]. They are probably the remains of ruptured lysosomal membranes which previously contained glycosphingolipids inclusions [*Gubler et al 1978, Fischer et al. 2006*]. The basement membranes and mesangial matrix are normal initially. With progression of the disease there is a wrinkling and thickening of the basement membranes, which is often associated with peripheral migration and interposition of the mesangium, probably resulting from increasing arteriolar and arterial involvement [*Tisher & Brenner (ed) 1989*]. "Free-floating" myelin figures are often present in Bowman's space and the tubular lumina [*Faria 1970, Gubler et al 1978*].

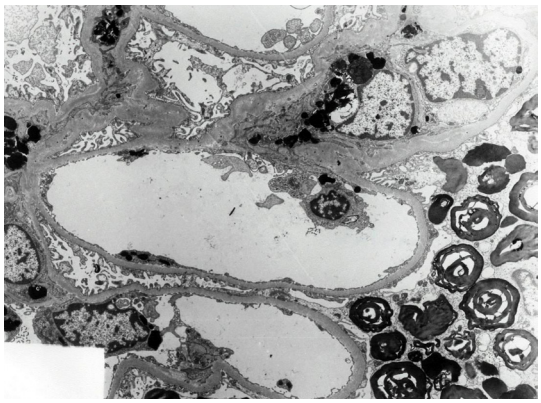

Figure 14 · "Myelin figures" and some "zebra bodies" in podocytes. Endothelial cells and mesangial cells have amorphous osmiophilic inclusion bodies. Focal and partial effacement of foot processes of the podocytes are also seen (Electron microscopy, 1,800x3).

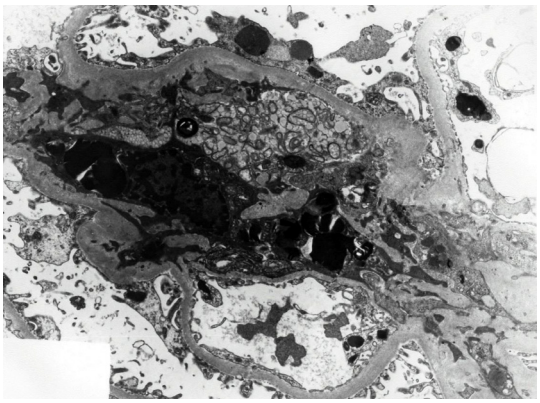

Figure 15 · Subendothelial membranofibrillary structures in relation with amorphous and myeloid osmiophilic bodies located in mesangial cell. Endothelial cells have amorphous osmiophilic bodies in their cytoplasm (Electron microscopy, 3,400x3).

In the **tubules**, the cells of the distal tubules and of the loops of Henle are the more frequently affected. They are enlarged and contain very large glycosphingolipids inclusions. Inclusions are rarer in proximal tubular cells, where they appear to be more frequently found in the proteinuric patients. Inclusions are also found in collecting duct cells [*Tisher & Brenner (ed) 1989*].

Endothelial cells of peritubular capillaries, arterioles, arteries, and veins contain inclusions. These glycosphingolipids structures, however, tend to be more varied in size and shape and may be elongated and racket or amorphously shaped. The cytoplasm is swollen, protrudes into the lumen of the vessel, and thereby decreases the caliber of the vessel [Figures 16 and 17] [*Tisher & Brenner (ed) 1989*].

Smooth-muscle cells of arteries and arterioles and **pericytes** also contain inclusion bodies [Figures 16 and 17]. There is a variable involvement from cell to cell, with the more severely affected myocytes displaying loss of myofilaments and other normal organelles. In advanced stages, the walls of arteries and arterioles are permeated by extracellular masses composed of a combination of granular dense material and striated membranous structures. The smooth-muscle cells may be absent and necrotic, suggesting that this material represents remnants of these cells [*Gubler et al 1978*].

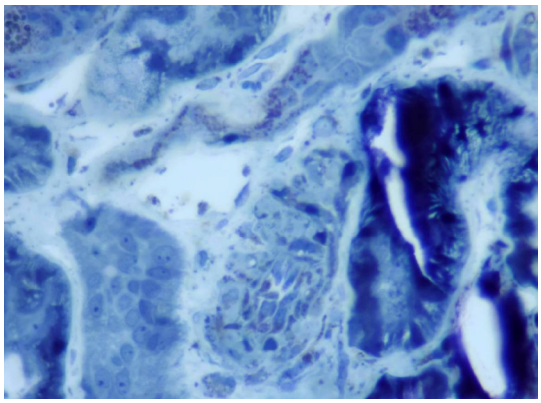

Figure 16 · Reactive endothelial cells with osmiophilic inclusions protruding and occluding vascular lumen in this arteriole. There are also many smooth muscle cells with osmiophilic inclusions. Tubular epithelial, peritubular capillary and interstitial cells have also osmiophilic inclusions (Semi-thin section, methylene blue-stained, x600).

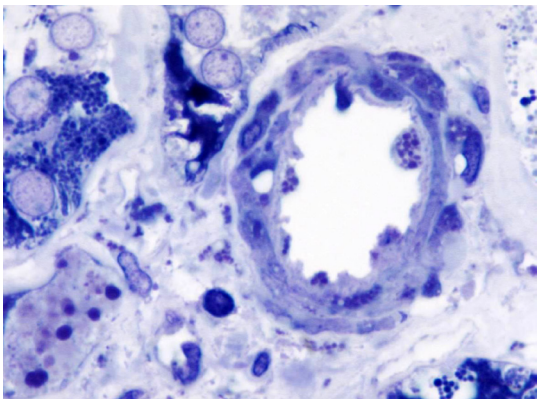

Figure 17 · Arteriolar vessel with reactive endothelial cells filled with osmiophilic inclusions, the arteriolar smooth muscle cells show also osmiophilic inclusions. Some interstitial cells have also osmiophilic inclusions in their cytoplasm. (Semi-thin section, toluidine blue-stained, x600).

Almost all **interstitial cells** contain lipid inclusions in hemizygous cases, although are they generally sparse in distribution but quite dense in appearance [Figures 13, 16 and 17] [*Tisher & Brenner (ed) 1989*]. It is believed that the involvement of the interstitial cells plays a critical role in the progression to ESRD; in fact, disease of the interstitium may result in renal scarring [*Sessa et al. 2003*].

In patients with the cardiac variant of Fabry disease, glycosphingolipids inclusions are extensive in podocytes, rare in epithelial tubular cells and in parietal epithelial cells in Bowman's capsule, and absent in mesangial, interstitial and vascular endothelial and smooth-muscle cells [*Meehan et al. 2004*].

Other renal diseases can occur concurrently with Fabry nephropathy. IgA deposits, with or without clinical expression of IgA nephropathy, are a frequent feature in kidney biopsies of Fabry patients [*Yoshida et al. 1994, Kawamura et al. 1997, Whybra et al. 2006, Waldherr et al. 1989, Pisani et al. 2005, Shimohata et al. 2009, Kakita et al. 2010*]. Crescentic glomerulonephritis, [*Shimazu et al. 2002, Singh et al. 2001, Kriegsmann et al. 2003*], granulomatous interstitial nephritis [*Hiraizumi et al. 1995*] and lupus nephritis [*Majima et al. 1992, Rosenmann et al. 1983*] are also reported as occurring simultaneously with Fabry disease.

Differential diagnosis is important in Fabry nephropathy. Although the structural abnormalities are quite consistent and specific, assay of α-galactosidase in hemizygotes and molecular studies in heterozygotes should be undertaken to confirm the diagnosis. The light microscopic lesions of vacuolated glomerular and tubular epithelial cells are largely nonspecific but suggest a storage process. Although very few storage diseases are characterized by renal involvement with the same cellular distribution as Fabrys disease, it is important to appreciate that vacuoles, per se, are not diagnostic and may be seen in many other medical diseases [Table 2]. The ultrastructural appearance of the inclusions, however, considerably narrows the many differential diagnostic considerations. But the structure of the individual inclusions, although distinctive, is not diagnostic at all. Similar inclusions have been described in the glomerular epithelium in a patient with pulmonary silicosis and proteinuria and haematuria [*Banks et al. 1983*], but plasma α-galactosidase activity was normal. Furthermore, similar structural features may be observed in renal glomerular cells of patients treated with chloroquine, in tubular cells in patients treated with aminoglycoside antibiotics and in patients treated with amioradone [*Katz et al. 1979, Roos et al. 2002, Albay et al. 2005, Woywodt et al. 2007, Pintavorn & Cook 2008*].

The Heart in Fabry Disease

Cardiac involvement is frequent and is caused by accumulation of glycosphingolipids in the myocardium, valves and conduction system. Gross cardiomegaly involving all chambers has been observed. Left ventricular hypertrophy is frequent and often associated with hypertrophy of the interventricular septum. Left ventricular hypertrophy appears similar to hypertrophic cardiomyopathy, is progressive and occurs earlier in males than females [*Kampmann et al. 2005, Frustaci & Chimenti 2007*]. Echocardiography demonstrates an increased thickness of the interventricular septum and the left ventricular posterior wall, and an increase of the aortic root diameter [*Pieroni et al. 2006*]. Right atrial and ventricular

Table 2 | Medical diseases affecting the renal parenchyma with histological vacuolation of renal cells.

	Podocytes	Mesangial	Glomerular capillary endothelium	Proximal tubular cells	Distal tubular cells	Interstitial cells	Vascular smooth--muscle cells	Vascular endothelium
Fabry disease	+	+	+		+	+	+	+
Gaucher disease						+		
Niemann-Pick disease	+		+	+	+	+		
Metachromatic leukodystrophy				+	+			
Neuronal ceroid cipofuscinosis	+		+	+	+			
GM1 gangliosidosis	+	+						
Sandoff disease	+			+				
I-cell disease	+			+				
Hurler's disease	+			+				
Fucosidosis	+							
Mannosidosis	+							
Aspartylglucosamine aciduria	+							
Alport syndrome						+		
Familial LCAT deficiency		+	+			+	+	+
Type III hyperlipoproteinemia		+						
Lipoprotein glomerulopathy						+		
Glycogen storage disease tipe I				+	+			
Nephrosialidosis	+			+	+	+		+
Minimal change disease				+	+	+		
Nephrotic range proteinuria	+					+		

LCAT: Lecithin-cholesterol acyltransferase. [*Finn 2007, Gubler 2007, Oslon 2007*].

dilatation and enlargement are variable findings. Mitral and tricuspid valves are frequently involved. The most common valvular defect is thickening and interchordal hooding of the leaflets of the mitral valve, with normal chordae tendineae and either normal or thickened and shortened papillary muscles. This defect may lead to the high incidence of mitral valve prolapse and insufficiency. The tricuspid valve may be similarly involved. Aortic valve could have mild stenosis or regurgitation; but the pulmonary valve is usually normal. Coronary artery disease is also found, and with age, a significant proportion of hemizygotes and heterozygotes complain of angina chest pain. Atherosclerosis is not uncommon in coronary arteries, and is aggravated by the hyperlipidemia and high blood pressure that often accompany the disease [*Desnick et al. 2001*].

In light microscopy of paraffin embedded tissue, there is extensive sarcoplasmic vacuolation of myocytes, leaving large clear spaces in the paraffin-embedded tissue stained with haematoxylin and eosin [*Nakao et al. 1995*]. These vacuoles do not stain well with conventional or special staining methods [*Owens et al. 2006*]. This histological picture is nonspecific as it can be seen in other lysosomal storage diseases [*Elleder 2003*]. Mild fibrosis is frequently present [*Roos et al. 2002*]. A gradient in myocardial disease severity was observed, the subendocardial layers were more severely affected than outer myocardial layers: subendocardial myocytes were bigger and characterised by larger intracellular glycolipid vacuoles than midwall layer myocytes. In patients with hypertrophic cardiomyopathy, histology showed the presence of severely hypertrophied cardiomyocytes, often in total disarray and frequently interrupted in short runs because of interstitial and various degrees of replacement fibrosis [*Pieroni et al. 2006*]. The coronary lesions are similar to typical atherosclerosis except that they are concentric with a white discoloration rather than the eccentric yellowish characteristics of atherosclerotic plaques [*Schiffmann et al. 2006*].

In semi-thin sections of epon embedded tissue, there is glycosphingolipids deposition around the nucleus and between the myofibrils. The vessels show marked hypertrophy of the endothelial cells and smooth-muscle cells secondary to glycosphingolipids deposition. Mitral and tricuspid valves have numerous lipid-laden cells embedded in fibrous tissue [*Desnick et al. 2001*].

Electron microscopy of endomyocardial biopsies shows prominent sarcoplasmic myeloid bodies within the centers of myocytes, causing substantial cellular enlargement in involved myocytes as compared to uninvolved ones [*Roos et al. 2002*]. Endothelial cells from interstitial capillaries are also frequently involved with glycosphingolipids. *Owens et al.* described the presence of abundant clumps and tangles of tubular crystals, and the disappearance of the concentric lamellar myelin bodies in a patient with Fabry disease treated with enzyme replacement therapy. They speculated that those crystalline structures might be breakdown products of the myelin bodies or the result of excess therapeutic enzyme that has become insoluble, but an artifact could not be entirely excluded. They also found some scattered eosinophilic bodies within the vacuolated areas which could represent giant secondary lysosomes or a collapsed aggregate of myelin figures, as seen by electron microscopy [*Owens et al. 2006*].

Differential diagnosis with chloroquine cardiotoxicity is important, because lamellar bodies similar to glycosphingolipids deposits are seen in the electron microscopy study [*Roos et al. 2002*].

The Nervous System

In both heterozygous and hemizygous patients, glycosphingolipids deposition in nervous tissue appears to be limited to perineural sheath cells of peripheral nerves, neurons of the peripheral and central autonomic nervous system and certain primary neurons of somatic afferent pathways. Glycosphingolipid deposition has been observed in Schwann cells. Vascular involvement is also prominent in the nervous system [*Cable et al. 1982, Desnick et al. 2001*]. Loss of small myelinated and unmyelinated fibers as well as small cell bodies of spinal ganglia of peripheral sensory neurons in sural nerves and spinal ganglia have been seen [*Kahn 1973, Scott et al. 1999*].

Ultrastructural examination of intradermal nerve fibers in Fabry disease has revealed signs of glycosphingolipids accumulation and small unmyelinated nerve fiber degeneration. Many axons were swollen, and their internal organelles were lost. In several damaged axons, dense inclusions, probably glycosphingolipids, were observed [*Cable et al. 1982, Toyooka et al. 1997*].

In the central nervous system, diffuse storage occurs in the cerebral vasculature, with more localized involvement of central neurons together with the dorsal root and autonomic ganglia in the peripheral nervous system. Autonomic centers throughout the neuro-axis show a predilection for glycosphingolipids storage, including the supra-optic and paraventricular nuclei, the parahippocampal gyrus, the vagal nuclei, Onuf's nucleus and the ganglion cells of the myenteric plexuses. The cerebrovascular manifestations, reported to affect predominantly the posterior circulation, consist of large vessel ectasia and large-vessel and small-vessel occlusive disease [*Kahn 1973, Kolodny & Pastores 2002*]. Basilar artery diameters, as measured by magnetic resonance angiography, could be used for early detection and monitoring of brain involvement in patients with Fabry disease [*Fellgiebel et al. 2009*]. Cerebral and cerebellar cortical infarcts have also been described. Brainstem centers in which glycosphingolipids deposition has been observed include the nuclei gracilis and cuneatus, the dorsal autonomic vagal nuclei, salivary nuclei, nucleus ambiguous, thalamus, reticular substance, mesencephalic nucleus of the fifth nerve and the substancia nigra. Hemisphere involvement has been noted in the amygdaloid, hypothalamic, and hippocampal nuclei [*Kahn 1973*]. Studies have revealed abnormal lipid deposits in the fifth and sixth cortical layers of the inferior temporal gyrus, the Edinger-Westphal nucleus, the parasympathetic cell column, and the midline nucleus [*Sung et al. 1975*]. Glycosphingolipid storage in neuronal cells of the anterior and posterior lobes of the pituitary has been described. Deposits of glycosphingolipids have also been found in the media of small arteries and arterioles of the leptomeninges of the brainstem and spinal cord, but were not found in the leptomeningeal vessels of the cerebral hemispheres [*Kahn 1973*].

The Eye and Occular Lesions

Macroscopically there are vascular lesions in conjunctiva and retina similar to the dilated vessels in the skin. The vessels of the bulbar and palpebral conjunctiva show ampullary and saccular aneurysms of the small venules with stasis and thrombosis in some cases [*Velzeboer & de Groot 1971*]. The vascular lesions in the retina consist of segmental dilatation and tortuosity of the venules. Tortuosity was also described in the arteries. There is a whorl-like corneal dystrophic pattern that may result from the formation of the series of subepithelial ridges or from the reduplication of the basement membrane [*Font & Fine 1972*].

Histologically, abnormal glycosphingolipids deposits are found in endothelial, perivascular and smooth-muscle cells of all ocular and orbital vessels, in smooth muscle of the iris and ciliary body, in perineural cells and in connective tissue of the lens and cornea [*Font & Fine 1972*]. Inclusions have been found in the epithelium of the conjunctiva, cornea, and lens, and by electron microscopy, in the basal layer of the conjunctival epithelial cells, in the surface epithelium and in the conjunctival globet cells [*Macrae et al. 1985*]. There may be hyperplasia and oedema of corneal epithelial cells. Bowman's membrane appears normal, basement membrane is reduplicated, and no deposits are observed in the stroma or endothelium under light and electron microscopy [*Velzeboer & de Groot 1971*]. Presence of amorphous material between basement and Bowman's membranes has been described [*Weingeist & Blodi 1971*].

Other Tissues

Many other organs, including the adrenal glands, gastrointestinal tract, liver, placenta, pancreas, prostate, spleen, testis, thyroid and urinary bladder show involvement of the blood vessels, smooth muscle, ganglia and nerves. In addition, vacuoles or glycosphingolipids stores have been demonstrated in epithelial cells, mucous glands, synovial membrane, smooth muscle of the bronchus, alveolar ciliated epithelial and goblet cells, type II alveolar epithelial pneumocytes and skeletal muscle. No inclusions have been found in alveolar macrophages. Involvement of reticuloendothelial cells has been noted in the bone marrow, liver, spleen, lymph nodes and liver, including hepatocytes and liver sinus endothelial and Kupffer cells [*Faria 1970, Elleder 1985, Desnick et al. 2001*].

PATHOGENESIS AND PATHOPHYSIOLOGY OF FABRY NEPHROPATHY

The pathogenesis of Fabry disease is not well understood. It is attractive to assume that the clinical manifestations are the direct result of accumulation of neutral glycosphingolipids, especially Gb3, within a range of cell types and tissues, leading to disturbed cell/organ function, but the sub-cellular mechanisms linking glycosphingolipids accumulation to the clinical and pathological manifestations of Fabry nephropathy are not clearly understood [*Sessa et al. 2002, Sessa et al. 2003a, Warnock et al. 2010*]. It is well recognized that tissue damage in Gaucher disease, the commonest lysosomal disorder, results from inflammation, cytokine release from macrophages engorged with storage material and abnormal intra- and inter-cellular signaling. Similar processes, triggered by the presence of excessive tissue Gb3 storage, are likely to be equally important in Fabry disease. The absence of infantile major manifestations of Fabry disease, despite deficient α-galactosidase and Gb3 accumulation, supports the hypothesis that Gb3 is not solely responsible for the disease manifestation. However, different hypotheses have been postulated about the mechanisms by which Gb3 can contribute to cellular/tissue damage in Fabry disease.

As in other chronic nephropathies, the progression of Fabry nephropathy is characterized by segmental and global glomerular sclerosis, tubular atrophy, interstitial fibrosis and vascular sclerosis [*Gubler et al 1978, Fogo et al. 2010*].

There are some pathogenic theories linking glycosphingolipids deposition in renal cells to the signs and symptoms of Fabry nephropathy:

Microvascular disease and ischaemic injury: The main pathogenic theory of the classical phenotype assigns its complications predominantly to ischaemic tissue damage resulting from microvascular endothelial disease and/or necrosis of vascular smooth-muscle cells and/or pericyte injury [*Desnick et al. 2001, Desnick et al. 2001a, Desnick 2003*]. Back in 1947, Pompen et al. suggested that Fabry disease might be a disease of the entire vascular system with compromise of muscle cells of the heart and vessels [*Pompen et al. 1947*]. Later, Gubler et al. made the observation that in Fabry patients, the progressive renal pathologic changes

in the glomeruli, tubules and interstitium could be related to ischaemic change [*Gubler et al 1978*]. These changes include glomerulosclerosis, often with wrinkled and partially collapsed glomerular basement membrane, tubular atrophy, interstitial fibrosis and vascular thickening. In particular, these investigators noted that the earliest and most consistent degenerative alteration was arterial fibrinoid deposits and suggested that these were due to necrosis of smooth-muscle cells fatally overloaded with Gb3 deposits. But the pathogenesis of the Fabry vasculopathy is poorly understood, and it involves a variety of mechanisms, including cell proliferation and luminal stenosis, acceleration of focal intravascular pressure, dilatation and disturbances of vascular auto-regulation secondary to endothelial dysfunction [*Altarescu et al. 2001, Desnick et al. 2001, Moore et al. 2001, Puccio et al. 2005, Moore et al. 2007*]. Some theories are trying to explain the ischaemic role in disease progression:

- A prothrombotic state, characterized by increased levels of soluble endothelial prothrombotic factors and of leukocyte adhesion-molecule expression has been described in patients with Fabry disease [*DeGraba et al. 2000*];

- In Fabry disease there is also an increased oxidative stress and circulating myeloperoxidase, which is associated with vasculopathic events in male patients [*Kaneski et al. 2006*];

- Globotriaosylceramide also appears to be a critical mediator of the hemolytic uraemic syndrome, which is characterised by widespread thrombosis [*Moake 1994*];

- Globotriaosylceramide storage in Fabry mice correlates with the development of a complex vasculopathy, characterised by thrombosis, atherogenesis and endothelial dysfunction [*Eitzman et al. 2003, Shu & Shayman 2007, Park et al. 2008*];

- Deacylated Gb3 (lyso-Gb3), also known as globotriaosylsphingosine, a biologically active cationic amphiphile with a large polar sugar moiety, have been observed in classically affected male Fabry patients and also in plasma and tissues of Fabry mice [*Aerts et al. 2008*]. Lyso-Gb3 promoted vascular smooth-muscle cell proliferation and also inhibit α-galactosidase activity, suggesting a role in the pathogenesis of Fabry disease by provoking hypertrophic vascular walls with possible ischaemic consequences [*Sanchez-Niño et al. 2011*].

Altogether, these structural and functional changes may lead to tissue ischemia and infarction, which are considered the main pathogenic mechanisms underlying many of the late complications of classical Fabry disease [*Desnick et al. 2001*].

Podocyte injury: The development of proteinuria and of late-onset renal dysfunction in patients with the cardiac phenotypic variant, which have significant renal Gb3 deposits confined to podocytes, suggests that these cells may have a role in the pathogenesis of Fabry nephropathy [*Meehan 2004*]. Different mechanism could involve podocytes in Fabry disease:

- Podocytes are involved in early stages of the disease, and it is reasonable to assume that glycosphingolipid inclusions within these cells may damage the glomerular filtration barrier by compromising the functional role of the slit diaphragms of the podocyte foot processes, initially causing microalbuminuria and subsequently overt proteinuria. Focal and diffuse effacement of foot processes of the podocytes could be seen in many cases and in the early stages of Fabry nephropathy; the relative preservation of the fine structures of the podocytes foot processes and of the slit diaphragms could be a major determinant of the amount of proteinuria [*Gubler et al 1978, Sessa et al. 2002, Sessa et al. 2003a, Tøndel et al. 2008, Najafian & Mauer 2011, Najafian et al. 2011a*]. In young Fabry patients, the width of the podocyte foot process and the fractional volume of Gb3 inclusions within the podocytes correlated with urinary protein excretion rates [*Najafian et al. 2011a*];

- Podocytes are postmitotic cells and fail to undergo proliferation under most pathologic circumstances, which means that they are generally not replaced when they are lost due to lethal injury. Gb3-overloaded podocytes suffer lethal injury and are lost. The denuded glomerular basement membrane contacts the parietal epithelial cells and forms synechiae [*Kriz & Lemley 1999, Alroy et al. 2002, Warnock 2005*]. Within the synechiae, there is activation and proliferation of cells, especially mesangial cells, the entry of immune cells, including macrophages, and the accumulation of extracellular matrix protein. This repair response may be driven in part by the leakage from the circulation into the synechiae of macromolecules, including cytokines, chemokines and growth factors, via the impaired glomerular filtration barrier. Matrix expansion and subsequent collapse of the capillary loop appears as the focus of solidification [*Kriz & Lemley 1999*]. This constitutes the lesion of segmental glomerulosclerosis, which progresses to global glomerulosclerosis;

- Increased hydraulic stress upon endothelial cells in the vicinity of damaged podocytes is other possible mechanism of glomerular sclerosis related to podocyte injury [*Sessa et al. 2003*];

- Lyso-Gb3 in podocytes engaged secondary mediators of podocyte injury, such as transforming growth factor beta (TGF-β1), a critical mediator of extracellular matrix production, fibrosis and podocytes injury, and CD74, a macrophage migration inhibiting factor (MIF) receptor that regulates the expression of lethal cytokines [*Sanchez-Niño et al. 2011*].

Tubulointerstitial injury: Deposition of glycosphingolipids within tubular epithelial cells may lead to focal tubular atrophy and interstitial fibrosis. As this process evolves, the glomeruli upstream of more severely affected tubules may function poorly or not at all. Other glomeruli may undergo hypertrophy to compensate, and hyperfiltration in these glomeruli may trigger a secondary form of focal segmental glomerulosclerosis [*Alroy et al. 2002*]. Further, by causing tubular injury, proteinuria has a direct role in chronic kidney disease progression and may provide a link between the aforementioned primarily glomerular pathologic processes and the development of tubulointerstitial disease [*Palmer 2007*].

Mesangial injury: Mesangial cell necrosis could also contribute to the development of glomerular sclerosis [*Gubler et al 1978*].

Inflammatory responses: Secondary effects of Gb3 accumulation which might be responsible for disease pathology include also inflammatory processes. Gb3 has been shown to be the cell membrane receptor CD77, which is believed to play an important role in apoptosis and necrosis [*Thomaidis et al. 2009*]. Rozenfeld and co-workers reported abnormal leukocyte function in Fabry disease patients as compared to healthy controls, and abnormal numbers of different immune cells, including lymphocytes, monocytes, CD8+ cells, B cells and dendritic cells [*Rozenfeld et al. 2009*].

ANIMAL MODEL IN FABRY DISEASE

Mouse models are particularly helpful to study the pathology of rare disorders such as Fabry disease, in view of the limited availability of human tissues and the ethical and legal restraints to experimental interventions in patients. Ohshima et al. generated a *GLA*-deficient C57BL/6 mouse by gene targeting ("Fabry mice") [*Ohshima et al. 1997*]. The Fabry mice display a complete lack of *GLA* activity in tissues of hemizygous males and homozygous females, but clinical signs of Fabry disease were not apparent, and organ failure was not detected up to 80 weeks of age [*Ohshima et al. 1999*]. Fabry mice accumulate Gb3 inside vascular endothelial and smooth-muscle cells, as well as in the plasma membranes of endothelial cells. Gb3 storage at these sites correlates with the development of a complex vasculopathy, characterized by thrombosis, atherogenesis and endothelial dysfunction, which is a hallmark of these knockout mice [*Ohshima et al. 1999, Eitzman et al. 2003, Shu & Shayman 2007, Park et al. 2008, Park et al. 2009, Shu et al. 2009*]. This provides an attractive *in vivo* model to investigate the pathophysiology of the cardiac and cerebrovascular complications of Fabry disease.

Marked Gb3 accumulations are observed in plasma and lysosomes of the kidneys, liver, spleen, heart and skin of these knockout mice, whereas Gb3 is present only in small amounts in wild-type mice [*Ohshima et al. 1999, Ioannou et al. 2001, Aerts et al. 2008, Rodrigues et al. 2009*]. The amounts of Gb3 in the kidneys, testes, lungs and heart of Fabry mice increase significantly between the ages of 24 to 48 weeks [*Rodrigues et al. 2009*]. Fabry mice are reported to accumulate ±25% of Gb3 in kidney tissue as compared to the level measured in a male Fabry patient [*Ohshima et al. 1999*].

Preclinical studies of enzyme replacement therapy for Fabry disease have been performed in the Fabry mice. The pharmacokinetics and biodistribution of administered α-galactosidase were evaluated. In spite of the lack of clinical signs, the reduction of substrate in various tissues and plasma was found to be dose dependent, and re-accumulation rates were determined for the liver, myocardium and spleen, providing an *in vivo* rationale for ERT in patients with Fabry disease [*Ioannou et al. 2001*].

Various gene therapy studies have been performed in the Fabry mice, including *in vitro* transduction of bone marrow cells with a retroviral vector and transplantation into radiation-conditioned knockout mice [*Takenaka et al. 2000, Qin et al. 2001*]; intravenous or intramuscular injection of an adeno-associated virus vector [*Jung et al. 2001, Takahashi et al. 2002, Park et al. 2003*]; and pulmonary instillation [*Li et al. 2002*] or intravenous injection of an adenovirus vector [*Ziegler et al. 1999, Ziegler et al. 2002*]. Increased activity of α-galactosidase has been documented, with substrate reduction in various tissues, which was dependent upon the vector and mode of administration.

DIAGNOSIS OF FABRY DISEASE

Demonstration of the absence or the deficient activity of the α-galactosidase in body fluids or skin cultured fibroblast and/or identifying the mutation of the *GLA* gene, are the main methods for diagnosing Fabry disease.

Performing the diagnosis of *de novo* cases is difficult. Several studies on the natural history of Fabry disease as well as analyses of the two available patient registries have shown that diagnosis is often carried out more than a decade after onset of first symptoms [*Mehta et al. 2004, Eng et al. 2007*]. Indeed, there are patients whose diagnosis is not made before irreversible organ damage has taken place. Several authors suggested early institution of enzyme replacement therapy in order to prevent irreversible organ damage, making early diagnosis mandatory [*Schiffmann 2007, Warnock 2007, Oqvist et al. 2009*].

Genetic counseling should be offered to all families in which the diagnosis of Fabry disease is made. In each extended family, all at-risk males and females should be examined clinically, biochemically and molecularly for diagnosis [*Desnick et al. 2001*].

Clinical Diagnosis

There are no specific clinical signs or symptoms in Fabry disease, but the pattern of association of the different clinical manifestations should help in the diagnosis. Clinical onset is usually during childhood or adolescence, but it may be delayed until the twenties or thirties [*Desnick et al. 2001*]. Fabry disease should be considered in males and females with the following signs [*Metha e3 Hughes 2008*]:

- Periodic crises of severe pain in the extremities (acroparesthesias);
- Vascular cutaneous lesions (angiokeratomas);
- Hypohidrosis;
- Characteristic corneal and lenticular opacities;
- Stroke;
- Left ventricular hypertrophy;
- Renal insufficiency of unknown etiology.

Biochemical Diagnosis

Enzime Assay

Biochemical diagnosis of Fabry disease is based on the measurement of α-galactosidase activity in plasma, leukocytes, urine, cultured skin fibroblasts or single hair roots [*Desnick et al. 2003, Bekri 2008*].

Due to its high sensitivity and specificity, measuring the α-galactosidase enzyme activity in leukocytes using 4-methylumbelliferyl-α-d-galactopyranoside fluorogenic substrate has become the gold standard enzyme assay for diagnosing Fabry disease in males [*Kint 1970, Desnick et al. 1973, Caudron et al. 2007, Andrade et al. 2008, Wilcox et al. 2008*]. However, the enzymatic assay is of limited value for the detection of heterozygous females [*Bekri 2008*].

Enzymatic diagnosis and screening of Fabry disease can also be done in dried blood spots on filter paper [*Chamoles et al. 2001*].

Storage Products

The measurement of the storage products, Gb3 and lyso-Gb3 in plasma or urine can provide supporting evidence for the diagnosis and is useful for monitoring treatment [*Winchester & Young 2010, Auray-Blais et al. 2010*].

All classic hemizygotes and over 90% of heterozygotes have an elevated level of urinary Gb3. Methods to measure Gb3 using tandem mass spectrometry have been developed for urine and plasma [*Mills et al. 2005, Young et al. 2005, Kitagawa et al. 2005*]. The method to measure Gb3 in urine has been adapted for use in urine dried on filter paper [*Auray-Blais et al. 2007*]. Urine samples dried on filter paper are much easier to store and ship than liquid specimens and make the method attractive for Fabry disease screening initiatives.

Plasma lyso-Gb3 can be determined quantitatively in plasma by high performance liquid chromatography using an external standard of authentic lyso-Gb3. Heterozygous female carriers have mild increases of lyso-Gb3 [*Aerts et al. 2008*].

The determination of urinary excretion of lyso-Gb3 allowed accurate measurement of lyso-Gb3 in urine samples of Fabry disease patients. Moreover, analysis of urine lyso-Gb3 is particularly useful for the diagnosis of Fabry disease because it is consistently absent in the urine of healthy controls. In addition, the close correlation of urine lyso-Gb3 with genotype suggests that it may have a value for predicting clinical severity [*Auray-Blais et al. 2010*].

Pitfalls in Biochemical Diagnosis

- Classically affected hemizygotes usually have no detectable α-galactosidase activity, but some hemizygotes, for example those with the p.N215S mutation, may have significant residual activity in plasma and/or leukocytes [*Desnick et al. 2001*].

- Atypical hemizygotes may be detected by the presence of residual α-galactosidase activity ranging from less than 5 to 35 percent of normal [*Desnick et al. 2001*]. Patients with an atypical clinical presentation and residual α-galactosidase activity should be investigated thoroughly before a definitive diagnosis is made. This should include characterization of the residual α-galactosidase activity, measurement of urinary Gb3/lyso-Gb3 and sequencing of the whole *GLA* gene.

- The biochemical identification of female heterozygotes is less reliable because of the effects of random X-chromosomal inactivation. Female heterozygotes can express enzyme activity levels varying significantly from the normal range (25-40%) to very low or even absent α-galactosidase activity [*Germain 2000, Desnick et al. 2001, Desnick et al. 2003*]. A more accurate but also much more laborious and time-consuming method to establish heterozygosity is by demonstration of both mutant and normal cell populations for α-galactosidase activity using, for example, single hair roots or cloned fibroblasts [*Germain 2000, Desnick et al. 2001*]. High-performance liquid chromatography of urinary sediment glycosphingolipids is a more sensitive test for the detection of heterozygotes. An additional limitation of the enzymatic diagnosis of female patients is the possible age-related reactivation of inactivated X-chromosomal genes, which could explain the not infrequent observation that older obligate heterozygotes have levels of α-galactosidase activity in the high end of the heterozygote range or within the normal range [*Desnick et al. 2001*]. DNA analysis permits precise heterozygote detection and is preferred in families in which the molecular defect has been identified.

Molecular Genetic Analysis

The detection of a disease-causing mutation in the *GLA* gene is now considered an essential step in the diagnosis of Fabry disease in both male and female patients [*Germain 2000, Desnick et al. 2011, Desnick et al. 2003, Caudron et al. 2007, Whinchester & Young 2010*]. Although there are some recurrent mutations, most are private to a family and are confined to individual pedigrees. About 75% of affected families have point mutations, including missense (around 56%), nonsense and splicing mutations [*Pastores & Lien 2002*], about 21% are short length deletions and/or duplications and the remainder are gross deletions and/or duplications and complex rearrangements. In a small minority of patients with biochemically confirmed Fabry disease, no *GLA* mutation can be found [*Schäfer et al.*

2005]. In those families in which the *GLA* mutation is already known, precise molecular diagnosis should be offered to all at-risk individuals, using mutation-specific detection methods [*Desnick et al. 2001*]. In patients with no family history, finding the pathologic *GLA* mutation requires gene sequencing, which can be eventually guided by the results of an appropriate mutation screening method [*Germain 2000*].

Depending on the clinical context, genotyping can be performed by sequencing the entire *GLA* gene or the relevant sequences thereof [*Germain 2000, Desnick et al. 2001, Desnick et al. 2001a*]. The most reliable way to identify novel and known single base changes and small deletions and insertions is to sequence the entire coding sequence and flanking regions in both directions [*Winchester & Young 2010*]. The mutation screening methods include single-strand conformation polymorphism analysis, chemical cleavage of mismatches with fluorescent detection and denaturing high performance liquid chromatography. The amplified exons plus flanking regions that contain sequence changes are then sequenced to identify the mutation precisely [*Shabbeer et al. 2005, Winchester & Young 2010*]. Another approach to the rapid identification of known mutations in individuals and populations is the use of disease-specific chips or microarrays. Large gene rearrangements, which only account for 3-4% of the mutations, can be detected by Southern blot hybridization using full-length cDNA as the probe or by multiplex PCR of the gene in 4 fragments [*Winchester & Young 2010*].

It is difficult to detect deletions of one or more exons or the entire gene, especially in heterozygous females. Current technologies to detect deletion/duplication mutations include quantitative polymerase chain reaction (PCR), real-time PCR, multiplex amplifiable probe hybridization, multiplex ligation-dependent probe amplification (MLPA) and array comparative genome hybridization [*Oqvist et al. 2009, Winchester & Young 2010*].

If a mutation is not found in the exonic or flanking regions and there is no large gene rearrangement, the possibility of a splice site mutation (≅5% of known mutations) should be investigated by the use of reverse-transcriptase polymerase chain reaction or real-time PCR to analyze total RNA. This is important because an alternatively spliced α-galactosidase transcript is expressed at a level of 1-10% in normal tissues. A deficiency of α-galactosidase activity may result from disturbing the balance between the normal and alternative transcripts [Winchester & Young 2010].

Polymorphisms in the *GLA* gene that increase or decrease the α-galactosidase activity can complicate the diagnosis of Fabry disease. Three polymorphisms, g.1150G>A, g.1168G>A, g.1170C>T, in the untranslated sequence before the initiation sequence in the first exon, have a combined frequency of 10%. First one is associated with a 3-fold increase in plasma α-galactosidase activity in hemizygotes. In contrast, the g.1170T allele polymorphism is associated with lower α-galactosidase expression [*Winchester & Young 2010*].

Histological Diagnosis

Histological demonstration of the stored glycosphingolipids in affected tissues, using light microscopy [*Scriba 1950*] and electron microscopy [*Henry & Rally 1963, Hartley et al. 1964*], has been used as an adjuvant for the diagnosis of Fabry disease for the last 60 years. The histopathological identification of glycosphingolipids in tissues of patients with Fabry disease, its technical details and the limitations of its use in diagnosis, was thoroughly discussed in a previous chapter of this thesis.

Diagnosing Fabry disease in urine samples using phase-contrast microscopy and immunocytochemistry, after a proper training period, is not difficult and is highly sensitive and specific, permitting easy, cheap and a rapid diagnosis [*Selvarajah et al. 2011*].

The histopathological diagnosis of Fabry disease in a biopsy should imply its confirmation by enzyme analysis or by identifying the causative *GLA* gene mutation, the latter being particularly important in heterozygous female patients [*Oqvist et al. 2009, Ferreira et al. 2009*].

Pedigree Analysis

As recommended by the National Society of Genetic Counselors in the United States of America (USA), and others, pedigree analysis in families of Fabry patients should be used to identify relatives who need diagnostic testing [*Bennet et al. 2002, Merta et al. 2007, Laney & Fernhoff 2008*]. Laney et al. found that, on average, for every index case of Fabry disease diagnosed, five family members with Fabry disease were identified through family history [*Laney & Fernhoff 2008*]. Support services from geneticists and genetic counselors should be made available to healthcare professionals in order to carry out a comprehensive pedigree analysis. Software tools, such as the 'Fabry Indicator' (distributed without charge by Genzyme Corp.) or the 'Fabry Pedigree Facilitator' (distributed without charge by TKT Europe - 5S AB), have been developed to aid healthcare professionals in pedigree analysis.

Prenatal Analysis

The optimal time for determination of genetic risk, clarification of carrier status and discussion of the availability of prenatal testing is before pregnancy. It is appropriate to offer genetic counseling (including discussion of potential risks to offspring and reproductive options) to young adults who are affected, carriers or at risk of being carriers [*Mehta & Hughes 2008*].

Prenatal diagnosis of Fabry disease in a male foetus can be made directly in chorionic villi and in cultured chorionic villi cells and cultured amniotic cells by measuring α-galactosidase activity and/or mutation analysis if the family mutation is known. Chorionic villus

sampling is usually performed at approximately 10-12 weeks' gestation and amniocentesis is performed at approximately 15-18 weeks' gestation. Preimplantation diagnosis of Fabry disease by mutation analysis in blastomeres is technically feasible and has been performed in some cases [*Winchester & Young 2010*]. With the advent of enzyme replacement therapy there are now very few requests for prenatal diagnosis of Fabry disease.

Newborn Screening

Screening newborns within a few days of birth for a panel of treatable diseases is internationally recognized as an important public health programme. Newborn screening has the potential to identify Fabry disease patients early, before irreversible tissue and organ damage occur. Therefore, newborn screening allows timely therapeutic intervention and also permits families to make informed reproductive choices.

A Fabry disease newborn screening program in Italy tested 37,104 consecutive male infants for α-Galactosidase enzyme activity in dried blood spots [*Spada et al. 2006*]. According to these data, the prevalence of Fabry disease in the Italian population (1:4600 live newborns, counting only known disease-causing mutations) is much higher than previously estimated. Pedigree analysis of affected infants identified additional undiagnosed cases of Fabry disease among their relatives. The newborn screening of Chinese population of Taiwan found *GLA* mutations in 1:1400 male newborns, and concluded that the diagnosis of Fabry disease should be considered in all patients with idiopathic hypertrophic cardiomyopathy [*Lin et al. 2009*]. Newborn screening allows also discovering of new mutations [*Hwu et al. 2009*]. These results confirm the feasibility of newborn screening for Fabry disease and support the importance of pedigree analysis in conjunction with newborn screening programs. Newborn screening may result in the identification of a broader clinical spectrum of Fabry disease [*Zarate & Hopkin 2008*]. The early identification of undiagnosed patients allows timely medical intervention providing a better clinical outcome [*Lin et al. 2009*].

TREATMENT OF PATIENTS WITH FABRY DISEASE

The chronicity of the clinical events in Fabry disease, causes severe debilitation and incapacity that extends over years. Fabry disease management strategies should be individually tailored, according to patient age and disease stage. These strategies include the use of medication to alleviate the symptoms, disease-specific treatment to delay and prevent possible serious organ damage, and adherence to standard health care measures and a healthy lifestyle.

Treatment and Prevention of Symptoms and Organ Damage

Table 3 summarizes the therapeutic options for symptoms and organ damage in Fabry disease [table 3].

Enzime Replacement Therapy

The availability of enzyme replacement therapy with recombinant alpha-galactosidase since 2001 [*Eng et al. 2001, Schiffmann et al. 2001*], offers the promise of altering the natural history of this rare form of proteinuric chronic kidney disease [*Schiffmann 2007, Warnock 2007*].

Currently, there are two forms of enzyme replacement therapy available for the treatment of Fabry disease: (1) Replagal® (agalsidase alfa; Shire Human Genetic Therapies, Inc., Cambridge, MA, USA) and (2) Fabrazyme® (agalsidase beta; Genzyme Corporation, Inc., Cambridge, MA, USA). The primary amino acid sequences of the gene products are the same; however, the structures of the oligosaccharide side chains are slightly different, resulting in distinct glycosylation patterns [*Blom et al. 2003, Lee et al. 2003*]. Agalsidase alfa is produced in a continuous human cell line by gene activation and is used at a dose of 0.2 mg/kg infused intravenously every other week [*Schiffmann et al. 2001*]. Agalsidase alfa is typically infused over a 40-min period without routine premedication. Agalsidase beta, which is produced in Chinese hamster ovary cells, contains a higher proportion of the

Table 3 | Treatment and prevention of symptoms and organ damage.

Symptoms or organ damaged	Treatment
Cardiovascular complications	Percutaneous transluminal coronary angioplasty, intra-aortic balloon pump, coronary artery bypass graft and valvular replacement are methods of treatment for the cardiac events. In those patients with mitral valve prolapse, taking prophylactic antibiotics when undergoing dental procedures or surgery is recommended. Permanent cardiac pacing or implantable cardioverter-defibrillators should be considered in high-risk patients.
Cerebrovascular complications	Prophylactic oral anticoagulants or antiplatelet medication are recommended for stroke-prone patients or in patients who have had transient ischemic attacks. Reducing general stroke risk factors (e.g. hypertension, diabetes mellitus, smoking, dyslipidemia).
Depression	Anti-depressant drugs (e.g., selective serotonin reuptake inhibitors,selective norepinephrine reuptake inhibitors).
Gastrointestinal complications	Metoclopramide (for patients with gastrointestinal manifestations due to delayed gastric emptying). Pancrelipase, H2-blockers, loperamide can ameliorate gastrointestinal symptoms in some patients. Some patients may benefit from a change in eating habits (small meals).
Hearing loss	Hearing aids. Patients should avoid excessive noise exposure.
Hyperlipidemia	Regular routine surveillance and statin therapy.
Lymphedema	Compression stockings and lymph drainage in some case.
Lung complications	Avoidance of smoking in patients with obstructive lung disease.
Nephropathy	· **Mild reductions in kidney function:** A "renal diet" is recommended, consisting in low sodium (2.4 g/day) and low protein diet. Angiotensinconverting enzyme inhibitors / Angiotensin receptor blockers for anti-proteinuric therapy. · **Severe compromise of kidney function:** dialysis (hemodialysis or peritoneal dialysis) and/or renal transplantation have become lifesaving procedures.
Pain	Avoid the precipitating factors of pain. Diphenylhydantoin, carbamazapine, gabapentin and/or nonsteroidal anti-inflammatory drugs. Some patients may require more potent analgesics (e.g., opioids) for pain management.
Skin lesions	Angiokeratoma can be removed by argon laser treatment or intense pulsed light. Lesions that are more pedunculated may be treated with a series of liquid nitrogen treatments prior to laser therapy.
Visual symptoms	Ocular manifestations in patients with Fabry disease rarely, if ever, cause significant impairment of vision and, as a rule, do not require treatment.

mannose-6-phosphate residues and is administered at a dose of 1.0 mg/kg intravenously every other week [*Eng et al. 2001*]. Patients are usually premedicated with an antipyretic before infusion, which initially may take 4-6 h (initial infusion); subsequent infusions may be administered at a rate of 3-5 mg/min.

Agalsidase beta has been approved for the treatment of Fabry disease in the US, while both agents are available for clinical use in over 40 countries worldwide, including the European Union [*Desnick 2004a*].

Agalsidase alfa in adult males has been reported to reduce plasma Gb3 levels and urinary excretion of Gb3, stabilize kidney function, reduce the severity of neuropathic pain, improve gastrointestinal symptoms, reduce left ventricular mass in adult males with left ventricular hypertrophy, and improve quality-of-life measures [*Sciffmann et al. 2001, Hoffmann et al. 2005, Schiffmann et al. 2006a, Hoffmann et al. 2007, Hughes et al. 2008*]. Agalsidase beta has demonstrated similar biochemical responses, and it also appears to stabilize kidney function [*Eng et al. 2001, Germain et al. 2007*]. In a phase IV study, agalsidase beta tended to reduce the incidence of major adverse events (i.e., progression of kidney dysfunction, myocardial infarction, stroke, death) in patients with advanced disease at baseline [*Banikazemi et al. 2007*].

When should enzyme replacement therapy be initiated?

Gb3 accumulation begins during fetal development, but signs and symptoms of Fabry disease take years to appear [*Desnick et al. 1973, Elleder et al. 1998, Tsutsumi et al. 1985*]. The issue of when to initiate enzyme replacement therapy for Fabry disease remains to be determined. Recently, a panel of expert physicians in Fabry disease recommended that enzyme replacement therapy should be initiated in all patients with Fabry disease as soon as clinical signs and symptoms, such as pain or isosthenuria, are observed. Current guidelines indicate that enzyme replacement therapy should be initiated in all males older than 16 years and in pediatric males at the time of development of significant symptoms [*Eng et al. 2006*].

These guidelines also state that enzyme replacement therapy in female patients should be started only after the onset of significant symptoms [*Pintos-Morrel & Beck 2009*]. The following signs and symptoms suggest serious implications of Fabry disease in females that warrant enzyme replacement therapy: (a) Uncontrolled pain at any age that requires alteration of lifestyle and interferes with quality of life; (b) Presence of and a progressive increase in proteinuria, exceeding 300 mg per 24 hours or renal biopsy findings that suggest significant renal involvement; (c) Patients on dialysis or who have undergone transplantation; (d) Ischaemic heart disease with or without cardiac dysfunction; (e) Moderate-to-severe heart enlargement (i.e., left ventricular hypertrophy); (f) Heart rhythm abnormalities; (g) Significant brain involvement or magnetic resonance imaging changes; (h) Frequent severe vertigo episodes; (i) Severe fatigue.

Patients on renal replacement therapy:

In Fabry patients who are undergoing dialysis or have received a kidney transplant, enzyme replacement therapy is indicated in order to prevent cardiac, cerebrovascular and neurological complications such as transient ischaemic attacks and stroke [*Mignani et al. 2004, Pisani et al. 2005a, Mignani et al. 2008*].

Markers for controlling enzyme replacement therapy efficacy:

There is no consensus at present on the specific measures that should be incorporated in the assessment of burden of disease, but likely factors would include decreased kidney function, proteinuria, urinary Gb3 excretion, pathologic changes on renal biopsy and the extent and severity of glycosphingolipid deposits [*Fervenza et al. 2008*]. Until now, microvascular Gb3 clearance has been considered the target marker for the follow-up of enzyme replacement therapy in Fabry patients, but the validation of endothelial Gb3 clearance as a surrogate outcome measure for the clinical efficacy of enzyme replacement therapy has not been convincingly established [*Thurberg et al. 2002, Fervenza et al. 2008*]. The question remains which organ(s) should be selected for biopsy to monitor the clearance of glycosphingolipid deposits with enzyme replacement therapy. Skin biopsies are easy to obtain and have a low rate of complications. The role of renal biopsy has become more relevant in recent years, not only for the diagnosis of Fabry nephropathy, but also because offering a spectrum of alterations in the tissue that, even without clinical signs of Fabry nephropathy, could favour the institution and follow-up of enzyme replacement therapy [*Tøndel et al. 2008, Ortiz et al. 2008*].

Anti-agalsidase antibodies and adverse reactions to enzyme replacement therapy:

Despite differences in glycosylation that could affect tissue delivery and uptake, agalsidase alfa and agalsidase beta appear to have identical immunogenicity and specific activity [*Lee et al. 2003, Linthorst et al. 2004*]. Enzyme replacement therapy may induce the production of IgG and IgE antibodies and cause allergic reactions, which are preventable by premedication with hydrocortisone and/or antihistamines before intravenous infusion. Infusion-related events (i.e., fever, rigors, hypertension) may be reduced or eliminated by a slower rate of administration or interruption of treatment [*Fervenza et al. 2008*].

Therapies under Investigation

Chaperone Therapy

It has been demonstrated that some *GLA* gene mutations result in destabilisation, aggregation and premature degradation of a mutant enzyme polypeptide that is catalytically active. Strategies directed at preventing premature degradation by pharmacologic stabilising of the mutant protein may substantially increase residual alpha-galactosidase activity. Because the level of enzyme activity necessary to prevent disease is relatively low (< 10%), even a modest increase in chaperone-induced enzyme activity might be expected to arrest the progression of Fabry disease. A novel approach uses small molecules designed to enhance the residual enzyme activity by protecting the mutant enzyme from misfolding and degradation in the cell. Phase II trials are now in progress in males and females

with Fabry disease with a pharmacologic chaperone (1-deoxygalactonorijimycin; DGJ; AmigalTM; Amicus Therapeutics, NJ, USA) [*Schiffmann et al. 2008*].

Gene replacement Therapy

Gene replacement therapy has been investigated in the mouse model of Fabry disease but no human trials have been undertaken to date [*Ziegler et al. 1999, Takenaka et al. 2000, Li et al. 2002, Ziegler et al. 2002, Ziegler et al. 2004, Ogawa et al. 2009*].

2

AIMS AND SCOPE OF THE THESIS

Vítor Faria, a pathologist of the Department of Pathology of *Hospital de São João* (HSJ) and Faculty of Medicine, University of Porto (FMUP), published in 1970, in a Portuguese medical journal, the results of his notable light microscopy and electron microscopy studies of skin, liver and kidney biopsies of the first patient with Fabry disease diagnosed in Portugal. Since then, a relatively large series of kidney biopsies of patients with Fabry disease was collected at the Department of Pathology of HSJ, comprising patients of both genders either with the classical, severe phenotype (including a young boy), or with milder, late-onset variant phenotypes. In addition, paraffin-embedded tissue samples of the spleen removed from a classically affected Fabry patient were available in archive. The indication for splenectomy was to treat severe hypersplenism complicating long-term hemodialysis for therapy of ESRD due to Fabry nephropathy. A second major contribution to the study of Fabry disease in HSJ/FMUP were the first diagnoses of Fabry disease by mutational analyses of the *GLA* gene ever done in Portugal, including in amniotic fluid cells for prenatal diagnosis, which were carried out at the Genetics Department of FMUP in the late nineties. So far, the cumulative number of families with classical Fabry disease diagnosed, screened and followed-up at the Nephrology and/or Genetics departments of HSJ is the largest in Portugal, and a total of twelve of those patients have so far received ERT. In this background, thanks to the outstanding organization of the HSJ renal biopsy registry due to Dr. Elísio de Carvalho, there was the opportunity to study the aforementioned histological material to address some unresolved issues regarding the pathology of Fabry disease, particularly of the kidney.

Although the complications of Fabry nephropathy, particularly ESRD, are among the leading causes of morbidity and premature mortality of patients with Fabry disease, systematic descriptions of the renal pathology, of its evolution, and of the corresponding clinico-pathological correlations are scarce, particularly in the heterozygotes. Furthermore, the pathogenic mechanisms involved in the development and progression of the Fabry nephropathy and the roles of the different kidney cell types in these processes are not well understood. Therefore, the standardization of the description of kidney biopsies of patients

with Fabry disease and a reproducible scoring system for the most pathogenically relevant histopathological lesions would be an invaluable tool to identify prognostic criteria and to assess the response to therapeutic interventions, including ERT.

The histological diagnosis of Fabry disease has been mostly based on the demonstration of the characteristic intralysosomal glycosphingolipids deposits in affected tissues, by electron microscopy, particularly on kidney biopsies. However, many pathology laboratories do not have access to, or do not routinely perform electron microscopy studies. As both, the cytoplasmic vacuolation and the chronic degenerative lesions that are associated with the progressive nephropathy of Fabry disease, are non-specific histological findings, its diagnosis disease may be missed in renal biopsies exclusively processed for light microscopy. Not recognising the diagnosis of Fabry disease may delay the timely institution of specific treatment and to fail the screening of at-risk relatives of affected individuals. An immuno-histochemical method to demonstrate the presence of Gb3 deposits in tissue samples processed for light microscopy would be a most useful diagnostic tool for Fabry disease, including for retrospective evaluation of archive material, and could render electron microscopy unnecessary.

The alpha-galactosidase knock-out model of Fabry disease was invaluable for studying the pathophysiology of the disease and for the development and testing of the new therapies, that address the underlying enzyme deficiency and storage defect. However, because the Fabry mice do not manifest progressive chronic kidney disease, not even at advanced ages, their value as a model for human Fabry nephropathy is questionable. Still, it can speculated that a comparative study of the pathology of Fabry nephropathy and the kidney pathology of Fabry mice might suggest clues to the pathogenesis of the human disease and to the mechanisms involved its progression.

Although lymph node and spleen enlargement have been occasionally reported in patients with Fabry disease, the clinical involvement of the reticuloendothelial system is relatively rare in Fabry disease as compared to other metabolically related glycosphingolipidosis (e.g., Gaucher disease). However, it is possible that the involvement of reticuloendothelial system in Fabry disease is not recognised in many patients because it is subclinical and not properly sought.

In this context, the major tasks of this PhD Thesis project were the following:

- Histological study and description of all renal biopsies of patients with Fabry disease available at the Department of Pathology of HSJ, using a semi-quantitative scoring approach to both the storage-related (e.g., vacuolation, glycosphingolipids inclusions)

and the non-specific degenerative chronic lesions (e.g., glomerular sclerosis, interstitial fibrosis, arterial sclerosis and/or hyalinosis);

- Assess the utility of light microscopy reading of toluidine blue- or methylene blue-stained semi-thin sections to diagnose Fabry disease in kidney biopsies, and for scoring the renal lesions of Fabry nephropathy;

- Compare the renal lesions observed in the heterozygous females with those of hemizygotes, in order to identify differences in pathology that might explain the significantly lower risk of ESRD in the heterozygotes;

- Analyze the morphological characteristics of the renal lesions, including the distribution of Gb3 deposits per cell type, and correlate these findings with the clinical presentation and evolution of Fabry nephropathy, seeking to identify useful prognostic factors;

- Analyze the pattern of residual immunohistochemical expression of Gb3 in paraffin-embedded renal biopsies, in order to validate this approach as a further histological method to diagnose Fabry disease in kidney tissue samples processed for routine light microscopy examination; describe the pattern of immunohistochemical co-expression of Gb3 and a lysosomal membrane marker;

- Describe the kidney pathology of the Fabry knock-out mice using the semi-quantitative scoring approach developed for the human kidney biopsies, and compare these findings with those in Fabry patients, seeking to identify factors that might be pathogenically relevant to explain the interspecies difference in the development of progressive chronic kidney disease associated with Fabry disease;

- Describe the histopathology of the spleen removed from a classically affected male Fabry patient, on long term maintenance hemodialysis, in order to elucidate whether the severe hypersplenism that justified the indication of splenectomy might be pathogenically related to Fabry disease.

3

BOOK CHAPTERS AND PAPERS

RENAL MANIFESTATIONS OF FABRY DISEASE

David G. Warnock, Carmen Valbuena, Michael West, João Paulo Oliveira

Fabry Disease, 1st edn. Springer, 211-214

Chapter 12
Renal Manifestations of Fabry Disease

David G. Warnock, Carmen Valbuena, Michael West, and João Paulo Oliveira

Abstract The majority of Fabry patients have kidney involvement (nephropathy), with significant proteinuria and progressive loss of kidney function leading to end-stage-renal-disease. Baseline assessment of the severity of Fabry nephropathy, institution of appropriate therapy, and close monitoring of the subsequent course is essential. Once present, target kidney damage is not reversed, so stopping further progression is the treatment goal. Fabry nephropathy is treatable, even in patients with fairly advanced disease. While the cornerstone of therapy remains enzyme replacement therapy with agalsidase preparations, this treatment alone does not reduce urine protein excretion. Treatment with angiotensin converting enzyme inhibitors or angiotensin receptor blockers must be added to enzyme replacement therapy to reduce urine protein excretion with the goal that this will stabilize kidney function. Kidney function, with estimated glomerular filtration rate based on serum creatinine, and measurements of urinary protein should be obtained at every clinic visit, and the rate of loss of function followed over time. Anti-proteinuric therapy can be dosed to a pre-specified urine protein target rather than a specific blood pressure goal, recognizing that successful therapy will usually lower the blood pressure below the goal of 130/80 mmHg used for other forms of kidney disease. The overall goal for treating Fabry nephropathy is to reduce the rate of loss of glomerular filtration rate to ≤ -1.0 ml/min/1.73 m^2/year, which is that seen in the normal adult population.

Keywords Chronic kidney disease · Focal glomerular sclerosis · Glomerular filtration rate · Proteinuria · Renal biopsy

Abbreviations

ACEI	Angiotensin converting enzyme inhibitor
ARB	Angiotensin receptor blocker
CKD	Chronic kidney disease

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eGFR	Estimated GFR
EM	Electron microscopy
ESRD	End-stage renal disease
ERT	Enzyme replacement therapy
GFR	Glomerular filtration rate
GLA	The α-galactosidase A locus
GL-3	Globotriaosylceramide
mGFR	Measured GFR

12.1 Fabry Nephropathy

Chronic kidney disease (CKD), progressing to end-stage renal disease (ESRD), has been a major cause of morbidity and early mortality in adult male with the classical Fabry phenotype [1, 2]. As is typical for X-linked genetic diseases, heterozygous females with α-galactosidase A gene (*GLA*) mutations generally have a more variable, less severe and later-onset clinical course compared to males Fabry patients [1, 2]. However, a substantial proportion of heterozygous females ultimately develop significant clinical complications, including CKD and ESRD [3–9].

Although the patients originally described in 1898 by William Anderson and by Johannes Fabry had proteinuria, and despite subsequent reports of urinary abnormalities in typically affected patients, Fabry disease was long regarded as a dermatologic condition – '*Angiokeratoma Corporis Diffusum*'. It was not until the first autopsy studies [10, 11] that Fabry disease was recognized as a systemic lipoid storage disorder, with major involvement of the cardiovascular system and the kidneys [12]. Although the majority of classically affected patients were males [13–15], the kidney pathology was identical for males and females [16].

The main clinical features of Fabry nephropathy were described in the early 1960s [14, 17, 18]: proteinuria developing in childhood and adolescence, followed by continued decline of renal function in early adulthood, eventually reaching ESRD in the third to the fifth decade of life. It was later recognised that heterozygous females occasionally present with the classical phenotype of Fabry disease, including ESRD [19], and that patients who lacked the neuropathic, cutaneous and ocular manifestations of classical Fabry disease could still develop progressive CKD [20].

Not infrequently, patients without a family history of the Fabry disease are first diagnosed by nephrologists, in the investigation of proteinuric CKD, most often on renal biopsy [2]. Therefore, any physician involved in the diagnosis and treatment of Fabry patients should be aware of the renal manifestations of Fabry disease, just as nephrologists should be familiar with the most common extra-renal features.

12.2 Diagnosis of Fabry Nephropathy

In patients with the diagnosis of Fabry disease the finding of microalbuminuria, overt proteinuria and/or progressive CKD are presumptive evidence of Fabry

nephropathy. Increased urinary excretion of globotriaosylceramide (GL-3) further supports the diagnosis of Fabry nephropathy in both genders, including children [21], although some females may have normal GL-3 excretion. Atypical clinical presentations for Fabry nephropathy, including acute nephritic syndrome, acute onset of the severe nephrotic syndrome, severe hypertension early in the course of progressive CKD, rapidly progressive loss of function, and macroscopic hematuria, are indications for diagnostic kidney biopsy [9, 22]. Minimal change nephropathy [23], IgA nephropathy [24–27], crescentic glomerulonephritis [28–30], granulomatous interstitial nephritis [30] and lupus nephritis [31, 32] have been described as coexisting renal disorders in patients with Fabry nephropathy, again requiring kidney biopsy for definitive diagnosis. The finding of multiple parapelvic renal cysts in the diagnostic workup of a patient with CKD also suggests consideration of Fabry disease in the differential diagnosis [33].

Electron microscopy intracellular lamellated inclusions similar to that seen in Fabry nephropathy have been reported in patients with silicosis [34, 35] or who have been exposed to certain drugs, including chloroquine [36–38]. Aminoglycoside antibiotics can cause lysosomal changes referred to as ‘myelin bodies’, but these are typically located in proximal tubules and not in the glomeruli [39]. The finding of typical glycosphingolipid deposits in a kidney biopsy specimen, especially when the glomerular epithelial cells are involved, is an important histologic clue for Fabry nephropathy, but the definitive diagnosis must be confirmed by identifying the causative *GLA* gene mutation, particularly in female patients [9, 40].

12.3 Clinical and Laboratory Manifestations of Fabry Nephropathy

The clinical and laboratory manifestations of Fabry nephropathy are expression of the accumulation of GL-3 and other neutral glycosphingolipids in the renal parenchyma and its consequences upon glomerular and tubular function, as reflected in the urine composition.

Even in early stages of Fabry nephropathy, red blood cells, white blood cells, and hyaline and granular casts can be found in the urine sediment [1, 41]. Birefringent lipid globules, with the characteristic ‘Maltese cross’ appearance, may also be noted with polarised light microscopy. These globules are present either free in the urine or within desquamated uroepithelial cells. Lipid-laden renal tubular cells account for about 75% of the exfoliated cells in the urine sediment of classically affected Fabry patients [42].

Studies of tubular reabsorption, and secretion were first described 50 years ago [16, 43, 44]. Defects of the proximal and distal tubules, out of proportion to the decrease of GFR, have also been demonstrated [44]. Signs of proximal tubular dysfunction included low tubular maximum reabsorption for glucose and low tubular maximum secretion for para-aminohippuric acid. Defective renal acidifying mechanisms, with reduced net acid excretion, particularly of urinary ammonium excretion,

signify line distal tubule dysfunction. Hypokalemia has also been rarely observed as a very early manifestation of Fabry nephropathy [45], and can be accentuated when lymphedema is mistakenly treated with aggressive diuretic therapy [J-P Oliveira, personal observation]. It is possible that glycosphingolipid deposits in the principal and intercalated cells of the renal collecting ducts alter tubular potassium reabsorption, and enhance net potassium secretion [46]. The heterogeneity of the renal tubular disorders associated with the early stages of Fabry nephropathy may be related to the variable degrees of glycolipid accumulation in the tubule epithelial cells [44].

An inability to maximally concentrate the urine, and even isosthenuria, has been the most commonly reported tubular function abnormality in Fabry disease, and was demonstrated even in patients with normal glomerular filtration rate (GFR) [16, 43]. The urinary concentration defect may be the earliest functional manifestation of Fabry renal disease, leading to polyuria, nocturia, and polydipsia [2]. Vasopressin-resistant (nephrogenic) diabetes insipidus has been demonstrated in adult males with Fabry disease [16] as well as in children [45].

Most patients with classic Fabry disease develop proteinuria [1, 41], although the severity of the proteinuria is more marked in males than females [5, 8]. Before the age of 18 years, microalbuminuria or overt proteinuria are present in a minority of patients of both genders [47–49], even in children with residual α-galactosidase activity >1% normal [47]. By the age of 35 years, about 50% of the affected males are estimated to have proteinuria, and all patients who survive into the 6th decade of life eventually develop proteinuria [2]. In almost all cases, proteinuria is of glomerular origin, containing ≥50% albumin. Nephrotic range proteinuria develops in less than 20% of male Fabry patients with CKD [2, 5, 8]. However, the full clinical and laboratory presentation of nephrotic syndrome is not frequent [2]: hypoalbuminemia and hyperlipidemia were respectively seen in only 26 and 21% of patients with nephrotic-range proteinuria [50].

Progressive decline of renal function is a common complication of Fabry disease, particularly of the classically affected males [1, 2, 41]. Figure 12.1 shows the clinical course of a young male patient, who starting at age 14, rapidly progressed to ESRD. He had microalbuminuria, until age 18, with loss of nearly half of his GFR. He then developed hypertension and overt proteinuria, with continued inexorable decline in GFR at –11.5 ml/min/1.73 m^2/year [9]. Enzyme replacement therapy (ERT) was started very late in the course, but without control of his hypertension and proteinuria, he progressed to ESRD by the age of 23.4 years.

A minority of Fabry patients have decreased GFR before the age of 18 years [47, 49, 51], but cases of ESRD have been reported as early as in the second decade of life [52]. By 43 years of age, an estimated 50% of male Fabry patients have serum creatinine levels ≥1.5 mg/dl (estimated GFR (eGFR) <60 ml/min/1.73 m^2 by the CKD-EPI equation [53]), and an estimated 50% develop ESRD by the age of 53 years [2]. After serum creatinine reaches ≥1.5 mg/dl, the time of progression to ESRD is variable, ranging from ~1 year to >12 years [2]. The mean age of patients who have estimated GFR <30 ml/min/1.73 m^2 is not significantly different between genders and some of these patients reach the most advanced CKD stages only in

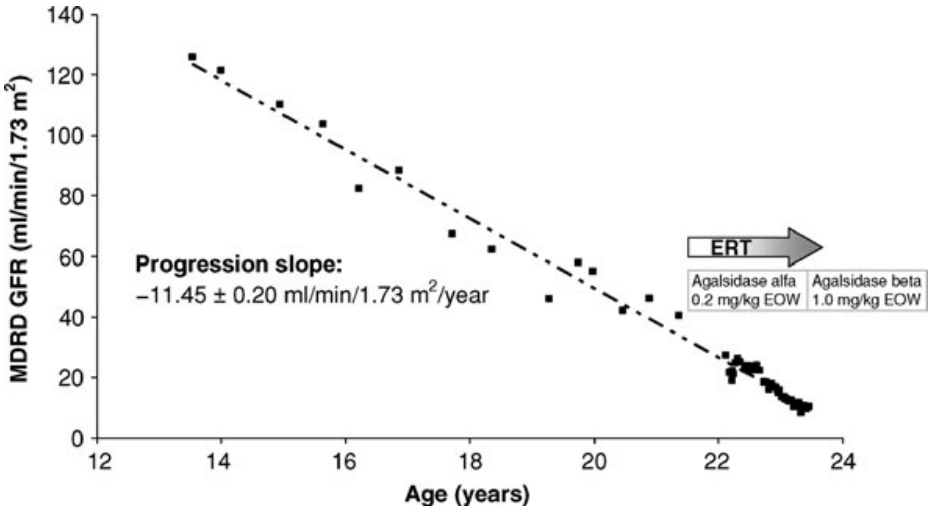


Fig. 12.1 Progressive, severe loss of glomerular filtration in a young male with Fabry disease. The urine albumin/creatinine ratio averaged 25 mg/mmol (221 mg/g) until age 18 and 188 mg/mmol (1,663 mg/g) thereafter (normal 3.8 mg/mmol; 33.6 mg/g). The systemic blood pressure averaged 114/76 until age 18, and 139/92 thereafter despite multiple antihypertensive agents. Agalsidase alfa was instituted at age 21.6 at 0.2 mg/kg every other week when the eGFR was 41 ml/min/1.72 m^2, and then switched to agalsidase beta at 1 mg/kg every week at age 23 when the eGFR was 15 ml/min/1.73 m^2. Kidney function steadily declined, attributable to late institution of enzyme replacement therapy, and inadequate control of the blood pressure and proteinuria. The rate of decline of kidney function was -11.45 ± 0.2 ml/min/1.73 m^2/year between the ages of 13.5 and 23.4 years (reprinted with permission from Oqvist et al. [9])

the eighth decade of life [5, 9]. The prevalence of anemia is higher among CKD patients with Fabry disease as compared to the general CKD population, at similar CKD stages [54, 55].

Regular assessment of eGFR and proteinuria is mandatory, with a frequency that should be related to the baseline severity. Changes in eGFR can be followed over time, recognizing the limitations of the estimating equations, especially when 'hyperfiltration' [56] >135 ml/min/1.73 m^2 is present [57]. Similar concerns have been expressed about estimates of GFR in children with Fabry disease. In a recent study from the Fabry Registry [49], the majority of children had estimated GFR values (using the Schwartz eGFR formula from 1976 [58]) that were higher than expected for healthy children and adolescents of the same age. The study by Tøndel et al. reported a significant increase in eGFR when compared to GFR measured as plasma iohexol clearance [59].

Plasma clearance techniques with clearance markers or radionuclide are currently viewed as the most accurate methods for measuring GFR, as indirect methods have previously overestimated GFR above 90 ml/min/1.73 m^2 [53]. It can be expected that the extended validation range of the CKD-EPI equation for eGFR [53] will reduce the prevalence of apparent hyperfiltration in patients with Fabry nephropathy. Figure 12.2 presents comparisons of estimated GFR versus measured GFR, comparing the Modification of Diet in Renal Disease eGFR equation [60] in Fig. 12.2a to the CKD-EPI equation for eGFR [53] in Fig. 12.2b; there is less variability around

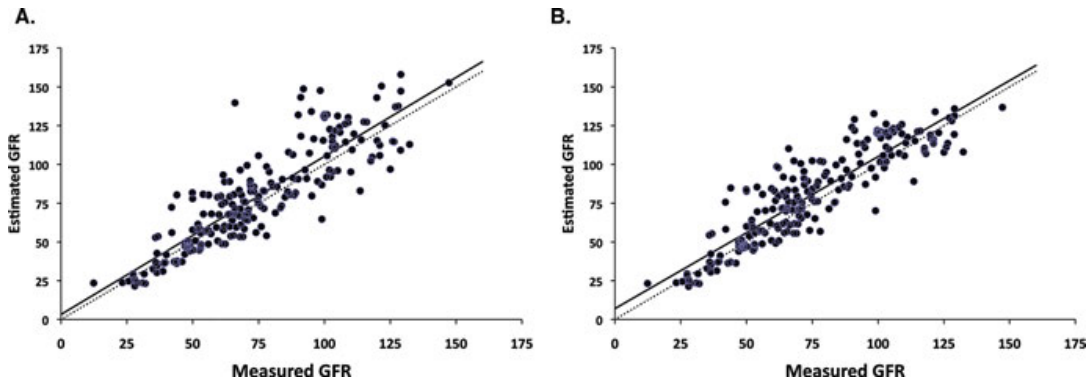


Fig. 12.2 Linear Regression Plots Comparing Measured Glomerular Filtration Rate to estimated GFR with 214 paired measurements. GFR was measured with ^{99}technetium DTPA clearance (data provided by M. West, personal communication). (**A**) Using the Modification of Diet in Renal Disease equation [60], the regression slope was 1.019 ± 0.038 (SE; 95% CI, 0.982–1.057, $P < 0.0001$; $r^2 = 0.772$), and the intercept was 3.229 ± 3.304 ml/min/1.73 m^2. (**B**) Using the CKD-EPI estimating equation [53], the regression slope was 0.980 ± 0.033 (SE; 95% CI, 0.948–1.013, $P < 0.0001$; $r^2 = 0.812$), and the intercept was 6.984 ± 2.587 ml/min/1.73 m^2

the regression with the CKD-EPI equation, and more importantly, there is a balanced distribution of data points above and below the solid regression line, especially for eGFR >90 ml/min/1.73 m^2.

The variability of eGFR compared to measured GFR is evaluated with Bland-Altman plots [61] in Fig. 12.3. The 2 Standard Deviation confidence band was narrower for the CKD-EPI estimating equation [53] compared to the Modification of Diet in Renal Disease equation [60]. The improved accuracy of the CKD-EPI estimating equation is not surprising because the Modification of Diet in Renal Disease equation had never been validated for GFR values >60 ml/min/1.73 m^2 [60]. Figure 12.3 shows that there were fewer outliers and a better distribution of values above and below the solid horizontal line, especially for GFR values >90 ml/min/1.73 m^2 for the CKD-EPI equation compared to the Modification of Diet in Renal Disease equation. It appears that much of the 'hyperfiltration' described in Fabry nephropathy can be explained by the limitations of the previous estimating equation, providing of course that these observations can be confirmed with other cohorts. For adult patients, the CKD-EPI equation [53] for eGFR should be used, and for children, the newly described Schwartz equation [62] appears to be the preferred approach for estimating GFR. Nonetheless, there remain a few adults with Fabry nephropathy and true hyperfiltration based on measured GFR [57]. Whether hyperfiltration presages a later fall in GFR in unknown; during ERT these patients had a rapid decline in GFR to the normal range consistent with disease progression or with treatment benefit. This normalization of GFR was also observed in children during ERT albeit with GFR calculated by the Counahan-Barratt formula [63] with the attendant overestimate of GFR [64].

For both genders, the proportion of patients with overt proteinuria, the magnitude of the proteinuria and the prevalence of nephrotic range proteinuria are higher with more advanced CKD stages [5, 8]. However, the role of proteinuria upon the rate

Fig. 12.3 Bland Altman Plots Comparing measured Glomerular Filtration Rate to estimated GFR with 214 paired measurements. GFR was measured ^{99}technetium DTPA clearance (data provided by M. West, personal communication). (**A**) Using the Modification of Diet in Renal Disease equation [60], the estimated GFR exceeded measured GFR by 4.36 ml/min/1.73 m^2 at the zero intercept (bias), and the ±2 standard deviation range was –26.6 to 36.0 ml/min/1.73 m^2. (**B**) Using the CKD-EPI estimating equation [53], the estimated GFR exceeded measured GFR by 5.11 ml/min/1.73 m^2 at the zero intercept (bias), and the ±2 standard deviation range was –21.4 to 32.3 ml/min/1.73 m^2

of CKD progression has been questioned [2, 4, 50]. Proteinuria develops in most patients with Fabry nephropathy, and there seems to be a general relation between the rate of loss of kidney function and the magnitude of the proteinuria, especially in male patients [8]. When adult males and females are stratified by baseline eGFR and proteinuria values, the rates of loss of kidney function are similar (Fig. 12.4). Among adult patients with an estimated GFR ≥60 ml/min/1.73 m^2, albuminuria can be demonstrated in 55% of the males and 35% of the females [5]. Nevertheless, about 10% of hemizygous males and 30% of heterozygous females reach stage 3 CKD without developing overt proteinuria [5], suggesting that other risk factors for progressive loss of GFR need to be considered, especially in females.

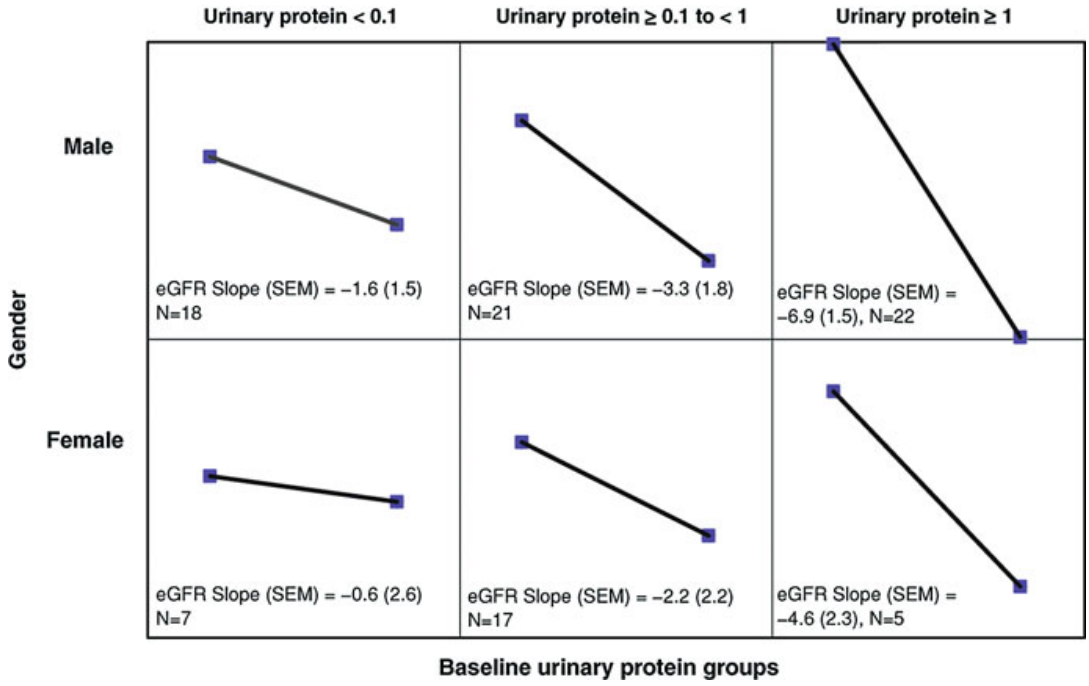


Fig. 12.4 Natural History of Fabry Nephropathy. eGFR progression slopes (ml/min/1.73 m^2/year) for male and female patients stratified by baseline 24-h urinary protein excretion (g/24 h). The *y*-axis represents eGFR (ml/min/1.73 m^2) and the *x*-axis in each panel represents a 12-month span. SEM = standard error of the mean (reproduced with permission from Schiffmann et al. [8])

Hypertension is less prevalent in CKD Fabry patients than in the general CKD population, at comparable levels of GFR [5]. More than 60% of the Fabry patients who developed hypertension did so concurrently with, or after serum creatinine reached ≥1.5 mg/dl (eGFR <60 ml/min/1.73 m^2) [2]. Proteinuria positively correlates with systolic blood pressure in adult males and females [5]. Kleinert et al. [65] observed systolic blood pressure ≥130 mmHg or diastolic blood pressure ≥80 mmHg in 57% of men and 47% of women among 391 patients with Fabry disease who were participating in the Fabry Outcome Survey. In both the Fabry Outcome Survey and the Fabry Registry, blood pressures were more elevated with more severe Fabry nephropathy as reflected in the eGFR [5, 65].

Among classically affected males and female carriers, renal ultrasonography and magnetic resonance imaging abnormalities, including renal atrophy, cortical and parapelvic cysts, decreased cortical thickness, increased echogenicity, and decreased corticomedullary differentiation, were detected in 64.5 and 60%, respectively [66], although this high prevalence has not been confirmed at other centers (D. Warnock, personal observation). No imaging abnormalities were present in males aged <12 years and females aged <20 years. In both genders, the occurrence and number of abnormalities increased with age in males and females. Cysts, particularly parapelvic cysts, were more common and appeared earlier than in the general population [66]. With magnetic resonance and computed tomography imaging, multiple renal sinus cysts were documented in 50% classically affected males with Fabry disease, aged 20–49 years [33]. An example of large non-communicating parapelvic cysts in a 42-year old male patient is shown in Fig. 12.5. The cause and significance of such cysts remain unknown.

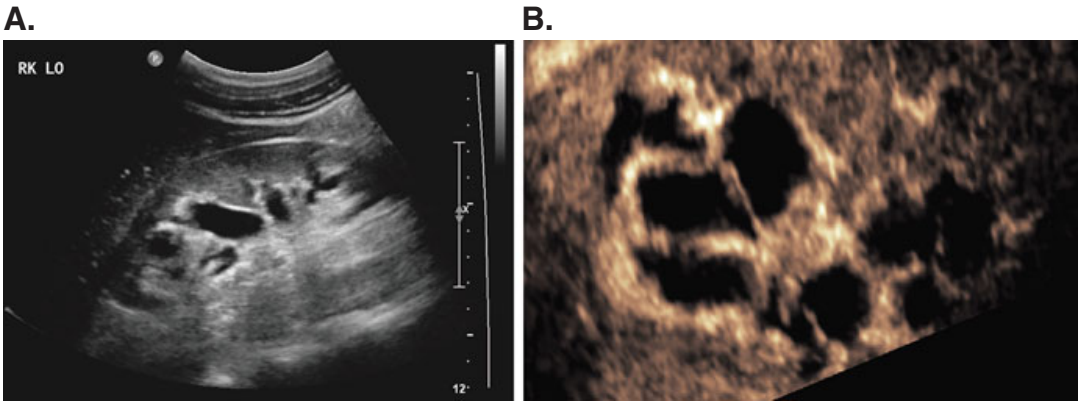


Fig. 12.5 Kidney ultrasonography of 42 year old male with Fabry disease (blood pressure 120/80; eGFR = 73 ml/min/1.73 m^2; urine protein/c reatinine ratio = 0.10 g/g on 10 mg/day lisinopril). (**A**) Conventional gray-scale saggital image. (**B**) Coronal reformatted ultrasound false colored image generated from a volume acquisition showing multiple non-communicating parapelvic cysts embedded in the central echo complex of the right kidney (Images courtesy of Franklin Tessler, MD CM, Professor of Radiology, University of Alabama at Birmingham)

12.4 The Epidemiology of Fabry Disease and Nephropathy

Before the availability of kidney transplantation and dialysis for long-term renal replacement therapy, survival of classically affected males beyond the 5th decade of life was exceptional and most patients died in uremia [14, 67], at an average age of 41 years [68]. More recently, median [69] and mean [2] survival times of affected males were estimated as 50 years and death was attributed to a large variety of causes, other than ESRD. In patients undergoing long-term renal replacement therapy, the late cardiac and cerebrovascular complications of Fabry disease may become the major causes of morbidity and mortality [1, 70].

The prevalence of Fabry disease among patients on renal replacement therapy was 18.8/100,000 and 16.7/100,000, respectively in the European [71] and the United States ESRD registries [72]. These figures are about 10- to 45-fold higher than prevalence estimates in the general population [73, 74]. In both registries, approximately 12% of patients with the diagnosis of Fabry disease were women. The mean age of Fabry patients at the beginning of renal replacement therapy, was 38 years in Europe [71] and 42 years in the United States [72]. Overall, when renal replacement therapy was initiated, roughly 50% of the Fabry patients were aged between 35 and 44 years, slightly less than 10% were younger than 24 years and 10% were aged 55 or more years; these estimates that have not changed over the intervening 20 years [9].

In a large cohort of US patients who initiated dialysis between 1985 and 1993, the 3-year survival of Fabry patients was significantly lower (63% vs. 74%) and their median survival was roughly 2 years less as compared to matched non-diabetic hemodialysis controls [72]; diabetic and non-diabetic controls were matched with Fabry patients for age, gender, race, year of dialysis initiation and modality. The survival curves for Fabry patients and non-diabetic controls began to diverge after approximately 1.5 years. A comparable 3-year survival estimate of 60% was reported in Europe [71]: patient survival on dialysis was 41% at 5 years, compared to 68% in patients with non-diabetic ESRD. These studies need to be revisited now that the awareness of Fabry disease as a cause of ESRD is better recognized in the nephrology community [9].

Ten-year renal allograft and patient survival were similar in patients with Fabry disease and matched controls, in Europe [71] as well as in the United States [75]. In the American cohort, 1-, 5-, and 10-year graft survival (respectively 91, 76, and 56%) was statistically similar to the graft survival in control patients, reflecting the transplant experience in the late 1990s. Cumulative patient survival was estimated as 83% at 5 years and 67% at 10 years post-transplantation, also not significantly different from matched controls [75]. The analysis of a much larger cohort of Fabry patients who received a kidney transplant between 1997 and 2007 in the United States demonstrated 5-year graft and patient survival rates respectively of 74 and 81% [70]. Graft survival was similar, and dialysis-free patient survival was superior for patients with Fabry disease compared to a matched cohort of patients with other causes of ESRD, implying that Fabry disease did not recur in the allografts in any significant fashion [76]; the risk of returning to dialysis was 40% lower for the Fabry patients compared with the matched cohort, but their risk of death was roughly two-fold higher [70]. Overall, these data favour kidney transplantation as the renal replacement therapy of choice for appropriate patients with ESRD due to Fabry nephropathy, either pre-emptively or within a few years following the start of dialysis. In addition, the fact that Fabry patient survival was decreased despite equivalent graft survival and better dialysis-free survival could be used to argue for prospective studies of the efficacy of ERT for incident cardiovascular and cerebrovascular events in Fabry patients who have undergone renal transplantation.

12.5 Phenotype/Genotype Correlations and Residual Enzyme Activity

A broad correlation exists between the level of α-galactosidase A activity and the clinical manifestations of Fabry disease [1]. Residual α-galactosidase A activity is inversely related with the rate of deterioration of renal function in males with Fabry disease; patients with undetectable enzyme expression, or residual α-galactosidase activity in leukocytes below 1% normal, reached serum creatinine concentrations ≥1.5 mg/dl (eGFR <60 ml/min/1.73 m^2) at a younger age than patients with higher residual α-galactosidase activities [2]. In the former group, creatinine levels ≥1.5 mg/dl were already reached by patients <25 years of age, while none with residual α-galactosidase activities >1% normal reached that creatinine level before the age of 45 years. By the age of 55 years, none of the surviving patients with α-galactosidase activity <1% normal had serum creatinine <1.5 mg/dl, whereas a number of patients with higher residual enzyme activities had maintained their eGFR >60 ml/min/1.73 m^2 [2].

As expected from structural predictions [77, 78], the type of *GLA* mutation correlated with the level of α-galactosidase activity, thereby influencing the age of onset and progression of CKD, at least in male patients. Conservative missense mutations were associated with significantly higher mean residual α-galactosidase activity (6.8% of normal) as compared to non-conservative missense mutations (1.0% of normal) or with nonsense, deletion or insertion mutations (0.8% of normal) [2]. None of the male patients with missense conservative mutations had eGFR ≤60 ml/min/1.73 m^2, in contrast to male patients with the other types of mutations for whom eGFR ≤60 ml/min/1.73 m^2, could be observed as soon as the third decade of life. As mentioned before, there is a fairly large subset of female patients with Fabry nephropathy but minimal proteinuria [5]. With the exception of the distinctly unusual compound heterozygotes or homozygotes, female patients usually have some residual enzyme activity [79]. The association of measurable α-galactosidase activity, as well as other risk factors for progression (e.g., proteinuria, systolic hypertension, smoking, hyperlipidemia) needs to be carefully considered for female as well as male patients with Fabry nephropathy.

12.6 The Renal and Cardiac Variant Phenotypes

The residual α-galactosidase A activity threshold for progressive kidney disease appears to be lower than for heart disease, as patients with the 'renal phenotype' and progressive CKD also develop left ventricular hypertrophy, but patients with the 'cardiac phenotype' may have proteinuria but generally do not develop progressive CKD. Patients lacking the classic early presentation with neuropathic, cutaneous and ocular manifestations of Fabry disease, but who otherwise developed progressive CKD, have long been recognized [20]. More recently, screening

of 514 Japanese adult males on maintenance hemodialysis led to the diagnosis of Fabry disease in six patients whose ESRD had been originally attributed to 'chronic glomerulonephritis' [80]. Their median age at the start of hemodialysis was 49 years, ranging from 25 to 56 years. Expressed as a percentage of the normal control mean, residual α-galactosidase A activity ranged from 2 to 28% (median: 4.5%) in lymphocytes and from 7 to 28% (median: 12%) in plasma. None of the patients had angiokeratomas or corneal dystrophy but five of six had left ventricular hypertrophy of moderate to severe degree. In one of the cases, a kidney biopsy was initially classified as 'glomerulosclerosis', but re-evaluation of the original biopsy specimen, with appropriate fixation for electron microscopy, demonstrated typical features of Fabry nephropathy; in retrospect, this patient appeared to have had classical Fabry disease and progressed to ESRD [80].

The 'renal variant' phenotype, especially in the absence of known family history of Fabry disease, may be particularly difficult to recognize. Even when a kidney biopsy is available, the diagnosis may be missed if the histological examination is limited to routine light microscopy [9]. In CKD or ESRD patients diagnosed with chronic glomerulonephritis, hypertension, or with an unknown cause for the ESRD, the presence of otherwise unexplained left ventricular hypertrophy of moderate to severe degree may be a clinical indication to specifically screen for Fabry disease [1].

In large scale, nationwide Fabry disease screening programs among patients with ESRD, either on chronic dialysis or transplanted, the frequency of undiagnosed cases of Fabry disease in males ranged from 0.07 to 0.15%, respectively in Austrian chronic dialysis [81] and transplant [82] cohorts, to 0.26% in Czech hemodialysis patients [83]. However, in both countries, the overall prevalence of Fabry disease among males on dialysis or transplanted, including the previously known cases as well as the newly discovered, was 263/100,000. These data are in agreement with results of a screening programme of 37,104 consecutive Italian male neonates [84], which demonstrated a 7- to 11-fold higher prevalence of *GLA* mutations associated with variant phenotypes of Fabry disease, as compared to mutations known to cause classic phenotype. In the combined analysis of several smaller case-finding studies among Japanese patients in maintenance hemodialysis [80, 85–87] the frequency of Fabry disease in males was as high as 0.72% (720/100,000), with an 80% rate of formerly unsuspected diagnoses. Other screening studies in Canada have failed to identify a single case of Fabry disease in over 1,300 patients [88] [M. West, personal communication]. Taken together, these data suggest that the prevalence of Fabry disease may vary widely in different populations and that many patients remain under-diagnosed, even in those with severe kidney involvement. It is also possible that *GLA* mutations associated with the 'renal variant' phenotype may have a non-homogeneous geographic distribution. While the prevalence, especially of 'late onset' variants reported by Spada et al. [84] was somewhat surprising, the concordance between the prevalence reported in that study, and the results from screening hemodialysis patients for Fabry disease has several important implications: the strategy of screening high risk cohorts for Fabry disease has merit [9]; the mutations associated with some residual activity that present with clinical findings later in life clearly have the potential of causing ESRD in some of the patients. The benefit

of ascertaining index cases, even though they have already reached ESRD, is the opportunity to diagnose affected relatives at earlier stages of disease.

A 'cardiac variant' of Fabry disease, mainly presenting as left ventricular hypertrophy, has also been described [1, 89, 90]. Patients with hypertrophic cardiomyopathy represent an important population that merits systematic screening for Fabry disease [89, 90]. Patients with cardiac variant Fabry disease can develop proteinuria as early as the third decade, and usually have glycosphingolipid accumulation limited to podocytes. These patients do not usually have progressive CDK or reach ESRD. It has been postulated [1] that the development of progressive CKD in Fabry disease results from glycosphingolipid storage in the renal vascular endothelial cells and, to a lesser extent, in the interstitial and mesangial cells, and is not explained by glycosphingolipid inclusions limited to the podocytes. A mechanistic explanation is offered by the fact that patients with the cardiac variant phenotype are more likely to have residual α-galactosidase activities ranging from ~1 to 10% of normal, with cases reported with up to 30% residual enzyme activity in plasma [91]. Altarescu and Elstein [92] posited that cardiac involvement is the sine qua non of Fabry disease, and hence will be seen in all male patients in an age-related association.

Males with the cardiac variant maintain renal function longer than classically affected males, and usually do not progress to ESRD. Their demise is attributable to severe left-sided heart failure and/or ventricular arrhythmias. Takenaka et al. [93] described severe left ventricular dysfunction and tachyarrhythmias in a group of 7 elderly males Fabry patients with the late-onset cardiac variant. From data in that report, it is possible to determine that their kidney function was well preserved in the terminal phases of their disease with an eGFR of 72.6 ml/min/1.73 m^2 (Table 12.1). On average, this group had 11.9 ± 1.8 (SEM) percent of residual enzyme activity, emphasizing that preservation of kidney function requires less residual α-galactosidase A enzyme activity than is required for protection against Fabry cardiomyopathy.

Table 12.1 Kidney function in males with end-stage cardiac variant Fabry disease*

Patient ID	Age (years)	Serum creatinine (mg/dl)	Plasma α-galactosidase A activity (% normal)	eGFR*
1	68	1.4	0.90	87.5
2	66	1.2	1.10	69.6
3	63	1.5	0.90	90.6
4	64	0.4	1.10	70.6
5	83	0.7	1.20	55.6
6	78	1.7	1.00	71.8
7	66	1.4	1.20	62.6
Mean ± SEM	69.7 ± 2.9	1.2 ± 1.8	1.10 ± 0.05	72.6 ± 4.8

*Data obtained from reference [93]. Estimated glomerular filtration rate (eGFR) estimated with the CKD-EPI equation [53]; expressed as ml/min/1.73 m^2

12.7 Glycosphingolipid Accumulation in Fabry Nephropathy

GL-3 and galabiosylceramide, the major neutral glycosphingolipids that accumulate in patients with Fabry disease, were first identified in extracts of kidney tissue obtained at the autopsy of a 28-year-old male with classic disease, who had died in renal failure [94]. The relative concentration of galabiosylceramide was one-third that of GL-3, which accumulates in all affected tissues of Fabry patients, while galabiosylceramide has more limited organ distribution [95]. The kidneys are major sites of glycosphingolipid deposition in Fabry disease and electron microscopy (EM) shows inclusions in all types of kidney cells, despite their normal appearance by conventional histology, even in patients with normal renal function and no proteinuria [96, 97]. The accumulation of glycosphingolipids in the kidneys starts in prenatal life and rudimentary deposits have been observed in podocytes of an aborted male foetus, at 19 weeks of gestational age [98]. Although the description of the pathology and most of the current concepts of Fabry nephropathy have been derived from autopsy and biopsy studies of affected males, it has since long been recognized that heterozygous females develop the same kidney lesions observed in males [96].

GL-3 is one of the most abundant glycolipids in the human kidney [99] and globoside, its metabolic precursor, is also a normal constituent of kidney tissue [100]. In all the neutral glycosphingolipid fractions isolated from the human kidney, C18-sphingosine constitute the predominant fraction and the fatty acid composition shows a characteristic common pattern with C22:0, C24:0, and C24:1 as the predominant isoforms [99]. GL-3 synthase is strongly expressed in the kidney [101] while the globoside synthase is expressed at a relatively low level [101]. Inhibitors of the first step in the biosynthetic pathway, glucosylceramide synthase, are currently being developed as small molecules for the treatment of lysosomal storage disease [102, 103].

GL-3 is a cell surface receptor for the B-subunit of Shiga-toxins (Verotoxins) [104]. It is expressed in the kidney [105], by many different cell types including podocytes, mesangial cells, endothelial cells, and proximal and distal tubular cells [106]. With standard immunohistochemistry of paraffin section, expression of GL3 can be observed in <1% of human tubular cortical cells [C. Valbuena, personal observation]. In human uroepithelial cells, GL-3 and globoside are iso-receptors for different molecular variants of the adhesin molecules of uropathogenic *Escherichia coli* [35]. In cultured human glomerular and tubular kidney epithelial cells, GL-3 expression at the cell membrane is inversely related to α-galactosidase expression [107].

Taken together, these data suggest that most of the neutral glycosphingolipids that accumulate in the kidneys of patients with Fabry disease is endogenously produced by normal metabolic and catabolic pathways. The fatty acid profiles of plasma GL-3 [108] and in the urine [109] are consistent with this assumption. Furthermore, the observations that Fabry nephropathy does not clinically recur in transplanted kidneys [76], and that the typical baseline kidney pathology of the transplanted

kidney from an asymptomatic heterozygous donor to her non-affected daughter did not change over time [110], also supporting the foregoing hypothesis.

12.8 Pathology of Fabry Nephropathy

Glycosphingolipids consist of a hydrophilic carbohydrate moiety linked to a hydrophobic ceramide tail, which is composed of a sphingoid long-chain base in amide linkage to a fatty acid. Glycosphingolipids are important structural and functional components of cell membranes and their physiological breakdown occurs stepwise within lysososomes, by the action of acid hydrolases [108]. As ceramide tails are dissolved during the paraffin-embedding procedure, the most characteristic finding on routine light microscopy of kidney biopsies (Fig. 12.6) is vacuolation of podocytes, of parietal epithelial cells of Bowman's capsule, and of Henle's loop and distal tubular cells [96, 111–113]. Mesangial widening, focal segmental glomerular sclerosis and global sclerosis, tubular atrophy, interstitial fibrosis and other non-specific lesions are additionally seen, even at the early stages of Fabry nephropathy [96]. In adolescents, these changes have been shown to be present in kidney biopsies even before any clinical signs of Fabry nephropathy are detected [59].

While in paraffin-embedded specimens the vacuoles look empty, and staining with lipid-soluble dyes is negative [96, 114, 115], the presence of GL-3 within vacuoles may still be demonstrated in formalin-fixed, paraffin-embedded kidney biopsy sections [116, 117] using immunostaining with a murine monoclonal antibody generated against the carbohydrate moiety of GL-3 [118]. On silver-stained ultra-thin sections of epoxy-embedded specimens, the stored glycosphingolipids appear as argyrophilic granules within the cytoplasmic vacuoles [96]. On light microscopy, the cellular distribution of glycosphingolipid deposits is best assessed in methylene blue or toluidine blue-stained semi-thin sections of epoxy-embedded tissue (Fig. 12.7) [27, 112, 119, 120]. In the glomeruli, the largest amounts of lipid material are seen in podocytes, followed by the parietal epithelial, mesangial, and glomerular endothelial cells [59, 96, 112, 119]. Progressive involvement of the parietal epithelial cells, of mesangial cells, of endothelial cells, and of the afferent and efferent glomerular arterioles, occurs as the disease worsens [46]. In heterozygous females, the parietal epithelium may be more extensively involved than the podocytes [27, 121].

On electron microscopy (Fig. 12.8), the largest inclusions are seen in podocytes and in cells of the proximal and distal tubules, and Henle's loop [96]. In these tubular segments, affected cells can be strikingly enlarged with giant inclusions measuring up 10 μm in diameter. The tubular involvement is not homogeneous and normal and affected cells may be found contiguously [96]. Proximal tubules and interstitial cells are frequently involved in Fabry nephropathy, and the presence of glycosphingolipid deposits in proximal tubular cells has been correlated with overt proteinuria in females with normal or only slightly decreased renal function [27]. Dense osmiophilic bodies have also been observed in the Bowman's space [96] and in the proximal tubular lumen [122].

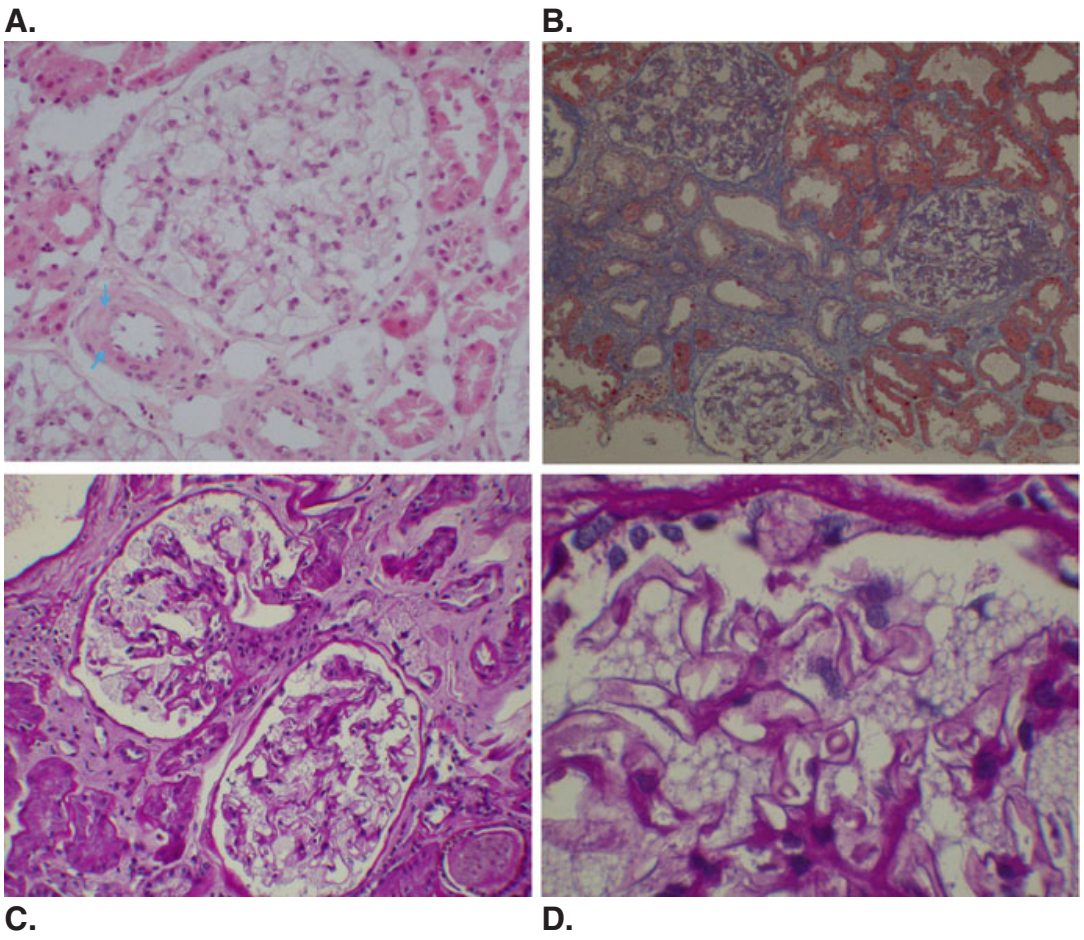


Fig. 12.6 Glomeruli showing extensive vacuolation of podocytes and GL-3 deposits in Fabry nephropathy. (**A**) Podocytes with extensive cytoplasmic vacuolation, and arteriolar vessel with hyaline droplets and reactive endothelial cells (*blue arrows*, 19 years old male patient. Hematoxylin and Eosin, magnification ×20 objective). (**B**) Focal and segmental glomerulosclerosis and localized tubular atrophy and interstitial fibrosis in a 45 years old heterozygous female patient (Special trichrome, magnification ×20 objective). (**C**) 41 year-old male patient; showing segmental sclerosis, vacuolation of visceral epithelial cells (podocytes) and parietal epithelial cells lining Bowman's capsule. There is also periglomerular and interstitial fibrosis (Periodic acid-Schiff stain. Magnification ×20 objective). (**D**) 41 year-old male patient; higher magnification of *panel* **C**, showing vacuolation of visceral epithelial cells (podocytes) and parietal epithelial cells lining Bowman's capsule (Periodic acid-Schiff stain. Magnification ×60 objective)

Most of the pathologic characterization of Fabry nephropathy has been derived from autopsy and biopsy studies of affected males [10, 16, 18, 43] but the heterozygous females develop the same type of kidney lesions described in males [16, 19, 96]. Descriptions of kidney pathology in heterozygous females are scarce and the largest published series describing kidney biopsy findings in affected females included only three [96] five [113] and four patients [27]. Valbuena et al. [27] recently reviewed the kidney biopsies of four affected females who had normal or only mildly impaired kidney function, two of them with overt proteinuria. Chronic

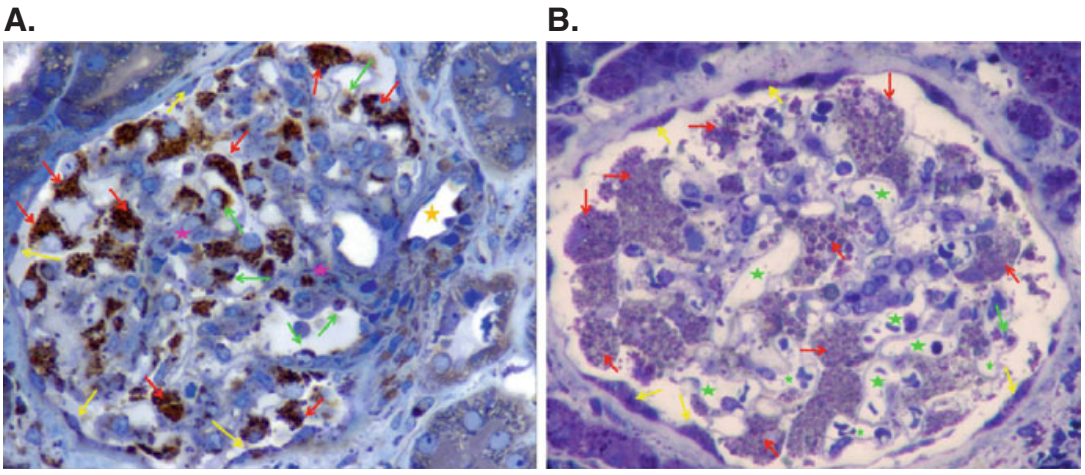


Fig. 12.7 Glomeruli showing extensive GL-3 deposits in Fabry nephropathy; plastic embedded tissue, semi-thin sections. (**A**) 7 year-old male patient; glomeruli show GL-3 osmiophilic inclusions in podocytes (*red arrows*), parietal epithelial cells along Bowman's capsule (*yellow arrows*), mesangial cells (*pink star*), and capillary endothelial cells (*green arrows*). (**B**) 19 year-old male patient; glomeruli show GL-3 osmiophilic inclusions in podocytes (*red arrows*), parietal epithelial cells along Bowman's capsule (*yellow arrows*), and capillary endothelial cells (*green arrows*), with glomerular capillary lumen marked by green star (Toluidine blue. Magnification ×60 objective)

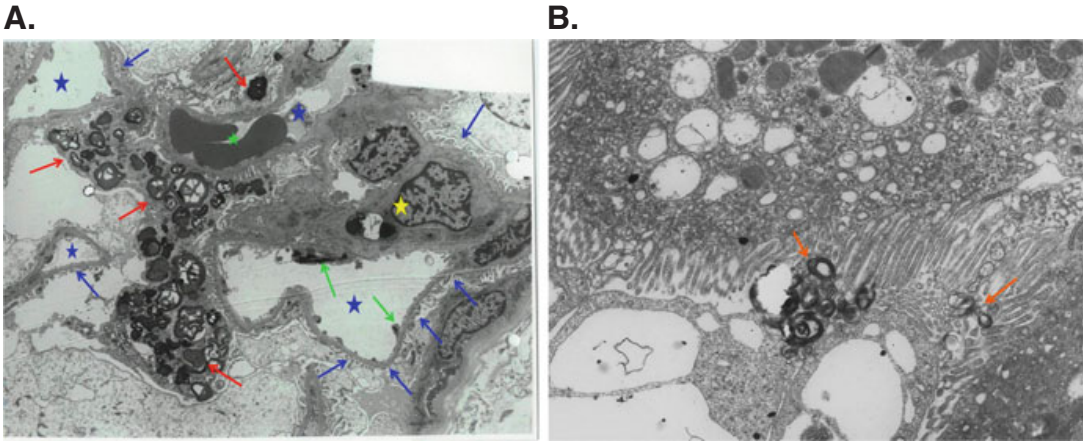


Fig. 12.8 Electron micrographs of glomerular and proximal tubule involvement in Fabry nephropathy. (**A**) Electron micrograph from a 42 years old female patient showing lamellar and amorphous osmiophilic inclusions in glomerular visceral epithelial cells (*red star*), endothelial (*green arrow*) and mesangial cells (*yellow star*). Glomerular capillary lumen are marked with a *blue star*, one of them is filled with two erythrocytes (*green star*). The podocyte foot processes remain intact (*blue arrows*. Magnification ×5,400). (**B**) Electron micrograph from a 19 years old male patient with nephrotic-range proteinuria showing lamellar osmiophilic inclusions in proximal tubular epithelial cells (*orange arrows*. Magnification ×15,900)

non-specific degenerative lesions and glycosphingolipid accumulation per cell type were semiquantitatively assessed by light and electron microscopy. Their data were combined with those of the previous series to identify significant clinico-pathologic correlations [27].

Non-specific degenerative lesions, including glomerular hyaline, increased mesangial matrix and widening of the mesangial stalks, focal segmental and global glomerular sclerosis, wrinkling of the capillary walls leading to capillary collapse, tubular atrophy and interstitial fibrosis are evident since the early stages of Fabry nephropathy [59, 96, 120]. Early signs of focal segmental glomerular sclerosis have been observed even in a female teenager with normal renal function and normal urinary albumin excretion [59]. Hyaline material or fibrinoid deposits in the media of arterioles and small and large renal arteries have been observed on routine light microscopy sections of kidney biopsies of teenagers of both genders, who had normal glomerular filtration rate and no overt proteinuria [96] or either normal or only a slightly increased urinary albumin excretion rate [59]. These fibrinoid deposits may result from necrosis of severely involved muscular cells. Valbuena et al. [27] found no histopathological evidence supporting a major role of vascular damage in the early pathogenesis of Fabry nephropathy in females. Females may develop similar degrees of arteriolar and arterial hyalinosis as males, although at an older age than males [27]. In older patients, intimal thickening can be superimposed on the vascular medial abnormalities [96]. In both genders, arterial sclerosis is generally more severe with more advanced CKD [120], but arterial and arteriolar lesions can even be observed in young males (Fig. 12.9).

The fine structure of the slit diaphragms of the foot processes may be spared despite the evidence of massive glycosphingolipid deposits in the cytoplasm of podocytes [46]. The ultrastructure of the glomerular basement membrane is usually

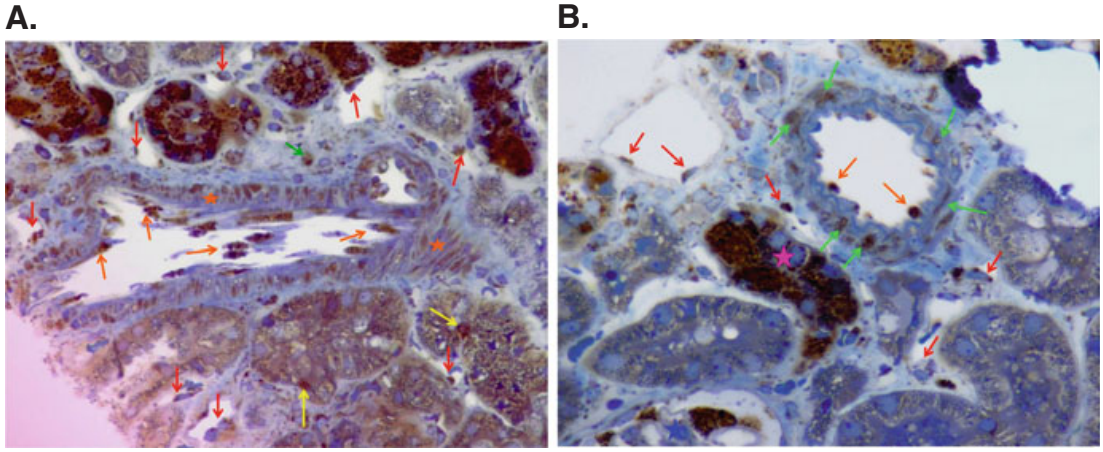


Fig. 12.9 Vascular lesions in a 7-year old male patient with Fabry nephropathy. (**A**) Artery showing osmiophilic inclusions in reactive endothelial cells (*orange arrows*) and smooth muscle cells (*orange star*). Inclusion bodies were also seen in peritubular capillary cells (*red arrows*), interstitial cells (*green arrows*) and proximal (*yellow arrows*) and distal tubular cells (Toluidine blue. Magnification ×40 objective). (**B**) Arteriolar vessel showing osmiophilic inclusions in endothelial cells (*orange arrows*) and smooth muscle cells (*green arrows*). Inclusion bodies were also seen in peritubular capillary cells (*red arrows*) and distal tubular cells (*pink star*). Part of a glomerulus with inclusions in podocytes and parietal epithelial cells is seen in botton left corner (*blue star*. Toluidine blue. Magnification ×60 objective)

normal, even alongside heavily affected podocytes. However, focal thickening and wrinkling of the glomerular basement membrane and extensive fusion of podocyte foot processes are associated with tortuosity, wrinkling and collapse of the capillary walls [96]. Furthermore, in both genders effacement of podocyte foot processes is correlated with the clinical presence of proteinuria or the nephrotic syndrome [27, 59, 113]. The basement membranes of atrophic tubules also appear thickened and wrinkled [96].

As in other chronic nephropathies, the progression of Fabry nephropathy is characterized by segmental and global glomerular sclerosis, tubular atrophy and interstitial fibrosis [46, 96, 97, 112, 113, 123]. In males, the presence of glomerular segmental or global sclerosis is the only significant pathologic association of proteinuria at early stages of Fabry nephropathy [2, 96, 120, 123]. Furthermore, negative correlations could be demonstrated between inulin clearance and histologic scores of either chronic glomerular pathology or tubulointerstitial pathology [2, 120]. In females, glomerular sclerosis and tubulointerstitial fibrosis were predictors of proteinuria and CKD stage [27].

The development of proteinuria and of late-onset CKD in some but not all patients with the cardiac variant, who have significant renal GL-3 deposits confined to podocytes [124], suggests that these cells may have a role in the pathogenesis of Fabry nephropathy. Lethal injury to GL-3-overloaded podocytes, through the formation of focal adhesions between the glomerular tuft and the Bowman's capsule [112, 125], loss of GL-3-laden podocytes in the urine, and increased hydraulic stress upon endothelial cells in the vicinity of damaged podocytes [46] are other possible mechanisms of glomerular sclerosis in Fabry nephropathy. By causing tubular injury, proteinuria can have a direct role in CKD progression and may provide a link between the aforementioned primarily glomerular pathologic processes and the development of tubulointerstitial disease [126, 127]. Mesangial cell necrosis [96], direct toxic injury to tubular cells [112] and diffuse involvement of interstitial cells [46] have also been suggested to play a role in the progression of Fabry nephropathy.

Quantitative approaches to assess the kidney pathology in biopsies of patients with Fabry disease [50, 119] have been developed as investigational tools to evaluate the short term [128, 129] and long term [130, 131] effects of agalsidase therapy in clinical trials. Thurberg et al. [119] developed a scoring system for changes in GL-3 deposits on semi-thin sections and electron micrographs in patients treated with agalsidase-beta. Figure 12.10 shows the characteristic GL-3 inclusions in vascular endothelial cells of peritubular capillaries. Clearance of these deposits, as well as form other cell types was fully achieved by 12 months of therapy [131], and maintained on follow-up biopsies after 5 years of ERT [130]. Most recently, Fogo et al. [120] systematically scored histologic lesions in kidney biopsies of a set of 35 males (mean age 36.4 years) and 24 females (43.9 years) who mostly had clinically mild Fabry nephropathy. Average serum creatinine was 1.3 mg/dl; eGFR was 82 ml/min/1.73 m^2, and urine protein to creatinine ratio was 1.08 g/g. A morphometric index of chronic damage was obtained for each biopsy. A validated scoring

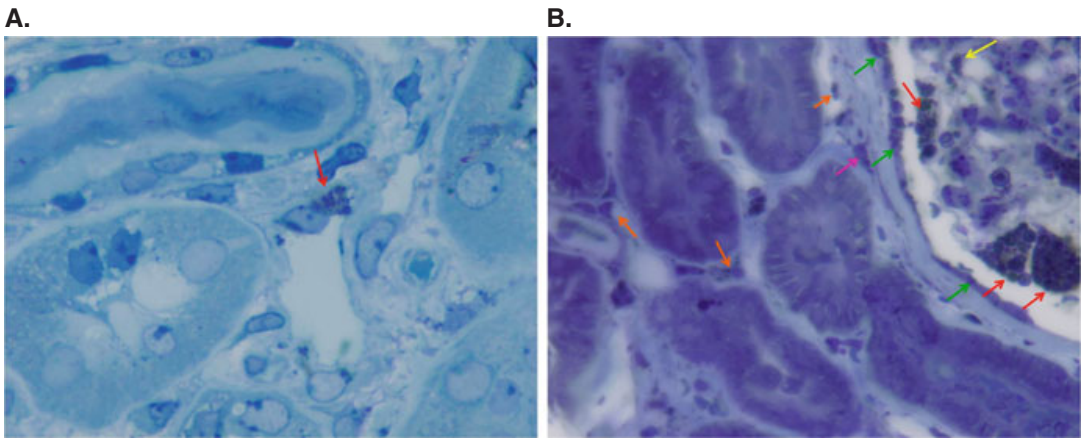
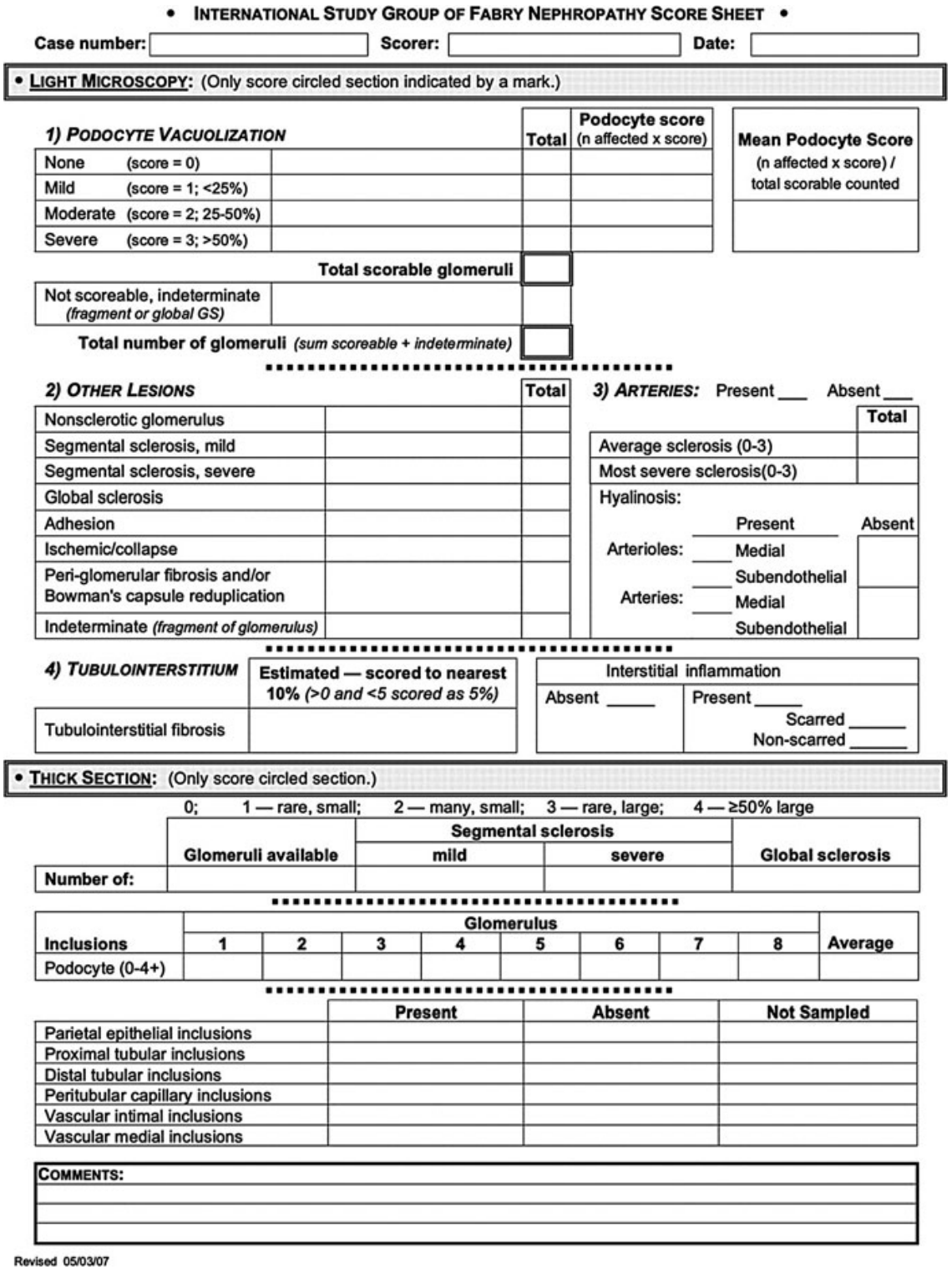


Fig. 12.10 Peritubular Capillary GL-3 Inclusions. (**A**) Plastic embedded tissue from a 32 years old female patient, showing osmiophilic inclusions in peritubular capillary cells (*Red arrow*. Methylene blue. Magnification ×40 objective). (**B**) Plastic embedded tissue from a 40 years old male patient. Tubulo-interstitial area and part of a glomerulus with round and spherical inclusion bodies in podocytes (*red arrows*), parietal epithelial cells (*green arrows*) and endothelial cells (*yellow arrow*). Inclusion bodies were also seen in peritubular capillary cells (*orange arrows*) and some interstitial cells (*pink arrow*) (Toluidine blue. Magnification ×60 objective)

system was developed, with the long-term goal to determine whether baseline histologic information can be related to the rate of progression and/or response to ERT in Fabry nephropathy. The development of a standardized scoring system of both disease-specific lesions, i.e., lipid deposition-related, and general lesions of progression, i.e., fibrosis and sclerosis, showed a spectrum of histologic appearances even in early clinical stage Fabry nephropathy. Males had greater podocyte vacuolization on light microscopy (mean score) and larger and more frequent glycosphingolipid inclusions on semi-thin sections than females. Males also had significantly more proximal tubule, peritubular capillary and vascular intimal inclusions. Arteriolar hyalinosis was similar, but females had significantly more arterial hyalinosis. Chronic kidney disease stage correlated with arterial and glomerular sclerosis scores. Significant changes, including segmental and global sclerosis, and interstitial fibrosis were seen even in patients with stage 1–2 chronic kidney disease with minimal proteinuria [120]. These findings support the role of kidney biopsy in the baseline evaluation of Fabry nephropathy, even with mild clinical disease. The validated scoring instrument developed by Fogo et al. [120] is shown in Fig. 12.11.

12.9 Dysfunction of the Microcirculation in Fabry Disease

The evidence supporting abnormal cerebral, coronary and renal blood flow, vessel architecture, endothelial function, and auto-regulation has been reviewed [44, 132–135]. The sub-cellular mechanisms linking glycosphingolipid accumulation to



• INTERNATIONAL STUDY GROUP OF FABRY NEPHROPATHY SCORE SHEET •

Case number: Scorer: Date:

• LIGHT MICROSCOPY: (Only score circled section indicated by a mark.)

1) PODOCYTE VACUOLIZATION

		Total	Podocyte score (n affected x score)
None	(score = 0)		
Mild	(score = 1; <25%)		
Moderate	(score = 2; 25-50%)		
Severe	(score = 3; >50%)		

Mean Podocyte Score (n affected x score) / total scorable counted

Total scorable glomeruli

Not scoreable, indeterminate *(fragment or global GS)*		

Total number of glomeruli *(sum scoreable + indeterminate)*

2) OTHER LESIONS

		Total
Nonsclerotic glomerulus		
Segmental sclerosis, mild		
Segmental sclerosis, severe		
Global sclerosis		
Adhesion		
Ischemic/collapse		
Peri-glomerular fibrosis and/or Bowman's capsule reduplication		
Indeterminate *(fragment of glomerulus)*		

3) ARTERIES: Present ___ Absent ___

		Total
Average sclerosis (0-3)		
Most severe sclerosis(0-3)		
Hyalinosis:		
Arterioles:	Present ___ Medial	Absent
	___ Subendothelial	
Arteries:	___ Medial	
	Subendothelial	

4) TUBULOINTERSTITIUM

	Estimated — scored to nearest 10% *(>0 and <5 scored as 5%)*
Tubulointerstitial fibrosis	

Interstitial inflammation: Absent ___ Present ___ Scarred ___ Non-scarred ___

• THICK SECTION: (Only score circled section.)

0; 1 — rare, small; 2 — many, small; 3 — rare, large; 4 — ≥50% large

	Glomeruli available	Segmental sclerosis mild	Segmental sclerosis severe	Global sclerosis
Number of:				

Inclusions	Glomerulus 1	2	3	4	5	6	7	8	Average
Podocyte (0-4+)									

	Present	Absent	Not Sampled
Parietal epithelial inclusions			
Proximal tubular inclusions			
Distal tubular inclusions			
Peritubular capillary inclusions			
Vascular intimal inclusions			
Vascular medial inclusions			

COMMENTS:

Revised 05/03/07

Fig. 12.11 Scoring sheet for Fabry nephropathy by light microscopy. Biopsy material was assessed on periodic acid-Schiff-stained slides. Of note, more than one lesion could be present on light microscopy in any one glomerulus, e.g. segmental sclerosis and ischemia/collapse could coexist in the same glomerulus, and both features were then scored as 'present' for that glomerulus. Optimal slides for scoring included >10 glomeruli for light microscopic assessment, and at least 3 glomeruli for semi-thin section scoring (reproduced with permission from Fogo et al. [120])

the clinical manifestations of Fabry disease are not clearly understood. The classical theory emphasized ischemic tissue damage resulting from microvascular endothelial disease and/or necrosis of vascular smooth muscle cells and/or pericyte injury [1, 136]. Recent studies provided evidence that GL-3 induces oxidative stress in vascular endothelial cells [137] and that circulating myeloperoxidase levels are significantly increased in males with Fabry disease, predicting the risk of subsequent vasculopathic events [138].

12.10 Progressive Loss of Glomerular Filtration in Fabry Nephropathy

An open-label extension study of the phase III study of agalsidase-beta has been published [130]. After 54 months of treatment, 8 patients had protocol kidney biopsies, which showed complete maintenance of GL-3 clearance of renal capillary endothelial cells. eGFR remained stable in 41 patients, but 6 patients with significant baseline proteinuria (>1.0 g/day) or segmental and global sclerosis in more than half of their glomeruli had rapid loss of GFR (Fig. 12.12).

The progression rates that have been reported for a number of prospective studies, trials and case reports have recently been reviewed [57, 139]. Very few of these studies have achieved slowing of progression to the optimal rate of –1.0 ml/min/

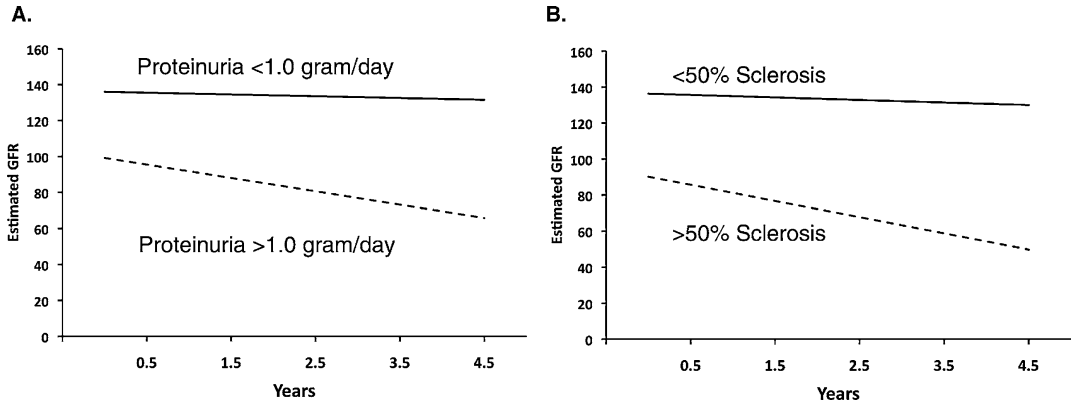


Fig. 12.12 Association between overt proteinuria, glomerular sclerosis and loss of glomerular filtration rate in Fabry nephropathy. (**A**) The 42 participants in the Phase III extension study with baseline estimated GFR of 136 ml/min/1.73 m^2 and less than 1.0 g of urine protein/day had a rate of loss of estimated GFR equal to –1.001 ml/min/1.73 m^2/year, and the 10 participants who had baseline estimated GFR of 99.2 ml/min/1.73 m^2 and more than 1.0 g of urine protein/day had a rate of loss of estimated GFR equal to –7.40 ml/min/1.73 m^2/year. The difference between these two slopes was 6.39, p-value = 0.004. (**B**) The 32 participants in the Phase III extension study with baseline estimated GFR of 136.4 ml/min/1.73 m^2 and focal or global sclerosis in less than 50% of their glomeruli had a rate of loss of estimated GFR equal to –1.40 ml/min/1.73 m^2/year, and the 8 participants who had baseline estimated GFR of 90.2 ml/min/1.73 m^2 and focal or global sclerosis in more than 50% of their glomeruli had a rate of loss of estimated GFR equal to –8.96 ml/min/1.73 m^2/year. The difference between these two slopes was 7.55, p-value = 0.003 (data re-plotted from Germain et al. [130])

1.73 m^2/year [57, 60]. Variability in the rates of change of GFR could reflect differences in baseline characteristics of the different patient populations, treatment duration, the prevalence of angiotensin converting enzyme inhibitor (ACEI) or angiotensin receptor blocker (ARB) use, achieved urine proteinuria reduction, as well as techniques of GFR measurement that complicate the comparison of different treatment groups.

12.11 Proteinuria, ERT and Progression of Fabry Nephropathy

Studies in CKD show that reno-protective treatments effectively limit the progressive decline in GFR to the extent that proteinuria is reduced [140, 141]. The rate of eGFR decline, especially for male patients largely depends on the level of proteinuria achieved during anti-proteinuric therapy [127]. With this approach, the goal is to achieve a rate of GFR decline equal to that observed in the general population [142]. It is not certain that proteinuria is as important in the progressive loss of GFR in females as it has been shown to be in male Fabry patients. Ortiz et al. [5] described a significant proportion of adult females with eGFR <60 ml/min/1.73 m^2 at baseline evaluation who did not have overt proteinuria, suggesting that other factors may be important determinants of loss of kidney function for females patients.

ERT does not reduce proteinuria, and pediatric patients have developed overt proteinuria despite ongoing ERT [59], emphasizing the need to monitor and treat proteinuria in patients already receiving ERT.

Treatment with ACEIs and/or ARBs was undertaken in an open-label study of 10 patients, most of whom had control of their proteinuria before initiation of ERT [143]. When urine protein excretion was controlled to ≤0.50 g/day in patients treated with agalsidase-beta at 1.0 mg/kg every 2 weeks, the rate of loss of eGFR was not significantly different from zero [143]. The response was much better than previously reported for patients with overt proteinuria and eGFR <60 ml/min/1.73 m^2 [144–147], but requires confirmation in an ongoing multi-center study [148]. There are patients described with progressive loss of kidney function and controlled urine protein excretion who showed greatly slowed rates of loss of function when ERT dosing was increased from 0.2 mg/kg of agalsidase-alfa given every other week to weekly dosing [146], or dosing with agalsidase-beta at 1 mg/kg given every 2 weeks [125].

Effective ACEI/ARB therapy is challenging for the physician and patient if the baseline blood pressure is relatively low. In addition, hyperkalemia and anemia, and other side effects, such as persistent cough with ACE inhibitors, can complicate the use of anti-proteinuric therapy in patients with CKD. Recent studies of Fabry disease reveal disappointing rates of use of renin-angiotensin-aldosterone system inhibitors under 50% despite multiple indications: hypertension, proteinuria, reduced GFR, hyperfiltration and others [14, 15, 17, 18]. This suggests that real barriers limit the use of ACEIs and ARBs in Fabry disease. As most physicians who care for patients with Fabry disease are not nephrologists and may not be familiar with use of these

agents, education about the optimal use of these therapies is paramount. Referral of all Fabry disease patients with any of the above indications to a nephrologist is strongly recommended.

The response to ACEI/ARB therapy has to be optimized by adjusting the doses to achieve reduction of proteinuria to a pre-defined target level, but the optimal target has not been established for Fabry nephropathy. In addition, it is not known whether stabilization of kidney function can be achieved with control of proteinuria and ERT given at lower doses than used in the pilot study [143] or the FAACET trial [148].

Whether differences between 0.2 and 1.0 mg/kg, or between agalsidase-alfa (Replagal®, Shire Human Genetic Therapies, Cambridge, MA USA) versus agalsidase-beta (Fabrazyme®, Genzyme Therapeutics, Cambridge, MA USA) have an effect on renal outcomes is as of yet unknown. For example, in comparing the low risk groups of male patients with Fabry nephropathy and baseline proteinuria <1.0 g/day treated with agalsidase-alfa at 0.2 m/kg body weight every 2 weeks, West et al. [57] reported a progression rate of -2.1 ± 1.3 ml/min/1.73 m^2/year using a nucleide tracer technique. Germain et al. [130] used agalsidase-beta at 1.0 mg/kg every 2 weeks, and reported a progression rate of -1.01 ± 0.97 ml/min/1.73 m^2/year using the Modification of Diet in Renal Disease 4 variable equation to estimate GFR [149]. Statistically these rates are not different [57] by t-test. However, comparison is difficult due to non-randomized allocation of subjects to either treatment arm and different techniques for GFR determination.

The ongoing Canadian Fabry Disease Initiative study [150] is a randomized study that will evaluate the effects on progressive loss of GFR of agalsidase-alfa given at 0.2 mg/kg every 2 weeks compared to agalsidase-beta given at 1.0 mg/kg every 2 weeks. All patients will receive ACEI or ARB therapy at doses to achieve reduction of proteinuria to a similar degree. It is also important to demonstrated the progression rate in Fabry nephropathy be reduced to the 'normal' rate [60, 151] with optimal ACEI or ARB therapy and agalsidase-alfa given at 0.2 mg/kg every 2 weeks as has been demonstrated with agalsidase-beta given at 1.0 mg/kg every 2 weeks and control of urine protein excretion [130, 143].

The clinical significance of an absolute reduction of the progression rate by –1.0 ml/min/1.73 m^2/year could be questioned, but to put this change in perspective, this is exactly the magnitude of the reduction or the progression rate reported in the RENAAL [152] and IDNT [153] studies, which are generally accepted as important advances in the treatment of diabetic renal disease [154]. A similar effect was reported in the recent study of strict blood pressure control and progression of kidney disease in children [155]. In this study, there was not a significant difference between the eGFR slopes, but the intensively treated group of children had a significant delay in reaching the primary composite endpoint, which was defined as a 50% reduction in the eGFR or progression to ESRD. In all forms of chronic kidney disease, the beneficial effect of blood pressure control versus control of proteinuria has not been determined [156, 157]. The optimal goal for proteinuria reduction, and the effects of gender and age on setting and achieving these goals in Fabry nephropathy needs to be better defined [149].

12.12 Other Considerations for Treating Fabry Nephropathy

In addition to proteinuria, other potentially treatable factors in patients with CKD, including hypertension, smoking, and hyperlipidemia may contribute to progressive loss of GFR [126]. There is every reason to expect that this approach will apply to patients with Fabry nephropathy, so correction of these other risk factors should also be considered for interventions to slow CKD progression. Systemic blood pressure is generally lower in patients with Fabry disease [5, 158]. Beta-blockers and diuretics may cause hypotension and limit the use of anti-proteinuric therapy.

12.13 Treatment Goal for Fabry Nephropathy

The goal for treatment of Fabry nephropathy is reduction in the rate of loss of GFR to ≤ -1.0 ml/min/1.73 m^2/year [60, 151]. What can be done if this goal is not reached? Measuring proteinuria and urinary sodium excretion are important first steps. Control of dietary sodium is important to ensure ACEI/ARB effectiveness. Repeated measures of eGFR are needed; basing treatment decisions on only 2 measurements of serum creatinine is not recommended. If proteinuria is not controlled, then anti-proteinuric dosing increases are recommended, with the understanding that systemic blood pressure may further decrease. The doses can be split to minimize acute falls in blood pressure, and small dose increments are preferable. It may be necessary to reduce the dose of other anti-hypertensive agents (e.g., beta blockers, diuretics, calcium channel blockers) so that ACEI or ARB dosing can be increased. Aldosterone receptor blockers may lower proteinuria with modest effects on blood pressure [159]. Switch from one form of agalsidase to another, or increasing ERT dosing or frequency of administration has not been well studied, and would best done in the setting of a prospective clinical trial. The possibility of another form of kidney disease should be addressed with a kidney biopsy [160].

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KIDNEY BIOPSY FINDINGS IN HETEROZYGOUS FABRY DISEASE FEMALES WITH EARLY NEPHROPATHY

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ERRATUM

Kidney biopsy findings in heterozygous Fabry disease females with early nephropathy

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There was an error in Table 6. The error was limited to the data shown in the table and did not affect any of the statistical analyses presented in the Results and Discussion, which were based on a SPSS file that the authors have double-checked for accuracy. The corrected table is reproduced here.

The authors very much regret their error.

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Table 6 Comparison of the three largest kidney biopsy series reporting pathology findings in females with Fabry disease

	Gubler et al. [10]			Gubler et al. [10]				Present series				
Age at kidney biopsy (years)	8	22	51	30	37	54	55	73	32	42	45	50
CKD stage	0	0	2	?	2	4	5	4	2	1	2	2
Proteinuria	0	0	0	+++	+++	+++	++++	++++	++++	0	++	0
No. of evaluated glomeruli	8	15	25	2	4	17	23	5	12	31	29	17
No. of sclerotic glomeruli	0	0	5	1	2	17	22	3	9	2	7	4
(% of sclerotic glomeruli)	0%	0%	20%	50%	50%	100%	96%	60%	75%	6%	24%	24%
Tubular atrophy/interstitial fibrosis	0	0	+	0	+	++	+++	+	+/++	+	+	+

CKD stages classified according to the clinical practice guidelines of the National Kidney Foundation [33], using reported Creatinine Clearance or available algorithms to estimated GFR (eGFR): "0" — no evidence of kidney disease, "1" — eGFR ≥ 90 ml/min/1.73m2, "2" — eGFR = 89–60 ml/min/1.73m2, "3" — eGFR = 59–30 ml/min/1.73m2, "4" — eGFR = 29–15 ml/min/1.73m2, "5" — eGFR < 15 ml/min/1.73m2.

No. of sclerotic glomeruli is the total number of evaluated glomeruli that showed either segmental or global sclerosis.

Proteinuria was graded as: "0" = no overt proteinuria, "+" = 0.3–0.5 g/day, "++" = 0.5–1.0 g/day, "+++" = 1.0–3.5 g/day, "++++" ≥ 3.5g/day.

Tubular atrophy/interstitial fibrosis graded as: "0" = absent, "+" = mild, "++" = moderate, "+++" = severe.

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ORIGINAL ARTICLE

Kidney biopsy findings in heterozygous Fabry disease females with early nephropathy

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Abstract Fabry disease is an X-linked glycosphingolipidosis caused by deficiency of alpha-galactosidase. Progressive chronic kidney disease (CKD) is a major cause of morbidity and mortality in males. Although 40% of heterozygous females may develop renal involvement, pathologic data on Fabry nephropathy in heterozygotes are scarce. We reviewed the kidney biopsies of four affected females who had normal to slightly sub-normal renal function, two of them with overt proteinuria. Chronic non-specific degenerative lesions and glycosphingolipid accumulation per cell type were semi-quantitatively assessed by light and electron microscopy. Cellular distribution of glycosphingolipid deposits was best assessed on semithin sections. Podocyte effacement was seen only in proteinuric patients. Combined analysis of our data with those of two earlier series showed that glomerular sclerosis and tubulointerstitial fibrosis are predictors of proteinuria and CKD stage. There was no histopathological evidence supporting a major role of vascular damage in the early pathogenesis of Fabry nephropathy in females.

Keywords Fabry disease · Heterozygous females · Kidney pathology

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Introduction

Fabry disease is a rare X-linked lysosomal storage disease due to deficiency of α-galactosidase (αGal) enzyme activity. This metabolic defect results in multi-systemic accumulation of neutral glycosphingolipids with terminal α-galactosyl residues, predominantly globotriaosylceramide (Gb3) [1]. Complications of renal, cardiac and cerebrovascular involvement are the major causes of late morbidity and mortality [1–3].

The severity of clinical expression in males is inversely correlated with αGal residual activity and the classical phenotype of Fabry disease typically develops in individuals with <1% residual enzyme activity [2]. In these patients, chronic kidney disease (CKD) is an invariable complication, usually progressing to end-stage renal failure (ESRF) in the third to fifth decades of life. Low-grade proteinuria precedes deterioration of renal function in most cases, and the prevalence and degree of proteinuria increase

with CKD stage [4]. Before the availability of effective renal replacement therapies, ESRF was the main cause of death of classically affected patients [1, 2].

In females, the expression of Fabry disease is modulated by the effect of X chromosome inactivation [5] and the resulting proportions of normal and enzyme-deficient cells in individual organs. Heterozygous women may be completely asymptomatic or only have mild symptoms [1], but the majority ultimately develop substantial clinical manifestations [6, 7] and are at higher risk of premature death [8]. Evidence of renal involvement, including proteinuria and decreased renal function, may be found in up to 40% of known adult heterozygotes [4, 6]. A small proportion of female patients can be as severely affected as males with the classical phenotype [1, 9], reaching ESRF approximately at the same age [4].

The kidneys are major sites of glycosphingolipid deposition in Fabry disease and electron microscopy (EM) shows inclusions in all types of kidney cells, despite their normal appearance by conventional histology, even in patients with normal renal function and no proteinuria [10, 11]. As Gb3 deposits are dissolved by the paraffin-embedding procedure, the most characteristic finding on routine light microscopy (LM) of kidney biopsies is vacuolation of podocytes, of parietal epithelial cells and of Henle's loop and distal tubular cells [10, 12–14]. Mesangial widening, segmental and global glomerular sclerosis, tubular atrophy, interstitial fibrosis and other non-specific lesions are additionally seen, even at the early stages of Fabry disease nephropathy [10, 12, 13].

The sub-cellular mechanisms linking glycosphingolipid accumulation to the clinical manifestations of Fabry disease are not clearly understood. The main pathogenic theory of the classical phenotype assigns its complications predominantly to ischaemic tissue damage resulting from microvascular endothelial disease and/or necrosis of vascular smooth muscle cells and/or pericyte injury [1–3].

The development of proteinuria and of late-onset renal dysfunction in patients with the cardiac phenotypic variant, who have significant renal Gb3 deposits confined to podocytes [2, 15], suggests that these cells may have a role in the pathogenesis of Fabry nephropathy. Lethal injury to Gb3-overloaded podocytes, through the formation of focal adhesions between the glomerular tuft and the Bowman's capsule [12, 16], or increased hydraulic stress upon endothelial cells in the vicinity of damaged podocytes [17] are other possible mechanisms of glomerular sclerosis in Fabry nephropathy. By causing tubular injury, proteinuria has a direct role in CKD progression and may provide a link between the aforementioned primarily glomerular pathologic processes and the development of tubulointerstitial disease [18]. Mesangial cell necrosis [10], direct toxic injury to tubular cells [12] and diffuse involvement of interstitial cells [17] have also been suggested to be relevant in the pathogenesis of Fabry nephropathy progression.

Most of the current concepts and pathologic description of the Fabry nephropathy have been derived from autopsy and biopsy studies of affected males [19–24], but it has been recognised since long that affected females develop the same type of kidney lesions described in males [20, 25]. However, data on kidney pathology in heterozygous females are scarce and the largest published series describing kidney biopsy findings in affected females included only three [10] or five patients [13].

As in other chronic nephropathies, the progression of Fabry nephropathy is characterised by segmental and global glomerular sclerosis, tubular atrophy and interstitial fibrosis [10–14, 17]. In males, the presence of glomerular segmental or global sclerosis is the only significant pathologic association of proteinuria at early stages of Fabry nephropathy [10, 27]. Furthermore, negative correlations could be demonstrated between inulin clearance and LM composite scores of either chronic glomerular pathology or tubulointerstitial pathology [26, 27]. Contrastingly, a composite score of Gb3 inclusions did not correlate with either renal function or LM pathology scores [27]. Whether these findings also apply to females with Fabry disease is not known.

We have reviewed the kidney biopsies of four females with Fabry disease, with normal or mildly sub-normal renal function, using a specifically developed approach to assess Gb3 accumulation in different cell types and to quantify chronic non-specific degenerative lesions. The latter findings were combined with similar data of previously published series [10, 13] and correlated with proteinuria and renal function.

Subjects, materials and methods

Following approval by the institutional Health Ethics Board, retrospective clinical details and laboratory data were retrieved from each patient's hospital files. Hypertension was diagnosed and classified according to recommended criteria [28]. Serum creatinine (sCr) and proteinuria values reported as at the time of kidney biopsy were those obtained on protocol evaluation prior to the biopsy. Proteinuria was expressed as the concentration ratio of total protein to creatinine (mg/g) in routine 24-h urine samples. Glomerular filtration rate was estimated (eGFR) as the creatinine clearance calculated by the Cockcroft–Gault equation [29], adjusted for tubular creatinine secretion and normalised to the body surface area (BSA) of 1.73 m^2 [30, 31]. BSA was derived from height and weight [32]. CKD was staged by eGFR [33].

Leukocyte αGal activity was measured according to a standard method [34]. Mutations of the αGal gene (*GLA*)

were identified by amplifying all its seven exons and respective intronic boundaries, using routine polymerase chain reaction (PCR) methods, followed by automatic sequencing of each PCR amplicon.

Case summaries

Case 1 had proteinuria first detected on a routine medical checkup, at the age of 30 years. She was asymptomatic, reported no relevant past medical history and had normal blood pressure. Two of her brothers, followed elsewhere, had received kidney transplants as young adults, for ESRF attributed to "focal glomerular sclerosis". Her sCr was 0.8 mg/dl, proteinuria was quantified as 1,400 mg/day and the urine sediment was normal. A comprehensive diagnostic workup for proteinuria showed no abnormalities and an angiotensin-converting enzyme inhibitor (ACEI) was eventually prescribed. She stayed asymptomatic and normotensive but her sCr increased to 1.1 mg/dl and proteinuria reached nephrotic level (4.5 g/day) within 2 years of the first observation, prompting a diagnostic kidney biopsy.

The other three patients were known heterozygotes for Fabry disease and underwent kidney biopsy on protocol baseline evaluation for enzyme replacement therapy (ERT) with recombinant human αGal (agalsidase). Case 2, sister of case 1, was identified on family screening. Case 3 asked for genetic screening at the age of 48 years, after a maternal first cousin died with complications of Fabry disease. Two years before, she had been diagnosed stage 2 hypertension and medicated accordingly including with an ACEI. Case 4 was identified following the diagnosis of Fabry disease nephropathy in her two young adult sons. She was not aware of previous cases of CKD in the family.

Kidney biopsy procedure and processing

Tissue samples had all been obtained by percutaneous kidney biopsy according to routine clinical procedures [35, 36]. Two or three core samples were taken in each case, which were appropriately processed for LM, EM and immunofluorescence (IF) study, according to standard histopathology protocols [37, 38].

Fragments selected for LM were fixed in 10% buffered formalin, embedded in paraffin, and 3-μm thick serial sections were cut from the paraffin block and stained with haematoxylin and eosin, periodic acid Schiff and modified trichrome.

Fragments selected for EM were fixed in 3% glutaraldehyde, post-fixed in osmium tetroxide and embedded in epoxy resin (Epon™). Methylene blue or toluidine blue-stained 1-μm semithin (ST) sections were prepared from the Epon™ embedded fragments. Ultrathin sections (80 nm) for EM examination were stained with uranyl acetate and lead citrate. Photographs encompassing glomerular capillary loops and mesangial areas were taken at various magnifications.

Fragments selected for IF were snap-frozen in isopentane at −80°C. Tissue sections thereof were cut at 5 μm in a cryostat and processed for indirect IF staining, with a panel of antibodies directed to immunoglobulins (IgG, IgA, IgM), complement factors (C3, C1q) and fibrinogen.

Kidney biopsy reading, scoring and interpretation

Archive LM and ST slides, as well as IF and EM photographs, were reviewed by a single nephropathologist (CV). Available LM and ST slides were re-stained and/or new sections were prepared from the original paraffin blocks as needed. Counting of a minimum of ten "scorable glomeruli" was prerequisite for further biopsy reading. The total number of "scorable glomeruli" in each biopsy was the sum of the highest number counted in any of the conventional LM sections with the number counted in the ST section. Glomeruli showing less than three fourths of the Bowman's capsule circumference and void Bowman's capsules were not counted. In each case, two glomeruli were reviewed ultra-structurally.

The biopsies were read using standard criteria for glomerular, tubulointerstitial and vessel morphology evaluation [37–39]. The total numbers of scorable glomeruli showing segmental sclerosis or global sclerosis were counted and their proportions calculated for each biopsy. Interstitial fibrosis and tubular atrophy were estimated in LM sections as the proportion of cut section affected, scored to the nearest 5%.

Arterial sclerosis, arteriolar or arterial hyalinosis and hypertrophy of the smooth muscle layer of arteries and arterioles were recorded as present or absent in LM sections. Arterial sclerosis was further assessed according to the ratio of vessel wall thickness to lumen diameter [40].

Specific Fabry disease changes—i.e. vacuoles in LM sections, dark blue cytoplasmic inclusions in ST sections and electrondense osmiophilic intracellular deposits in EM (Fig. 1) [10, 11, 13, 24] —were recorded as present or absent in different types of glomerular, tubular, interstitial and vascular cells. The extension of glycosphingolipid accumulation was further graded in the ST sections according to the proportion of cells of the specified type that showed any deposits.

The histopathological findings were compared among the four patients and related to CKD progression and blood pressure history as well as to renal function and proteinuria at the time of biopsy. Furthermore, in an attempt to establish significant correlations between CKD stage and proteinuria level and specific LM histopathological parameters, we have statistically analysed our data in combination

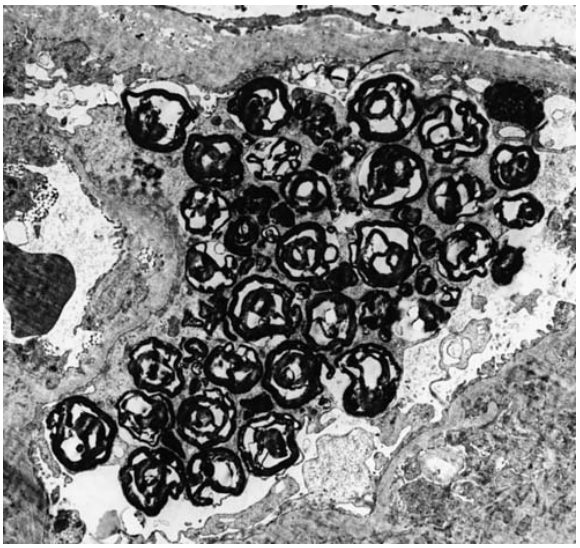

Fig. 1 Multiple large Gb3 inclusions in a podocyte, with the typical concentric lamellated ultra-structural appearance—"myelin figures" (case 1; EM, 1,800×3)

with those of previous series [10, 13]. Non-parametric correlations and stepwise regression analyses were performed with the SPSS for Windows® statistics software.

Results

Demographic and other relevant clinical and laboratory data of the four patients included in this study are shown in Table 1. Mutation p.R220X is known to cause the classical phenotype of Fabry disease [41]. Mutation p.239ΔI has not been previously described, but a deceased first cousin of case 3 had the classical Fabry disease phenotype. Although in case 4 no exonic *GLA* mutation was found and the pathogenic role of the double intronic molecular variants identified on sequence analysis is uncertain, her two sons, who share the same genotype, presented with the classical Fabry disease phenotype. In all cases, residual leukocyte αGal activity was between 30% to 35% of the normal mean. At the time of kidney biopsy, all patients had eGFR in the slightly sub-normal or the low normal range, but only in case 1 was a significant increase in sCr retrospectively documented. Overt proteinuria (>300 mg/day) was present in two patients (cases 1 and 2), reaching nephrotic level in case 1; cases 3 and 4 had proteinuria below 300 mg/day. Hypertension was diagnosed in a single patient (case 3) but two others had pre-hypertensive blood pressure levels.

All biopsies were of good quality and the minimum number of scorable glomeruli was 12 (Table 2). In case 3, the IF study was not performed.

Results of LM and ST assessment of chronic non-specific glomerular and tubulointerstitial lesions and of arteriolar and arterial lesions are respectively presented in Tables 2 and 3. The degrees of interstitial fibrosis, tubular atrophy and the percentage of sclerotic glomeruli seemed to correlate with eGFR, and the percentage of segmentally sclerotic glomeruli roughly correlated with the degree of proteinuria. The degrees of interstitial fibrosis and of tubular atrophy observed in each biopsy were identical in three of the patients. However, case 3 had a disproportionately higher degree of interstitial fibrosis; of note, she was the eldest patient in this series and the only one with systemic hypertension and hypercholesterolaemia. The presence of vascular hyalinisation and/or of medial hypertrophy did not appear to correlate with either renal function or proteinuria: these lesions were not observed in case 1, who had the severest renal involvement, with CKD progression, but were present in case 4, who had the mildest clinical presentation of Fabry nephropathy.

Podocyte and tubular cell vacuolation was a universal finding on conventional LM—although in case 1 (Fig. 2) and case 4 it was scarce and focally distributed. Vacuolated interstitial cells were observed only in case 1.

Assessment of Gb3 deposits in ST sections is summarised in Table 4. ST sections allowed a more accurate identification of glycosphingolipid deposits in glomerular (Fig. 3),

Table 1 Clinical and laboratory data at the time of kidney biopsy

Case	Age	Mutation	αGal (L)	sCr	eGFR	uProt/Cr	Urine sediment	BP status	Medication
1	32y	p.R220X	20	1.1	74	2,002	Normal	Pre-hypertension	ACEI, OC
2	45y	p.R220X	18	0.9	79	721	*E*=27.6/μl, *L*=22.3/μl[b]	Pre-hypertension	
3	50y	p.239ΔI	20	0.9	76	184	Normal	Hypertension, stage 1	TD, ACEI, S, OC
4	42y	Intronic[a]	18	0.7	92	139	Normal	Normal	

Age in full years

BMI body mass index, in kg per square meter, *αGal (L)* α-galactosidase residual activity in leukocytes (normal laboratory range, 36–80 nMol/h/mg), *sCr* serum creatinine, in mg/dl, *eGFR* estimated glomerular filtration rate, in ml/min/1.73 m^2, *uProt/Cr* urine total protein to creatinine concentration ratio, in mg/g, *BP* blood pressure, *E* erythrocytes, *L* leukocytes, *ACEI* angiotensin-converting enzyme inhibitor, *TD* thiazide diuretic, *S* statin, *OC* oral contraceptive

[a] Intronic *GLA* mutations: IVS4+1703A→G/IVS6+249C→T

[b] Erythrocytes/leukocytes; as counted by flow cytometry (normal values—*E*<27.1/μl, *L*<20.5/μl)

Table 2 Glomerular global and focal sclerosis and chronic non-specific tubulointerstitial lesions

Histological parameter	Cases			
	1	2	3	4
Conventional light-microscopy sections				
Number of glomeruli, *N*	7	17	13	17
Glomeruli with global sclerosis, *N* (%)	3 (43%)	0	2 (15%)	1 (6%)
Glomeruli with focal sclerosis, *N* (%)	3 (43%)	5 (29%)	2 (15%)	1 (6%)
Tubular atrophy, %[a]	30%	10%	5%	5%
Interstitial fibrosis, %[a]	30%	10%	20%	5%
Semithin sections				
Number of glomeruli, *N*	5	12	4	14
Glomeruli with global sclerosis, *N* (%)	1 (20%)	1 (8%)	0	0
Glomeruli with focal sclerosis, *N* (%)	2 (40%)	1 (8%)	0	0
Combined LM + ST total				
Number of glomeruli, *N*	12	29	17	31
Glomeruli with global sclerosis, *N* (%)	4 (33%)	1 (3%)	2 (12%)	1 (3%)
Glomeruli with focal sclerosis, *N* (%)	5 (42%)	6 (21%)	2 (12%)	1 (3%)
Glomeruli with any sclerosis, *N* (%)	9 (75%)	7 (24%)	4 (24%)	2 (6%)

LM light microscopy, *ST* semithin

[a] Tubular atrophy and interstitial fibrosis were estimated to the nearest 5%, by assessing the relative degree of involvement in each cortical field, at ×200 magnification, and averaging all available fields

interstitial and capillary endothelial cells (Fig. 4) and disclosed the presence of Gb3 deposits in cells without apparent vacuolation on conventional LM sections. The extension of glycosphingolipid deposition did not seem to correlate with the patient's age in none of the cell types analysed. Glomerular parietal epithelial cells were the most extensively affected in all cases, with more than one third of these cells showing at least one toluidine blue-stained deposit in ST sections. Podocytes, vascular smooth muscle cells and distal tubular cells were also involved in all cases. Distal tubules were affected in all cases but not all distal tubules were affected nor did every cell in affected distal tubules have Gb3 deposits (Fig. 5). The extension of

Table 3 Arteriolar and arterial lesions (light-microscopy sections)

Histological parameter	Cases			
	1	2	3	4
Hyalinisation	No	No	Yes	Yes
Intimal fibrosis	No	No	+++[a]	No
Hypertrophy of muscle layer	No	No	Yes	Yes

[a] Graded according to the criteria of Remuzzi et al. [40]

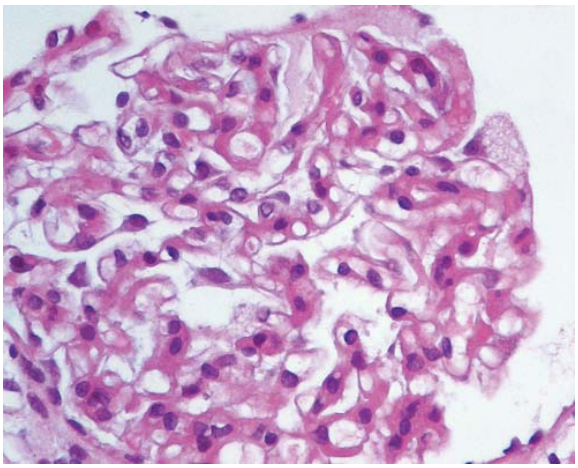

Fig. 2 Cytoplasmic vacuolation observed in a single podocyte (case 1; LM, haematoxylin and eosin, ×200)—in fact, this was the sole vacuolated podocyte observed in the entire biopsy

podocyte glycosphingolipid deposition in ST sections was broadly related with the degree of proteinuria: only the two patients with baseline proteinuria/creatinine concentration ratio above 500 mg/g had more than one third of podocytes affected. Furthermore, glomerular endothelial and proximal tubular cell deposits were exclusively seen in these two patients with moderate to severe proteinuria, who also showed the highest degrees of vascular smooth muscle cell involvement. Involvement of interstitial cells was a distinctive histological feature in case 1 (Fig. 4), who had the severest presentation of Fabry nephropathy, including

Table 4 Fabry disease intracellular glycosphingolipid inclusions as assessed in semithin sections under light microscopy

Cell types	Cases			
	1	2	3	4
Podocytes	++	++	+	+
Parietal glomerular cells	+++	++	+++	+++
Mesangial cells	+	+	+	0
Glomerular endothelial cells	+	+	0	0
Proximal tubular cells	+	+	0	0
Distal tubular cells	+++	+	+	+
Arterial endothelial cells	++	+	−	−
Arteriolar endothelial cells	0	0	+	0
Vascular smooth muscle cells	+++	+++	+	++
Vascular fibroblasts	++	+++	−	0
Peritubular capillary cells	++	0	+	+[a]
Interstitial cells	++	0	0	0

0 No deposits, + deposits present in few cells (according to the tertiles of the proportion of affected cells), ++ deposits present in some cells (according to the tertiles of the proportion of affected cells), +++ deposits present in many cells (according to the tertiles of the proportion of affected cells), − cell type not sampled

[a] Limited to a single vessel

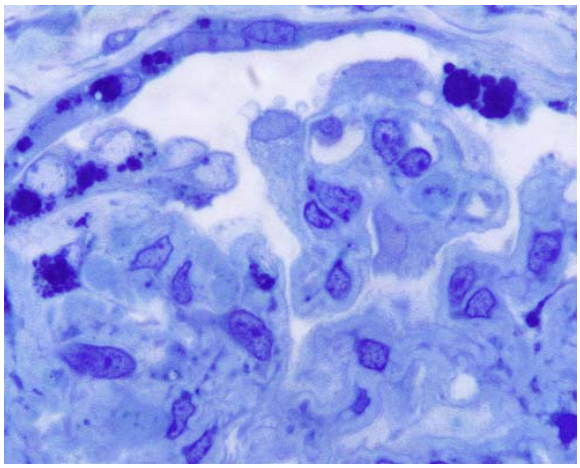

Fig. 3 Gb3 deposits in glomerular parietal and visceral epithelial cells (case 1; ST section, methylene blue-stained, ×400)

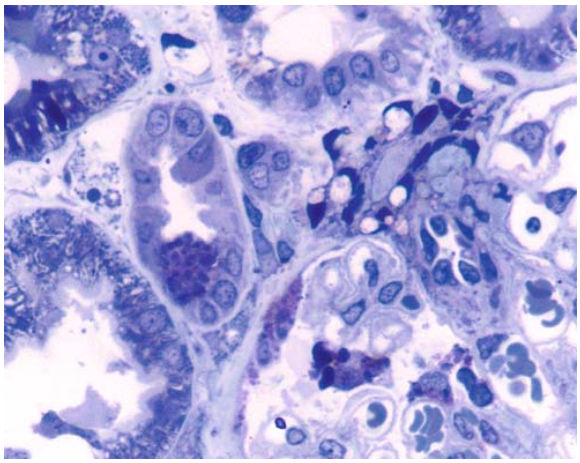

Fig. 5 Heavy Gb3 storage is visible in a single cell of a distal tubule (case 3; ST section, toluidine blue-stained, ×200)

nephrotic proteinuria and a significant increase in sCr along a short-term follow-up period. This patient also showed the highest degree of distal tubular as well as of peritubular capillary cell involvement. Case 4, who had normal renal function and minimal proteinuria, had relatively extensive vascular smooth muscle cell involvement.

EM features are shown in Table 5. EM revealed small glycosphingolipid deposits within mesangial cells in case 4 and within glomerular endothelial cells in cases 3 and 4, two cell types that seemed uninvolved in the corresponding ST sections. Podocyte effacement (Fig. 6) was a morphological feature in the two patients with moderate (case 2) or severe (case 1) proteinuria and was not seen in the remaining two patients, who had milder degrees of proteinuria. Additional notable EM findings in case 4 were the presence of extracellular membranofibrillary deposits in the mesangium (Fig. 7), as well as of electrondense immune deposits in sub-endothelial and mesangial location. The latter one stained intensively with IgA anti-serum in IF study. None of the other patients had immune deposits seen in EM or a positive IF study.

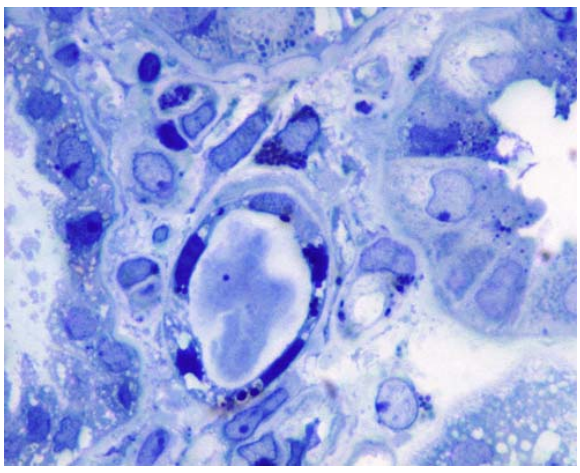

Fig. 4 Gb3 deposits in interstitial, peritubular capillary endothelial and tubular epithelial cells (case 1; ST section, methylene blue-stained, ×200)

Table 6 combines clinical and histopathological data of our patients and of those of previously reported series [10, 13]. Statistical analyses of these data demonstrated highly significant correlations ($p<0.005$) between proteinuria and glomerular sclerosis, between CKD stage and glomerular sclerosis and tubulointerstitial fibrosis, as well as between glomerular sclerosis and tubulointerstitial fibrosis. On stepwise logistic regression, glomerular sclerosis emerged as the most important predictor of proteinuria ($R^2=0.76$; $p<0.0005$), while tubulointerstitial fibrosis was the most important predictor of CKD stage ($R^2=0.69$; $p=0.002$).

Table 5 Non-specific cell lesions and intracellular glycosphingolipid deposits as assessed by electron microscopy

Cell types	Cases			
	1	2	3	4
Non-specific lesions				
Podocyte effacement	Focal	Focal	No	No
Osmiophilic intracellular deposits				
Podocytes	+	++	++	++
Parietal glomerular cells	++	+++	+++	+
Mesangial cells	+	+	+	+
Glomerular endothelial cells	+	+	+	+

+ Deposits present in few cells (according to the tertiles of the percentage of affected cells), ++ deposits present in some cells (according to the tertiles of the percentage of affected cells), +++ deposits present in many cells (according to the tertiles of the percentage of affected cells)

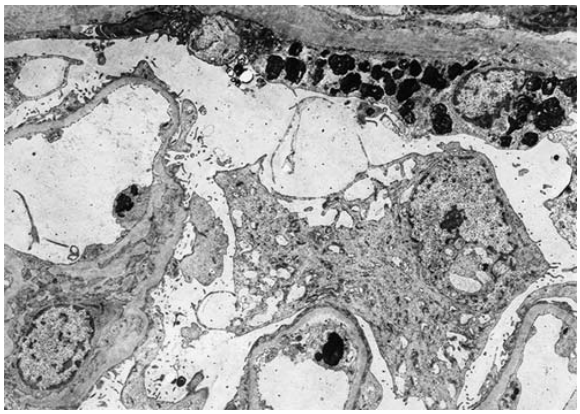

Fig. 6 Focal areas of podocyte effacement; amorphous Gb3 deposits are visible in glomerular parietal epithelial cells and in endothelial cells (case 2; EM, 1,800×3)

Discussion

To compare the severity of the kidney lesions observed in our patients and to identify meaningful clinicopathologic correlations, we defined a set of histopathologic and ultra-structural criteria for systematic reading and semi-quantification of glomerular, tubulointerstitial and vascular lesions, as well as the burden of glycosphingolipid accumulation in each kidney cell type. The items selected for biopsy reading and scoring were based on clinicopathologic correlations derived from published biopsy series [10, 13, 26] and on the hypotheses regarding the involvement of different types of cells on the pathogenesis of Fabry nephropathy [2, 10, 12, 17].

In clinical trials of agalsidases, the effect of ERT on the kidney was assessed by protocol repeat biopsies, but the scoring systems used in those settings [26, 42–44] were specifically designed to quantify treatment-induced changes and are not useful for broader general use. The usefulness of protocol kidney biopsies and of a scoring system to evaluate histologic markers of progressive Fabry nephropathy has been formerly suggested [12, 45], and a clinically oriented scoring system for quantitative analysis of kidney biopsies of patients with Fabry disease is currently undergoing validation [27].

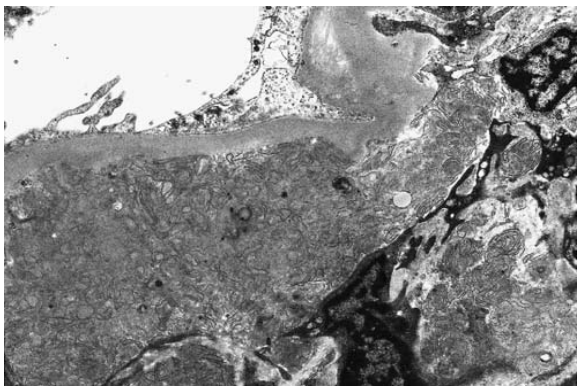

Fig. 7 Mesangial membranofibrillary deposits in sub-endothelial location (case 4; EM, 2,700×3)

Assessment of chronic non-specific glomerular and tubulointerstitial degenerative lesions

We searched for clinicopathologic correlations of chronic non-specific glomerular and tubulointerstitial lesions using the data shown in Table 6. In these 12 patients, the clinical expression of Fabry nephropathy as related to age is in accordance with cross-sectional data from a worldwide Fabry disease registry [4]. The series reported by Gubler et al. [10] included a child with very mild disease and an asymptomatic young adult without any pathologic evidence of Fabry nephropathy. Contrastingly, all females reported by Fischer et al. [13] had clinically severe nephropathy and the youngest patient in their series was 30 years old. In our patients, the severity of clinical expression of Fabry nephropathy stands between that of the two former series. The overall results of our statistical analyses of these data are entirely concordant with previous findings in males [27].

Assessment of arterial and arteriolar degenerative lesions

In our patients, the severity of the clinical expression of Fabry nephropathy or the degrees of interstitial fibrosis, tubular atrophy or glomerular sclerosis did not relate to the presence of intimal fibrosis, vascular hyalinosis or hypertrophy of the muscle layer (Tables 2 and 3). None of those vascular lesions were observed in case 1, who had the severest renal involvement and CKD progression, but were present in case 4, who had the mildest clinical presentation of Fabry nephropathy. Only case 3 had atherosclerotic intimal vascular lesions, in agreement with her clinical history of systemic hypertension and high serum cholesterol levels [46]. Non-concordant tubulointerstitial fibrosis and tubular atrophy scorings (20% vs. 5%, respectively) were the exclusive findings in this latter patient. A possible explanation could be the combined effects of hypertension and hypercholesterolaemia [46]. However, in patients with benign as well as malignant hypertensive nephrosclerosis, the degree of tubular atrophy has been reported to be higher than the degree of interstitial fibrosis [47].

Assessment of intracellular glycosphingolipid accumulation

Although usually referred to as a distinctive sign of Fabry nephropathy [10–14], diffuse cytoplasmic vacuolation of

Table 6 Comparison of the three largest kidney biopsy series reporting pathology findings in females with Fabry disease

	Gubler et al. [10]			Fischer et al. [13]			Present series					
Age at kidney biopsy (years)	8	22	51	30	37	54	55	73	32	42	45	50
CKD stage	0	0	2	?	2	4	5	4	2	1	2	2
Proteinuria	0	0	0	+++	+++	+++	++++	++++	++++	++	0	0
No. of evaluated glomeruli	8	15	25	2	4	17	23	5	12	31	29	17
No. of sclerotic glomeruli	0	0	5	1	2	17	22	3	9	2	7	4
% of sclerotic glomeruli	0%	0%	20%	50%	50%	100%	96%	60%	75%	31%	24%	24%
Tubular atrophy/interstitial fibrosis	0	0	+	0	+	++	+++	+	+/++	+	+	+

No. of sclerotic glomeruli is the total number of evaluated glomeruli that showed either segmental or global sclerosis. CKD stages: 0—no evidence of kidney disease, 1—eGFR≥90 ml/min/1.73 m^2, 2—eGFR=89–60 ml/min/1.73 m^2, 3—eGFR=59–30 ml/min/1.73 m^2, 4—eGFR=29–15 ml/min/1.73 m^2, 5—eGFR<15 ml/min/1.73 m^2. Proteinuria was graded as: 0=no overt proteinuria, +=0.3–0.5 /day, ++=0.5–1.0 g/day, +++=1.0–3.5 g/day, ++++≥3.5 g/day. Tubular atrophy/interstitial fibrosis graded as: 0=absent, +=mild, ++=moderate, +++=severe

podocytes and tubular cells was not evident in some of our patients and also was not related to the amount or the volume of Gb3 deposits, as evaluated on the ST sections (Figs. 2 and 3). As patchy cytoplasmic vacuolation of glomerular and tubular cells is a non-specific kidney pathology feature of massive proteinuria and Alport syndrome [48, 49], its diagnostic value for Fabry nephropathy in females is limited.

In line with Gubler et al. findings in male patients [10], EM showed the presence of Gb3 deposits in every glomerular cell type, even in those that seemed to be spared on ST sections. This disparity is most probably dependent on the size of the Gb3 deposits, which would be below the resolution of the light microscope in the apparently unaffected cells on the ST sections. Comparing data on Tables 4 and 5 for differences between EM and ST findings in the glomeruli, the semi-quantification of Gb3 deposits on ST sections seemed to be better related with the degree of proteinuria. In addition, the absence of deposits in mesangial cells and in glomerular endothelial cells was a feature exclusive of the patient with the mildest clinical expression of Fabry nephropathy.

In our patients, the epithelial cells of the Bowman's capsule showed the severest burden of Gb3 accumulation of all kidney cell types, as seen in ST as well as in EM evaluation. This finding is consistent with a recent report describing the presence of Gb3 deposits in 84% of glomerular parietal epithelial cells, as compared to only 42% of podocytes, in the ultra-structural study of the kidney biopsies of two affected sisters [50]. However, it is at odds with the more traditional view that podocytes are the earliest and most severely affected kidney cells in Fabry nephropathy, with the epithelial cells of Bowman's capsule becoming involved as the disease progresses [10–14]. Therefore, it might be speculated that predominant involvement of glomerular parietal as compared to glomerular visceral epithelial cells is a singular feature of Fabry nephropathy in females.

Tubular involvement was expected at the early stages of Fabry nephropathy [10–14]. Distal tubules were affected heterogeneously in all cases (Fig. 5). Although in females this could be the histological expression of X chromosome inactivation, as suggested before as an explanation for similar biopsy findings [10], this same pattern of distal tubular Gb3 storage has also been described in biopsies of affected males [10]. In proximal tubules, Gb3 deposits were exclusively observed in the two patients with overt proteinuria.

The finding of Gb3 deposits on peritubular capillaries grossly related to the clinical severity of Fabry nephropathy but did not correlate with the vascular arteriolar or arterial lesions and, in contrast to previous observations [51], did seem to occur as an early pathologic event. Although clearance of peritubular capillary Gb3 deposits in serial kidney biopsies has been used as a marker of ERT efficiency [44], the pathophysiologic relevance of this treatment outcome needs further demonstration.

Involvement of interstitial cells was seen only in case 1, who had the severest clinical and pathological expression of Fabry nephropathy. Thus, it might be speculated that Gb3-related interstitial cell damage could somehow contribute to, or be an aggravating factor of, the tubulointerstitial fibrosis seen in kidney biopsies of patients with Fabry disease, even before significant deterioration of renal function has occurred.

Additional findings

Podocyte effacement was apparent on EM sections only in the two patients with overt proteinuria, an observation that is consistent with previous data [10, 13] and adds to the hypothesis that in the early stages of Fabry nephropathy, the relative preservation of the fine structures of the podocyte foot processes and of the slit diaphragms is a major determinant of the amount of proteinuria [11, 14]. The membranofibrillary deposits observed in case 4 were

identical to those already described by Gubler et al. [10] and by Fischer et al. [13] in hemizygous males and might be the remnants of Gb3 inclusions of dead cells. We postulate that the rupture of Gb3 overloaded lysosomes into the cytoplasm of affected cells could be a crucial event leading to cell death. In podocytes and parietal glomerular epithelial cells, this would eventually set in motion the pathogenic cascade that ultimately result in focal adhesions of the glomerular tuft to the Bowman's capsule, synechiae and focal segmental sclerosis [52–55].

Mesangial deposition of IgA in case 4 was totally asymptomatic. Clinically overt IgA nephropathy has been reported as a coincidental diagnosis in males [56] as well as in females [57, 58] with Fabry disease, but in recently reported series, asymptomatic IgA deposits were identified in about 15% of patients [44, 59], a frequency that is significantly higher than in non-selected autopsy cases [60]. This may not be merely coincidental [57] and deserve further study.

In conclusion, despite the potential confounding effect of X chromosome inactivation, the overall findings in kidney biopsies of females with Fabry disease are not significantly different from those described in males, validating kidney biopsy as an important diagnostic tool for Fabry nephropathy also in heterozygotes. The more robust pathological predictors of proteinuria and CKD stage are non-specific chronic lesions identified in LM, respectively glomerular sclerosis and tubulointerstitial fibrosis. The cellular distribution and the burden of Gb3 accumulation per cell type are best determined in ST sections. Finally, in our series of patients, we could not find evidence supporting a major role of vascular damage in the early pathogenesis of Fabry nephropathy in females. Instead, we suggest that damage to visceral and parietal glomerular epithelial cells may be more relevant at this stage.

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Conflict of interest statement The authors declare that they have no conflict of interest.

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NOVEL HUMAN PATHOLOGICAL MUTATIONS. GENE SYMBOL: *GLA*

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Hum Genet 2009; 126: 352

352 Hum Genet (2009) 126:329–352

Gene symbol: LDLR

Disease: Hypercholesterolemia

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Input for Missense/Nonsense Mutations (*single base-pair substitutions*)

Accession	Codon number	Nucleotide substitution	Amino acid substitution
HM080120	317	TGCc-TGA	Cys-Term

Comments: The nomenclature we use is updated from what I see in the database. The nucleotide is c.1014C > A and changes the codon p.Cys338Term.

Gene symbol: GLA

Disease: Fabry disease

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Input for gross insertion and duplications

Accession	Description
HN080002	Tandem duplication of exons 2, 3 and 4

Comments: In a young adult male with the classic phenotype of Fabry disease, in whom sequencing of all GLA exons and exon-intron boundaries had been normal, a direct tandem duplication of exons 2–3–4 was found by cDNA analysis. This finding was confirmed by genomic DNA sequencing, which further showed that the duplicated segment most probably localized between exons 1 and 2. This interpretation is based on the demonstration of a normal intron 4 sequence, using a pair of primers in exons 4 (forward) and 5 (reverse), and an abnormal intron 4 sequence using a pair of primers in exons 4 (forward) and 2 (reverse). Both the 5′ and the 3′ ends of the duplicated segment localize to Alu repeats.

MLPA anaylsis confirmed the duplication of exons 2, 3 and 4. To the best of our knowledge this is the first duplication defect described in association with Fabry disease.

SCORRING SYSTEM FOR RENAL PATHOLOGY IN FABRY DISEASE: REPORT OF THE INTERNATIONAL STUDY GROUP OF FABRY NEPHROPATHY (ISGFN)

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Original Article

Scoring system for renal pathology in Fabry disease: report of the International Study Group of Fabry Nephropathy (ISGFN)

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Abstract

Background. In Fabry nephropathy, alpha-galactosidase deficiency leads to accumulation of glycosphingolipids in all kidney cell types, proteinuria and progressive loss of kidney function.

Methods. An international working group of nephrologists from 11 Fabry centres identified adult Fabry patients, and pathologists scored histologic changes on renal biopsies. A standardized scoring system was developed with a modified Delphi technique assessing 59 Fabry nephropathy cases. Each case was scored independently of clinical information by at least three pathologists with an average final score reported.

Results. We assessed 35 males (mean age 36.4 years) and 24 females (43.9 years) who mostly had clinically mild Fabry nephropathy. The average serum creatinine was 1.3 mg/dl (114.9 μmol/l); estimated glomerular filtration rate was 81.7 ml/min/1.73 m^2 and urine protein to creatinine ratio was 1.08 g/g (122.0 mg/mmol). Males had greater podocyte vacuolization on light microscopy (mean score) and glycosphingolipid inclusions on semi-thin sections than females. Males also had significantly more proximal tubule, peritubular capillary and vascular intimal inclusions. Arteriolar hyalinosis was similar, but females had significantly more arterial hyalinosis. Chronic kidney disease stage correlated with arterial and glomerular sclerosis scores. Significant changes, including segmental and global sclerosis, and interstitial fibrosis were seen even in patients with stage 1–2 chronic kidney disease with minimal proteinuria.

Conclusions. The development of a standardized scoring system of both disease-specific lesions, i.e. lipid deposition related, and general lesions of progression, i.e. fibrosis and sclerosis, showed a spectrum of histologic appearances even in early clinical stage of Fabry nephropathy. These findings support the role of kidney biopsy in the baseline evaluation of Fabry nephropathy, even with mild clinical disease. The scoring system will be useful for longitudinal assessment of prognosis and responses to therapy for Fabry nephropathy.

Keywords: chronic kidney disease; Fabry disease; pathology; sclerosis; scoring

Introduction

Fabry disease is an X-linked genetic disorder with cellular accumulation of globotriosylceramides (GL-3) due to lysosomal alpha-galactosidase enzyme activity deficiency [1,2]. Males classically develop chronic kidney disease

(CKD) and progress to end-stage renal disease (ESRD) before their fifth decade [1–3]. Enzyme replacement therapy (ERT) reduces GL-3 deposits [4–6] and slows progression of Fabry nephropathy [7].

Females have variable disease severity [1]; they can be asymptomatic, but most of them develop clinically apparent disease [8,9] and risk premature death [10]. Proteinuria and/or reduced glomerular filtration rate (GFR) may be found in 40% of adult females [8,11], and some are severely affected, reaching ESRD at the same median age as males [3,11].

Renal intracellular GL-3 deposits are present, even in patients with normal GFR and minimal proteinuria [12,13]. Vacuolization of podocytes and epithelial cells is a characteristic histologic finding [12,14–16]. Mesangial expansion, segmental and global glomerulosclerosis, tubular atrophy and interstitial fibrosis are also seen, even in early stages of the disease [12–17]. The original descriptions of Fabry nephropathy were in males [16]. Females can develop the same kidney histopathology as males [12,16,18], but fewer female cases have been described [12,15,16, 18–20].

We systematically scored histologic lesions in kidney biopsies in a set of 35 adult males and 24 adult females with Fabry disease, the majority with clinically mild nephropathy. In addition, a morphometric index of chronic damage was obtained for each biopsy [21]. A validated scoring system was developed, with the long-term goal to determine whether baseline histologic information can be related to the rate of progression and/or response to ERT in Fabry nephropathy.

Subjects and methods

The International Study Group of Fabry Nephropathy (ISGFN) was formed by pathologist and nephrologist pairs, from 10 different major referral centres for Fabry disease, and by a central nephropathology site (Dr A.B. Fogo, Nashville, TN, USA). The central site coordinated the collection, distribution and circulation of available biopsies among the participating centres and data entry into a computerized database. Kidney biopsies were from white adults with the diagnosis of Fabry disease, as confirmed by mutational analysis of the alpha-galactosidase gene.

Clinical assessments

Demographic and clinical data were abstracted from the clinical records closest to the time of the biopsy. Biopsies were performed between January 1999 and September 2006. GFR was estimated (eGFR) with the Modification of Diet in Renal Disease simplified equation [22]. Urine protein values were expressed as urine protein/creatinine ratios (UPCR). When only 24-h protein measurements were available, daily creatinine excretions were estimated as the product of eGFR and serum creatinine.

Histological scoring

General approach. Disease-specific GL-3 storage-related and nonspecific histologic features of Fabry nephropathy were included in the score sheet (see Appendix for details). The scoring matrix was developed and refined in several round-robin slide reviews and face-to-face scoring sessions, using a modified Delphi technique [23].

Biopsies were coded and the exact tissue sections to be scored were marked on the slides at the central site, and slides were distributed on a blinded basis for review. A subset of 20 representative biopsies was initially circulated to all centres for training and scoring validation. Subsequent scoring was done by three centres for each case.

Interstitial fibrosis and index of chronic damage. In addition to the semi-quantitative histologic assessment on interstitial fibrosis, the index of chronic damage was morphometrically determined for each biopsy.

Statistical analysis

The results are presented as mean ± standard deviation (SD), with medians and ranges where indicated. Bivariate determinations are presented as percentages of the number scored or enumerated. Trend analysis was done with linear regression of the median of variables for each CKD stage [24] against the stage ordinal (stages 4 and 5 were combined).

The reliability of the scoring among the pathologists was assessed with intraclass correlation coefficient (ICC) analysis [25,26] for the semi-quantitative assessment of interstitial fibrosis and morphometric assessment of the chronic damage index, and for the podocyte vacuolization on periodic acid Schiff-stained light microscopic sections and podocyte GL-3 deposits on semi-thin sections stained with toluidine blue. Because each case was not scored by every pathologist, a generalization of the standard linear model that allows for fixed and random effects was used for ICC analysis instead of analysis of variance [27].

The interclass correlation, using Spearman's correlation coefficient, was used to compare the overall assessment of interstitial fibrosis obtained with semi-quantitative scoring by the pathologists with the scores obtained for the same cases using a published morphometric technique [21]. The semi-quantitative and morphometric scores of interstitial fibrosis also underwent Bland–Altman analysis [28]. Interclass correlations, using Spearman's correlation coefficient, were also used to compare the overall assessment of podocyte vacuoles on light microscopy with podocyte GL-3 inclusions on semi-thin sections.

Statistical comparisons were done with unpaired two-tailed *t*-tests and two-tailed Fisher exact tests. The *F*-statistic was used to evaluate the significance of trends. *P*-values < 0.05 were considered to be significant. Calculations were carried out with SAS, version 9.1 (SAS Institute, Inc. Cary, NC, USA).

Results

Summary of clinical assessments

All patients had defined mutations, and five male patients had the p.N215S missense mutation, previously described in the late-onset cardiac variants of Fabry disease [1]. The mean age was 39.4 ± 13.2 years (range 16–70), and the serum creatinine was 1.3 ± 1.2 mg/dl (range 0.6–7.1) [114.9 ± 106.1 μmol/l (range 53.0–627)]. UPCR was 1.08 ± 1.34 g/g (range 0.01–5.62) [122.0 ± 151.4 mg/mmol (range 1.1–635.1)] (Table 1). Forty-six patients (78.0%, 25 males) had stage 1 or 2 CKD, 8 (13.6%, 7 males) had stage 3 CKD and 5 males (8.5%) had stage 4 or 5 CKD (Table 2). Males with CKD stage 1 were significantly younger than the females (28.1 ± 10.3 versus 42.4 ± 10.6 years, $P < 0.003$).

Average systolic and diastolic blood pressures were 125.7 ± 13.0 (range 100–167) and 75.6 ± 10.5 mmHg (range 54–105; Table 1), respectively. Systolic blood pressure was >135 mmHg in 13 (22%) patients, and diastolic blood pressure was >85 mmHg in 9 patients (15.3%). Angiotensin-converting enzyme inhibitors or angiotensin receptor blockers were given to 24 patients (40.7%).

Nine patients started ERT 1.5 ± 1.0 months (range 0.6–3.9, median 1.4) before biopsy at a dose of 0.2 ($n = 3$) or 1.0 mg/kg every 2 weeks ($n = 6$). Forty-six patients started ERT 8.2 ± 13.1 months (range 0.03–76.2, median 4.0) after biopsy at a dose of 0.2 ($n = 13$) or 1.0 mg/kg every 2 weeks ($n = 33$).

Table 1. Values are presented as mean ± SD and median, and (range; median) for UPCR

	All patients ($n = 59$)	Male ($n = 35$)	Female ($n = 24$)	P-value
Age (years)	39.4 ± 13.2 (40.0)	36.4 ± 14.0 (37.0)	43.9 ± 10.6 (43.5)	0.030*
Serum creatinine (mg/dl)	1.3 ± 1.2 (0.9)	1.6 ± 1.4 (1.2)	0.8 ± 0.2 (0.8)	0.007*
eGFR (ml/min/1.73 m^2)	81.7 ± 34.8 (81.9)	79.0 ± 42.3 (80.0)	85.8 ± 19.6 (84.0)	NS
UPCR (g/g)	1.08 ± 1.34 (0.01–5.62; 0.42)	1.28 ± 1.48 (0.04– 5.62; 0.51)	0.80 ± 1.09 (0.01–3.47; 0.17)	NS
Systolic BP (mmHg)	125.7 ± 13.0 (126.0)	126.1 ± 13.1 (125.0)	125.3 ± 13.3 (129.0)	NS
Diastolic BP (mmHg)	75.6 ± 10.5 (75.0)	74.3 ± 11.5 (73.0)	77.4 ± 8.9 (77.5)	NS

Values are presented as mean ± SD and median, and (range; median) for UPCR. To convert UPCR to mg/mmol, multiply by 113.12.
*P-values; males compared to females, two-tailed t-test.
eGFR, estimated glomerular filtration rate; UPCR, urine protein to creatinine ratio; BP, blood pressure; NS, non-significant.

Table 2. Summary of patient characteristics by CKD stage

	Stage 1	Stage 2	Stage 3	Stages 4, 5
Males (n)	15	8	7	5
Age (years)	28.1 ± 10.3	39.9 ± 14.1	46.4 ± 11.3	41.5 ± 16.6
eGFR	119.1 ± 21.9	73.4 ± 7.8	43.9 ± 8.9	16.3 ± 10.3
UPCR	0.73 ± 0.83 (0.04–2.39; 0.37)	0.81 ± 0.85 (0.04–2.18; 0.55)	0.86 ± 0.59 (0.20–1.97;0.71)	4.23 ± 1.25 (2.3–5.6; 4.29)
Females (n)	10	13	1	0
Age (years)	42.4 ± 10.6*	43.5 ± 9.9	63.0	NA
eGFR	104 ± 12.8	74.8 ± 8.4	46.0	NA
UPCR	0.44 ± 0.48 (0.04–1.46; 0.24)	1.00 ± 1.36 (0.01–3.47; 0.15)	1.83	NA

Values are presented as mean ± SD and (range; median).
eGFR, estimated glomerular filtration rate (ml/min/1.73 m^2); UPCR, urine protein to creatinine ratio (g/g; to convert to mg/mmol, multiply by 113.12.); NA, not applicable.
*$P < 0.003$; males compared to females, two-tailed t-test.

Biopsy findings

The histologic evaluations are summarized in Tables 3–6. On average, 16.1 ± 11.9 glomeruli were present for light microscopic assessment (range 1–67), and 2.6 ± 1.9 (range 0–8) glomeruli were examined on toluidine blue-stained semi-thin sections. Two cases were not scored on the semi-thin sections because of inadequate staining.

Glomerular sclerosis. There was a wide range of glomerular sclerosis scores (Table 3). Mild or severe segmental sclerosis was observed in 19 of 35 males (9 stage 1 CKD, 3 stage 2 CKD, remainder with more advanced CKD), and 11 of 24 females (2 stage 1 CKD, 8 stage 2 CKD, 1 patient with more advanced CKD). The average extent of segmental sclerosis was 4.7 ± 7.8% of glomeruli, with mild and severe lesions seen in the same biopsy. Global sclerosis was present in 14.8 ± 22.7% of glomeruli. Ten of the 15 males (66.7%) with stage 1 CKD had segmental or global sclerosis in 6.2 ± 8.7% (median 2.9) of their glomeruli, and 7 of the 10 females with stage 1 CKD had segmental or global sclerosis in 5.7 ± 9.0% (median 3.0) of their glomeruli. On average, 43.5 ± 23.0% of the glomeruli had global sclerosis for the 13 patients with eGFR <60 ml/min/1.73 m^2, with only 1 without global sclerosis.

Interstitial fibrosis and chronic damage index. The semi-quantitative and morphometric scores for fibrosis were closely correlated (top section, Table 4; Spearman's coefficient = 0.871, $P < 0.001$). Bland–Altman analysis

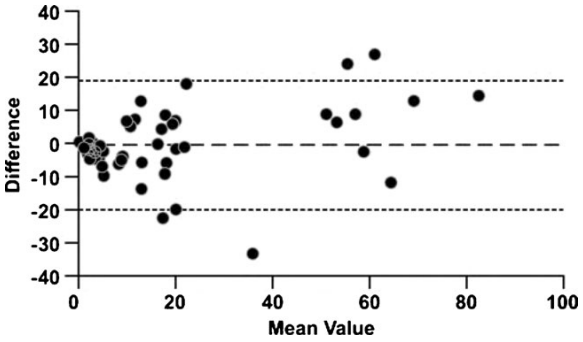

Fig. 1. Interstitial fibrosis (%): comparison of semi-quantitative scoring to morphometric assessment. Bland–Altman plot compares the difference (index of chronic damage – semi-quantitative score; solid horizontal line) with their mean. The limits of agreement (±2 SD) are shown as dashed horizontal lines.

(Figure 1) [28] showed that the semi-quantitative assessment had no significant bias compared to the morphometry (mean difference of average score minus index of chronic damage = +0.79%; 95% Confidence Interval; −1.74 to +3.32%). The limits of agreement (mean difference ± 2 SD) ranged between −18.6% and 20.2%.

The reliability of scoring among the pathologists was assessed with ICC analysis [25,26]. The variance in scoring attributable to the cases and the residual variance were used to estimate the ICC (0.6539). The variance within cases was 52.9% of the variance between cases (Table 4), indicating reasonably good agreement between the different

Table 3. Kidney morphologic findings in Fabry patients

	All ($n = 59$)	Male ($n = 35$)	Female ($n = 24$)
Glomeruli, number in biopsy (light microscopy sections)	16.1 ± 11.9 (13.6)	17.3 ± 12.8 (14.3)	14.3 ± 10.3 (13.6)
Segmental sclerosis; (mild + severe) (%)	4.7 ± 7.8 (0.9)	5.1 ± 8.5 (1.9)	4.0 ± 6.7 (0.0)
Global sclerosis (%)	14.8 ± 22.7 (2.9)	18.3 ± 24.3 (5.9)	9.8 ± 19.5 (0.5)
Glomeruli without sclerosis (%)	69.9 ± 27.3 (75.2)	66.3 ± 30.4 (75.2)	75.2 ± 21.6 (77.7)
Interstitial fibrosis (%)	16.2 ± 22.9 (6.0)	20.4 ± 25.0 (10.0)	10.1 ± 18.3 (2.5)
Arterial sclerosis (0–3)	0.71 ± 0.72 (0.43)	0.75 ± 0.77 (0.33)	0.64 ± 0.66 (0.47)
Podocyte vacuoles (light microscopy; 0–3)*	2.2 ± 0.8 (2.5)	2.3 ± 0.7 (2.6)	1.9 ± 0.8 (1.9)
Podocyte inclusions (semi-thin sections; 0–4)*	3.0 ± 1.2 (3.5)	3.3 ± 1.1 (3.9)	2.4 ± 1.2 (2.8)

Values are presented as mean ± SD and (median). Assessment from standard light microscopy sections unless otherwise indicated. Percentage represents proportion of glomeruli with lesion. Of note, glomeruli with periglomerular fibrosis, extensive corrugation, ischaemia or adhesion were not scored as 'non-sclerotic', and thus the sum of global or segmental sclerosis and 'non-sclerotic' glomeruli does not necessarily equal 100%.
*$P < 0.03$, two-tailed *t*-test, females compared to males.

Table 4. Interclass and intraclass correlation coefficients for interstitial fibrosis, podocyte vacuoles and podocyte GL-3 inclusions

Scoring (*n*)	Mean (%)	SD	Median	Minimum	Maximum
Morphometric score (59)	16.22	22.94	6.00	0	89.0
Average semi-quantitative score (59)	17.01	10.56	8.33	0	75.0
Spearman's interclass correlation coefficient = 0.871, $P < 0.001$					
Scoring (scale)	Mean (%)	SD	Median	Minimum	Maximum
Podocyte vacuoles (1–3)	2.19	0.80	2.5	0	3.00
Podocyte inclusions (1–4)	2.95	1.21	3.4	0	4.00
Spearman's interclass correlation coefficient = 0.5774, $P < 0.0001$					
Scoring (*n*)	Variance of cases (V) (SE)	Residual variance (R) (SE)	ICC (V/V+R)	Within case variance(1-ICC)/ICC	
Interstitial fibrosis score (59)	340.3 (71.8)*	180.1 (18.9)*	0.6539	52.9%	
Podocyte vacuoles (59)	0.557 (0.119)*	0.235 (0.0310)*	0.7030	42.2%	
Podocyte inclusions (57)	1.344 (0.278)*	0.3443 (0.047)*	0.7961	25.7%	

*$P < 0.0001$.
ICC, intraclass correlation coefficient; R, residual variance; V, variance of cases.

pathologists with respect to the semi-quantitative scoring of interstitial fibrosis.

Podocytes vacuoles and inclusions. Vacuoles were frequently observed in podocytes (Figure 2A), corresponding to extracted GL-3 deposits. Vacuole average severity (0–3 scale) was 2.3 ± 0.7 (median 2.6) for males and 1.9 ± 0.8 (median 1.9) for females ($P < 0.03$, Table 3). GL-3 inclusions were scored for the semi-thin sections stained with toluidine blue (0–4 scale). The distribution of GL-3 inclusions was quite variable (Figure 2B and C). Women had significantly fewer deposits than men (2.4 ± 1.2 versus 3.3 ± 1.1; $P < 0.03$; Table 3). The most common pattern in male patients was a mixture of large expanded deposits with concurrent small deposits.

Of patients with glomeruli available on semi-thin sections, one 46-year-old woman did not have any GL-3 deposits, and seven additional female patients and one male patient had small deposits (i.e. inclusion score <1.5); none had received ERT. The female patient without podocyte deposits had distal tubular and arterial medial deposits and was known to have mutation p.R363P. Parietal epithelial inclusions were present (Figure 2B), but were not reliably scored.

The podocyte vacuole scores and GL-3 inclusion scores agreed reasonably well (Table 4). The reliability of the scoring among the pathologists was assessed with ICC analysis [25,26]. The variance attributable to the cases, and the residual variance were used to estimate the ICC. The variance component within cases was 42.2% and between cases was 25.7%, respectively, for the scorings of podocyte vacuoles on light microscopy and GL-3 inclusions on semi-thin sections. Of the three ICC comparisons, the reliability of the scoring of the podocyte GL-3 inclusions appeared to be superior to the semi-quantitative scoring of interstitial fibrosis or podocyte vacuoles.

Tubular inclusions. Distal tubule inclusions were present in 42 of the 56 cases (75.0%) (Table 5, Figure 2D–F). Proximal tubule inclusions were more prevalent in males than females (47.1% versus 19.0%, $P = 0.046$). Inclusions in both proximal and distal tubules were present in 14 males and 4 females, and 14 males and 9 females only had distal tubule inclusions.

Capillary and arterial inclusions. Peritubular capillary inclusions were identified in 26 males and 7 females ($P = 0.002$). Intimal vascular inclusions were more prevalent in males than females. Vascular medial inclusions were present in 25 out of 38 patients (Figure 3). Lesions included typical arteriosclerotic lesions and severe medial injury and necrosis with inclusions, often associated with luminal dilatation. Arterial sclerosis was mild (Table 3). Hyalinosis was present in arterioles of 34 patients and in arteries of 22 patients out of 55 evaluated (Table 5). Arteriolar hyalinosis was scored in 22 of 34 males (64.7%) and 12 of 21 females

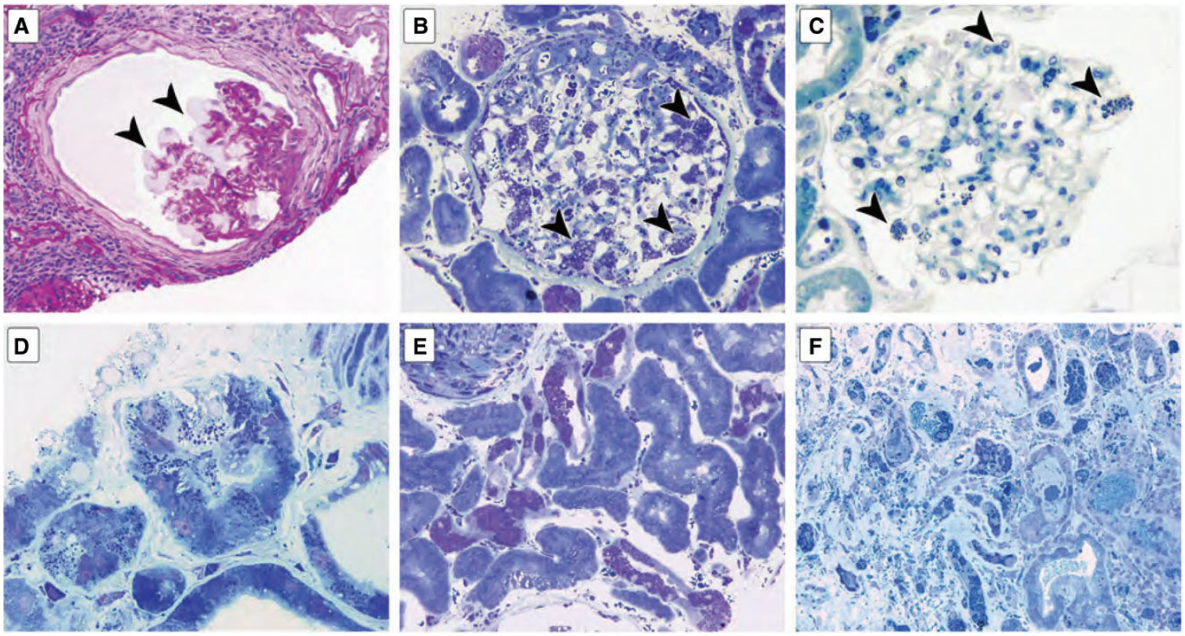

Fig. 2. Vacuolization and glycosphingolipid deposits in Fabry nephropathy. (**A**) The glomerulus shows vacuolization of podocytes (arrowheads), and mild corrugation of glomerular basement membranes, with periglomerular and interstitial fibrosis (periodic acid-Schiff, x400). (**B**) The glomerulus shows massive expanded deposits in most podocytes (arrowheads) and also in mesangial and endothelial cells and parietal epithelial cells in this male Fabry patient (toluidine blue, ×400). (**C**) The glomerulus shows occasional small deposits in podocytes (arrowheads) and rare deposits in mesangial areas in this female patient (toluidine blue, ×400). (**D**) There are prominent deposits in some proximal tubular cells and peritubular capillary endothelium (toluidine blue, ×1000). (**E**) Numerous deposits in distal tubules and very rare deposits in proximal tubules. The vascular smooth muscle of a large artery (top left corner) also shows deposits, as do parietal epithelial cells lining the Bowman's capsule (bottom) (toluidine blue, ×1000). (**F**) Numerous deposits in distal tubules with rare deposits in proximal tubules and frequent deposits in peritubular capillaries and interstitium (toluidine blue, ×200).

Table 5. Kidney morphologic findings in Fabry patients

	All ($n = 59$)	Male ($n = 35$)	Female ($n = 24$)	P-value[a]
Distal tubule inclusions	42/14 (75.0%)	28/6 (82.4%)	14/8 (63.6%)	NS
Proximal tubule inclusions	20/35 (36.4%)	16/18 (47.1%)	4/17 (19.0%)	0.046
Peritubular capillary inclusions	33/23 (58.9%)	26/8 (76.5%)	7/15 (31.8%)	0.002
Vascular intimal inclusions	19/19 (50.0%)	16/9 (64.0%)	3/10 (23.1%)	0.038
Vascular medial inclusions	25/13 (65.8%)	17/8 (68.0%)	8/5 (61.5%)	NS
Arteriolar hyalinosis (light microscopy sections)	34/21 (61.8%)	22/12 (64.7%)	12/9 (57.1%)	NS
Arterial hyalinosis (light microscopy sections)	22/33 (40.0%)	9/25 (26.5%)	13/8 (61.9%)	0.012

Data are presented as present/absent and percentage present/(present + absent);
Excluded cases are those that could not be scored due to insufficient sampling for that variable. Lesions scored on semi-thin sections except as noted.
[a]Fisher exact two-tailed test statistic, females compared to males;
NS, non-significant.

(57.1%). Arterial hyalinosis was present in 9 of 34 males (26.5%) and 13 of 21 females (61.9%; $P = 0.012$).

Trend analysis. Arterial sclerosis was more severe with advanced CKD ($P = 0.0003$), but blood pressure was not correlated (not shown). Glomerular sclerosis was more severe with more advanced CKD. In stage 1 CKD, 7.8 ± 17.6% (median 0.85) of the glomeruli had segmental or global sclerosis despite minimal proteinuria.

Discussion

We have described the renal biopsies of mild Fabry nephropathy in adult patients. Histologic evidence of kidney involvement precedes clinical signs in early Fabry nephropathy, confirming previous reports [16,17,19,29]. Glomerulosclerosis and interstitial fibrosis were observed in both genders, even in patients with minimal or no proteinuria in our series. In the evaluation of early Fabry nephropathy, clinical assessments lack sensitivity, potentially delaying initiation of ERT [30], highlighting the utility of kidney biopsies as part of the baseline assessment.

A similar scoring strategy was used for lupus nephritis [31] and focal segmental glomerulosclerosis [32]: inclusion of pathologists and clinicians; multiple iterations; validation and adjudication of difficult lesions; and refinement of definitions using a modified Delphi technique [23]. We evaluated disease-specific GL-3 lesions and secondary

Table 6. Summary of trend analyses by CKD stage

	Stage 1 ($n = 25$)	Stage 2 ($n = 21$)	Stage 3 ($n = 8$)	Stages 4, 5 ($n = 5$)	P-value*
eGFR	113.1 ± 20.0 (110.0)	74.3 ± 8.0 (72)	44.2 ± 8.3 (45.5)	16.3 ± 10.3 (13.6)	0.002
Arterial sclerosis, 0–3	0.46 ± 0.68 (0.00)	0.62 ± 0.57 (0.50)	0.93 ± 0.39 (1.00)	1.93 ± 0.72 (1.66)	0.003
No sclerosis, % glomeruli	82.3 ± 18.7 (88.4)	74.5 ± 20.9 (78.8)	50.2 ± 28.9 (55.1)	20.7 ± 14.2 (21.7)	0.027
Mild + severe sclerosis, % glomeruli	2.8 ± 5.5 (0.00)	4.3 ± 6.5 (1.06)	6.9 ± 10.9 (1.35)	11.9 ± 13.2 (5.26)	NS
Global sclerosis, % glomeruli	5.0 ± 17.1 (0.00)	8.7 ± 11.3 (5.9)	35.9 ± 24.9 (32.7)	55.5 ± 14.6 (50.0)	0.024
Mild + severe + global sclerosis, % glomeruli	7.8 ± 17.6 (0.85)	13.1 ± 15.7 (7.1)	42.9 ± 26.9 (46.4)	67.4 ± 11.6 (69.8)	0.030
Interstitial fibrosis, %	8.4 ± 15.9 (2.0)	9.4 ± 16.2 (3.0)	27.8 ± 19.7 (21.5)	65.6 ± 13.9 (61.0)	0.027

Values are presented as mean ± SD and (median).
eGFR, estimated glomerular filtration rate (ml/min/1.73 m^2); NS, non-significant.
*P-value for the regression calculated with the F-test; interstitial scores transformed as log(score) before analysis.

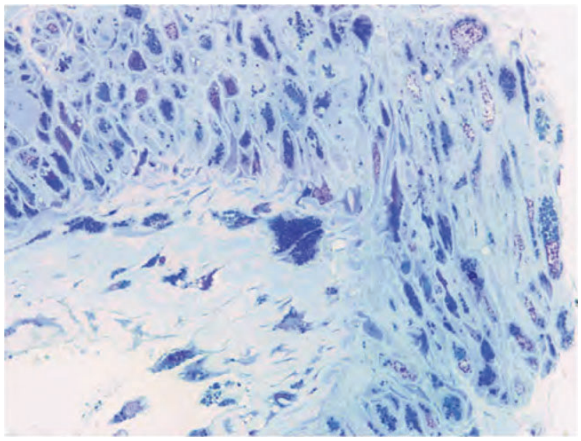

Fig. 3. Vascular lesions in Fabry nephropathy. Numerous inclusions in endothelial cells and vascular smooth muscle cells in this large artery are seen in the toluidine blue-stained semi-thick scout section (toluidine blue, ×1000).

alterations that occur in Fabry nephropathy [33], as well as any other form of CKD (e.g. arterial and glomerular sclerosis, and interstitial fibrosis). Interstitial fibrosis, global and segmental sclerosis and podocyte inclusions were robustly scored. The latter parameter showed the highest degree of inter-individual agreement. The semi-quantitative scoring of interstitial fibrosis showed similar reliability when compared to the morphometric assessment. Other lesions (e.g. parietal epithelial cell inclusions, interstitial inflammation) could not be reliably scored with the current approaches.

Glomerulosclerosis occurs early in Fabry nephropathy and is more severe with more advanced CKD. Only 37% of our patients with eGFR >60 ml/min/1.73 m^2 did not have any glomerular sclerotic lesions, and ∼13% had sclerosis in >20% of glomeruli. Although proportions of segmental and global sclerosis varied among cases, no significant histologic differences between genders could be identified in early CKD. This finding may have important prognostic implication; Germain *et al.* [6] have described a marked loss of GFR in male Fabry patients with segmental and global glomerular sclerosis in >50% of their glomeruli.

GL-3 inclusions in podocytes were larger in males than in females, despite the 7.5-year higher mean age of females. Even in early CKD, half of the males had large podocyte inclusions involving >50% of the glomerular tuft. The podocyte scores for vacuolization and inclusions were significantly correlated, with only two patients having discordant findings.

Most of our patients (78%) had mild nephropathy (stage 1–2 CKD). Nearly half of the stage 1–2 CKD patients were females. Some conspicuous gender differences were observed. Clinical disease was milder in the female cohort, with corresponding lesser degree of global sclerosis and less podocyte, peritubular, vascular and proximal tubule inclusions than in the males. However, segmental and global glomerulosclerosis, interstitial fibrosis, distal tubular inclusions, arteriolar hyalinosis and vascular medial inclusions were of similar degrees in females as in males. Arterial hyalinosis was the only lesion more prevalent in females than males, which may be related to their higher mean age. These gender differences indicate that prognostic scoring elements may be assessed differently in male and female Fabry patients. Arterial sclerosis increased with CKD stage, and there appears to be a non-linear relationship between interstitial fibrosis and CKD stage, with minimal fibrosis scores in the stage 1–2 biopsies (Table 6).

Attempts to develop prognostic markers and/or pathogenic insights from renal biopsies have been undertaken in other forms of CKD. Glomerulosclerosis, interstitial fibrosis, endocapillary proliferation and surprisingly, mesangial proliferation were signs of poor prognosis in IgA nephropathy [34]. In the African-American Study of Kidney Diseases, global sclerosis was minimally related to mean arterial pressure, but was predicted by systolic blood pressure, serum cholesterol and reciprocal of serum creatinine [35]. With longitudinal studies of Fabry patients, a similar strategy may support the development of a chronicity index that reflects the long-term outcome. The variables summarized in Tables 3–5 may serve as the basis for such studies. The amount of chronic renal damage at the time of biopsy is likely to be related to the rate of loss of kidney function [23]. In addition, some lesions may influence the rate of decline, as well as baseline GFR. There may also be an indication as to response to therapy for some of the scored variables.

Several limitations of our study are evident. (1) We have assigned CKD stages to the patients based on baseline serum creatinine values and calculated eGFR. The

majority of patients had GFR >60 ml/min/1.73 m^2, beyond the current validation range of the MDRD. UPCR was used as an index of proteinuria. KDOQI [24] defined CKD as either kidney damage or eGFR <60 ml/min/1.73 m^2 for ≥3 months, with kidney damage defined as pathologic abnormality or a marker of damage. Since all of our patients had biopsy-proven Fabry nephropathy, we feel justified in using CKD staging to describe the severity of their kidney damage. Furthermore, the significant increases in the various sclerosis scores with CKD stage, with more severe scores in stage 2 than stage 1 (Table 6), lend further credence to the use of CKD staging, bearing in mind the reservations about using estimating equations for GFR. (2) Nine patients received ERT before biopsy, but the median time before biopsy was only 1.4 months, with none more than 4 months. It is not likely that short-term ERT would influence sclerotic lesions. (3) Biopsies were performed according to the local standard of care, and the criteria for submitting cases for this study were not restrictive. This retrospective, cross-sectional design raises a possible selection bias for inclusion in the study. (4) Few patients with advanced CKD were included, limiting the trend analysis as well as comparisons between genders. Nevertheless, the pathology was similar in both genders, although developing at a later age in females.

The present study focused on histologic sections for developing the scoring system. For this purpose, standard histologic sections provided sufficient glomeruli and more extensive vascular and interstitial sampling, overcoming the limited tissue sampling with semi-thin sections used in previous studies [5]. On the other hand, histological findings suggestive of Fabry nephropathy (e.g. vacuolization) may be missed on routine histologic sections; semi-thin sections are best for characterizing podocyte GL-3 inclusions. This conclusion is strengthened by the ICC analysis in Table 4.

In conclusion, our description and validation of a scoring system of histologic involvement in Fabry nephropathy are notable because of the inclusion of a large number of adult females. Chronic glomerular and interstitial damage develop early in the course of Fabry disease, and the absence of typical clinical signs of CKD does not rule out Fabry nephropathy. Overall, our results are in agreement with previous, smaller series of Fabry patients, showing significant histologic changes before renal function is decreased [12,16,19]. Access to detailed illustrations and definitions of lesions (see Appendix) should enhance the utility and application of the scoring system for future studies of Fabry nephropathy. Future development of a chronicity index based on longitudinal data may be useful for describing severity of pathologic changes and prognostic implications for progressive Fabry nephropathy. For now, we conclude that important information is provided by kidney biopsy in Fabry nephropathy that is not available from routine assessment of kidney function and proteinuria. Our results support the role of kidney biopsy in the baseline evaluation of all Fabry patients, even with mild clinical disease.

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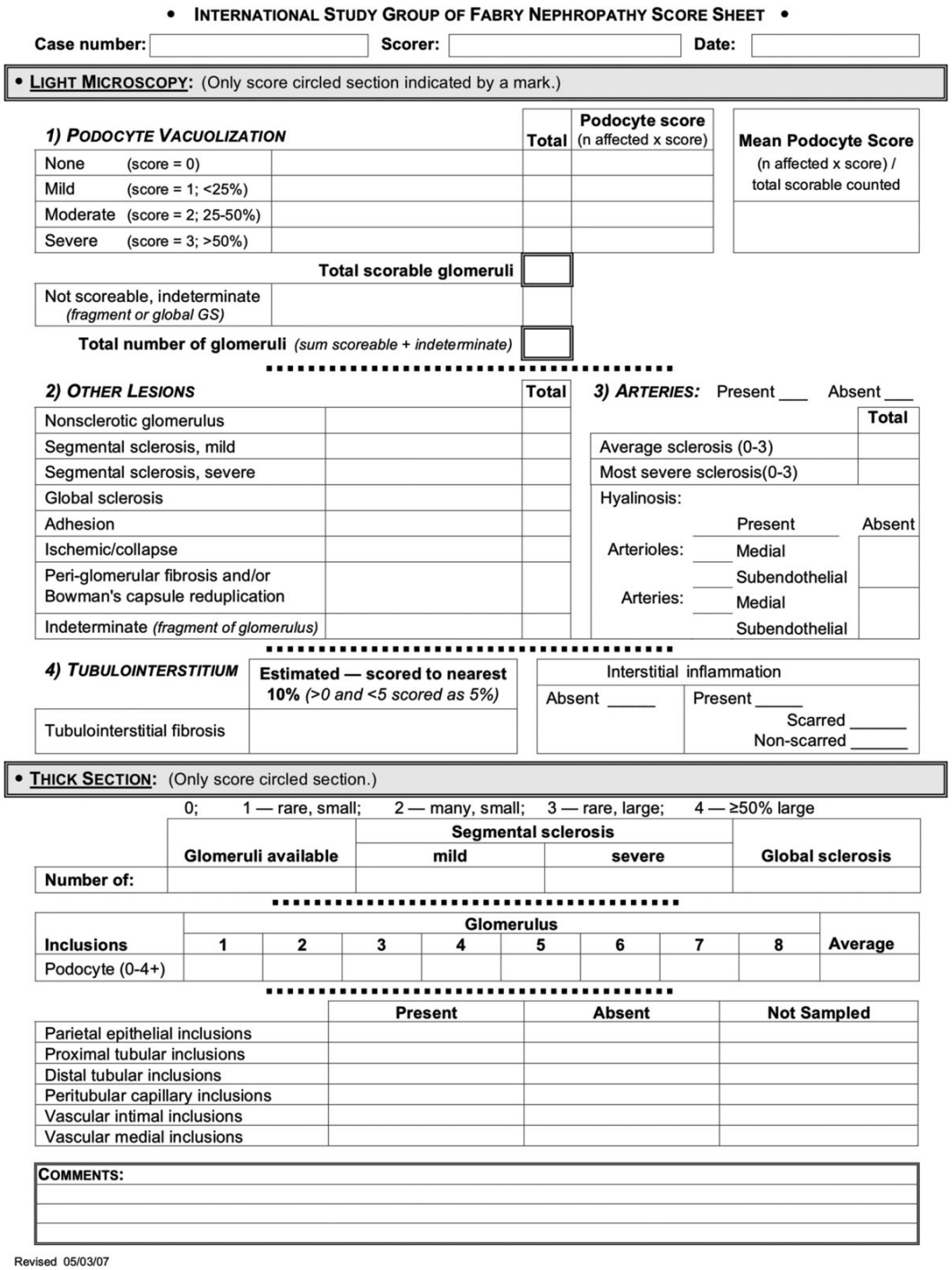
Appendix. Histologic scoring

General approach

The initial effort involved six cases coded and blinded as to origin of slides and was intended to identify common features in the biopsies (fibrosis, sclerosis, vascular changes, GL-3 deposits, etc.) that would be included in the final scoring sheet. Round-robin slide reviews and two face-to-face scoring sessions with a modified Delphi technique [23] were used to refine the scoring system. Then, slides from 20 cases were circulated, with each group scoring each element specified in the score sheet (Figure A1). In a face-to-face meeting, cases with difficult lesions were discussed for further refinement and clarification of definitions. This sequence of scoring, Delphi consensus process and



• INTERNATIONAL STUDY GROUP OF FABRY NEPHROPATHY SCORE SHEET •

Case number: Scorer: Date:

• LIGHT MICROSCOPY: (Only score circled section indicated by a mark.)

1) PODOCYTE VACUOLIZATION		Total	Podocyte score (n affected x score)	Mean Podocyte Score (n affected x score) / total scorable counted
None	(score = 0)			
Mild	(score = 1; <25%)			
Moderate	(score = 2; 25-50%)			
Severe	(score = 3; >50%)			
	Total scorable glomeruli			
Not scoreable, indeterminate (fragment or global GS)				
Total number of glomeruli (sum scoreable + indeterminate)				

2) OTHER LESIONS	Total
Nonsclerotic glomerulus	
Segmental sclerosis, mild	
Segmental sclerosis, severe	
Global sclerosis	
Adhesion	
Ischemic/collapse	
Peri-glomerular fibrosis and/or Bowman's capsule reduplication	
Indeterminate (fragment of glomerulus)	

3) ARTERIES: Present ___ Absent ___

	Total
Average sclerosis (0-3)	
Most severe sclerosis(0-3)	

Hyalinosis:

		Present	Absent
Arterioles:	Medial		
	Subendothelial		
Arteries:	Medial		
	Subendothelial		

4) TUBULOINTERSTITIUM	Estimated — scored to nearest 10% (>0 and <5 scored as 5%)
Tubulointerstitial fibrosis	

Interstitial inflammation

Absent _____ Present _____ Scarred _____ Non-scarred _____

• THICK SECTION: (Only score circled section.)

0; 1 — rare, small; 2 — many, small; 3 — rare, large; 4 — ≥50% large

	Glomeruli available	Segmental sclerosis		Global sclerosis
		mild	severe	
Number of:				

Inclusions	Glomerulus 1	2	3	4	5	6	7	8	Average
Podocyte (0-4+)									

	Present	Absent	Not Sampled
Parietal epithelial inclusions			
Proximal tubular inclusions			
Distal tubular inclusions			
Peritubular capillary inclusions			
Vascular intimal inclusions			
Vascular medial inclusions			

COMMENTS:

Revised 05/03/07

Fig. A1. Scoring sheet for Fabry nephropathy by light microscopy.

rescoring, was adopted to validate the scoring system. The final 59 cases were then circulated with each biopsy scored by three different centres. Final scores of all 59 cases represent the average in each case from all validated scorings from these centres. An average for each parameter was calculated, with a range. For lesions scored as present or absent, cases with divergent scoring were reviewed for a final score by A.B.F. Electron micrographs were not examined in the current project. Morphometry was done on all slides by one centre (see below).

Lesions were scored on the same sections on the circulated slide, so that all observers assessed the exact same

tissue. For most cases, tissue was assessed on periodic acid-Schiff (PAS)-stained slides. In a few cases, when PAS-stained slides were not available, Jones' stained slides were scored instead. Of note, more than one lesion could be present on light microscopy in any one glomerulus, e.g. segmental sclerosis and ischaemia/collapse could coexist in the same glomerulus, and both features were then scored as 'present' for that glomerulus. From our experience, optimal slides for scoring should ideally include >10 glomeruli for light microscopic assessment, and at least 3 glomeruli for semi-thin section scoring.

Light microscopic slide scoring

Glomeruli that could not be scored due to, for instance, incomplete sections or global sclerosis were indicated in the box as 'indeterminate' for scoring. Glomeruli with <25% of the tuft represented in the section were also marked as 'indeterminate' for vacuolization scoring. The total was calculated as a sum of all scored and non-scored glomeruli.

Podocyte vacuolization was scored for each individual glomerulus ('0' none, and '1', '2', or '3', respectively, when <25%, 25–50%, and >50% of podocytes showed cytoplasmic vacuoles). A final average score was calculated for each biopsy.

The total number of glomeruli without any degree of sclerosis, as well as with no adhesions, evidence of ischaemia or periglomerular fibrosis, was counted and reported as nonsclerotic glomeruli. Segmental sclerosis was defined as obliteration of capillary lumen with a increased matrix involving a portion of the glomerulus (<50%, 'mild'; or ≥50% of the tuft affected, 'severe'). Global sclerosis was scored when the entire glomerular tuft was sclerosed. Adhesions were scored when there was continuity of connective tissue between the glomerular tuft and Bowman's capsule without concomitant well-defined sclerosis. Ischaemia/collapse was scored when >50% of the glomerular tuft showed corrugation of the glomerular basement membrane and retraction of the tuft. Periglomerular fibrosis and/or Bowman's capsule reduplication were defined as present when >25% of the circumference of Bowman's capsule was affected by these processes, in the absence of global glomerulosclerosis.

Of note, as a consequence of our definition of non-sclerotic glomeruli, the sum of the percentages of globally and segmentally sclerotic glomeruli and non-sclerotic glomeruli is not mathematically 100%.

Arterial sclerosis was scored based on lesions in vessels as an average and also as a separate score for the most severely affected as described by Remuzzi *et al.* [36]: '0', vascular lesions absent; '1+', wall thickness increased but to a degree less than the diameter of the lumen; '2+', wall thickness equal or slightly greater than the diameter of the lumen; and '3+', wall thickness far exceeding the diameter of the lumen with extreme luminal narrowing or occlusion. Arteriolar or arterial hyalinosis was scored as 'present' or 'absent' and specified as involving the media or subendothelial location.

Interstitial fibrosis was semi-quantitatively estimated on cortical tissue. Interstitial inflammation was noted as 'present' or 'absent'. If present, its location in scarred areas or non-scarred areas or both was noted. However, this parameter was variably scored. Any area involved with either tubular atrophy and/or interstitial fibrosis was scored as 'involved with interstitial fibrosis'. Each ×20 field was assessed, and degree of involvement with interstitial fibrosis estimated. An average of all cortical fields was then calculated, and scored as the nearest 10%, except for minimal fibrosis of ≤5%, which was scored as 5%. The results were compared with morphometric assessment on the same slides by the method published by Howie *et al.* [21].

Additional scoring was done on the epoxy-embedded toluidine blue-stained semi-thin section slides. The number of glomeruli available on the single section was designated. The extent of segmental and global sclerosis, defined as above, was noted. A podocyte inclusion score (see below) was given separately for each glomerulus, and an average score was thereby calculated for each biopsy. Podocyte inclusions were scored as follows: '0', no deposits; '1+', rare small inconspicuous deposits; '2+', more frequent small deposits; '3+', <50% of the tuft involved with large expanded deposits in the presence or absence of concurrent small deposits; and '4+', ≥50% of the tuft involved with large expanded deposits. Inclusions in parietal epithelial, proximal and distal tubular, peritubular capillary, vascular intimal and vascular medial cells were scored as 'present', 'absent' or 'not sampled' in the specimen.

Morphometric assessment of chronic renal damage

Each biopsy specimen was examined to give a measure of the amount of chronic damage called 'the index of chronic damage' [21]. Briefly, images of the cortex were captured on a computer, and areas with chronic damage, defined as globally sclerosed glomeruli, atrophic tubules and interstitial fibrosis, were outlined using an interactive image analysis program. The index of chronic damage in a specimen was the total cross-sectional area with chronic damage expressed as a percentage of the cortical cross-sectional area.

The mean estimates of the extent of interstitial fibrosis were compared with the index of chronic damage in two ways, firstly by calculation of the Spearman's correlation coefficient, and secondly by assessment of the agreement between them using the Bland–Altman method [28]. This method gave the mean difference, or bias, with 95% confidence interval, and the limits of agreement, which were two standard deviations of the difference on each side of the mean difference. Preliminary analysis of 20 specimens showed that exclusion of the area of globally sclerosed glomeruli, which was not included in semi-quantitative estimation of the extent of interstitial fibrosis, made little difference to the index of chronic damage, because inclusion of globally sclerosed glomeruli added only 1% to the index. Subjective scoring of interstitial fibrosis was generally recorded to the nearest 10%, or to 5% for minimal fibrosis, and so 1% on the index was considered insignificant. The values of the morphometric index used were those including the area of globally sclerosed glomeruli.

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KIDNEY HISTOLOGIC ALTERATIONS IN α-GALACTOSIDADE-DEFICIENT MICE

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ORIGINAL ARTICLE

Kidney histologic alterations in α-Galactosidase-deficient mice

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Abstract Fabry disease is a rare X-linked disorder caused by mutations in the α-galactosidase gene (*GLA*), the resultant deficiency of lysosomal α-galactosidase enzyme activity leading to systemic accumulation of globotriaosylceramide and other glycosphingolipids. *GLA* knockout mice ("Fabry mice") were generated as an animal model for Fabry disease but, as they do not manifest progressive chronic kidney disease (CKD), their relevance as a model for human Fabry nephropathy is uncertain. We evaluated the histological alterations in the kidneys of Fabry mice at different ages, as contrasted to those observed in wild-type mice. Furthermore, we compared the renal histological alterations of Fabry mice to the kidney pathology reported in patients with Fabry disease at comparable age ranges and across different CKD stages, using a scoring system that has been developed for Fabry nephropathy. Fabry mice are phenotypically different from wild-type mice, displaying progressive age-related accumulation of glycosphingolipids in all types of renal cells. There were no statistically significant differences between Fabry mice and Fabry patients in the prevalence of glycosphingolipid storage per renal cell type with the exceptions of mesangial (higher in humans) and proximal tubular cells (higher in mice). However, Fabry mice lack the nonspecific histological glomerulosclerotic and interstitial fibrotic renal lesions that best correlate with progressive CKD in Fabry patients, and do not develop large podocyte inclusions. We postulate that the elucidation of the mechanisms underlying these species differences, may contribute important clues to a better understanding of the pathogenesis of Fabry nephropathy.

Keywords Fabry disease · α-Galactosidase-deficient mice · Histology · Kidney · Glycosphingolipids · Score

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Introduction

Fabry disease [OMIM #301500] is an X-linked inborn error of glycosphingolipid (GSL) catabolism, caused by mutations in the α-Gal gene (*GLA*) [1]. Deficient or absent activity of the lysosomal enzyme α-galactosidase (α-Gal) [2, 3] leads to systemic accumulation of neutral GSL with terminal α-D-galactosyl residues, predominantly globotriaosylceramide (Gb3), within a variety of cell types and organic fluids [4]. Classically affected patients often present

in childhood with acroparesthesias due to small-fibre peripheral neuropathy and angiokeratomas [4, 5]. Complications arising during adulthood include progressive renal failure, cardiac disease, and cerebrovascular disease [4, 5].

In humans, the Fabry nephropathy is characterized by initial (micro) albuminuria and development of proteinuria between the second to third decades of life. Glomerular filtration rate gradually declines and classically affected males progress to end-stage renal disease before their fifth decade [4–7]. A spectrum of histological lesions can be observed in the kidneys, even in cases with normal glomerular filtration rate and minimal proteinuria [8–15]. These lesions may include intracellular GSL deposits and nonspecific chronic glomerular, vascular, and tubulointerstitial fibro-sclerotic lesions.

A range of kidney biopsy studies have been undertaken to gain a better understanding of the natural history of Fabry nephropathy [8, 12–15] and to evaluate the response to enzyme replacement therapy (ERT) [16–18]. Fogo et al. aimed to standardize the reading of kidney biopsies and quantification of the histological alterations [15]. Kidney biopsies from 59 adult Fabry patients, both males and females, were assessed using a validated scoring system. The investigators concluded that important information is provided by kidney biopsy in Fabry nephropathy that is not available from routine assessment of kidney function and proteinuria.

An appropriate animal model of Fabry disease would be invaluable to foster the study of the pathophysiology of Fabry disease, and to assess the efficacy of ERT and other therapeutic strategies. Ohshima et al. generated a *GLA*-deficient ("Fabry") mouse by gene targeting a mutated allele [19]. Marked Gb3 accumulations have been observed in plasma and lysosomes of the kidneys, liver, spleen, heart, and skin of these knockout mice, whereas Gb3 was present only in small amounts in wild-type mice [20–23]. The knockout mice have been reported to accumulate ±25% of Gb3 in kidney tissue as compared to the level measured in a single male patient [20]. Data on progression of Gb3 accumulation in the kidneys of Fabry mice have been inconclusive one group having reported stabilization between age 20 and 40 weeks [20], whereas others found significant increases between the ages of 24 and 48 weeks [23].

Fabry mice accumulate Gb3 in vascular endothelial and smooth muscle cells and in plasma membranes of endothelial cells. Gb3 storage at these sites correlates with the development of a complex vasculopathy, including thrombosis, atherogenesis, and endothelial dysfunction [20, 24–28]. Nevertheless, these mice have no clinical phenotype of Fabry disease [20].

Our study aimed to characterize the kidney pathology of this *GLA*-deficient mouse using a standardized histological scoring method that has been developed for Fabry nephropathy in humans [14, 15]. The results were compared with observations in wild-type mice, and with similar data of previously published series in human Fabry patients.

Subjects, materials, and methods

Fabry knockout and wild-type mice

Alpha-Gal$^{(-)}$ males and α-Gal$^{(-/-)}$ females were provided by the National Institutes of Health (Bethesda, MD, USA), and a colony was maintained at "Instituto de Biologia Celular e Molecular" (IBMC), University of Porto (Porto, Portugal). Given the genetic background of this Fabry mice colony, C57BL/6 wild-type mice were used as controls. The genotype of each mouse was confirmed by polymerase chain reaction (PCR) analysis of genomic DNA extracted from tail biopsies using a standard method [29]. PCR amplification was done according to previously described protocols [23]. Mice were kept in group cages with woodchip bedding under 12/12 h light–dark cycle (lights on at 08:00). Food and water were provided *ad libitum*. Mice were bred under standard conditions established by the Institute Committee on the Use and Care of Animals. Euthanasia was done by cervical dislocation followed by decapitation, at ages 12, 24, and 48 weeks. Tissue samples of all sacrificed animals were collected and processed for microscopy according to published procedures [20].

This project was approved by the Portuguese Veterinary Authority–"Direcção-Geral de Veterinária"—under the name of "Estudo de patofisiologia da doença de Fabry", DGV 2005-03-09 003758.

Processing of kidney parenchyma for microscopy

Immediately following euthanasia, the kidneys were dissected from the involving tissues, immersion fixed in 2% paraformaldehyde/2% glutaraldehyde in 0.1-M cacodylate buffer for 1 h, and preserved in the refrigerator at 4°C until histological processing.

One whole kidney of each animal was collected for this study with the exception of two Fabry and one wild-type mice, of which there was only one-half kidney available. Each kidney or half kidney was equally divided in two parts: one half was preserved for later studies, while the other was divided in two fragments: one was embedded in paraffin, and the other was processed for semi-thin sectioning. Thick serial sections (3 μm) were cut from each paraffin block and stained with hematoxylin and eosin (HE), periodic acid Schiff (PAS), and modified trichrome (MT). The fragment selected for the preparation of semi-thin sections was further cut into a minimum of five 1×1 mm pieces, which

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were post-fixed in 1% osmium tetroxide and embedded in epoxy resin (Epon™). Semi-thin sections (1 μm) were obtained from each of these Epon-embedded kidney tissue samples and stained with methylene blue (MB) and toluidine blue (TB).

Reading and scoring of kidney samples

A single nephropathologist (CV), experienced in evaluating and scoring kidney biopsies of Fabry patients, assessed and scored all the histological sections, blinded as to the genotype, gender, and age of the mice. The slides were evaluated according to standard methods of kidney pathology reading and to scoring methods recently used for Fabry nephropathy [14, 15]. In short, disease-specific GSL storage-related, as well as nonspecific histological features of Fabry nephropathy, including glomerular sclerosis, interstitial fibrosis, and vascular abnormalities, were assessed and semi-quantified.

The involvement of the various types of renal cells was estimated in each of the TB- and MB-stained semi-thin cuts and scored according to the percentage of cells of each particular type containing any visible deposits of GSL: a storage score of "1+" was given to a cell type when less than 1/3 of all cells of that type had cytoplasmic GSL inclusions; a score of "2+" was given when between 1/3 to 2/3 of those cells were involved; and the score of "3+" was given when GSL inclusions were present in more than 2/3 of the cells. A final intermediate storage scoring could be assigned to a specific cell type when the scores assigned to that cell in the different semi-thin cuts were not entirely concordant. As a surrogate for the degree of GSL accumulation in the kidney parenchyma, a combined GSL storage score was calculated for each mouse, by averaging the storage scores assigned to the various renal cell types. The prevalence of involvement of the different renal cell types in each mouse cohort or sub-cohort was estimated as the percentage of mice in that specific series that were

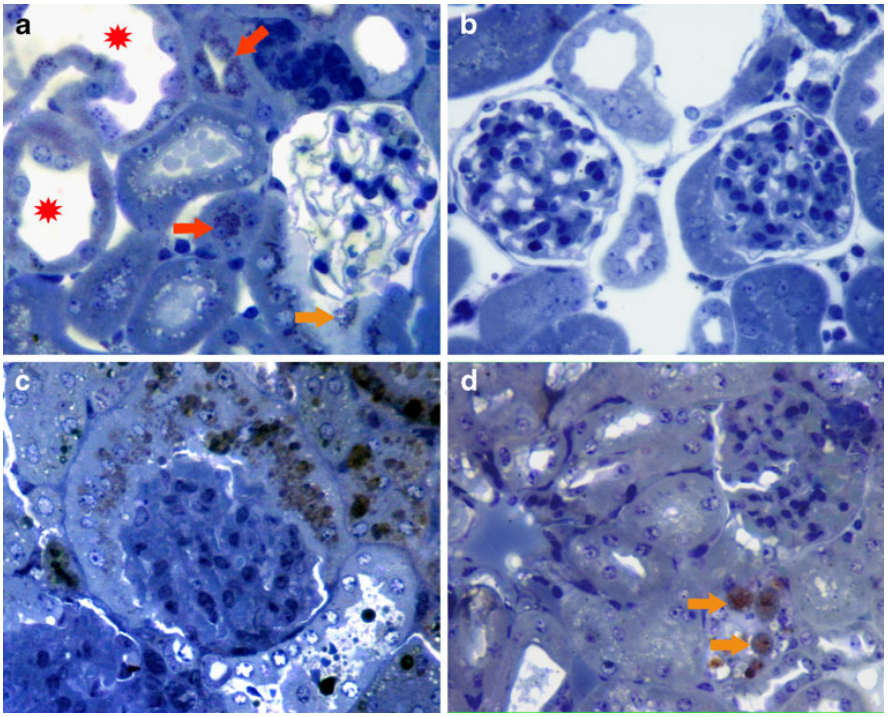

Fig. 1 Representative photomicrographs of cross sections of kidney tissue stained with toluidine blue. **a** Female Fabry mouse aged 24 weeks. —Glomerulus opening in a proximal tubule, showing osmiophilic inclusions in epithelial tubular cells; a podocyte contains small and dark-stained osmiophilic inclusions (*orange arrow*). Some tubuli show all epithelial cells with small and dark-stained deposits (*red arrows*), and others show all epithelial cells with light-stained deposits (*red stars*). **b** Female wild-type mouse aged 24 weeks. —Two glomeruli, both with squamous parietal epithelium, without any visible osmiophilic inclusions. No osmiophilic inclusions were visible in any of the renal cells of this mouse. **c** Male Fabry mouse aged 48 weeks. —Glomerulus limited by prominent cuboidal parietal epithelium containing osmiophilic inclusions. Tubular cells are also filled with large and dark-stained osmiophilic deposits. **d** Female wild-type mouse aged 48 weeks. —Glomerulus with squamous parietal epithelium without any visible osmiophilic inclusions. Some tubular cells contain osmiophilic inclusions (*orange arrows*). Sections stained with toluidine blue. Magnification ×600

assigned a storage scoring of at least "1+" for the particular cell type.

The presence of nonspecific histological features was assessed in TB-and MB-stained semi-thin sections and in HE-, PAS-, and MT-stained paraffin sections. The degree of interstitial fibrosis was reported as the estimated percentage of affected cut section, at ×200 magnification.

An atlas of the microanatomy and histology of the mouse available online was used for histological guidance on evaluation of the murine renal parenchyma [30].

Statistical analyses

The Fisher exact, Mann–Whitney *U*, and Kruskal–Wallis tests were used as appropriate for statistical comparisons of the prevalence and scoring of renal histological features in wild-type and Fabry mice of both genders across different age groups, and to compare the prevalence of GSL inclusions in the various renal cell types between Fabry mice and Fabry patients. All statistical calculations were carried out with the software PASW® (Predictive Analytics SoftWare; SPSS Inc., Chicago, IL, USA), version 18.0. *P* values<0.05 were reported as statistically significant.

Results

Characterization of GSL deposits

GSL deposits were seen as grey to black osmiophilic inclusions in semi-thin cuts. Three different types of osmiophilic inclusions were present in all Fabry mice: (1) small, diffuse, finely granular ("sand-like"), and light-stained; (2) small, punctiform, and dark-stained; (3) round, large, and dark-stained. These three kinds of inclusions were found in Fabry mice of all age groups,

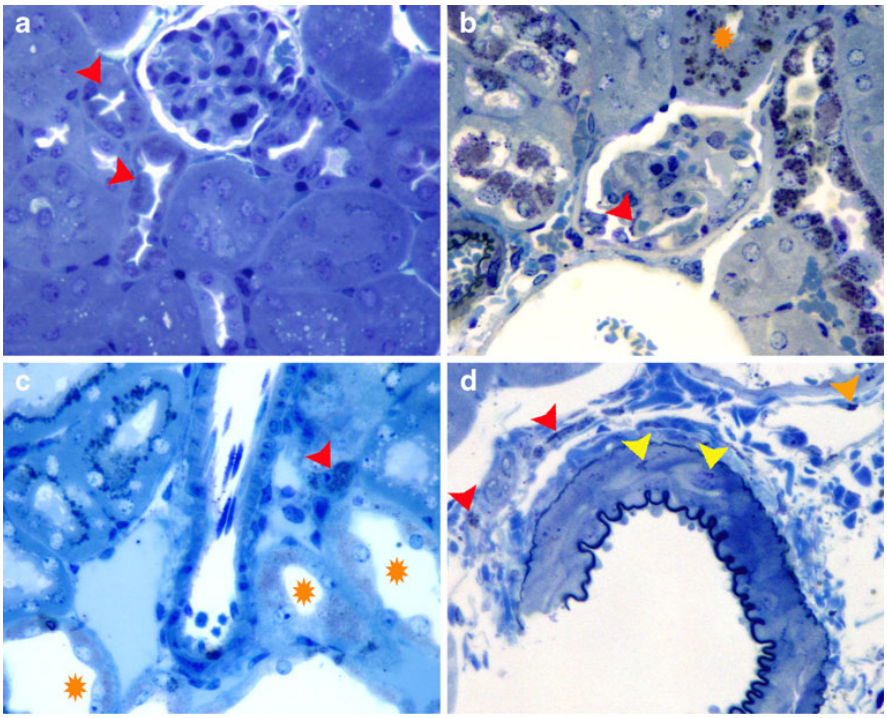

Fig. 2 Glycosphingolipid deposits in different renal cell types in Fabry mice. **a** Glomerulus with squamous parietal epithelial cells, without any visible osmiophilic inclusions. All the epithelial cells of two adjacent distal tubules are filled with finely granular ("sand-like") osmiophilic deposits (*red arrows*). **b** Glomerulus with squamous parietal epithelial cells containing small intracytoplasmic osmiophilic inclusions and one podocyte with small and inconspicuous osmiophilic inclusions (*red arrow*). The tubular epithelial cells are filled with small dark-stained osmiophilic inclusions and/or large and dark-stained osmiophilic inclusions (*orange star*). **c** Tubulo-interstitial area with small dark-stained osmiophilic inclusions in proximal tubular epithelial cells and finely granular ("sand-like") osmiophilic deposits in distal tubular epithelial cells (*orange stars*). Interstitial cell with intracytoplasmic osmiophilic inclusions (*red arrow*). Arteriolar vessel with prominent and reactive endothelial cells containing osmiophilic inclusions. **d** Perivascular interstitial area with osmiophilic inclusions visible in interstitial cells (*red arrows*) and in capillary endothelial cells (*orange arrow*); inclusions are also visible in vascular smooth muscle cells of an arteriolar vessel (*yellow arrows*). Sections stained with toluidine blue (**a, d, c**) or methylene blue (**b**). Magnification ×600

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Table 1 Distribution of osmiophilic inclusions per renal cell type in male and female Fabry and wild-type mice

Age (weeks)	Strain	Osmiophilic inclusions in renal cells	
		Males	Females
12	Control	Absent	DT
12	Control	PT, DT	Absent
12	Fabry	G, PT, DT, I, PTC	G, PT, DT, PTC,V
12	Fabry	G, PT, DT	G, PT, DT, I, PTC, V
24	Control	Absent	PT
24	Control		Absent
24	Fabry	G, PT, DT, V	G, PT, DT, I, V
24	Fabry	G, PT, DT, I, PTC, V	G, PT, DT, I, PTC, V
24	Fabry	G, PT, DT, PTC, V	
48	Control	DT	Absent
48	Control	Absent	Absent
48	Fabry	G, PT, DT, I, PTC, V	G, PT, DT, I, PTC, V
48	Fabry	G, PT, DT, V	G, PT, DT, I, PTC, V

G glomerular; *PT* proximal tubular; *DT* distal tubular; *I* interstitial; *PTC* peritubular capillary; *V* vascular arterial/arteriolar

but the first type was more frequent in the 12-week-old mice and the third type in the older age group, suggesting that the size of inclusions may increase with age (Figs. 1 and 2).

Tables 1 and 2 and Figs. 1 and 2 summarize the histological findings in the various mice sub-cohorts evaluated in this study.

Evaluation of the glomerular compartment

No osmiophilic inclusions were found in the glomerular cells of wild-type mice (Table 1, Fig. 1). All Fabry mice showed GSL inclusions in some of the glomerular cells (Table 1, Figs. 1 and 2). Parietal epithelial cells showed the highest prevalence and amount of cytoplasmic inclusions,

Table 2 Semi-quantification of osmiophilic deposits per renal cell type in the semi-thin cuts of Fabry mice

Age (weeks)	Gender	Glomerular cells				Tubular cells		Interstitial cells	PTC cells	Arterial / arteriolar vascular cells	
		Podocytes	Mesangial	Endothelial	Parietal	Proximal	Distal			Endothelial	Smooth muscle
12	♂	+	−	+	++	+	++	+	+	−	−
12	♂	+	−	−	−	+	+/++	−	−	−	−
12	♀	+	−	−	+	+	++	−	+	−	+
12	♀	+	−	−	++	++	++	++	+	+	+
24	♂	−	−	−	+	+	++	−	−	−	+
24	♂	+	+	−	+	+	++	+	++	−	+
24	♂	+	−	−	+	+	++	−	+	−	+
24	♀	+	+	−	+	++	+	+	−	+	+
24	♀	+	+	+	+	+	++/+++	+	++	+	++
48	♂	+	+	−	+/++	+	++/+++	++	++	++	−
48	♂	+	−	−	++	+	++	−	−	−	+
48	♀	+	−	−	+	+	++	++	++	+	+/++
48	♀	−	−	−	+	+/++	++	+	+	−	+/++

PTC peritubular capillary cells

The involvement of the different renal cell types was semi-quantitatively scored ("−" through "+++"). There were no statistically significant differences between gender and age groups in the prevalence and storage score per cell type

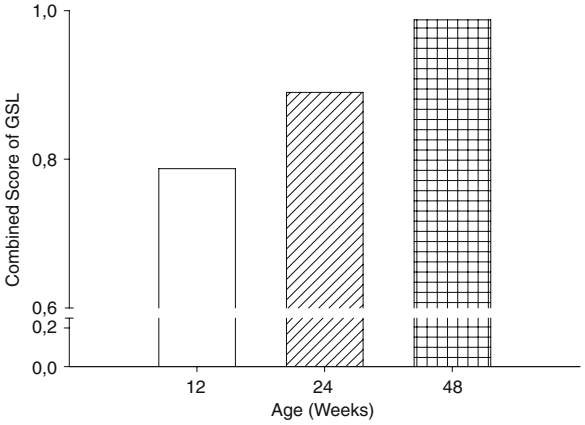

Fig. 3 Combined glycosphingolipids storage score in Fabry mice. There is an increase of the combined semi-quantification scoring of osmiophilic inclusions related to age

followed by podocytes, mesangial, and endothelial cells (Table 2). In all 11 mice showing podocyte involvement, the podocyte inclusions were small and inconspicuous (Fig. 2). There were no statistically significant differences between gender and age groups in the prevalence and semi-quantification of osmiophilic inclusions per glomerular cell type (Table 2).

Evaluation of tubular compartment

Four out of 11 wild-type mice presented scattered punctiform osmiophilic inclusions in some tubular epithelial cells (Table 1). These osmiophilic bodies were visible only at ×400 or ×600 magnification under light microscopy (Fig. 1). Semi-quantification showed very low amounts of this type of inclusions in wild-type mice. All Fabry mice had osmiophilic inclusions in proximal and distal tubular epithelial cells, visible even at ×40 magnification in mice aged more than 12 weeks, and at ×200 magnification in 12-week-old mice (Tables 1 and 2, Figs. 1 and 2). Indeed, these tubular cells were the renal cell types with the highest prevalence and amount of cytoplasmic inclusions, showing an age-related tendency to increase in distal tubular cells and to decrease in proximal tubular cells. There were no statistically significant differences between gender and age groups of Fabry mice in the prevalence and semi-quantification of osmiophilic inclusions per tubular cell type (Table 2).

Evaluation of interstitial compartment

No osmiophilic inclusions were found in interstitial cells of wild-type mice (Table 1). Eight out of 13 Fabry mice showed accumulation of osmiophilic inclusions in these cells (Tables 1 and 2, Fig. 2). There were no statistically significant differences between gender and age groups in the prevalence and semi-quantification of osmiophilic inclusions in interstitial cells (Table 2), although the semi-quantification of osmiophilic inclusions in these cells showed a trend to increasing from the ages 24 to 48 weeks.

Evaluation of vascular compartment

No osmiophilic inclusions were found in vascular cells of wild-type mice (Table 1). Twelve out of 13 Fabry mice contained osmiophilic inclusions in some of the vascular cells—peritubular capillary cells (PTC), endothelial cells of arterial or arteriolar vessels, and/or smooth muscle vascular cell (Tables 1 and 2, Figs. 1 and 2). Vascular endothelial (glomerular capillary, PTC, and/or arterial/arteriolar) and/or arterial or arteriolar smooth muscle cells inclusions were

Table 3 Presence of GSL inclusions in each renal cell type: comparison between Fabry mice and Fabry patients

	Glomerular cells				Tubular cells		Interstitial cells	PTC cells	Arterial / arteriolar vascular cells	
	Podocytes	Mesangial	Endothelial	Parietal	Proximal	Distal			Endothelial	Smooth muscle
Gubler et al., 1978 (n=12) [8]	91.7%	NA	16.7%	91.7%	33.3%	91.7%	91.7%	NA	83.3%	91.7%
Tøndel et al., 2008 (n=9) [13]	100%	88.9%	77.8%	NA	NA	100%	NA	NA	NA	NA
Valbuena et al., 2008 (n=4) [14]	100%	75%	50%	100%	50%	100%	25%	75%	75%	100%
Fogo et al., 2010 (n=59) [15]	98.3%	NA	NA	NA	36.4%	75%	NA	58.9%	50%	65.8%
Human cases, combined	97.6%	92.3%	44%	93.3%	36.6%	81.5%	81.3%	60%	59.2%	74.1%
Fabry mice (n=13)	84.6%	30.8%	15.4%	92.3%	100%	100%	61.5%	69.2%	46.2%	76.9%
P value	0.09	0.004	0.15	1.0	0.0001	0.12	0.38	0.75	0.53	1.0

NA data not available

Data are presented as the percentages of Fabry mice or Fabry patients with GSL deposits in each specific renal cell type. P values are for the statistical comparison of the prevalence of GSL deposits per cell type in Fabry patients and in Fabry mice

found in ten cases (76.9%). Eight Fabry mice showed inclusions in both vascular cell types (Table 2). There were no statistically significant differences between gender and age groups in the prevalence and semi-quantification of osmiophilic inclusions per vascular cell type, although the semi-quantification of osmiophilic inclusions in endothelial vascular cells showed a trend to increase with age (Table 2).

Comparison of the prevalence, semi-quantification, and histological characteristics of GSL deposits in Fabry mice versus wild-type mice and their evolution with age

In all types of renal cells, the semi-quantification of osmiophilic inclusions was significantly different between the Fabry mice and wild-type mice ($p<0.0001$).

Although none had deposits in other renal cell types, 36.4% of the wild-type mice showed osmiophilic inclusions in tubular cells (Table 1). These tubular inclusions were inconspicuous, scant, lightly stained and visible only at a minimum magnification of ×400.

All Fabry mice showed osmiophilic inclusions in tubular and glomerular cells, 92.3% had deposits in PTC and/or arterial or arteriolar cells, and 61.5% had inclusions in interstitial cells (Table 1). The combined semi-quantification scoring of osmiophilic inclusions in Fabry mice increased with age (Fig. 3). In all Fabry mice, different proportions of the three types of osmiophilic inclusions were found in tubular cells. Tubular inclusions were seen at a minimum ×200 magnification at 12 weeks of age and at minimum ×40 magnification at 24 and 48 weeks of age. Deposits in the other renal cells were almost always small and dark stained.

Comparison of renal GSL deposits in Fabry mice and Fabry human patients

Table 3 shows the prevalence of GSL deposits in the different renal cells in Fabry patients and in Fabry mice. The prevalence of GSL deposits in proximal tubular cells in Fabry mice was significantly higher as compared to human patients, whereas the reverse was true for mesangial cells. In all other renal cell types, the prevalence of GSL deposits was similar in both Fabry mice and Fabry patients.

Nonspecific histological alterations in the kidney parenchyma

Table 4 and Fig. 4 summarize the nonspecific renal histological alterations observed in the two mice cohorts. Twelve Fabry mice out of 13 (92.3%) and 10 wild-type mice out of 11 (90.9%) had some morphologic nonspecific alterations in the kidney parenchyma, including hypertrophy of arteriolar vascular wall, venous dilatation, interstitial

Table 4 Nonspecific histological alterations in the kidney parenchyma of male and female Fabry and wild-type mice

Age (weeks)	Strain	Males					Females				
		Glomerular alterations	Tubular alterations	Inflammatory cells	Interstitial fibrosis	Vascular alterations	Glomerular alterations	Tubular alterations	Inflammatory cells	Interstitial fibrosis	Vascular alterations
12	Control	–	–	–	<1%	VD	–	–	–	–	–
12	Control	–	UC	+	–	VD	–	–	–	–	VD
12	Fabry	–	–	–	–	VD	–	TC	+	–	VD
12	Fabry	–	–	–	–	–	–	–	+	<1%	VD
24	Control	–	TC	+	–	VD	–	DTL	–	–	–
24	Control						–	TC	–	–	–
24	Fabry	–	TC	+	–	VD	–	TC, DTL	+	–	VD
24	Fabry	–	TC	–	<1%	VD	–	–	+	–	VD
24	Fabry	–	TC	–	–	VD					
48	Control	–	TC	+	<1%	–	–	UC	–	–	–
48	Control	–	TC	+	–	VD	–	UC	–	–	VD
48	Fabry	–	–	+	<1%	VD	–	–	–	–	VD
48	Fabry	–	TC	+	–	–	–	UC	+	<1%	HVAA

–/+ feature absent/present; *DTL* dilated tubular lumen; *HVAA* hypertrophy of arteriolar vascular wall; *TC* tubular casts; *UC* urinary cyst; *VD* vein dilatation

Interstitial fibrosis reported as the percentage of affected cut section, as estimated at ×200 magnification

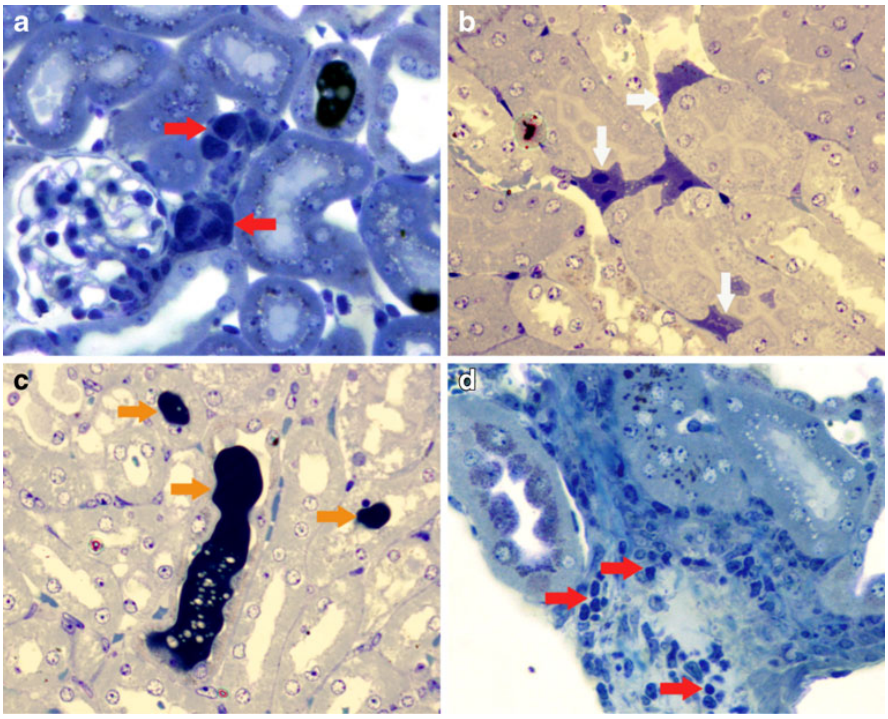

Fig. 4 Nonspecific lesions found in Fabry mice. **a** Cluster of amorphous, undifferentiated cells (*red arrows*) in the glomerular vascular pole and in periglomerular location. Dark osmiophilic cast within the tubular lumen. **b** Prominent stellate interstitial cells visible in the tubulo-interstitial area (*white arrows*). **c** Dark casts within tubular lumina (*orange arrows*). **d** Inflammatory cells (*red arrows*) in perivascular and peritubular interstitial location. Sections stained with toluidine blue (**a**) or methylene blue (**b–d**). Magnification ×600 (**a**, **d**) and ×560 (**b, c**)

fibrosis, presence of inflammatory cells in the tubulo-interstitial area, tubular dilatation, tubular casts, and urinary cysts. There were no statistically significant differences between the Fabry and the wild-type mice regarding the prevalence of any of these features.

Discussion

We describe the renal histological phenotype of α-Gal-deficient mice and compare these findings with observations in wild-type mice and reported biopsy data from Fabry patients.

The histological phenotypes of GSL accumulation in the kidneys of Fabry mice and wild-type mice were clearly distinct. Inconspicuous, finely granular osmiophilic inclusions, possibly composed of intrinsically expressed GSL or of other lipid-related deposits [20, 23], were observed in tubular cells of some wild-type mice. In the Fabry mice, osmiophilic inclusions were more abundant, larger, and more darkly stained as compared to the wild-type mice. Small inclusions were observed in all types of renal cells, and both small and large inclusions were seen in tubular and cuboidal parietal epithelial glomerular cells (Fig. 1). Diversity of size of GSL deposits in renal cell types has also been observed in kidney biopsies from Fabry patients, the largest inclusions being present in epithelial tubular cells and podocytes [8]. However, despite the sexual dimorphism of murine glomerular parietal cells, with cuboidal cells more prevalent in males and squamous cells more prevalent in females [30], there were no statistically significant gender differences in the prevalence of GSL deposits per renal cell type.

The patterns of distribution of GSL deposits in the different types of renal cells in the Fabry mice and in male and female patients of comparable age ranges were not statistically different, except in proximal tubular and mesangial cells. Fabry mice had a significantly higher prevalence of inclusions in proximal tubular cells as compared to Fabry patients, whereas mesangial cell inclusions were more prevalent in Fabry patients. Notably, large GSL podocyte inclusions, which are a distinctive feature of the human Fabry nephropathy, were not present in the Fabry mice.

The combined GSL storage score increased with age. The size and staining intensity of the osmiophilic inclusions

were, in general, more prominent in 48-week-old mice as compared to 12-week-old mice. Although this finding lacked statistical significance, it may suggest that accumulation of GSL in the kidneys of Fabry mice is progressive over time. This is in agreement with an earlier report describing a significant increase of GSL concentration in extracts of kidney tissue in 48-week-old Fabry mice as compared to 24-week-old mice [23]. It has been postulated that the Fabry nephropathy in humans results from progressive accumulation of GSL in the kidney parenchyma [31]. However, we are not aware of any biopsy studies in Fabry patients demonstrating an age-related increase in the size of deposits in renal cells, as we have observed in Fabry mice.

Glomerular sclerosis and tubulo-interstitial fibrosis are the histological features that best correlate with the progression of Fabry nephropathy in humans [14, 15, 31]. In our study, such nonspecific lesions were virtually absent in both the Fabry and wild-type mice in all three age groups studied. This represents a major difference between the kidney pathology of Fabry mice and human Fabry disease, and may correlate with the lack of clinical kidney disease in the Fabry mice [20].

Ischaemic damage resulting from microvascular endothelial disease and/or necrosis of vascular smooth muscle cells and/or pericyte injury is the major theory for the pathogenesis of the classical phenotype of Fabry disease [4, 8, 10, 32]. However, we found a largely comparable prevalence of GSL deposits in the vascular and endothelial cells in Fabry mice and patients. These findings do not support a major role of ischaemic injury in the initiation of Fabry nephropathy, and is in agreement with observation by Valbuena et al. who concluded that vascular damage did not seem to have a major role in the early pathogenesis of Fabry nephropathy in females [14].

The prevalence of histological involvement of podocytes in Fabry mice and in Fabry patients was similar. However, the size of GSL deposits was smaller in Fabry mice than is typically seen in humans with Fabry nephropathy. Therefore, progressive increase of lysosomal GSL deposits in podocytes, ultimately causing irreversible cell injury [10, 14], may be a critical mechanism in the pathogenesis of human Fabry nephropathy.

We found a relatively low prevalence of GSL accumulation in mesangial cells of Fabry mice as compared to Fabry patients. Additional studies will have to be performed to investigate a possible role of mesangial cell necrosis in the pathogenesis of glomerular sclerosis and deterioration of renal function in humans with Fabry disease [8].

In conclusion, Fabry mice show a distinct phenotype with regard to the accumulation of GSL in the kidney parenchyma, if compared to wild-type mice. The pattern of involvement of the different renal cell types is mostly similar to that observed in Fabry patients but the amount of accumulated GSL is smaller. The absence of nonspecific sclerotic and fibrotic kidney lesions represents a major difference between the kidney pathology of Fabry mice and human Fabry disease, and explains the lack of clinical kidney disease in the Fabry mice. This may be related to genetic background of the Fabry mice, since C57BL/6 mice are particularly resistant to all types of kidney disease [33]. However, as human tissues from Fabry patients are limitedly available and experimental interventions are constrained by ethical and legal issues, this Fabry knockout mouse model remains valid and useful for new lines of specific research into the mechanisms underlying the species differences and factors driving development of the Fabry nephropathy.

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IMMUNOHISTOCHEMICAL DIAGNOSIS OF FABRY NEPHROPATHY AND LOCLIZATION OF GLOBOTRIAOSYLCERAMIDE DEPOSITS IN PARAFFIN--EMBEDDED KIDNEY TISSUE SECTIONS

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ERRATUM

Erratum to: Immunohistochemical diagnosis of Fabry nephropathy and localisation of globotriaosylceramide deposits in paraffin-embedded kidney tissue sections

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The original version of the above mentioned manuscript, unfortunately, contained a mistake. Table 1 was published with the wrong version.

The authors very much regret their error.

The following is the updated version of Table 1.

The online version of the original article can be found at http://dx.doi.org/10.1007/s00428-011-1182-y.

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Table 1 Medical diseases affecting the kidney parenchyma, with histological vacuolization of cells [8–10]

Disease	Podocytes	Mesangial cells	Glomerular capillary endothelium	Proximal tubular cells	Distal tubular cells	Interstitial cells	Vascular smooth-muscle cells	Vascular endothelium
Aspartylglycosaminuria	+							
Fabry disease	+	+	+		+	+	+	+
Fucosidosis	+							
Gaucher disease						+		
Glycogen storage disease type I				+	+			
GM1 gangliosidosis	+	+						
Hurler's disease	+			+				
I-cell disease	+			+				
LCAT deficiency		+	+			+	+	+
Lipoprotein glomerulopathy						+		
Mannosidosis	+							
Metachromatic leukodystrophy				+	+			
Nephrosialidosis	+			+	+	+		+
Neuronal ceroid lipofuscinosis	+		+	+	+			
Niemann-Pick disease	+		+	+	+	+		
Sandhoff disease	+			+				
Type III hyperlipoproteinemia		+						
Alport disease						+		
Nephrotic range proteinuria	+					+		
Minimal change disease				+	+	+		

GM1: monosialotetrahexosylganglioside. LCAT: Lecithin:cholesterol acyltransferase. "+" vacuolization present

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ORIGINAL ARTICLE

Immunohistochemical diagnosis of Fabry nephropathy and localisation of globotriaosylceramide deposits in paraffin-embedded kidney tissue sections

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Abstract Fabry disease (FD) is a rare X-linked lysosomal storage disorder of glycosphingolipids, mostly globotriaosylceramide (Gb3). Proteinuric chronic kidney disease develops frequently, and recognition of Fabry nephropathy on a kidney biopsy may be the first clue to the underlying diagnosis. Since the accumulated glycosphingolipids are largely extracted by the paraffin-embedding procedure, the most characteristic feature of Fabry nephropathy on routine light microscopy (LM) is nonspecific cell vacuolization. To test whether residual Gb3 in kidney tissue might be exploited for the specific diagnosis of Fabry nephropathy, paraffin-embedded kidney biopsies of nine FD patients (one boy, four men, four women) and of a female carrier of a mild genetic mutation, with no evidence of Fabry nephropathy, were immunostained with an anti-Gb3 antibody. The adult biopsies were additionally co-stained with a lysosomal marker (anti-lysosomal-associated membrane protein 2 (anti-LAMP2) antibody). The distribution of Gb3 deposits was scored per cell type and compared to the histological scorings of glycosphingolipid inclusions on semi-thin sections. FD patients had residual Gb3 in all types of glomerular, tubular, interstitial and vascular kidney cells. The highest expression of LAMP2 was seen in tubular cells, but there were no meaningful associations between LAMP2 expression and prevalence of Gb3 deposits on different kidney cell types. The histological scorings of glycosphingolipid inclusions were relatively higher than the corresponding immunohistochemical scorings of Gb3 deposits. In the mildly affected female, Gb3 expression was limited to tubular cells, a pattern similar to controls. Gb3 immunostaining allows the specific diagnosis of Fabry nephropathy even in kidney biopsies routinely processed for LM.

Keywords Fabry disease · Kidney biopsy · Immunohistochemistry · Globotriaosylceramide (Gb3) · Lysosomal-associated membrane protein 2 (LAMP2)

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Introduction

Fabry disease (FD) is an X-linked storage disorder of neutral glycosphingolipids (GSL) caused by deficient activity of the lysosomal enzyme α-galactosidase [1, 2]. The pathogenic basis of FD is continuous accumulation of, in particular, globotriaosylceramide (Gb3), but the mechanisms underlying tissue injury and disease progression are still incompletely

understood. Chronic kidney disease (CKD), cardiomyopathy and stroke are major causes of morbidity and early mortality in affected adults [1, 2]. Although the phenotypic expression in females is often less severe and more variable, up to two thirds of the heterozygotes ultimately become symptomatic and have major organ involvement [2, 3]. The diagnosis is often missed or delayed, particularly if early-onset neuropathic and/or cutaneous manifestations, or a positive family history, are lacking [2].

Identification of GSL deposits in affected organs by light microscopy (LM) [4] or electron microscopy (EM) [5, 6] has been regarded as an ancillary diagnostic method of FD, particularly useful in patients with renal involvement [6]. However, as GSL are almost entirely extracted by non-polar solvents (e.g., ethanol, xylene) used in the standard paraffin-embedding procedure [7], cellular vacuolization remains as the most distinctive LM feature of FD. Vacuolization of kidney cells is also a non-specific feature of several other disorders affecting the kidneys (Table 1), some of them more prevalent than FD [8–10]. Staining of formalin-fixed paraffin-embedded (FFPE) LM specimens with lipid-soluble dyes or periodic acid-Schiff (PAS) is also negative [6, 7]. Frozen sections allow the widest range of histochemical methods available for GSL identification to be carried out [7] but freezing leads to loss of fine cellular details and staining reactions are not specific for Gb3. Thus, even though the lamellate, myelin-like ultrastructure of Gb3 inclusions is not pathognomonic, EM is the most reliable approach to identify GSL deposits in Fabry patients' tissues including the kidneys [1, 11]. On semi-thin (ST) toluidine blue- or methylene blue-stained EM scout sections evaluated by LM, GSL deposits are easily identified as dark blue inclusions [12]. However, freezing of tissue samples and/or processing for EM are often not routinely performed, and the diagnosis of FD can easily be missed on standard LM assessment of kidney biopsies.

Highly specific anti-Gb3 monoclonal antibodies [13] have been employed to confirm Gb3 accumulation in the kidney biopsy of a female with FD, using an indirect immunofluorescence technique in frozen sections [14], and to detect Gb3 in FFPE sections of the kidney and other organ samples collected at autopsy of a classically affected Fabry male [15]. The latter study provided robust evidence that a

Table 1 Medical diseases affecting the kidney parenchyma, with histological vacuolization of cells [8–10]

Disease	Podocytes	Mesangial cells	Glomerular capillary endothelium	Proximal tubular cells	Distal tubular cells	Interstitial cells	Vascular smooth muscle cells	Vascular endothelium
Aspartylglycosaminuria	±							
Fabry disease	±	±	±		±	±	±	±
Fucosidosis	±							
Gaucher disease						±		
Glycogen storage disease type I				±	±			
GM1 gangliosidosis	±	±						
Hurler's disease	±			±				
I-cell disease	±			±				
LCAT deficiency		±	±			±	±	±
Lipoprotein glomerulopathy						±		
Mannosidosis	±							
Metachromatic leukodystrophy				±	±			
Nephrosialidosis	±			±	±	±		±
Neuronal ceroid lipofuscinosis	±		±	±	±			
Niemann–Pick disease	±		±	±	±	±		
Sandhoff disease	±			±				
Type III hyperlipoproteinemia		±						
Alport disease						±		
Nephrotic range proteinuria	±					±		
Minimal change disease				±	±	±		

GM1 monosialotetrahexosylganglioside, *LCAT* lecithin/cholesterol acyltransferase, + vacuolization present

significant amount of Gb3, or parts thereof containing the antigen specificity recognized by the monoclonal antibodies, remained within cells even after standard tissue processing for LM.

We aimed to assess whether these monoclonal antibodies could be effectively used for the immunohistochemical identification of Gb3 in archive FFPE kidney biopsies of Fabry patients, as a routine diagnostic tool for Fabry nephropathy. Further goals were to estimate the relative abundance of lysosomes in different kidney cell types of adult Fabry patients, using an anti-lysosomal-associated membrane protein 2 (anti-LAMP2) antibody as lysosomal marker and to analyse whether Gb3 deposits always colocalised with LAMP2.

Subjects, materials and methods

The protocol of this study was approved by the institutional Health Ethics Board.

Patients and biopsies

All kidney biopsies of patients diagnosed with FD processed at the Pathology Department of a large university hospital between January 1, 1977 and December 31, 2007 were identified using a computerised database search. Cases for whom paraffin blocks were still available in archive were selected for this study. The original haematoxylin–eosin stained microscope slides, EM photomicrographs and ST (1 μm) scout sections were reviewed by the same pathologist (CV) highly experienced in the kidney pathology of FD.

Relevant clinical details and laboratory data were retrieved from the patient's hospital records. In all cases, the diagnosis of FD had been established by the demonstration of deficient leukocyte or plasma α-galactosidase activity and/or identification of a mutation of the α-galactosidase gene (*GLA*).

Immunohistochemical protocols

Double immunohistochemical staining was performed on 10% buffered FFPE 2-μm-thick sections, newly prepared from the archive paraffin blocks. An endomyocardial biopsy from an affected male with cardiac involvement was used as positive control. Kidney tissue samples of two adult males without evidence of kidney disease were used as tissue negative controls. Negative control sections were additionally prepared by replacing the primary antibodies in the immunohistochemical protocol with normal rabbit immunoglobulin (negative control rabbit immunoglobulin fraction, 20 mg/ml; Dako, Denmark). All immunohistochemical procedures were carried out at room temperature. Commercial reagents and reaction kits were used according to the manufacturer's instructions.

The microscope slides were initially deparaffinized in xylene, rehydrated through an ethanol gradient and washed for 20 min in phosphate-buffered saline (PBS). Endogenous peroxidase activity was quenched by 3% hydrogen peroxide in methanol for 30 min. Inhibition of tissue non-specific epitopes was achieved by incubating the slides for 5 min with a blocking solution (UltraVision Block; Lab Vision Corp., Fremont, CA, USA).

Double immunohistochemical detection of Gb3 and LAMP2 in kidney biopsies of adults

The murine anti-Gb3 primary antibody (anti-Gb3(CD77), IgG, 1 mg/ml, clone BGR23; Seikagaku, Tokyo, Japan) was diluted to 1:100 with an antibody diluent (Lab Vision Antibody Diluent; Lab Vision Corp.), applied to the slides and let to incubate overnight in a humidity control chamber. Detection of bound primary antibody was achieved with a commercial kit (UltraVision Detection System Anti-Polyvalent, Alk-Phos/Fast Red; Lab Vision Corp.). In short, the slides were washed three times for 5 min in PBS, incubated with the biotinylated goat anti-polyvalent secondary antibody for 15 min and rinsed twice for 5 min in PBS. The streptavidin–alkaline phosphatase complex was then applied and incubated for 15 min. After rinsing twice for 5 min in PBS, the slides were incubated for 90 min with the chromogen/substrate solution (Fast Red/Naphthol Phosphate Substrate).

LAMP2 immunoreactivity was identified with a commercial kit (DAB + Chromogen//REAL™ EnVision™ Detection System, Peroxidase/DAB+, Rabbit/Mouse; Dako). The slides were initially washed in PBS for 10 min and then let to incubate for 120 min with a mouse monoclonal anti-LAMP2 antibody (Anti-LAMP2(H4B4), IgG, 200 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA, USA), diluted to 1:100 with an antibody diluent (Lab Vision Antibody Diluent). Following 30 min incubation with the detection reagent (REAL™ EnVision™/HRP, Rabbit/Mouse; Dako), the slides were washed in PBS for 10 min and incubated for 1.5 min with a diaminobenzidine chromogen (REAL™ DAB + Chromogen; Dako) in a buffered hydrogen peroxide solution (REAL™ Substrate Buffer; Dako). After fast washing in distilled water and PBS, the slides were lightly counterstained with Mayer's haematoxylin and mounted with 10% buffered glycerine in PBS. Gb3 immunoreactivity was identified by a bright red precipitate, and LAMP2 immunoreactivity was identified by a crisp brown precipitate.

Immunohistochemical detection of Gb3 in the kidney biopsy of a child

Immunohistochemical detection of Gb3 was also performed in the kidney biopsy of a young boy but using a higher dilution

Table 2 Demographic, clinical and laboratory data of patients with Fabry disease at the time of kidney biopsy

Case ID	Gender	Age	*GLA* mutation	αGal(L)	CKD stage	uProt	Biopsy indication
H07-13445	M	7	p.C94S	0.54	1	–	Baseline assessment pre-ERT
H04-4366	M	19	c.305_749[2]dupEx2_Ex4	0.07	1	+++	Proteinuria
H05-9169	M	20	c.305_749[2]dupEx2_Ex4	0.59	1	+++	Baseline assessment pre-ERT
H05-6791	M	21	p.239ΔI	0.38	1	+	Baseline assessment pre-ERT
H05-7176	M	40	p.153ΔD	0.25	2	+++	Baseline assessment pre-ERT
H99-4183	F	32	p.R220X	20	3	++++	Proteinuria (nephrotic)
H05-9180	F	42	c.305_749[2]dupEx2_Ex4	18	1	+	Baseline assessment
H03-3708	F	45	p.R220X	18	2	++	Baseline assessment pre-ERT
H05-6994	F	50	p.239ΔI	20	2	+	Baseline assessment pre-ERT
H05-9092[a]	F	29	p.R118C[a]	44	1	+++	Proteinuria

Age in full years.

M male, *F* female, *GLA* α-galactosidase gene, α *Gal (L)*α-galactosidase residual activity in leukocytes (Normal laboratory range 36-80 nMol/h/mg), *CKD* chronic kidney disease (Stages according to estimated glomerular filtration rate (eGFR), based on serum creatinine levels, race, gender and age: "1" = eGFR ≥ 90 ml/min/1.73 m^2 , "2"=eGFR 89–60 ml/min/1.73 m^2 and "3"=eGFR 59–30 ml/min/1.73 m^2), *uProt* proteinuria (Highest level recorded before the kidney biopsy, expressed in milligrams per day: "–" <150 mg/day, "+" 150–300 mg/day, "++" 300–1,000 mg/day, "+++" 1,000–3,500 mg/day, "++++" >3,500 mg/day), *ERT* enzyme replacement therapy

[a] Urine Gb3 levels were found to be consistently within the normal range in this female; hemizygous males for the *GLA* p.R118C mutation excrete normal levels of lysoGb3 in the urine

of the primary antibody (1:300) and the DAB + Chromogen// REAL™ EnVision™ Detection System, Peroxidase/DAB+, Rabbit/Mouse commercial kit to detect bound antibody. After washing three times for 2 min in PBS, the slides were incubated with the primary antibody overnight, in a humidity control chamber. Following washing three times for 5 min in PBS, the slides were incubated with the Dako REAL™ EnVision™/HRP, Rabbit/Mouse detection reagent for 30 min and then washed again twice in PBS for 5 min. Finally, the slides were incubated with the buffered hydrogen peroxide diaminobenzidine chromogen solution for 5 min (REAL™ DAB + Chromogen/REAL™ Substrate Buffer). After washing 10 min in PBS, the slides were counterstained with Mayer's haematoxylin and then mounted with a permanent mounting medium (SUB-XTM Mounting Medium; Surgipath Europe, Peterborough, UK). Gb3 immunoreactivity was identified by a crisp brown precipitate.

Gb3 and LAMP2 immunostaining assessment

The quality of the double immunostaining technique was first checked on the positive and the negative controls. Gb3 immunoreactivity was semi-quantitatively graded according to the tertiles of the proportion of cells of each type that showed positive reactions. LAMP2 immunoreactivity was semi-quantitatively graded according to the relative average intensity staining of each cell type.

Toluidine blue- or methylene blue-stained, plastic-embedded EM scout sections were used to semi-quantify the GSL inclusions in the different kidney cell types, as published elsewhere [16]. The semi-quantification of GSL inclusions in the EM scout sections was correlated to the Gb3 immunostaining data.

Statistics

Ages at the time of kidney biopsy are presented as mean± standard deviation and were compared between genders with the unpaired Student's *t* test. Spearman correlation was used to measure the association between the Gb3 and LAMP2 immunoreactivity scores and selected demographic, clinical and histological parameters. Statistical significance was assumed for test p values <0.05.

Results

Patients and kidney biopsy material

A total of ten paraffin blocks of kidney biopsies from ten patients (five males, mean age 21.4±11.8 years; five females, mean age 39.6±8.8 years; p=0.02) were available for the immunohistochemical studies. The biopsies had been performed as baseline assessment for enzyme replacement therapy (ERT; n=7, including a 7-year-old boy) or as diagnostic workup for proteinuria (n=3). Two further cases were excluded because the archive paraffin block could not be located or did not contain enough tissue. Demographic and

Table 3 Semi-quantification of immunohistochemical Gb3 and LAMP2 staining and comparison of the semi-quantification of Gb3 immunostaining with the semi-quantification of Gb3 inclusions in semi-thin sections, according to their prevalence per kidney cell type

Case ID	Glomerular cells				Tubular cells		Interstitial cells		Peritubular capillary cells		Arterial and arteriolar vessels			
	Podocytes		Parietal cells								Endothelial cells		Smooth muscle cells	
H07-13445	+	ND	++	ND	+	ND	+	ND	++	ND	++	ND	+	ND
	+	+++	++	++ / +++	+	++	+	+/++	++	+++	++	+++	+	+++
H04-4366	+++	++	+++	+++	++	+++	++	++	++	++	+	+	+	+
	+++	+++	+++	+++	++	++	++	+++	++	+++	+	+++	+	+++
H05-9169	++	+	+	++	+/++	+++	++	+	++	-	NS	NS	NS	NS
	++	+++	+	+++	+/++	+	++	+++	++	+++	NS	+++	NS	+++
H05-6791	+++	++	+++	++	+	+++	++	-	++	-	++	+	+	-
	+++	+++	+++	+++	+	+++	++	+	++	++	++	+	+	+++
H05-7176	+	+	++	++	+/++	++/+++	+/++	+	+/++	+	+	+	++	+
	+	+++	++	+++	+/++	+++	+/++	+++	+/++	+	+	+++	++	+++
H99-4183	+	-	++	+/++	+/++	++	+	-	+	-	+	-	+	-
	+	++	++	+++	+/++	+++	+	++	+	++	+	++	+	+++
H05-9180	+	+	+	+	+	+++	+	+	+	+	-	+	+	+
	+	+	+	+++	+	+	+	-	+	+	-	-	+	++
H03-3708	NS	NS	NS	NS	+	+++	+	-	+	+	NS	NS	NS	NS
	NS	++	NS	++	+	+	+	-	+	-	NS	+	NS	+++
H05-6994	NS	NS	NS	NS	+	+++	+	-	-	-	+	-	+	-
	NS	+	NS	+++	+	+	+	-	-	-	+	NS	+	+
H05-9092	-	+/++	-	++	+	+++	-	+/++	-	+/++	-	+/++	-	+
	-	-	-	-	+	-	-	-	-	-	-	-	-	-

Left upper quadrant: semi-quantification of immunohistochemical Gb3 staining. Immunostaining was scored semi-quantitatively according to the proportion of affected cells of each type: "–" no staining, "+" staining of <1/3 of cells, "++" staining of 1/3–2/3 of cells and "+++" staining of >2/3 of cells. "Controls" (autopsy specimens) were all tissue negative, except for "+" for tubular cells.

Right upper quadrant: semi-quantification of immunohistochemical lysosomal-associated membrane protein 2. Immunostaining was scored semi-quantitatively according to the relative average of the staining intensity of cells of each type: "–" absent, "+" weak, "++" moderate and "+++" strong.

Left and right lower quadrants: comparison of the semi-quantification of Gb3 immunostaining with the semi-quantification of glycosphingolipid inclusions in semi-thin cuts, according to their prevalence per kidney cell type. Left: paraffin-embedded LM sections, immunohistochemically stained with an anti-Gb3 monoclonal antibody. Right: semi-thin plastic embedded sections, stained with toluidine blue or methylene blue. Gb3 staining and GSL deposits scored semi-quantitatively according to the proportion of affected cells of each type: "–" no staining, "+" staining of <1/3 of cells, "++" staining of 1/3–2/3 of cells, "+++" staining of >2/3 of cells

NS cell type not sampled in the biopsy section, *ND* not done

diagnostic data of the patient cohort are summarised in Table 2. One female who had a *GLA* mutation (p.R118C) associated with a mild clinical phenotype had no histopathological or biochemical evidence of Fabry nephropathy. All others had severe FD mutations as evaluated by disease severity and/or minimal residual α-galactosidase activity in the males [1, 2].

Gb3 immunoreactivity

Table 3 and Fig. 1 summarise the immunohistochemical features and semi-quantification of Gb3 deposits in study biopsies, as well as in autopsy control specimens. In three cases, there were no glomeruli and/or arterial and arteriolar vessels available in the biopsy section for scoring. The carrier of mutation p.R118C showed Gb3 immunoreactivity only in a few tubular cells, a pattern similar to that observed in autopsy control specimens.

Glomerular cells All biopsies of patients carrying severe FD mutations which had glomeruli available for scoring (n=7; five males, including the boy) showed Gb3 deposits in podocytes and in parietal epithelial cells (Fig. 1a). In these males, there were no significant correlations between the extent of Gb3 immunoreactivity and either age, degree of proteinuria or CKD stage.

Tubular cells All patients showed Gb3 immunoreactivity in the tubular epithelium with a rather variable pattern of Gb3 immunoreactivity, either involvement of all cells in some

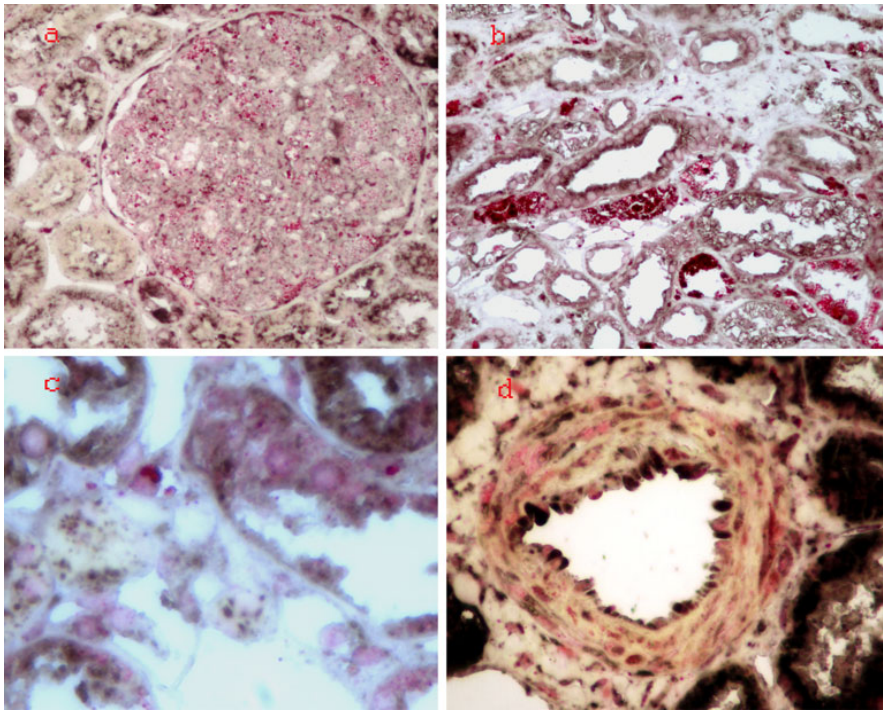

Fig. 1 Gb3 immunoreactivity on different kidney cell types. Gb3 (bright red precipitate)/LAMP2 (crisp brown precipitate) double immunohistochemistry. Magnification × 200 (**a**, **b**), ×600 (**c**), ×400 (**d**). **a** Glomerulus showing extensive Gb3 immunoreactivity, particularly in podocytes and parietal epithelial cells. Some interstitial cells are also stained. **b** Tubulointerstitial area showing variable degrees of Gb3 immunoreactivity among different tubules: In a few tubules, all the epithelial cells have Gb3 deposits while others have faint or no visible Gb3 immunoreactivity. Some of the peritubular capillaries are dilated and have Gb3 deposits in the endothelium. **c** Higher magnification of an interstitial area showing strong Gb3 immunoreactivity within an endothelial cell of a peritubular capillary. **d** Arterial vessel showing Gb3 immunoreactivity both in endothelial and smooth muscle cells

tubules or only of a few cells in other tubules (Fig. 1b). Tubular Gb3 deposits were relatively more abundant in patients with moderate/severe proteinuria (three males, one female). In the remainder, tubular Gb3 immunoreactivity was present only in a minority of cells, which was not different from the autopsy control specimens or the carrier of mutation p.R118C.

Interstitial cells Gb3 deposits were found in at least a few interstitial cells in all patients carrying severe FD mutations (Fig. 1a) but not in autopsy control specimens or in the carrier of mutation p.R118C.

Peritubular capillary cells With the notable exception of the 50-year-old woman, all patients carrying severe FD mutations showed Gb3 deposits in at least a few peritubular capillary cells (Fig. 1c). Such deposits were not found in the autopsy control specimens or in the carrier of mutation p.R118C.

Arterial and/or arteriolar vessels All evaluable biopsies of patients carrying severe FD mutations (n=7; four males, including the boy) showed Gb3 immunoreactivity in endothelial and/or smooth muscle cells (Fig. 1d). In one of the females, Gb3 immunoreactivity was identified only in smooth muscle cells. No Gb3 immunoreactivity was observed in these vessels in autopsy control specimens or in the carrier of mutation p.R118C.

LAMP2 immunoreactivity

Table 3 summarises the LAMP2 immunoreactivity data per kidney cell type in the adult patients (n=8; four males) carrying severe FD mutations and in the heterozygous carrier of mutation p.R118C. Linear (membrane-like) and punctiform LAMP2 immunoreactivity was identified in all types of kidney cells (Fig. 2a–d). Expression varied from high in tubular cells, variably in apical (Fig. 2b) or basal, or in both locations; intermediate in parietal glomerular epithelial cells; intermediate to low in podocytes (Fig. 2a) or low or absent in interstitial, peritubular capillary endothelial, arterial/arteriolar endothelial and smooth muscle cells (Fig. 2a–c). No meaningful associations could be identified between LAMP2 immunoreactivity and any demographic or clinical variables.

Relation between immunohistochemical detection of Gb3 and LAMP2

In all kidney cell compartments, Gb3 deposits were visible either co-localising with or independent of LAMP2 immunoreactivity (Figs. 2d and 3a–d). Three different (co-)

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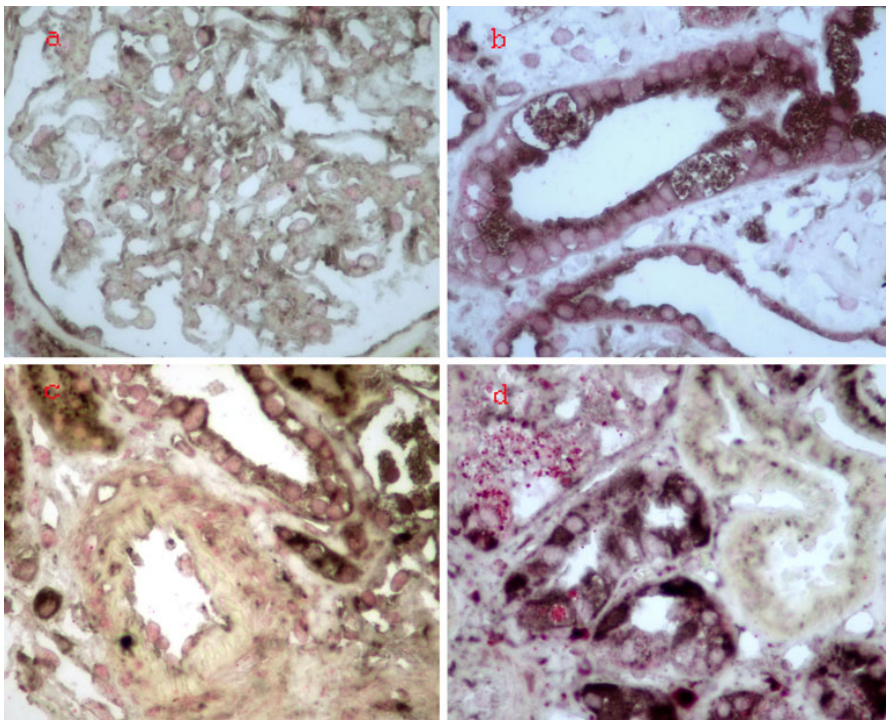

Fig. 2 LAMP2 immunoreactivity on different kidney cell types. Gb3 (bright red precipitate)/LAMP2 (crisp brown precipitate) double immunohistochemistry. Magnification × 400. **a** Glomerulus showing diffuse LAMP2 immunoreactivity in parietal epithelial cells. In podocytes, the distribution of LAMP2 immunoreactivity is limited to a few cells. **b** Tubules showing LAMP2 immunoreactivity in most cells, localised predominantly to the apical pole of the epithelium. Strong LAMP2 immunoreactivity is also seen in the endothelial cells of some of the peritubular capillaries. **c** Arteriole showing weak LAMP2 immunoreactivity in a few reactive endothelial cells. **d** Gb3 immunoreactivity in tubular cells and in podocytes. In one of the tubular epithelial cells, Gb3 deposits appear to be aggregated within a vacuole and surrounded by linear LAMP2 immunoreactivity. In podocytes (*upper left corner*) Gb3 deposits seem to be freely distributed within the cytoplasm, not co-localising with LAMP2 immunoreactivity

staining patterns could be identified, which were particularly evident in podocytes and tubular cells: (1) granular Gb3 staining surrounded by linear LAMP2 staining (Fig. 3a); (2) both Gb3 and LAMP2 staining, either in scattered or diffuse patterns, but topographically independent within the same cells (Fig. 3b) and (3) isolated Gb3 staining (Fig. 3c). No meaningful associations could be identified between the expression of LAMP2 immunoreactivity and the density of Gb3 deposits on the different kidney cell types.

Immunohistochemical detection of Gb3 as compared to histological identification of GSL deposits on semi-thin sections

We have compared the immunohistochemical semi-quantification of Gb3 per kidney cell type to the histological assessment of GSL inclusions on toluidine blue- or methylene blue-stained plastic-embedded EM scout sections (Table 3). In general, the scorings of the prevalence per different kidney cell types and/or the amount of GSL deposits identified on the ST sections were higher than the corresponding scorings of Gb3 immunoreactivity. These differences were particularly obvious on the glomerular epithelial and on the arterial/arteriolar vascular endothelial and smooth muscle cells (Fig. 4a–d). In three females carrying severe FD mutations, the immunohistochemical method allowed the identification of cytoplasmic Gb3 accumulation on kidney cells where GSL inclusions were not seen on the ST sections, specifically on interstitial cells and endothelial cells of peritubular capillaries. The female carrier of mutation p.R118C had no visible GSL deposits on the ST sections but showed Gb3 immunoreactivity on a small proportion of tubular cells. Gb3 was immunohistochemically detected on all kidney cell types that had GSL inclusions visible on the semi-thin sections.

Discussion

The most characteristic finding on routine LM of kidney biopsies of Fabry patients is the vacuolization of affected cells, especially of podocytes, parietal epithelial cells of Bowman's capsule, distal tubular and smooth muscle cells

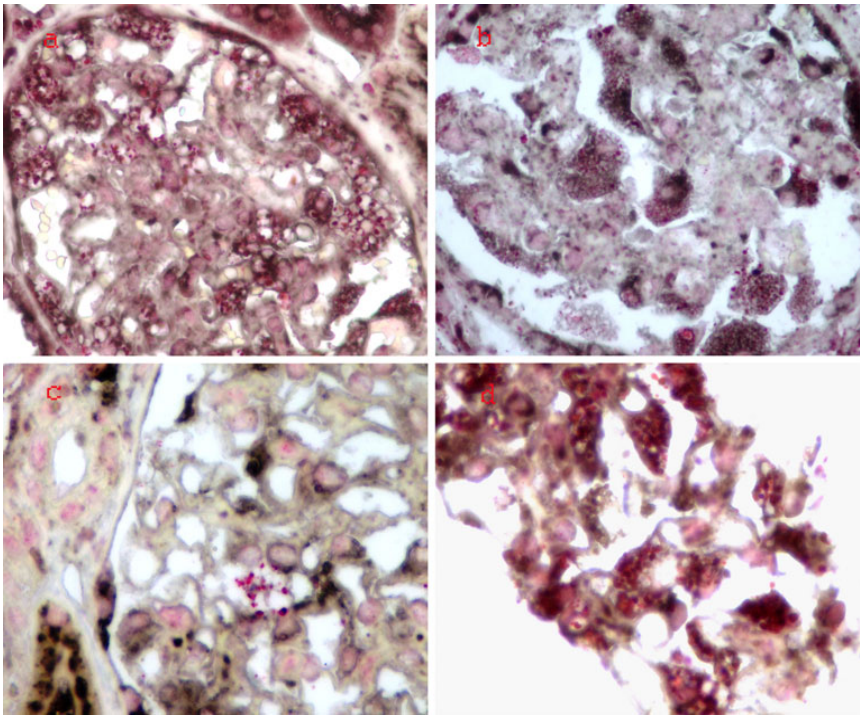

Fig. 3 Topographical relations between Gb3 and LAMP2 immunostaining. Gb3 (bright red precipitate)/LAMP2 (crisp brown precipitate) double immunohistochemistry. Magnification × 400 (**a**, **b**), ×600 (**c**, **d**). **a** In this glomerulus, granular Gb3 deposits within podocyte vacuoles are surrounded by linear LAMP2 staining ("fried egg" or "owl eye" pattern). **b** Gb3 immunostaining within podocytes additionally appears as granular or punctiform cytoplasmic deposits topographically unrelated to LAMP2 immunostaining. **c** Glomerulus showing granular Gb3 deposits in rare podocytes with no evidence of LAMP2 immunoreactivity. **d** Gb3 deposits seemingly breaking into the glomerular urinary space

[6, 16–20]. The vacuoles are histological artifacts resulting from removal of GSL deposits during clearing and paraffin embedding of the tissue. Cytoplasmic vacuolization is a non-specific feature of several other, often more prevalent kidney diseases [8–10], and although the pattern of cellular involvement may be suggestive of FD, the diagnosis of Fabry nephropathy can be overlooked on routine LM evaluation.

Gb3 and galabiosylceramide, the main GSL that accumulate in the kidneys of Fabry patients [21], are amphipathic molecules. The ceramide moiety is lipophilic and soluble in non-polar solvents, while the oligosaccharide chains are hydrophilic and may remain within the vacuoles in tissue sections routinely processed for LM. These residual carbohydrates can be non-specifically identified as argyrophilic granules in methenamine silver-stained [18, 22] and in PAS-stained (Valbuena, personal observation) [1] FFPE tissue.

Using murine monoclonal antibodies [13] formerly employed for immunohistochemical staining of Gb3 in FFPE skin biopsies [23], Askari et al. [15] have demonstrated the presence of residual Gb3 immunoreactivity in glomeruli, tubules, interstitial cells and blood vessels in FFPE kidney tissue sections of a deceased adult male with FD. With the same anti-Gb3 monoclonal antibody as primary antibody, we have now shown that immunostaining of Gb3 using standard immunohistochemical procedures can be helpful for the specific diagnosis of Fabry nephropathy, even in archive kidney tissue specimens routinely processed for LM study.

Gb3 and galabiosylceramide are cell surface receptors for the B-subunit of Shiga-toxins (Verotoxins) [24] and are both intrinsically expressed in the human kidney with the highest expression levels observed in cortical tubular epithelial cells, but also occurring in podocytes, mesangial cells and endothelium of renal vessels, including the microvasculature [25–28]. This constitutive expression of Gb3 in the kidney could hinder the specificity of immunostaining for the diagnosis of Fabry nephropathy. However, the staining of Gb3 in kidney tissue is temperature sensitive, becoming erratic, weak and indistinct unless the samples are kept continuously refrigerated (≤4°C). Indeed, kidney sections stained at room temperature had ~90% reduction in staining intensity [25]. Therefore, our immunohistochemical protocol was carried out at room temperature. In such conditions, ≤1% of tubular cortical cells of tissue negative control samples displayed faint Gb3 immunostaining and none of the other parenchymatous kidney cell types bound any anti-Gb3 antibodies at all. An identical Gb3 immunostaining pattern was observed in the female carrying the *GLA* p.R118C mutation.

In males, Gb3 immunostaining was seen in all cells types sampled in each biopsy specimen (Table 3), glomerular epithelial cells being most extensively involved. In comparison to males, females had smaller proportions of cells of each type that stained with the anti-Gb3 monoclonal antibody, which is consistent with the expected effect of X-chromosome inactivation [29].

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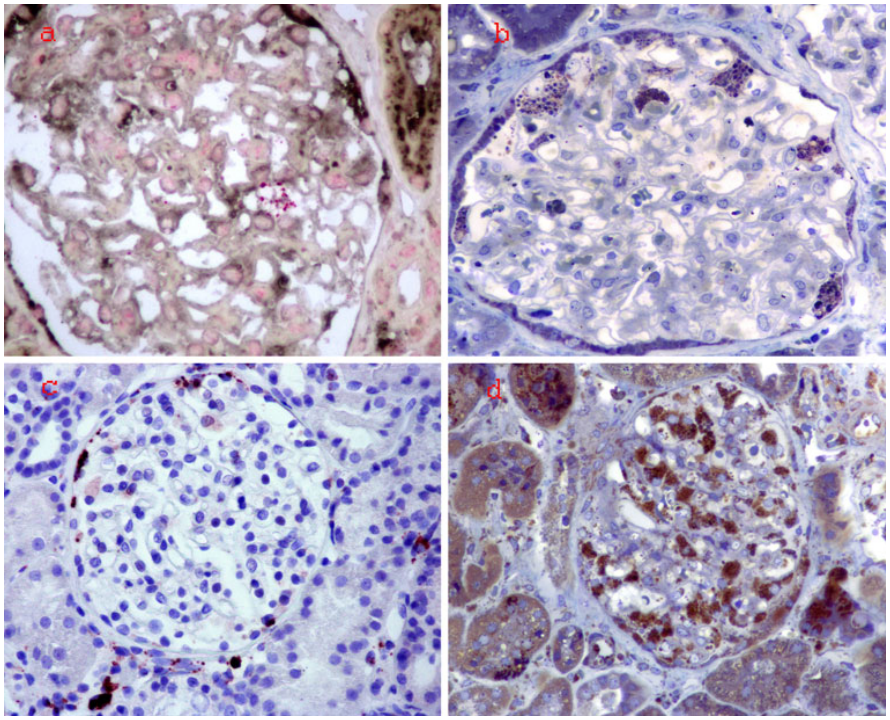

Fig. 4 Comparison of Gb3 immunostaining with glycosphigolipid histological identification in semi-thin sections. Gb3 (bright red precipitate)/LAMP2 (crisp brown precipitate) double immunohistochemistry (**a**); toluidine blue-stained semi-thin section (**b**); Gb3 (crisp brown precipitate) immunohistochemistry (**c**); toluidine blue-stained semi-thin section (**d**). Magnification×400 (**a**, **b**), ×600 (**c**, **d**). Case H05-9180. **a** Glomerulus showing granular Gb3 immunoreactivity in the cytoplasm of a single podocyte. **b** Glomerulus showing cytoplasmic glycosphingolipid inclusions in all parietal epithelial cells and in several podocytes. Case H07-13445. **c** Glomerulus showing strong Gb3 immunoreactivity in some of the parietal epithelial cells and weak staining of a few podocytes. Gb3 deposits are also visible in some interstitial cells but none of the tubular cells is Gb3 immunoreactive. **d** Large amounts of glycosphingolipid inclusions are present in most of the glomerular parietal epithelial cells and podocytes and in many interstitial and peritubular capillary cells. Glycosphingolipids are also visible in endothelial and smooth muscle cells of an arteriole. Tubular cells contain significantly lesser amounts of glycosphingolipid inclusions

With the exception of the interstitial and peritubular capillary endothelial cells, the extent of involvement of each kidney cell type, whether evaluated immunohistochemically by Gb3 staining or in EM scout sections by GSL inclusions, was closely correlated (Table 3). However, the prevalence of affected cells estimated by immunohistochemistry was lower for most cell types, particularly glomerular epithelial cells and vascular endothelial and smooth muscle cells of arteries and arterioles. Apart from removal of GSL by the histological procedure, an additional explanation for the difference in relative methodological sensitivity might be that GSL inclusions identified in ST sections may also contain galabiosylceramide, which is not recognized by the anti-Gb3 monoclonal antibody [13]. In females, the immunohistochemical approach was apparently more sensitive to detect Gb3 accumulation in interstitial cells (Table 3), but an alternative explanation is the tissue patching effect of X-chromosome inactivation [30].

We performed double Gb3 and LAMP2 immunostaining to analyse whether Gb3 could also be demonstrated in extralysosomal locations in kidney cells of Fabry patients. Testing that hypothesis was the aim of a previous study [15] that used immunofluorescence to identify the subcellular localisation of Gb3 in Fabry kidney cells; Gb3 was found to be widely distributed in other cellular structures, co-localising with lysosomal, endoplasmic reticulum and nuclear markers [15]. That study used LAMP1 as the lysosomal marker, and incubation with the anti-LAMP1 primary antibody was the first step in the immunofluorescence protocol. Although our finding that Gb3 could be detected in kidney cells not co-localising with LAMP2 is in agreement with their results, the same criticisms made to that study [31] also apply to our histological protocol.

LAMP2 immunostaining allowed an estimation of the relative abundance of lysosomes in each kidney cell type. The largest amounts of anti-LAMP2 antibody binding were

seen in the tubular epithelial cells followed by the parietal glomerular epithelial cells and podocytes. The observation of Gb3 deposits scattered in the cytoplasm of glomerular epithelial cells not co-localising with the lysosomal marker is in agreement with the postulate that rupture of Gb3 overloaded lysosomes into the cytoplasm might be involved in the pathogenesis of glomerular sclerotic lesions of Fabry nephropathy [16]. Of note, peritubular capillary cells and vascular endothelial and smooth muscle cells, which are thought to be critically involved in the pathogenesis of renal complications of FD [6, 17, 20], at most stained faintly with the lysosomal marker. The double immunostaining resulted in loss of cellular contours and the different types of tubular epithelial, mesangial and glomerular capillary endothelial cells could not be discriminated in some cases. This limits its usefulness in diagnostic pathology and research.

In conclusion, immunohistochemical staining of Gb3 is a relatively simple and specific diagnostic method for FD in the routine LM study of kidney biopsies, which can be used even for retrospective diagnosis of Fabry nephropathy in archive paraffin blocks, particularly when cell vacuolization is a prominent finding. Furthermore, it allows the exclusion of the diagnosis of Fabry nephropathy in patients carrying *GLA* mutations associated with high residual enzyme activity.

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SPLENOMEGALY, HYPERSPLENISM AND PERIPHEAL BLOOD CYTOPAENIAS IN PATIENTS WITH CLASSICAL ANDERSON-FABRY DISEASE

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ERRATUM

Erratum to: Splenomegaly, hypersplenism and peripheral blood cytopaenias in patients with classical Anderson–Fabry disease

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The original version of this article unfortunately contained a mistake. The reference citations in Table 5 are incorrect.

The following is the updated version of Table 5.

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Table 5 Summary of previous case reports of patients with Anderson-Fabry disease and symptomatic reticuloendothelial organ involvement or thrombocytopaenia

Ref. #	Organ enlargement				Thrombocytopaenia[a]	Additional notes
	Age	L	LN	S		
[6]	22		X		45.0×10^9/l	Platelet count normalised following splenic X-ray irradiation.
[6]	18		X		72.0×10^9/l	Platelet count normalised following splenic X-ray irradiation.
[7]	32	X		X	29.8×10^9/l	
[8]	30			X		Splenomegaly later confirmed at laparotomy for appendicitis and at autopsy [3].
[9]	28		X			LNBx showing lipid deposits in macrophages.
[9]	25		X			
[4, 32]	23		X	X		Surgical mesenteric LNBx and SBx showing vacuolated cells.
[10]	18		X			LNBx showing typical lamellated Gb3 deposits in EM examination.
[11, 33]	10	X	X	X		LNBx showing histiocytes with foamy cytoplasmic appearance.

Ref. # reference number. Age in years. *LN* lymph node; *L* liver; *S* spleen

[a] Platelet count, in patients with thrombocytopaenia."*X*" manifestation present. *Bx* biopsy. *EM* electron microscopy

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ORIGINAL ARTICLE

Splenomegaly, hypersplenism and peripheral blood cytopaenias in patients with classical Anderson–Fabry disease

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Abstract A 39-year-old male with classical Anderson–Fabry disease (AFD) and long-standing idiopathic splenomegaly, who had been on haemodialysis since the age of 24, was splenectomised for symptomatic pancytopaenia. Spleen enlargement was first noted at clinical presentation, at age 16, but despite thorough investigation its cause remained unclear. Anaemia, leukopaenia and thrombocytopaenia were first observed a few years thereafter, but well before the start of dialytic treatment. On gross pathological examination the spleen weighed 700 g and had a fibrocongestive appearance. Histologically, it showed expansion of the red pulp and decreased white pulp. Some histiocytes and many of the endothelial cells lining the sinusoids had vacuolated cytoplasm with argyrophilic material within, suggesting their involvement in the storage pathology of AFD. In a retrospective review of our cohort of patients with classical AFD ($n=10$), complete blood counts showing anaemia, leukopaenia or thrombocytopaenia were found in five, two and four patients, respectively, including a 6-year-old boy, whose spleen was also enlarged. Data from AFD international registries show that peripheral blood cytopaenias, particularly anaemia, are prevalent among these patients. Sinusoidal endothelial involvement resulting in compromise of splenic blood flow may be the cause of congestive splenomegaly and hypersplenism in classical AFD.

Keywords Anderson–Fabry disease · Splenomegaly · Hypersplenism · Thrombocytopaenia

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Introduction

Anderson–Fabry disease (AFD) is a rare X-linked inherited sphingolipidosis due to deficient activity of the lysosomal enzyme α-galactosidase (α-Gal) [1]. The α-Gal defect results in multi-systemic accumulation of neutral glycosphingolipids containing terminal α-galactosyl residues, mostly globotriaosylceramide (Gb3).

Classical AFD is clinically characterised at the early stages by acroparesthesias, decreased sweating and a distinctive skin rash of angiokeratomas, followed later by progressive chronic kidney disease (CKD), hypertrophic and/or ischaemic cardiac disease and premature cerebrovascular disease [1]. Involvement of endothelial, perithelial

and smooth-muscle cells of blood vessels, as well as of cardiomyocytes, dorsal root ganglia and cells of the autonomic nervous system are regarded as the major factors in the pathogenesis of the classical AFD phenotype [1].

Storage of Gb3 in cells of the reticuloendothelial system also occurs in AFD [1] and has been known since the original bone marrow aspirate and autopsy studies of patients with AFD demonstrated the presence of vacuolated or lipid-laden cells in the bone marrow, lymph nodes, spleen and liver [2, 3]. Data on the histopathology of the spleen is limited to a few early studies of autopsy specimens [2] and of biopsies taken at laparotomy [4].

In contrast to Gaucher disease—a sphingolipidosis in which glucosylceramide accumulates within macrophages, leading to massive hepatosplenomegaly and hypersplenism, often resulting in anaemia and thrombocytopaenia [5]—the reticuloendothelial involvement in patients with AFD is usually asymptomatic. Only a small number of cases have been reported who had enlargement of lymph nodes, spleen or liver as presenting sign(s) [3, 4, 6–11]. Liver enlargement, however, has been mostly regarded as a manifestation of congestive heart failure [3]. Anaemia is recognised as a common complication of AFD [3, 12], even in patients with normal renal function, and its pathogenesis has been ascribed to impaired renal function, heart failure, inflammation or to chronic bleeding [3, 12].

Herein, we report a classically affected AFD patient who had clinically evident reticuloendothelial involvement at diagnosis and describe the histopathological findings in his spleen, which had to be surgically removed 22 years later to treat symptomatic hypersplenism. In addition, a retrospective study of our series of patients with the classical AFD phenotype and analyses of the Fabry Registry database suggest that the prevalence of peripheral blood cytopaenias other than anaemia in AFD patients may be higher than previously recognised. Therefore, we propose that mild to moderate hypersplenism may be a relatively frequent subclinical manifestation of AFD.

Case report

At the age of 39 years, a male patient with the classical phenotype of AFD underwent splenectomy to treat symptomatic hypersplenism (see Table 1 for a summary of the patient's past clinical history). He had been for almost 15 years on maintenance haemodialysis and asymptomatic chronic Hepatitis B Virus (HBV) infection was known for more than 10 years. Although splenomegaly and hypersplenism had been recognised several years before reaching end-stage renal failure (ESRF), frequent epistaxis and recurrent cellulitis of the legs, along with platelet counts below 50×10^9/l and neutrophil counts below 1.0×10^9/l (see Table 2), eventually justified the surgical indication.

The patient was of slight build (height=1.52 m, "dry weight"=47.0 kg) and had the coarse facial features of AFD [3]. Blood pressure was normal without medication. Multiple 1.0–1.5 cm lymphadenopathies were palpable in

Table 1 Summary of the patient's clinical history

Age	Clinical events and laboratory data
10 y	No clinical evidence of disease on family screening, following the diagnosis of AFD in an older brother
16–17 y	Sought medical attention because of recurrent diarrhoea. Generalised lymphadenopathy, liver and spleen enlargement noted on physical examination. No evidence of a primary haematologic disorder
	Diagnosis of CKD and referral for further evaluation
17 y	Multiple angiokeratomas with the typical distribution of AFD noted on skin examination. A skin biopsy showed the typical histological features of AFD angiokeratomas
	Electron microscopy study of a cervical lymph node biopsy showed the distinctive lamellated appearance of Gb3 deposits within histiocytic cells
	CrCl=47.7 ml/min/1.73 m^2, sCr=1.7 mg/dl; urinalysis: trace proteinuria, normal sediment. Normocytic, normochromic anaemia (Hg=10.4 g/dl)
17–24 y	Progressive deterioration of renal function with proteinuria reaching nephrotic level (= 3.6 g/day).
	First notice of low WBC and platelet counts
24 y	ESRF and start of maintenance HD
25–28 y	Acute hepatitis B, progressing to asymptomatic chronic infection
29–38 y	Worsening of all blood cytopaenias with recurrent nose and gum bleeding
	No residual enzyme activity demonstrated on α-Gal leukocyte assay
	GLA gene mutation identified as p.C94S
39 y	Therapeutic splenectomy

y Age in full years, *AFD* Anderson–Fabry disease, *CKD* chronic kidney disease. *CrCl* creatinine clearance, *sCr* serum creatinine, *Hg* haemoglobin *WBC* white blood cell, *ESRF* end-stage renal failure, *HD* haemodialysis, *α-Gal* α-galactosidase, *GLA* α-galactosidase gene

Table 2 Evolution of the relevant haematological parameters along follow-up of the index patient

Clinical event	Date	Hg (g/dl)	WBC (× 10^9/l)	N (× 10^9/l)	P (× 10^9/l)
1st values in the nephrology clinic	Nov./1976	10.4[a]	6.4[a]	4.16[a]	150[a]
1st evidence of hypersplenism	Mar./1979	11.4[a]	4.8[a]	3.70[a]	72[a]
Lowest values observed before starting HD	1979–1983	9.8[a]	3.1[a]	2.17[a]	70[a]
Last values before the beginning of HD	Jun./1983	9.9[a]	4.0[b]	3.40[b]	120[a]
Lowest values observed on chronic HD	1983–1997	5.4[a]	1.3[b]	0.87[b]	19[a]
Last values prior to splenectomy	22/Feb./1998	9.1[a]	1.2[b]	0.72[b]	60[a]
2nd postoperative day	25/Feb./1998	10.9[a]	9.38[b]	8.37[b]	138[a]

Hg Haemoglobin, *WBC* white blood cells, *N* neutrophils; *P* platelets. *HD* haemodialysis
[a] Normal reference ranges, Hg=16±2 g/dl; WBC=5–10×10^9/l, N=3.0–5.8×10^9/l; P=290±150×10^9/l
[b] Normal reference ranges, WBC=7±3×10^9/l, N=2.0–7.5×10^9/l.

the neck, axillary and inguinal chains, the liver was palpable 2–3 cm below the right costal margin and the spleen was palpable 7–8 cm below the left costal margin. There were no clinical signs of portal hypertension and on ultrasound scan the liver size was at the upper limit of the normal range. Chronic lymphoedema was present in both legs. All three haematopoietic cell lines were represented in the bone marrow aspirate, with a ratio of myeloid to erythroid precursors of 1.5/1; many histiocytic cells showing a "foamy" cytoplasmic appearance were also noted.

At laparotomy, the spleen was described as greatly enlarged and congestive, with multiple capsular adhesions to the greater omentum, but no other abnormalities were recognised during the surgical exploration of the abdomen. On the second postoperative day, the white blood cell (WBC) and neutrophil counts had normalised and the platelet count had more than doubled as compared to the baseline preoperative control (see Table 2).

Methods, subjects and definitions

To assess the prevalence of generalised lymphadenopathy, hepatomegaly and splenomegaly, as well as of peripheral blood cytopaenias in male patients with classical AFD, we have retrieved all past and current clinical files of male patients with the diagnosis of AFD who have ever been admitted to, or followed-up at our hospital, since the first case was diagnosed in 1967 [13]. We have selected, for further review, only the files of patients which fulfilled the following criteria: (1) presentation with the classical AFD cutaneous phenotype and known or presumed hemizygosity for an α-Gal gene (*GLA*) mutation associated with residual α-Gal enzyme activity in plasma or in leukocytes less than 5% of the normal average (demonstrated in at least one affected male of the same family); and (2) clinical follow-up at our hospital for a minimum of 12 months with, at least, one complete blood count (CBC) available for review. Clinical and laboratory data obtained after the eventual start of enzyme replacement therapy (ERT) were excluded. These medical files were further analysed in detail for any record of the following clinical and haematologic features: generalized lymphadenopathy, palpable spleen or splenomegaly, palpable liver or hepatomegaly; anaemia, leukopaenia, neutropaenia or thrombocytopaenia. Any clinical events or circumstances that might have determined or contributed to those findings were also noted.

Adult age was defined as 18 or more years. Generalised lymphadenopathy was defined as enlarged lymph nodes palpable in two or more distinct anatomic regions [14]. Assessment of liver and of spleen size was done according to standard clinical [15–18] and sonographic criteria [19–22]. CKD stages were defined according to current clinical practice recommendations [23]. Glomerular filtration rate was estimated (eGFR) from sCr levels using standard algorithms [24, 25]. In adults, chronic renal insufficiency (CRI) was defined as a sCr value above 1.5 mg/dl, corresponding to stage 3 CKD or higher in most patients. In adult males, anaemia was defined as a haemoglobin level below 13 g/dl [26], leukopaenia was defined as a total WBC count below 4.3×10^9/l [27] and neutropaenia was defined as a neutrophil count below 1.5×10^9/L [28]. Appropriate age-related norms were used to define anaemia, leukopaenia and neutropaenia in children [26, 29]. Irrespective of age, thrombocytopaenia was defined as a platelet count below 150×10^9/l [30].

In addition, the prevalence of peripheral blood cytopaenias among adult patients enrolled at the Fabry Registry—a worldwide observational and voluntary programme tracking the natural course and outcomes of patients with AFD (https://www.lsdregistry.net/fabryregistry/)—was assessed using the above definitions for anaemia, leukopaenia and thrombocytopaenia. These prevalences were all reported as stratified by CKD stage, to allow for the confounding effect of advanced chronic renal failure upon haemoglobin levels [12, 31].

Results

Pathology findings in the spleen removed from the index patient

Grossly, the spleen measured 18.5×13.5×4.0 cm and weighed 700 g. The cut surface was homogeneous and of deep-red colour. On light microscopy examination, the parenchyma showed congestion and expansion of the red pulp, decreased white pulp and focal areas of fibrosis (Fig. 1). There were precursors of all haematopoietic cell lines, indicating extramedullary haematopoiesis (Fig. 2). Numerous macrophages had iron in the cytoplasm (Fig. 3) and a few showed cytoplasmic vacuolation (Fig. 4). A few scattered foci of haemophagocytosis were also observed (Fig. 5). Many endothelial cells of the sinusoids showed a reactive aspect with vacuolation and argyrophilic material within the cytoplasm (Fig. 6).

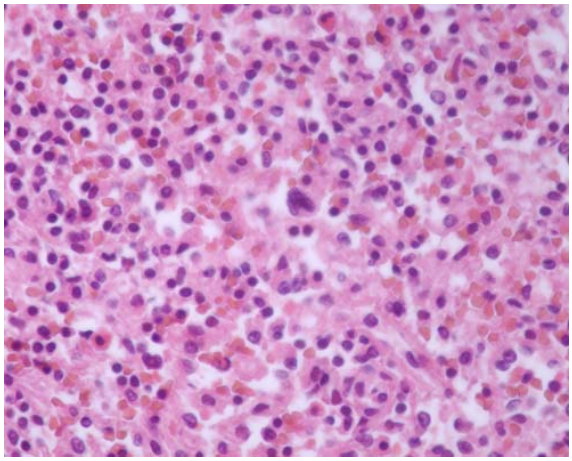

Fig. 2 Megakaryocyte in the red pulp; haematoxylin–eosin stain, ×400

Generalised lymphadenopathy, splenomegaly, hepatomegaly and peripheral blood cytopaenias in our patient cohort

We identified a total of 15 male patients with the diagnosis of AFD who have been admitted to our Hospital, or seen at outpatient clinics, at least once, during the last 40 years. Three patients were excluded from further analysis because they had a non-classical phenotype: one patient had presented at age 55 with a mild cardiac variant and other two, who had reached ESRF, respectively, at ages 58 and 61 years, were identified in a screening program of AFD among haemodialysis patients. At the time of this review, these latter two patients had already died. Two of the patients presenting with the typical cutaneous phenotype were excluded from further analysis because they had a total follow-up at our Hospital shorter than 12 months: one of them had been transplanted in another Hospital and the other refused dialysis and died in ESRF shortly after AFD diagnosis.

Demographic and relevant clinical data of the patients selected for review are presented in Table 3. Patient F1 had died at our hospital in 1976, of uraemic complications. Patient R1 was referred to another hospital after the beginning of chronic haemodialysis, received a kidney transplant a few years later and has recently died, but this review was limited to the data available in our hospital files.

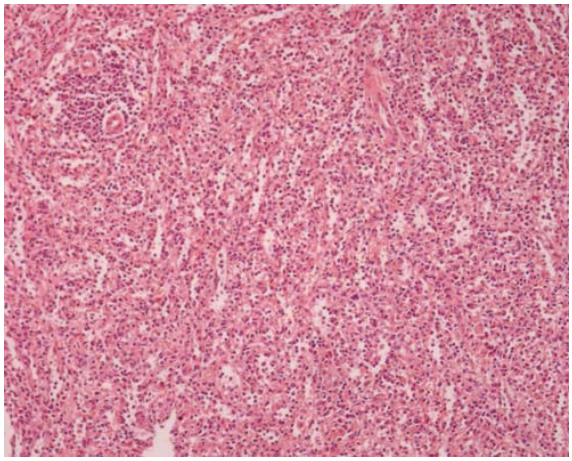

Fig. 1 Congestion and expansion of red pulp, reduced white pulp and focal fibrotic areas of the red pulp; haematoxylin–eosin stain, ×100

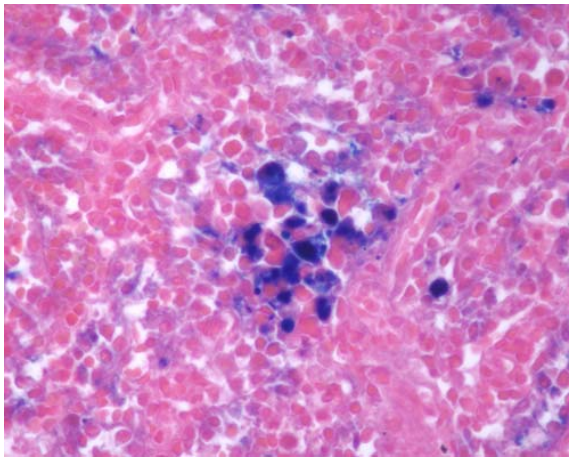

Fig. 3 Macrophages containing iron in the cytoplasm; Perls' Prussian blue stain, ×400

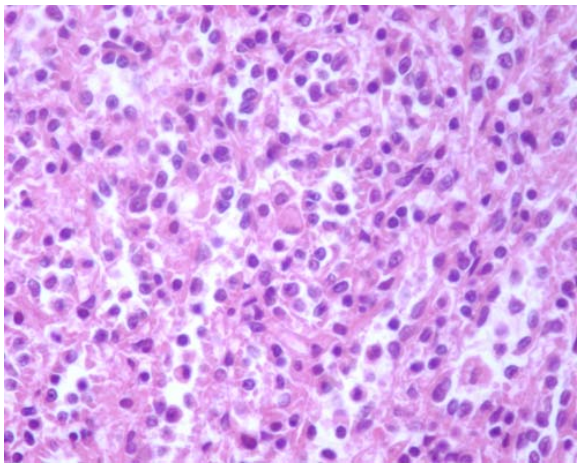

Fig. 4 Vacuolated histiocytes and reactive appearance of sinusoidal endothelial cells; haematoxylin–eosin stain, ×400

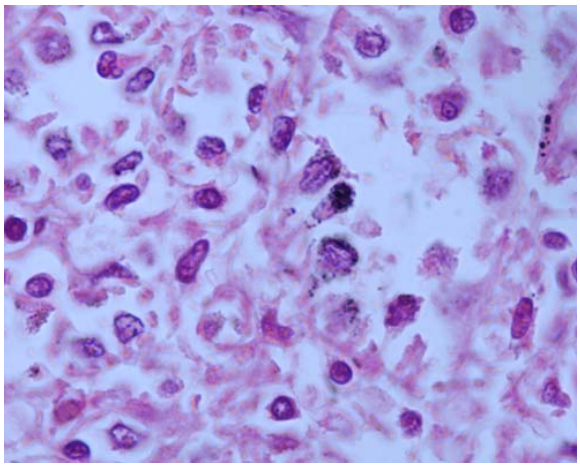

Fig. 6 Reactive endothelial cells containing argyrophilic material in the cytoplasm; methenamine silver stain, ×1,000

The liver was palpable only in the index patient (F2), but its size was not above the normal range. Enlarged lymph nodes were also found in patient R1 at age 27, in both axillary regions, when he was admitted for a kidney biopsy. Splenomegaly was incidentally diagnosed by ultrasonography in patient F3, at age 6, on the investigation of recurrent abdominal pain. Anaemia was found in all four adult patients with CRI but also in the 6-year-old child, whose eGFR was normal. Leukopaenia and/or neutropaenia were seen only in the two patients who had splenomegaly. Thrombocytopaenia was detected in the two relatives of the index patient, as well as in a third case of another family, adding up to a total prevalence of 40% in this cohort.

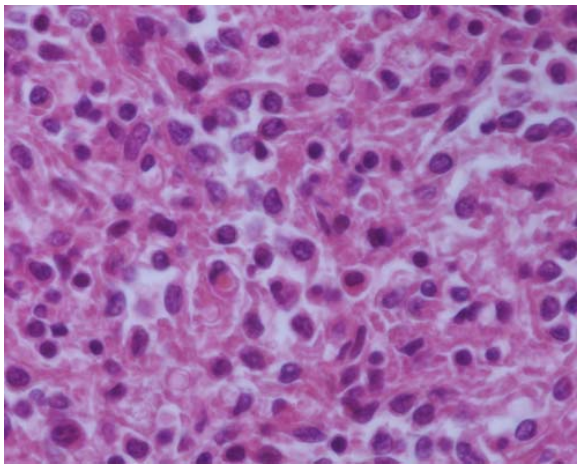

Fig. 5 Haemophagocytosis; haematoxylin–eosin stain, ×1,000

Peripheral blood cytopaenias in the Fabry Registry adult male cohort

The prevalences of anaemia, leukopaenia and thrombocytopaenia in adult males enrolled at the Fabry Registry, as stratified by CKD stage, are shown in see Table 4.

Discussion

Symptomatic involvement of the reticuloendothelial system in patients with AFD, manifesting by generalised lymphadenopathy and/or splenomegaly, although infrequently reported, has been recognised since long (see Table 5 for a summary of earlier case reports) [3, 4, 6–11, 32, 33]. Common to all previously published cases, as to our index patient, is male gender, the classical AFD phenotype, presentation up to the age of 35 years, and the absence of a more obvious clinical explanation for the splenomegaly or the lymphadenopathy. Additionally, there was evidence of Gb3 deposits in the lymph nodes and the spleen of all the patients who had biopsies taken for histological examination. However, while AFD is a recognised aetiology of generalised lymphadenopathy, even in the general medical literature [14], a recent review in a major textbook on metabolic diseases explicitly states that AFD is not a cause of splenomegaly [1].

In our patient, anaemia was first noted at age 17, when the eGFR was about 55 ml/min/1.73 m^2, a CKD stage where haemoglobin values below 12 g/dl are rarely found [31]. In addition, low platelet and WBC counts were recurrently observed since the age of 20, several years before the start of hemodialysis and of HBV infection. The development of peripheral blood cytopaenias in a patient

Table 3 Demographic and clinical data of the male patients with classic Anderson–Fabry disease selected for analysis

Case #	α-Gal activity	Age at diagnosis	Length of follow-up	Clinical outcome	Age at outcomes	Organ enlargement			Cytopaenia			
						L	LN	S	RBC	WBC	N	P
F1	NA	20 y	9 y	D (ESRF)	29 y				X			X
F2	0	17 y	32 y	HD>ER	24 y>43 y		X	X	X	X	X	X
F3	0	PN	7 y	ER	7 y			X	X	X		X
R1	0.1	27 y	5 y	HD	31 y		X		X			
T1	1.2	19 y	4 y 11 m	ER	20 y							
C1	0.4	20 y	3 y 11 m	ER	22 y							
P1	0.1	18 y	3 y 10 m	ER	20 y							X
P2	0.6	20 y	3 y 3 m	ER	21 y							
G1	1.1	52 y	1 y 9 m	HD>ER>D	52 y≫53 y				X			
G2	0.6[a]	18 y	2 y 6 m									

Each capital letter in the case identifiers denotes a different family; F2 is the patient reported herein. *α-Gal* α-Galactosidase activity measured in leukocytes (normal range: 36–80 nmol/h/mg), *y* Ages in full years, *m* months, Length of follow-up to a clinical outcome or to the last outpatient clinic visit, in full years and months up to 5 years, then in full years, *LN* lymph node, *L* liver, *S* spleen. *RBC* red blood cells, *WBC* white blood cells, *N* neutrophils, *P* platelets, *NA* data not available, *PN* prenatal diagnosis, *D* death, *HD* haemodialysis, *ER* enzyme replacement therapy, *ESRF* end-stage renal failure, *X* manifestation present.

[a] α-Galactosidase activity measured in plasma (normal range: 8.3–35.6 nmol/h/ml).

with an enlarged spleen and normal bone marrow cytology led to the clinical diagnosis of hypersplenism [34]. In patients with splenomegaly, thrombocytopaenia and/or leukopaenia are frequently associated with liver pathology [18, 21, 35, 36]. However, even after long-standing chronic HBV infection, our patient remained without any clinical or laboratory evidence of liver dysfunction or fibrosis.

The protracted dependence on haemodialysis may have contributed to the worsening of thrombocytopaenia as in these patients megakaryocytopoiesis decrease over time, regardless of increased plasma thrombopoietin levels, by an as yet unknown mechanism [37]. Peripheral destruction and splenic sequestration of platelets may have an additional role in the pathogenesis of haemodialysis-associated thrombocytopaenia [37, 38].

Notwithstanding the thorough diagnostic investigations and prolonged clinical follow-up, the underlying cause of splenomegaly in our patient could not be determined before surgery. According to a surgical classification [39], the removed spleen had a moderately increased size, and its gross appearance was essentially congestive and fibrotic. On histological examination, there was no evidence of any of the haematologic, infectious or inflammatory diseases most commonly associated with spleen enlargement [21, 40, 41]. The diagnostic yield of splenectomy is very high [40, 41] and, as occurred in our patient, up to 40% of all

Table 4 Prevalence of peripheral blood cytopaenias in adult males enrolled at the Fabry Registry, stratified by renal function

Cytopaenia	Estimated glomerular filtration rate (eGFR, ml/min/1.73 m^2)			
	≥90	≥60 to <90	≥30 to <60	<30
Anaemia[a]	80/250 (32%)	68/198 (34.3%)	80/142 (56.3%)	108/136 (79.4%)
Leukopaenia[b]	11/96 (13.1%)	12/80 (15%)	4/46 (8.7%)	8/46 (17.4%)
Thrombocytopaenia[c]	4/84 (4.8%)	6/67 (9%)	8/40 (20%)	13/41 (31.7%)

In these analyses, a patient was only represented once at each eGFR stage, using the smallest haemoglobin level, WBC or platelet count available at that particular level of renal function. The same patient may be represented in more than one eGFR stage, if renal function deteriorated during the course of follow-up. As patients have not been stratified by the level of α-Gal activity, nor by age ranges above 18 years, and the mean patient ages increased from the group with eGFR >=90 ml/min/1.73 m^2 to the groups with eGFR <60 ml/min/1.73 m^2, the apparent increase in the prevalence of thrombocytopaenia with decreasing eGFR could also be due to the severity of α-Gal deficiency, cumulative exposure to AFD, or any combination of these factors.

[a] Haemoglobin level <13 g/dl; data source: Steven Waldek, Fabry Registry Data Request Report, FDR-051025-01, 13Dec05.

[b] White blood cell count $<4.3\times10^9$ /l; data source: João P. Oliveira, Fabry Registry Data Request Report, FDR-060103-01, 13Jan06.

[c] Platelet count $<150\times10^9$ /l; data source: João P. Oliveira: Fabry Registry Data Request Report, FDR-060103-01, 12Jun06.

Table 5 Summary of previous case reports of patients with Anderson–Fabry disease and symptomatic reticuloendothelial organ involvement or thrombocytopaenia

Ref. #	Age	Organ enlargement			Thrombocytopaenia[a]	Additional notes
		L	LN	S		
[48]	22		X		45.0×10^9/l	Platelet count normalised following splenic X-ray irradiation.
[48]	18		X		72.0×10^9/l	Platelet count normalised following splenic X-ray irradiation.
[50]	32	X		X	29.8×10^9/l	
[8]	30			X		Splenomegaly later confirmed at laparotomy for appendicitis and at autopsy [49].
[46]	28		X			LNBx showing lipid deposits in macrophages.
[46]	25		X			
[43, 44]	23		X	X		Surgical mesenteric LNBx and SBx showing vacuolated cells.
[27]	18		X			LNBx showing typical lamellated Gb3 deposits in EM examination.
[17, 22]	10	X	X	X		LNBx showing histiocytes with foamy cytoplasmic appearance.

Ref. # Reference number, *Age* age in years, *LN* lymph node, *L* liver; *S* spleen, *X* manifestation present, *Bx* biopsy, *EM* electron microscopy.
[a] Platelet count, in patients with thrombocytopaenia.

cases will have a final diagnosis of "congestive splenomegaly" [41].

Spleens removed from patients with hypersplenism characteristically show prominent marginal zones of the malpighian corpuscles [42], a feature that may have been obscured in our patient by the atrophy of the white pulp. Although hypersplenism can result in splenic iron deposition, this rarely appears heavy and tends to be restricted to the sinusoidal lining cells [43]. Notably, massive siderosis was found in the spleens of the first two patients with AFD who underwent post-mortem pathological studies [44]. As the iron overload was not observed in any other site, including in the Kupffer cells of the liver, its pathogenesis was unclear. Reactive extramedullary haematopoiesis, as indicated by the increased number of haematopoietic cell precursors, has already been described in association with fibrocongestive splenomegaly [45].

The interpretation of the splenic pathology in our patient is potentially confounded by his chronically haemodialysed condition: before the availability of erythropoiesis stimulators to treat anaemia, the spleens removed from such patients were frequently enlarged and showed variable degrees of siderosis [43, 46]. However, even in haemodialysis-dependent patients that were splenectomised because of clinical hypersplenism, the weight of the spleen rarely exceeded 500 g [43, 46, 47]. The prominence of the red pulp in our patient was consistent with the red pulp hyperplasia previously described in haemodialysed patients [48], but we have not observed the lymphoid hyperplasia that was ascribed to chronic hepatitis in the same patient population [48].

Cytoplasmic vacuolation was seen in many of the endothelial cells lining the splenic sinusoids and in a small proportion of the macrophages, but the global burden of Gb3 accumulation, as histologically evaluated, could not account for the more than four-fold increase in spleen weight. This is similar to Gaucher disease, in which the amount of pathological lipid storage in the affected macrophages accounts for less than 2% of the increase in liver and spleen mass [49]. The argyrophilic material observed in sinusoidal endothelial cells probably corresponds to the trisaccharide side-chain of Gb3, which remains within the lysosomes despite removal of the ceramide moiety by the paraffin-inclusion procedure for histological analysis.

The improvement of all peripheral blood cytopaenias after splenectomy was a further argument to confirm the clinical diagnosis of hypersplenism in our patient [34]. Splenectomy was reported to resolve the cytopaenias in all patients with idiopathic splenomegaly and hypersplenism [40]. Splenectomy was also effective to decrease the blood transfusion requirements of chronically haemodialysed patients with evidence of increased splenic sequestration of red blood cells (RBC) [47, 50]; furthermore, the WBC and platelet counts also rose, reaching twice the pre-splenectomy values [50].

Taken together, these data strongly suggest that the most plausible aetiology of the generalised lymphadenopathy, splenomegaly and hypersplenism observed in our index patient was AFD or directly related to it. The hypersplenism may have been aggravated by the long-standing haemodialysis status. We hypothesise that partial or complete obstruction of the splenic sinusoids by the hypertrophied Gb3-laden endothelial cells is the basic mechanism leading to congestive splenomegaly in AFD. An identical mechanism has been proposed as the underlying cause of idiopathic fibrocongestive splenomegaly, in patients who had evidence of splenic platelet sequestration prior to splenectomy [51]. The increased RBC pooling in

the spleen could also explain the iron overload described in AFD patients, in the absence of Kupffer cell involvement [44].

In our cohort, anaemia was prevalent only among patients with CRI. However, low haemoglobin levels were observed since relatively early stages of renal dysfunction, at which the general CKD male patient population rarely develops anaemia [31]. In addition, the two patients with anaemia detected at early CKD stages also had thrombocytopaenia, an association that is even harder to explain in this clinical setting. The two other patients with thrombocytopaenia had normal renal function and no additional cytopaenias. One of these (and the only child included in our series) also had splenomegaly. Thrombocytopaenia, either alone or in combination, was the most frequent of the peripheral blood cytopaenias associated with hypersplenism among inpatients and outpatients seen in general hospital [35].

In our cohort, thrombocytopaenia—and, to a lower extent, generalized lymphadenopathy and splenomegaly—seemed to be associated with the lowest α-Gal enzyme activity levels, suggesting that it may only be a manifestation of severe α-Gal deficiency. However, given the small number of patients and families in our cohort, it cannot be excluded that other genetic factor(s) co-segregating within the index patient's family may be the real cause of these findings.

In a recent cross-sectional study of the Fabry Outcome Survey (FOS), a European AFD observational database, anaemia has been recognised as a highly prevalent complication of AFD [12]. Analyses of the adult male cohort of the Fabry Registry confirmed the high prevalence of anaemia, but have additionally shown that leukopaenia and thrombocytopaenia are also frequent. The real prevalence of generalised lymphadenopathy and splenectomy in the AFD population may have been underestimated so far, as they may be missed on a less than thorough and systematic physical examination. Asymptomatic enlarged lymph nodes are frequently palpable in healthy people [14] and their presence in AFD patients may be difficult to accept as a clinical sign of the disease. Furthermore, retrospective medical-record-based estimates of the prevalence of splenomegaly in hospitalised patients have consistently been more than two-fold lower than in prospectively oriented series and show a predominance of cases of massive splenomegaly [18, 21]. These observations suggest that mild spleen enlargements may pass unnoticed in the usual hospital clinical practice setting.

Given the wide range of normal values and the Gaussian distributions of haemoglobin levels and of WBC, neutrophil and platelet counts, the use of cut offs to identify cytopaenias may overlook disease processes which may affect haematopoiesis or the survival of peripheral blood cells, but are not of enough magnitude to be systematically recognised by such criteria. Of note, even in our index patient, who had clinically apparent splenomegaly, some of the CBC results were above the threshold levels for cytopaenias. Therefore, the most effective approach to demonstrate that AFD is associated with peripheral blood cytopaenias would be to compare the distributions of haemoglobin, and of WBC, neutrophil and platelet counts, in appropriately matched cohorts of AFD patients and of healthy subjects. The significantly lower ($p<0.0001$) haemoglobin levels in adult males with normal renal function enrolled at the Fabry Registry, as compared to large population databases of healthy individuals [52], seems to support this view, but similar comparisons of WBC, neutrophil and platelet counts are still lacking. The same reasoning should be applied to the sonographic evaluation and comparison of spleen dimensions in AFD patients and healthy individuals. Finding a correlation between peripheral blood cell counts and the sonographic size of spleen could be an approach to demonstrate that hypersplenism is the mechanism behind the development of peripheral blood cytopaenias in AFD.

In conclusion, the data presented and discussed herein suggest that AFD may be a cause of congestive splenomegaly and hypersplenism in males with the classical phenotype, plausibly related to endothelial Gb3 accumulation in the splenic sinusoids. It is possible that peripheral blood cytopaenias—most frequently anaemia and thrombocytopaenia—observed in a significant number of patients, are a sign of subclinical hypersplenism. These hypotheses need to be tested in larger patient cohorts. Screening for lymphadenopathy and for spleen and liver enlargement on physical examination, CBC and sonography evaluation of spleen size should be performed as part of the routine assessment of AFD patients.

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Conflict of interest statement We declare that we have no conflict of interest.

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4

GENERAL DISCUSSION

A significant collection of kidney biopsies of patients with Fabry disease, of both genders, either with the classical phenotype (including a male child) or with attenuated forms of the disease, was available for study at the Department of Pathology, *Hospital de São João* (HSJ) and the Faculty of Medicine, University of Porto (FMUP). In the absence of validated criteria to describe and score the kidney pathology of Fabry disease, it was necessary to standardise the reading and interpretation of the kidney biopsies of Fabry patients as a preliminary step to further pathological and clinico-pathological correlation studies. The histopathological evaluation system developed for this purpose was originally presented at the "Portuguese Symposium on Fabry Disease" in 2005, was used in all the subsequent studies of the kidney pathology of Fabry disease included in this Thesis, and became the rational for the scoring system eventually validated by the "International Study Group of Fabry Nephropathy (ISGFN)" [*Fogo et al. 2010*]. Based on considerations about the pathogenesis of chronic kidney disease progression, our approach to standardise the light microscopy assessment of kidney biopsies of patients with Fabry disease included not only the evaluation of histological parameters specifically related to the intracellular accumulation of glycosphingolipids (e.g., vacuolization and deposits) but also of non-specific degenerative glomerular, tubular, interstitial and vascular lesions (e.g. glomerular sclerosis, interstitial fibrosis, tubular atrophy, vascular sclerosis) which are a hallmark of chronicity and progression of kidney disease. To this end, paraffin-embedded tissue sections stained with hematoxilin-eosin, periodic acid Schiff and special trichrome, as well as plastic-embedded toluidine- or methylene-blue stained semi-thin sections were used. Archive electron microphotographs were also reviewed.

The kidney biopsies of the four females with Fabry disease available in our series were assessed according to that protocol [*Valbuena et al. 2008*]. All four patients were heterozygous for *GLA* gene mutations associated to the classical phenotype of Fabry disease, showed normal to slightly sub-normal renal function, and two of them had overt proteinuria. Like affected males, these Fabry females had glycosphingolipid deposits in all types of renal cells, with the largest amounts observed in glomerular parietal epithelial cells, followed by

podocytes. Proteinuria was associated with the presence of glycosphingolipid deposits in proximal tubular cells and effacement of the podocyte foot processes. The prevalence of glycosphingolipid deposits in interstitial cells seemed particularly striking in these patients, as compared to males. The presence of glycosphingolipid deposits in vascular cells and/or the presence of chronic vascular lesions could not be significantly related to chronic kidney disease stage or to glomerular sclerosis and/or interstitial fibrosis, suggesting that vascular damage does not play a major role in the early pathogenesis of Fabry nephropathy in females. Combined analysis of our data with those of two earlier series reporting renal biopsy findings in females with Fabry disease at diverse chronic kidney disease stages showed that glomerular sclerosis and tubular fibrosis are histopathological predictors of proteinuria and chronic kidney disease stage in females with Fabry nephropathy [*Valbuena et al. 2008*].

The ISGFN was formed in 2005, by nephrologists and pathologists from 11 referral centres for Fabry disease in Europe, the United States of America and Canada, with the major goal of developing a scoring system for Fabry nephropathy that should allow: (1) the recognition of prospective and retrospective correlations between kidney histopathology, clinical manifestations and renal function abnormalities; (2) the identification of prognostic factors; and (3) the quantification of therapeutic responses based on changes in histological lesions.

The ISGFN scoring system was developed by consensus and subsequently refined in round robin microscopy slide reviews and face-to-face scoring sessions, using a modified Delphi technique [*Fogo et al. 2010*]. It is based on light microscopy assessment of glomerular, tubulointerstitial and vascular lesions seen on standard as well as on toluidine blue- or methylene blue-stained semi-thin renal biopsy slides. The final version of the ISGFN scoring system [Attachment 1] was validated by blind scoring of 59 kidney biopsies of Fabry patients, males and females, allowing a detailed description of the renal pathology of Fabry disease, particularly at the initial chronic kidney disease stages [*Fogo et al. 2010*]. This was indeed the largest series of kidney biopsies of patients with Fabry disease ever collected and analysed using a standardized scoring approach. The major findings of the ISGFN study were that *(i)* males had greater podocyte vacuolization on light microscopy and glycosphingolipid inclusions on semi-thin sections than females; *(ii)* males had significantly more proximal tubular, peritubular capillary and vascular intimal inclusions; *(iii)* the degree of arteriolar hyalinosis was similar in both genders, but females had significantly more arterial hyalinosis (a difference that might be age-related, as females were significantly older); *(iv)* chronic kidney disease stage correlated with arterial and glomerular sclerosis scores; *(v)* significant changes, including segmental and global sclerosis, and interstitial fibrosis were seen even in patients with chronic kidney disease stage 1-2 with minimal proteinuria. Overall, these findings support the role of renal biopsy

in the baseline evaluation of Fabry nephropathy, even with mild clinical disease. The scoring system will be useful for longitudinal assessment of prognosis and responses to therapy for Fabry nephropathy.

The *GLA* knockout mouse ("Fabry mouse") was generated as an animal model for Fabry disease but, as the Fabry mice do not manifest progressive chronic kidney disease, their relevance as a model for human Fabry nephropathy is uncertain. We used the same renal biopsy reading and scoring protocol developed for Fabry nephropathy to describe the renal pathology of the Fabry mice at different ages and to contrast these findings with the renal pathology reported in patients with Fabry disease, at comparable age ranges and across different chronic kidney disease stages [*Valbuena et al. 2010*]. The major findings of this study were that *(i)* Fabry mice are phenotypically different from the wild-type mice, displaying progressive age-related accumulation of glycosphingolipids in all types of renal cells; *(ii)* the size and amount of inclusions in Fabry mice increase with age; *(iii)* no gender differences were found in Fabry mice regarding the accumulation of glycosphingolipids; *(iv)* Fabry mice lack the non-specific histological glomerulosclerotic and interstitial fibrotic renal lesions that best correlate with progressive chronic kidney disease in Fabry patients; *(v)* Fabry mice do not develop large podocyte inclusions as is usual in Fabry patients; *(vi)* there were no statistically significant differences between Fabry mice and Fabry patients in the prevalence of glycosphingolipid storage per renal cell type, with the exceptions of mesangial, which is higher in humans, and proximal tubular cells, which is higher in the Fabry mice. We postulate that the elucidation of the mechanisms underlying these species differences may contribute important clues to a better understanding of the pathogenesis of Fabry nephropathy.

The demonstration of stored glycosphingolipids in affected cells, by light microscopy or electron microscopy, has since long been regarded as an ancillary diagnostic criterion for Fabry disease. Due to the limitations of routine light microscopy techniques to detect glycosphingolipid deposits in tissue specimens, electron microscopy is generally considered the ideal approach to the morphological diagnosis of Fabry disease, even though the distinctive lamellated ultrastructure of Gb3 inclusions, while suggesting the diagnosis, is not pathognomonic. However, electron microscopy in not generally available and the diagnosis of Fabry disease has indeed been missed in kidney biopsies of patients routinely assessed by light microscopy.

Based on previous experiments using a highly specific murine anti-Gb3 monoclonal antibody to detect Gb3 in paraffin-embedded tissue specimens of Fabry patients, we tested the hypothesis that the residual glycosphingolipids remaining in tissues after standard histological processing for light microscopy evaluation allow the specific immunohistochemical diagnosis of Fabry nephropathy [*Valbuena et al. 2011,* in press].

Using the same anti-Gb3 murine antibody as the primary antibody in a conventional immunohistochemical protocol, we studied the kidney biopsies of nine patients with Fabry disease - including four men, four women and a male child -, and of a female carrier of a *GLA* gene mutation of low pathogenicity, without ultrastructural or biochemical evidence of Fabry nephropathy. Additional goals were to estimate the relative abundance of lysosomes in different renal cell types and to check whether Gb3 deposits were exclusively seen in colocalization with a marker of lysosomes. To this end, the biopsies of the adult patients were also marked with an antibody for a lysosomal membrane protein (LAMP2: Lysosomal Associated Membrane Protein 2). The distribution of Gb3 deposits was quantified per kidney cell type and compared with the corresponding histological measurements in semi-thin sections. .

The major findings of this study were that *(i)* patients with Fabry disease had residual Gb3 in all types of glomerular, tubular, interstitial and vascular kidney cells; *(ii)* the highest expression of LAMP2 was observed in tubular cells but no meaningful associations were identified between the expression of LAMP2 immunoreactivity and the prevalence of Gb3 deposits in the different kidney cells; *(iii)* the histological quantification of the glycosphingolipid deposits in the semi-thin sections was relatively higher than the corresponding immunohistochemical quantification; *(iv)* in the female carrying the low pathogenicity *GLA* mutation, the immunohistochemical expression of Gb3 was limited to tubular cells, in a pattern that was similar to that observed in biopsies of controls without Fabry disease.

The results of this study showed that the immunostaining of Gb3 allows the specific diagnosis of Fabry nephropathy, even in kidney biopsies processed for routine light microscopy evaluation. It also allows the exclusion of the diagnosis of Fabry nephropathy in patients carrying *GLA* mutations associated with high residual enzyme activity. It is possible, therefore, to make retrospective studies of paraffin-embedded kidney biopsies, either for diagnosis or for clinico-pathological correlation studies.

The importance of the renal biopsy for the diagnosis of Fabry disease was demonstrated in the study of a family in which the kidney biopsies of a male proband, of a similarly affected brother and of their mother, were all typical of Fabry nephropathy, but in whom the routine *GLA* gene mutational analysis revealed only non-pathogenic polymorphisms. Further biochemical and genetic studies motivated by the unequivocal histological evidence of Fabry disease ultimately led to the identification of a tandem duplication of three exons of the gene by cDNA sequencing [*Ferreira et al. 2009*], a type of mutation not previously described in Fabry disease.

The personal experience acquired in the kidney pathology of Fabry disease, from the evaluation and classification of a total of 72 kidney biopsies of men and women with Fabry nephropathy or α-galactosidase deficiency, resulted in the invitation to be co-author of the chapter dedicated to the renal manifestations of Fabry disease, in a monothematic textbook on Fabry disease [*Warnock et al. 2010*], to which I particularly contributed to the description of the morphological changes observed in the kidneys and corresponding illustrations.

The involvement of the reticuloendothelial system in patients with Fabry disease is usually asymptomatic. Only a small number of cases have been reported who had enlargement of lymph nodes, spleen or liver as presenting signs. Data on the histopathology of the spleen is limited to a few early studies of autopsy specimens and of biopsies taken at laparotomy. We had the opportunity to study the histopathology of an enlarged spleen removed from a classically affected male Fabry patient to treat symptomatic hypersplenism. The main histological abnormalities were *(i)* congestion and expansion of the red pulp; *(ii)* decreased white pulp; *(iii)* focal areas of fibrosis; *(iv)* abundant iron deposits in spleen parenchyma; *(v)* extramedullary haematopoiesis; *(vi)* hemophagocytosis; *(vii)* and the presence of argyrophilic granules within cytoplasmic vacuoles in endothelial sinusoidal cells. The endothelial cells appeared reactive, protruding into the lumen of the sinusoids. These findings led us to postulate that the argyrophilic granules might correspond to the carbohydrate moiety of residual Gb3 remaining within lysosomes and that the changes observed in the sinusoidal endothelial cells, which are probably due to the accumulation of glycosphingolipids, might be the cause of hypersplenism, by the entrapment and destruction of circulating blood cells. The observation of anemia, leukopenia and thrombocytopenia in a proportion of patients enrolled in an international registry of Fabry disease are in agreement with this hypothesis.

In conclusion, the histological alterations observed in the kidney biopsies of females with Fabry disease are similar to those described in affected males, but with minor degrees of involvement of the different types of renal cells. Non-specific glomerular sclerosis and interstitial fibrosis are the major correlates of proteinuria and chronic kidney disease stage in Fabry nephropathy, and should be routinely scored and reported. The amount and cell pattern of residual Gb3 in paraffin-embedded renal biopsy specimens following standard histological processing for light microscopy evaluation allows the specific immunohistochemical diagnosis of Fabry nephropathy. Furthermore, the histological diagnosis of Fabry nephropathy is a major diagnostic criterion of Fabry disease. Although *GLA* knockout mice accumulate Gb3 in all types of renal cells, as humans with Fabry disease do, they do not develop sclerotic and fibrotic degenerative lesions even at old ages. It is possible, therefore, that the elucidation of the molecular mechanisms underlying this critical species difference might contribute to the understanding of the pathogenesis of Fabry nephropathy and to identify additional therapeutic targets. The accumulation of

glycosphingolipids in the endothelium of the splenic sinusoids might be the cause of anemia and other peripheral blood cytopaenias described patients with Fabry disease.

Future studies will have to address the molecular mechanisms linking cellular pathology to chronic kidney disease progression. In many forms of kidney disease, macrophages are regarded as mediators of glomerular and tubular injury, as well as of the fibrotic events leading to chronic kidney disease [*Ricardo et al. 2008, Matsumoto et al. 2010*]. Lipid-laden macrophages in Fabry nephropathy resemble other glomerular and interstitial vacuolated cells and hence their presence may be overlooked on routine light microscopy evaluation of kidney biopsies. It is well recognized that tissue damage in Gaucher disease results from inflammation, cytokine release from macrophages engorged with accumulated glucosylceramide, and abnormal intra- and inter-cellular signaling [*Cox 2001*]. Similar processes, triggered by the presence of excessive tissue Gb3 storage, are likely to be equally important in Fabry disease.

To assess macrophage infiltration in Fabry nephropathy, paraffin-embedded kidney biopsies of Fabry patients were immunohistochemically stained for glycoprotein CD68, which is highly expressed by human tissue macrophages. The numbers of CD68-positive cells in the glomeruli, tubules and interstitium were semi-quantified and statistically correlated with demographic, clinical and light microscopy histopathological parameters, including the degree of glycosphingolipids storage in different cell types as evaluated on semi-thin sections. Although males had higher numbers of glomerular and interstitial CD68-positive cells, there was no gender difference in the amount of such cells in the tubules. As compared to non-sclerotic glomeruli, higher numbers of CD68-positive cells were observed in the glomeruli with sclerotic lesions. In the interstitium, the amount of CD68-positive cells was higher in fibrotic areas. In the glomeruli and the interstitium, but not in the tubules, the amount of CD68-positive cells correlated with the degree of glycosphingolipid storage in local cells. Glycosphingolipid storage in mesangial cells and in interstitial cells were the major predictors of the amounts of CD68-positive cells respectively in the glomeruli and the interstitium. These findings, which will soon be submitted for publication, suggest that like in other kidney diseases, the inflammatory response mediated by macrophages may play an important role in the development of glomerular sclerosis and interstitial fibrosis in Fabry nephropathy.

5

ABSTRACT

Fabry disease is a glycosphingolipidosis of neutral glycosphingolipids, mainly of globotriaosylceramide (Gb3), caused by deficiency of the lysosomal enzyme α-galactosidase. The absence or severe deficiency of α-galactosidase results in the classical, multi-systemic severe presentation of Fabry disease; when there is significant residual enzyme activity the disease has a more limited phenotypic expression and later clinical onset than the classical form. The prevalence of the classical phenotype of Fabry disease has been estimated of 1:117.000 live births. Given its rarity, Fabry disease is included among the "orphan diseases," which in Europe are defined as a life-threatening or chronically debilitating condition, affecting less than 5 in 10,000 individuals. The lack of interest of the pharmaceutical industry, under normal market conditions, in developing and marketing of drugs to treat rare diseases, because the costs of this investment would not be recoverable by the expected sales, led to the implementation of political and economic incentives to promote research into therapies for orphan diseases, of which the development of a specific therapy for the disease Fabry, with α-galactosidase produced by recombinant gene technology, is a paradigmatic example.

Over the last decade, following the approval of enzyme replacement therapy with recombinant α-galactosidase, and the issues raised by the monitoring of its effectiveness, there has been an exponential increase in basic and clinical research in Fabry disease, particularly aiming to clarify the pathogenic mechanisms underlying the systemic complications of the disease, understand its pathophysiology and identify therapeutic alternatives more convenient for patients and potentially more effective.

Since the late sixties that a significant collection of kidney biopsies of patients with Fabry disease, of both sexes, either with the classical phenotype (including a male child) or with attenuated forms of the disease, was gathered at the Department of Pathology, *Hospital de São João* (HSJ) and the Faculty of Medicine, University of Porto (FMUP). There was also

in archive a surgical specimen of splenectomy from a patient with classic Fabry disease, who developed symptoms of hypersplenism after several years in regular hemodialysis, as renal replacement therapy for end-stage renal disease (ESRD) due to Fabry disease nephropathy.

Victor Faria, a pathologist from the Pathology Department of HSJ and FMUP, published in 1970 the results of his exemplary light microscopy and electron microscopy studies of biopsies of skin, liver and kidney of the first patient with Fabry disease diagnosed in Portugal, pioneering the research in Fabry disease in HSJ/FMUP. A second important milestone in this history were the first diagnoses of Fabry disease by mutational analysis of the α-galactosidase gene (*GLA*) made in Portugal, including in a foetus for prenatal diagnosis, achieved by investigators of the Department of Genetics of FMUP, in the late nineties.

In this context, and thanks to the remarkable registry of kidney biopsies implemented by Dr. Elísio de Carvalho, the opportunity existed to review the histological material archived at the Department of Pathology of HSJ, aiming to describe in detail the kidney pathology of Fabry disease, seeking to identify morphological clues to the pathogenesis of Fabry nephropathy, elucidate the relative importance of the pathological involvement of the different types of cells in the progression of the kidney disease, and also to describe the splenic pathology coexisting in a dialysis-dependent ESRD patient with classic Fabry disease.

In the absence of validated criteria to describe and quantify the kidney pathology of Fabry disease, creating a system of classification of kidney biopsies to allow uniform reading and interpretation was a precondition for the development of the plan of the thesis. The classification system developed for this purpose was originally presented in 2005, at the "Portuguese Symposium on Fabry Disease" [1], became the basis for the classification system of the "International Study Group of Fabry Nephropathy (ISGFN)" [2] and was used in all studies of kidney pathology published in this thesis, including a description of the Fabry nephropathy pathology in women [3]; the demonstration the usefulness of an immunohistochemical method for specific diagnosis of Fabry disease nephropathy, even in paraffin-embedded tissue and processed for conventional optical microscopy [4]; and a comparative description of the renal pathology observed in knockout mice for the *GLA* gene [5].

The protocol study of kidney biopsies of patients with Fabry disease using the methods and scoring system validated by ISGFN and the analysis of their clinico-pathological correlations helped to understand the natural history of Fabry nephropathy. The results of this study [3] based on the classification of kidney biopsies of 35 men and 24 women with Fabry disease, mostly in the early stages of chronic kidney disease (CKD), recruited from 10 referral centers in Europe and North America, showed that lesions of chronic

glomerular sclerosis, interstitial fibrosis and tubular atrophy appear early in the natural history of disease and can be observed in patients in CKD stages 1 and 2 with minimal proteinuria. The degree of podocyte vacuolation in light microscopy and the amount and volume of deposits of glycosphingolipids in podocytes observed in the semi-thin sections were significantly higher in men than in women. Men also had higher amounts of glycosphingolipid inclusions in proximal tubules, peritubular capillaries and the vascular endothelium. The degree of arteriolar hyalinosis was similar in both genders but women had a higher degree of arterial hyalinosis, probably in relation with its significantly higher mean age (43.9 *versus* 36.4 years). Overall, the degree of arterial sclerosis also correlated significantly with CKD stage. These observations support the indication for renal biopsy in patients with Fabry disease, even in the early stages of nephropathy. Moreover, this system of histopathological classification of renal manifestations of Fabry disease may be useful to estimate prognosis and to assess the response to therapy of Fabry disease nephropathy.

The systematic study of the kidney biopsies from heterozygous women with pathogenic *GLA* gene mutations diagnosed in the HSJ [3], and their combined analysis with case series published in the literature, showed that the kidney pathology observed in women with Fabry disease is qualitatively similar to that described in affected males, and that the degrees of glomerulosclerosis and interstitial fibrosis are, respectively, the main predictors of proteinuria and CKD stage. The cellular distribution of the glycosphingolipid deposits could be more effectively evaluated on toluidine blue- or methylene blue-stained semi-thin sections. By electron microscopy, fusion of podocyte foot processes was observed only in patients with overt proteinuria. No consistent histopathological evidence of vascular involvement in the pathogenesis of early Fabry disease nephropathy could be detected in these women.

Since the intracellular glycosphingolipid deposits that accumulate in Fabry disease are largely removed during the histological processing of tissues fixed in formalin and embedded in paraffin for observation by light microscopy, vacuolization of the affected cells is the most typical feature (although nonspecific) of Fabry disease on routine histopathological evaluation. However, there was some histological evidence, including from personal observations, that a fraction of the glycosphingolipid deposits, probably the non-lipid soluble carbohydrate moiety, could remain within the lysosomes, even in tissues processed for standard light microscopy evaluation.

To test the hypothesis that the remaining fraction of the accumulated glycosphingolipids allow the specific immunohistochemical diagnosis of Fabry disease, we studied nine renal biopsies of patients with Fabry disease - including four men, four women and a male child -, as well as a heterozygous woman for a *GLA* gene mutation of low pathogenicity, without ultrastructural or biochemical evidence of Fabry nephropathy, using as primary antibody

a murine antibody highly specific for the human Gb3 [4].Biopsies of the adult patients were additionally marked with an antibody for a lysosomal membrane protein (LAMP2: Lysosomal Associated Membrane Protein 2). The distribution of Gb3 deposits was quantified per kidney cell type and compared with the corresponding histological measurements in semi-thin cuts. Patients with Fabry disease had residual Gb3 in all types of glomerular, tubular, interstitial and vascular kidney cells. The highest expression of LAMP2 was observed in tubular cells but no significant association could be identified between the immunohistochemical expression of LAMP2 and the prevalence of Gb3 deposits in the different types of kidney cells. The histological quantification of the glycosphingolipid deposits in the semi-thin cuts was relatively higher than the corresponding immunohistochemical quantification. In the heterozygous woman for the low pathogenicity *GLA* mutation, the immunohistochemical expression of Gb3 was limited to tubular cells, in a pattern that was similar to that observed in biopsies of controls without Fabry disease. The results of this study showed that the immunostaining of Gb3 allows the specific diagnosis of Fabry disease nephropathy, even in biopsies processed for routine light microscopy evaluation [4]. It is possible, therefore, to make retrospective studies of paraffin-embedded kidney biopsies, either for diagnosis or for clinico-pathological correlation studies.

The importance of the kidney biopsy for the diagnosis of Fabry disease was demonstrated in the study of a family in which kidney biopsies of a male proband, of a similarly affected brother and of their mother, were typical of Fabry disease nephropathy, but in whom the routine *GLA* gene mutational analysis revealed only non-pathogenic polymorphisms. Further genetic studies with cDNA sequencing allowed the identification of a tandem duplication of three exons of the gene [5], a type of mutation that had not previously been described in Fabry disease.

The *GLA* gene knock-out mouse ("Fabry mouse") was developed to provide an animal model of Fabry disease, but as these mice do not develop progressive CKD its relevance as a model for the human Fabry nephropathy is uncertain. To describe the renal pathology of Fabry mice, the histological changes observed in 24 kidneys of Fabry mice of different age groups were systematically evaluated and compared with control mice [6]. The histopathological changes observed in the kidneys of the Fabry mice were additionally compared with the kidney pathology reported in patients with Fabry disease, in similar age groups and at different CKD stages. With age, the Fabry mice gradually accumulate glycosphingolipids in all types of kidney cells, in a pattern that is similar to that observed in patients with Fabry disease nephropathy, with the exception of mesangial cells, where the prevalence of glycosphingolipid deposits is higher in patients with Fabry nephropathy, and of proximal tubular cells, where the prevalence of deposits is higher in Fabry mice. However, the Fabry mice do not develop the nonspecific histological lesions of glomerular sclerosis and interstitial fibrosis that best correlate with CKD progression in patients with

Fabry disease, nor express the bulky glycosphingolipid podocyte inclusions that are characteristic of Fabry disease nephropathy. The elucidation of the mechanisms underlying the inter-species histopathological differences may help to better understand the pathogenesis of Fabry disease nephropathy and to possibly identify additional therapeutic targets for prevention or treatment of CKD in patients with Fabry disease.

The experience acquired in the kidney pathology of Fabry disease, from the evaluation and classification of a total of 72 kidney biopsies of men and women with Fabry nephropathy or α-galactosidase deficiency, resulted in the invitation to be co-author of the chapter dedicated to the renal manifestations of Fabry disease, in a monothematic textbook on Fabry disease [7], to which I particularly contributed to the description of the morphological changes observed in the kidneys and corresponding illustrations.

I also had the opportunity to review the histopathology of the spleen of 39-year-old man with classical Fabry disease that had been on chronic hemodialysis treatment for nearly 15 years [8]. Splenectomy was performed as treatment for symptomatic hypersplenism with several years of evolution. On gross examination of the surgical specimen, the spleen weighed 700g and had a fibrocongestive appearance. Histologically, there was expansion of the red pulp and decreased white pulp. Some histiocytes and many endothelial cells lining the sinusoids showed cytoplasmic vacuolization but containing argyrophilic material inside, probably the residual carbohydrate fraction of glycosphingolipids accumulated in lysosomes. A review of published cases in the literature and data of an international registry of Fabry disease was suggestive that the involvement of the reticuloendothelial system is not uncommon in patients with Fabry disease and may be manifested by lymph node and spleen enlargement, and by peripheral blood cytopaenias, especially anaemia. The deposition of glycosphingolipids in the endothelium of the sinusoids, impairing the normal splenic blood flow, may be the cause of splenomegaly and hypersplenism in patients with classic Fabry disease.

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6

RESUMO

A Doença de Fabry é uma glicoesfingolipidose de glicoesfingolípidos neutros, sobretudo de globotriaosilceramida (Gb3), causada pela deficiência de atividade enzimática lisossómica de α–galactosidase. A ausência ou deficiência grave de α–galactosidase resulta na apresentação multi-sistémica clássica da Doença de Fabry; quando existe atividade enzimática residual significativa, a doença tem uma expressão fenotípica mais limitada e mais tardia do que a forma clássica. A prevalência do fenótipo clássico da Doença de Fabry foi estimada em cerca de 1:117.000 nados-vivos. Dada a sua raridade, a Doença de Fabry inclui-se entre as "doenças-órfãs", que na Europa se definem como doenças crónicas debilitantes ou que condicionem risco de vida, que afetem menos de 5 em 10.000 indivíduos. O pouco interesse da indústria farmacêutica, em condições normais de mercado, no desenvolvimento e comercialização de medicamentos para tratamento de doenças raras, porquanto os custos desse investimento não seriam recuperáveis pelas vendas esperadas, levou à implementação de políticas e de incentivos económicos para promover a investigação de terapêuticas para doenças-órfãs, de que o desenvolvimento de uma terapêutica específica para a Doença de Fabry, com α-galactosidase produzida por tecnologias de genética recombinante, constitui um exemplo paradigmático.

Ao longo da última década, na sequência da aprovação da terapêutica de reposição enzimática com α-galactosidase recombinante, e das questões levantadas pela monitorização da sua eficácia, registou-se um aumento exponencial de investigação básica e clínica em Doença de Fabry, visando sobretudo esclarecer os mecanismos patogénicos subjacentes às complicações sistémicas da doença, compreender a sua fisiopatologia, e identificar alternativas terapêuticas mais cómodas para os doentes e, eventualmente, mais eficazes.

Desde os finais da década de sessenta que se foi constituindo no Serviço de Anatomia Patológica do Hospital de São João (HSJ) e da Faculdade de Medicina da Universidade do Porto (FMUP) um importante acervo de biopsias renais de doentes com Doença de Fabry, de ambos os sexos, com formas graves (fenótipo clássico) ou com formas atenuadas

da doença (variantes tardias, com envolvimento orgânico mais limitado), incluindo uma criança de sexo masculino. Havia ainda em arquivo uma amostra de uma peça operatória de esplenectomia de um doente com Doença de Fabry clássica, que desenvolvera um quadro de hiperesplenismo sintomático depois de vários anos em programa regular de hemodiálise, para tratamento de substituição de função renal neste doente com nefropatia de Doença de Fabry.

Vítor Faria, patologista do Serviço de Anatomia Patológica do HSJ e da FMUP, publicou em 1970 resultados de estudos de microscopia óptica e de microscopia eletrónica das biopsias de pele, hepática e renal do primeiro doente com Doença de Fabry diagnosticado em Portugal, que ainda hoje são modelares, sendo pioneiro da investigação em Doença de Fabry no HSJ e na FMUP. Um segundo marco relevante dessa história, foi a realização no Serviço de Genética da FMUP, nos finais de década de noventa, dos primeiros diagnósticos de Doença de Fabry por análise mutacional do gene da α–galactosidase (*GLA*) que se fizeram em Portugal, incluindo num feto para diagnóstico pré-natal.

Neste contexto, e graças ao notável arquivo de biopsias renais implementado pelo Dr. Elísio de Carvalho, havia a oportunidade de fazer a análise sistematizada do material histológico existente no Serviço de Anatomia Patológica do HSJ, tendo por objetivos a descrição detalhada da patologia renal da Doença de Fabry, procurando identificar indícios morfológicos sobre a patogenia da doença renal e a importância relativa do envolvimento patológico dos diferentes tipos de células renais na progressão da doença, e ainda descrever a patologia esplénica coexistente num doente com Doença de Fabry clássica e insuficiência renal crónica terminal, dependente de diálise.

Dada a inexistência de critérios validados para descrever e quantificar a patologia renal da Doença de Fabry, a criação de um sistema de classificação das biopsias renais que permitisse uniformizar a sua leitura e interpretação era uma pré-condição para o desenvolvimento do plano da Tese. O sistema de classificação que desenvolvi para este efeito foi originalmente apresentado em 2005, no *Portuguese Symposium on Fabry Disease* [1], viria a servir de base para o sistema de classificação do *"International Study Group of Fabry Nephropathy* (*ISGFN*)*"* [2] e foi usado em todos os estudos de patologia renal publicados nesta Tese, incluindo a descrição da patologia renal em mulheres com nefropatia de Doença de Fabry [3], a demonstração da utilidade de um método imuno-histoquímico para diagnóstico específico da nefropatia da Doença de Fabry, mesmo em tecido incluído em parafina e processado para microscopia óptica convencional [4], e a descrição comparativa da patologia renal observada em ratinhos privados (*knock-out*) do gene *GLA* [5].

O estudo protocolado de biopsias renais de doentes com Doença de Fabry com o sistema de avaliação e classificação validado pelo *ISGFN*, e as respetivas correlações clínico-patológicas, ajudaram a compreender a história natural da nefropatia da Doença de Fabry. Os resultados deste estudo [3], baseado na classificação de biopsias renais de 35 homens e de 24 mulheres com Doença de Fabry, na sua maioria nos estádios iniciais de doença renal crónica, recrutados através de 10 centros de referência europeus e norte-americanos, mostraram que as lesões crónicas de esclerose glomerular, de fibrose intersticial e de atrofia tubular surgem precocemente na história natural da doença, podendo ser observadas em doentes nos estádios 1 e 2 de doença renal crónica, com proteinúria mínima. O grau de vacuolização podocitária em microscopia óptica e a quantidade e volume dos depósitos de glicoesfingolípidos em podócitos, observados nos cortes semifinos eram significativamente maiores nos homens do que nas mulheres. Os homens também apresentavam maiores quantidades de inclusões de glicoesfingolípidos nos túbulos proximais, nos capilares peritubulares e no endotélio vascular. Os graus de hialinose arteriolar eram semelhantes nos dois sexos mas as mulheres apresentavam maior grau de hialinose arterial, provavelmente em relação com a sua média etária significativamente mais elevada (43,9 *versus* 36,4 anos). Globalmente, o grau de esclerose arterial também se correlacionava significativamente com o estádio de doença renal crónica. Estas observações sustentam a indicação para biopsia renal em doentes com Doença de Fabry, mesmo nos estádios precoces de nefropatia. Ademais, este sistema de classificação histopatológica das manifestações renais da Doença de Fabry poderá ser útil para fazer uma estimativa de prognóstico e para avaliar a resposta à terapêutica da nefropatia da Doença de Fabry.

O estudo sistematizado das biopsias renais da série de mulheres heterozigotas para mutações patogénicas do gene *GLA* diagnosticadas no HSJ [3], e a sua análise combinada com casos de séries publicadas na literatura, permitiu demonstrar que a patologia renal observada em mulheres com Doença de Fabry é qualitativamente sobreponível à do homem, e que os graus de esclerose glomerular e de fibrose intersticial são, respetivamente, os principais preditores de proteinúria e do estádio de evolução da doença renal crónica. A distribuição celular dos depósitos de glicoesfingolípidos pôde ser mais eficazmente avaliada em cortes semifinos corados pelo azul de toluidina ou pelo azul-de-metileno. Em microscopia eletrónica, apenas em doentes com proteinúria patológica se observou fusão parcial dos processos pedicelares dos podócitos. Não se identificou evidência histopatológica consistente do envolvimento vascular na patogenia precoce da nefropatia da Doença de Fabry em mulheres.

Dado que os depósitos intracelulares de glicoesfingolípidos que se acumulam na Doença de Fabry são grandemente removidos no decurso do processamento histológico de tecidos fixados em formol e incluídos em parafina para observação por microscopia óptica, a vacuolização das células afetadas é a característica mais típica (embora inespecífica) da

Doença de Fabry em avaliação histopatológica convencional. Havia, no entanto, alguma evidência histológica, incluindo de observações pessoais, do que uma parte dos depósitos de glicoesfingolípidos, sobretudo a sua fração glucídica, não-lipossolúvel, pudesse permanecer nos lisossomas, mesmo em tecidos processados para avaliação por microscopia óptica de rotina.

Para testar a hipótese de que esta fração remanescente de glicoesfingolípidos permitisse o diagnóstico imuno-histoquímico de Doença de Fabry, estudaram-se 9 biopsias renais de doentes com Doença de Fabry - incluindo 4 homens, 4 mulheres e uma criança de sexo masculino -, e ainda a de uma mulher heterozigota para uma mutação do gene *GLA* de baixa patogenicidade, sem evidência bioquímica nem ultraestrutural de nefropatia de Doença de Fabry, usando como anticorpo primário um anticorpo murino altamente específico para a Gb3 humana [4]. As biopsias dos indivíduos adultos foram adicionalmente marcadas com um anticorpo para uma proteína da membrana lisossómica (LAMP2: *Lysosomal Associated Membrane Protein 2*). A distribuição dos depósitos de Gb3 foi quantificada por tipo de célula renal e comparada com as correspondentes quantificações histológicas em cortes semifinos. Os doentes com Doença de Fabry apresentavam Gb3 residual em todos os tipos de células glomerulares, tubulares intersticiais e vasculares do rim. A expressão mais elevada de LAMP2 foi observada em células tubulares mas não se identificou qualquer associação significativa entre a expressão imuno-histoquímica de LAMP2 e a prevalência de depósitos de Gb3 nos diferentes tipos de células renais. As quantificações histológicas dos depósitos de glicoesfingolípidos nos cortes semifinos foram relativamente mais elevadas do que as correspondentes quantificações imuno-histoquímicas. Na mulher heterozigota para a mutação *GLA* de baixa patogenicidade, a expressão imuno-histoquímica de Gb3 limitava-se a células tubulares, num padrão semelhante ao que foi observado em biopsias de controlos sem Doença de Fabry. Os resultados deste estudo demonstraram que a imunomarcação de Gb3 permite o diagnóstico específico da nefropatia da Doença de Fabry, mesmo em biopsias processadas para microscopia óptica de rotina [4]. Será possível, deste modo, o estudo retrospetivo de biopsias incluídas em parafina, quer para diagnóstico quer para estudos de correlação clínico-patológica.

A importância da biopsia renal para diagnóstico de Doença de Fabry foi evidenciada no estudo de uma família em que as biopsias renais de um probante de sexo masculino, de um seu irmão igualmente afetado e da mãe de ambos eram típicas de nefropatia de Doença de Fabry, mas em que a análise mutacional do gene *GLA* apenas revelara polimorfismos não patogénicos. O estudo genético adicional, com sequenciação de ADN complementar, permitiu identificar uma duplicação em tandem de três exões do gene [5], um tipo de mutação que nunca anteriormente fora descrito na Doença de Fabry.

O ratinho privado do gene *GLA* ("ratinho Fabry") foi produzido para constituir um modelo animal de Doença de Fabry, mas dado que estes ratinhos não desenvolvem doença renal crónica progressiva a sua relevância como modelo da nefropatia da Doença de Fabry humana é duvidosa. Para descrever a patologia renal dos ratinhos Fabry, avaliaram-se sistematizadamente as alterações histológicos observadas nos rins de 24 ratinhos Fabry de diversos escalões etários, por comparação com ratinhos controlo [6]. As alterações histopatológicas renais observadas nos ratinhos Fabry foram adicionalmente comparadas com a patologia renal reportada em doentes com Doença de Fabry, em faixas etárias equivalentes e em diferentes estádios de doença renal crónica. Com a idade, os ratinhos Fabry acumulam glicoesfingolípidos gradualmente em todos os tipos de células renais, num padrão que é semelhante ao que se observa em doentes com nefropatia da Doença de Fabry, com exceção das células mesangiais, em que a prevalência de depósitos é superior em doentes com nefropatia da Doença de Fabry, e das células dos túbulos proximais, em que a prevalência de depósitos é superior nos ratinhos Fabry. Porém, os ratinhos Fabry não desenvolvem as lesões histológicas inespecíficas de esclerose glomerular e de fibrose intersticial que melhor se correlacionam com a progressão da doença renal crónica em doentes com Doença de Fabry, nem expressam as volumosas inclusões de glicoesfingolípidos em podócitos que são características da nefropatia da Doença de Fabry. A elucidação dos mecanismos subjacentes às diferenças histopatológicas observadas entre espécies poderá contribuir para melhor compreender a patogenia da nefropatia da Doença de Fabry e, eventualmente, identificar alvos terapêuticos adicionais para prevenção ou tratamento da doença renal crónica na Doença de Fabry.

O conhecimento que adquiri na patologia renal da Doença de Fabry, decorrente da avaliação e classificação de um total de 72 biopsias renais de homens e mulheres com nefropatia de Doença de Fabry ou com deficiência de α-galactosidase, resultou no convite para ser coautora do capítulo dedicado às manifestações renais da Doença de Fabry, de um livro de texto monotemático sobre Doença de Fabry [7], no qual tive particular contribuição na descrição das alterações morfológicas observadas nos rins e nas respetivas ilustrações.

Tive ainda a oportunidade de rever o exame histopatológico do baço de um homem de 39 anos de idade com Doença de Fabry clássica, em programa regular de hemodiálise desde há cerca de 15 anos [8]. A esplenectomia fora efetuada para tratamento de hiperesplenismo sintomático com vários anos de evolução. No exame macroscópico da peça operatória, o baço pesava 700g e apresentava um aspeto fibrocongestivo. Histologicamente observou-se expansão da polpa vermelha e diminuição da polpa branca. Alguns histiócitos e muitas células do revestimento endotelial dos sinusoides apresentavam vacuolização citoplasmática porém contendo material argirofilo no seu interior, provavelmente a fração glucídica residual dos glicoesfingolípidos acumulados nos lisossomas. Uma revisão de casos publicados na literatura e dos dados de um registo

internacional de Doença de Fabry foi sugestiva de que o envolvimento do sistema reticuloendotelial não é invulgar em doentes com Doença de Fabry, podendo manifestar-se por linfadenomegalias e aumento do volume do baço, e por citopenias do sangue periférico, sobretudo anemia. A deposição de glicoesfingolípidos no endotélio dos sinusoides, comprometendo a normal circulação sanguínea esplénica, poderá ser causa de esplenomegalia e de hiperesplenismo em doentes com Doença de Fabry clássica.

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7

RESUMEN

La enfermedad de Fabry es una glicoesfingolipidosis donde se acumulan glicoesfingolípidos neutros, sobretodo globotriaosilceramida (Gb3), causada por la deficiente actividad del enzima lisosomal α–galactosidasa. La ausencia o deficiencia grave de α–galactosidasa produce la presentación multisistémica clásica de la enfermedad de Fabry; cuando la actividad enzimática residual es significativa, la enfermedad se presenta fenotípicamente de forma más limitada y tardía que la forma clásica. Se estima la prevalencia del fenotipo clásico de la enfermedad de Fabry en unos 1:117.000 nacidos vivos. Debido a su rareza, la enfermedad de Fabry se incluye entre las enfermedades huérfanas, definidas por la Unión Europea como aquellas enfermedades crónicas debilitantes o que condicionan riesgo de vida y que afectan menos de 5 en 10.000 individuos. Dado el escaso interés de la industria farmacéutica en invertir en el desarrollo y comercialización de medicamentos para tratar estas enfermedades, ya que el coste de la inversión no sería recuperado en las ventas subsecuentes, se han implementado políticas de desarrollo e incentivos económicos para promover la investigación de tratamientos para las enfermedades huérfanas. El desarrollo de una terapia específica para la enfermedad de Fabry con α-galactosidasa, mediante técnicas moleculares de genética recombinante, es un buen ejemplo de este problema.

A lo largo de la última década, después de aprobarse el tratamiento sustitutivo enzimático, con α-galactosidasa recombinante, y de los problemas surgidos acerca de la monitorización de su eficacia, se ha registrado un aumento exponencial de la investigación básica y clínica en la enfermedad de Fabry. Esclarecer los mecanismos etiopatogénicos causantes de las complicaciones sistémicas de la enfermedad, comprender su fisiopatología e identificar alternativas terapéuticas más cómodas y/o eficaces para los enfermos, son los principales objetivos de estas nuevas investigaciones.

Desde finales de la década de los sesenta, se fue generando en el Servicio de Anatomía Patológica del Hospital de São João (HSJ) y de la Facultad de Medicina de la Universidad do Porto (FMUP) un archivo considerable de biopsias renales de pacientes de ambos sexos con enfermedad de Fabry. Están incluidas tanto formas graves (fenotipo clásico) como

atenuadas o leves (variantes tardías, con alcance orgánico más limitado) de la enfermedad, así como la biopsia de un niño. Dicho archivo cuenta también con una pieza de esplenectomía de un paciente con enfermedad de Fabry clásica, que había desarrollado un cuadro de hiperesplenismo sintomático después de vários años en programa de hemodiálisis por fallo renal crónico debido a la nefropatía provocada por la enfermedad de Fabry.

Vítor Faria, patólogo del Servicio de Anatomía Patológica del HSJ y de la FMUP y pionero en la investigación de la enfermedad de Fabry en Portugal, publicó en 1970 unos resultados reveladores basados en el estudio de microscopía óptica y electrónica de las biopsias de piel, hígado y riñón del primer paciente con enfermedad de Fabry diagnosticado en Portugal. Por otro lado, a finales de los años noventa se realizaron en el Servicio de Genética de la FMUP los primeros diagnósticos moleculares de enfermedad de Fabry en Portugal, mediante el análisis de las mutaciones del gen de la α-galactosidasa (*GLA*), incluyendo el diagnóstico prenatal de un feto.

En este contexto, y gracias al notable trabajo de archivo de biopsias renales del Dr. Elísio de Carvalho, surgió la oportunidad de realizar el análisis sistematizado del material histológico existente en el Servicio de Anatomía Patológica del HSJ. Los objetivos se centraron en la realización de una descripción detallada de la patología renal de la enfermedad de Fabry, procurando identificar indicios morfológicos sobre la etiopatogénesis de la nefropatía de Fabry y la importancia relativa de la implicación patológica de cada una de las diferentes células renales en la progresión de la enfermedad. La descripción de la patología esplénica co-existente en el paciente con enfermedad de Fabry clásica e insuficiencia renal crónica en hemodiálisis, fue otro de los objetivos.

Debido a la inexistencia de criterios convalidados para describir y cuantificar la patología renal en la enfermedad de Fabry, fue imperativo y mandatorio para el desarrollo del plan de Tesis, crear un sistema de clasificación de las biopsias renales que permitiera uniformizar su lectura e interpretación. El sistema de clasificación que creé para este fin fue originalmente presentado en 2005, en el *Portuguese Symposium on Fabry Disease* [1], y posteriormente, sería la base del sistema de clasificación, actualmente aceptado y convalidado, del "*International Study Group of Fabry Nephropathy* (ISGFN)" [2]. Este sistema fue usado en todos los estudios de patología renal publicados en esta Tesis, que incluye la descripción de la patología renal en mujeres con nefropatía de Fabry [3], la demonstración de la utilidad de un método inmunohistoquímico para diagnóstico específico de la nefropatía de la enfermedad de Fabry en material incluido en parafina [4], y la descripción comparada de la patología renal observada en ratones knock-out para el gen *GLA* [5].

El estudio sistematizado de las biopsias renales de pacientes Fabry, con el sistema de evaluación y clasificación convalidado por el ISGFN, y las respectivas correlaciones

clínico-patológicas, ayudaron a comprender la historia natural de la nefropatía de la enfermedad de Fabry. En este estudio [3] se clasificaron 69 biopsias renales (35 hombres y 24 mujeres) de pacientes con enfermedad de Fabry, provenientes de 10 centros de referencia europeos y norteamericanos. Los pacientes se encontraban en su mayoría en un estadío inicial de enfermedad renal crónica. Los resultados revelaron que las lesiones crónicas inespecíficas de esclerosis glomerular, fibrosis intersticial y atrofia tubular surgen precozmente en la historia natural de la enfermedad (estadios 1 y 2 de enfermedad renal crónica y proteinuria mínima). El grado de vacuolización podocitaria en los cristales de los cortes de parafina, y la cantidad y volumen de los depósitos de glicoesfingolípidos en los podocitos observadas en los cortes semifinos, eran mayores en los hombres que en las mujeres ($p<0,05$). Los hombres presentaban también mayor cantidad de inclusiones de glicoesfingolípidos en los túbulos proximales, en los capilares peritubulares e en el endotelio vascular. El grado de hialinosis arteriolar era semejante en ambos sexos, pero las mujeres mostraron un mayor grado de hialinosis arterial, probablemente relacionado con la franja etaria (43,9 *versus* 36,4 años). Globalmente, el grado de esclerosis arterial también se correlacionaba con el estadio de la enfermedad renal crónica ($p<0,05$). Estas observaciones sustentaron la importancia de realizar biopsia renal de base en pacientes con enfermedad de Fabry, incluso en estadios clínicos precoces de nefropatía. Además, este sistema de clasificación histopatológica de las manifestaciones renales de la enfermedad de Fabry podrá ser útil en el futuro para realizar predicciones de pronóstico y evaluar la respuesta al tratamiento de la nefropatía de la enfermedad de Fabry.

El estudio sistematizado de las biopsias renales de la serie de mujeres heterocigotas para mutaciones patógenas del gene *GLA* diagnosticadas en el HSJ [3], y su análisis combinado con los casos de series publicadas en la literatura, permitió demostrar que la patología renal observada en las mujeres con enfermedad de Fabry es cualitativamente superponible a la del hombre, y que los grados de esclerosis glomerular y de fibrosis intersticial son, respectivamente, los principales factores predictivos de la protenuria y del estadio de enfermedad renal crónica. Se demostró asimismo, que los cortes semifinos teñidos con azul de toluidina o azul de metileno, permite evaluar de forma eficaz la distribución de los depósitos de glicoesfingolípidos en el tejido renal. Únicamente las pacientes con proteinuria patológica mostraron, en el estudio de microscopía electrónica, fusión parcial de los pedicelos de los podocitos. No se identificó evidencia histopatológica de la implicación de la vasculatura renal en la patogénesis de la nefropatía de Fabry en mujeres heterocigotas.

Las características bioquímicas de los glicoesfingolípidos acumulados en la enfermedad de Fabry, hacen que éstos desaparezcan mayoritariamente durante el proceso de inclusión en parafina de los tejidos fijados en formol para diagnóstico en microscopia óptica de rutina. Así la vacuolización de las células afectadas, se torna la característica histológica típica, aunque no exclusiva, de esta enfermedad. A pesar de todo, existían evidencias histológicas,

que incluían observaciones personales, de que una parte de los depósitos de glicoesfingolípidos, la fracción glucídica no liposoluble, pudiera permanecer en los lisosomas, incluso en tejidos incluidos rutinariamente en parafina.

Para probar la hipótesis de que esta fracción de los glicoesfingolípidos permitiese realizar el diagnóstico inmunohistoquímico de la enfermedad de Fabry, se estudiaron 9 biopsias renales de pacientes con esta enfermedad y afectación renal - 4 hombres, 4 mujeres y un niño de sexo masculino - y también la biopsia de una mujer heterocigota para una mutación del gene *GLA* con baja patogenicidad y sin evidencia bioquímica y ultraestructural de nefropatía de Fabry [4]. Se usó un anticuerpo de ratón altamente específico para la Gb3 humana como anticuerpo primario, y las biopsias de lo pacientes adultos fueron sometidas a doble marcación simultánea con un anticuerpo contra una proteína de membrana lisosomal (LAMP2: *Lysosomal Associated Membrane Protein 2*). Se cuantificó la distribución de los depósitos de Gb3 por tipo celular renal y se compararon los resultados con las correspondientes cuantificaciones histológicas realizadas en los cortes semifinos. Los pacientes con enfermedad de Fabry mostraron positividad para Gb3 en todos los tipos de células renales (glomerulares, tubulares, intersticiales y vasculares). Las células tubulares presentaban la mayor expresión de LAMP2, pero no se identificó ninguna asociación estadísticamente significativa entre la expresión inmunohistoquímica de LAMP2 y la prevalencia de depósitos de Gb3 en los distintos tipos celulares. Las cuantificaciones histológicas de los depósitos celulares de glicoesfingolípidos en los cortes semifinos, fueron relativamente mayores que las correspondientes cuantificaciones inmunohistoquímicas. La expresión inmunohistoquímica de Gb3 en la mujer heterocigota para la mutación *GLA* de baja patogenicidad, estaba limitada a las células tubulares, con un patrón similar al que fue observado en las biopsias de los casos control usados (sin enfermedad de Fabry).

Los resultados obtenidos demostraron que la identificación inmunohistoquímica de Gb3 permite el diagnóstico específico de nefropatía de enfermedad de Fabry en biopsias procesadas rutinariamente para estudios de microscopia óptica [4]. Con esto, se torna posible el estudio retrospectivo de material de archivo (biopsias incluidas en parafina), tanto para diagnóstico como para estudios de correlación clínico-patológica.

La importancia de la biopsia renal como elemento de diagnóstico de enfermedad de Fabry, fue resaltada en el estudio de una familia en las que las biopsias renales del probando de sexo masculino, de su hermano, igualmente afectado, y de su madre, mostraban alteraciones típicas de nefropatía de enfermedad de Fabry. Pero el análisis de las mutaciones del gene *GLA* sólo reveló polimorfismos no patogénicos. La evidencia histológica sustentó la realización de estudios genéticos adicionales, con secuenciación del ADN complementario, permitiendo identificar de esta forma una duplicación en tándem de tres exones del gene [5], mutación patogénica nunca anteriormente descrita en la enfermedad de Fabry.

El ratón *knockout* para el gen *GLA* ("raton Fabry") fue creado para investigar en un modelo animal de enfermedad de Fabry, pero ya que estos ratones no desarrollaban enfermedad renal crónica progresiva, su interés como modelo de la nefropatía de enfermedad de Fabry es polémico. Para descrivir la patologia renal de este modelo animal, estudiamos las alteraciones histológicas renales observadas en 24 ratones Fabry de diversas franjas etarias, comparándolas con las de los ratones control [6]. Asímismo, las alteraciones histopatológicas de los ratones Fabry fueron también comparadas con la patología renal publicada en los pacientes con enfermedad de Fabry, con franjas etarias equivalentes y en diferentes estadios de enfermedad renal crónica. La acumulación de glicoesfingolípidos en los diferentes tipos celulares renales en ratones Fabry, se incrementa gradualmente con la edad. El patrón de acumulación es similar al observado en pacientes con nefropatía de la enfermedad Fabry, a excepción de la acumulación en células mesangiales, donde la prevalencia de depósitos es superior en los humanos afectos por esta nefropatía, y en células de túbulos proximales, donde la prevalencia es superior en los ratones Fabry. Además, los ratones Fabry no desarrollan las lesiones histológicas crónicas inespecíficas de esclerosis glomerular y de fibrosis intersticial, que con significancia estadística en su correlación con la progresión de la enfermedad renal crónica en humanos. Los depósitos podocitarios en estos ratones son significativamente más pequeños si se comparan con las voluminosas inclusiones de glicoesfingolípidos en los podocitos glomerulares humanos. El esclarecimiento de los mecanismos subyacentes en estas diferencias histopatológicas observadas entre especies, podrá contribuir para comprender mejor la patogenesis de esta nefropatía, y, eventualmente, identificar nuevas dianas terapéuticas para prevenir o tratar la enfermedad renal crónica en la enfermedad de Fabry.

El conocimiento que adquirí en la patología renal de la enfermedad de Fabry, producto de la evaluación y clasificación de un total de 72 biopsias renales e hombre y mujeres con nefropatía de enfermedad de Fabry, o con deficiencia de α-galactosidasa, conllevó a la invitación para ser co-autora del capítulo dedicado a las manifestaciones renales de la enfermedad de Fabry, de un libro monotemático sobre enfermedad de Fabry [7], en el cual tuve particular contribución en la descripción de las alteraciones morfológicas renales y en la respectiva iconografía.

Tuve también la oportunidad de revisar el examen histopatológico del bazo de un hombre de 39 años con enfermedad de Fabry clásica, e programa de hemodiálisis desde hacía unos 15 años [8], cuya esplenectomía se realizo como tratamiento de hiperesplenismo sintomático de varios años de evolución. La macroscopía mostró una pieza de 700gr de peso con aspecto fibrocongestivo. Histológicamente se observó expansión de la pulpa roja y disminución de la pulpa blanca, vacuolización citoplasmática de algunos histiocitos así como de muchas células endoteliales que revestían los sinusoides. Estas células mostraron un material puntiforme y argirófilo en dicho citoplasma vacuolizado, probablemente la fracción

glucídica residual de los glicoesfingolípidos acumulados en los lisosomas de las mismas. Una revisión de casos publicados en la literatura y de datos de un registro internacional de enfermedad de Fabry contribuyó para levantar la hipótesis de que la implicación del sistema retículoendotelial no es rara en estos pacientes, pudiendo manifestarse con linfadenomegalias y esplenomegalia, y con citopenias en sangre periférica, sobretodo anemia. El depósito de glicoesfingolípidos en el endotelio sinusoidal, comprometiendo la normal circulación sanguínea esplénica, podría ser la causa de la esplenomegalia y del hiperesplenismo en los pacientes con enfermedad de Fabry clásica.

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8

ATTACHMENT

• **FABRY NEPHROPATHY WORKING GROUP SCORE SHEET** •

Case number: _____ **Scorer:** _____ **Date:** _____

• **LIGHT MICROSCOPY:** (Only score circled section indicated by a mark.)

Total number of glomeruli _____

1) PODOCYTE INCLUSIONS		**Total**	**Podocyte score** (n affected x score)
None (score = 0)			
Mild (score = 1; <25%)			
Moderate (score = 2; 25-50%)			
Severe (score = 3; >50%)			
	Total scorable glomeruli		
Indeterminate			

Mean Podocyte Score (n affected x score) / total scorable counted

2) OTHER LESIONS		**Total**
Nonsclerotic glomerulus		
Segmental sclerosis, mild		
Segmental sclerosis, severe		
Global sclerosis		
Adhesion		
Ischemic/collapse		
Peri-glomerular fibrosis and/or Bowman's capsule reduplication		
Indeterminate		

3) ARTERIES: Present ___ Absent ___

	Total
Average sclerosis (0-3)	
Most severe sclerosis(0-3)	

Hyalinosis:

	Present	Absent
Arterioles:	Medial	
	Subendothelial	
Arteries:	Medial	
	Subendothelial	

4) TUBULOINTERSTITIUM	**Estimated — scored to nearest 10%**
Tubular atrophy	
Interstitial fibrosis	

Interstitial inflammation:

Absent ____ / Present _____

Scarred _____

Non-scarred _____

• **THICK SECTION:** (Only score circled section.)

0; 1 — rare, small; 2 — many, small; 3 — rare, large; 4 — ≥50% large

	Glomeruli available	**Segmental sclerosis**		**Global sclerosis**
		mild	**severe**	
Number of:				

	Glomerulus								
Inclusions	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**Average**
Podocyte (0-4+)									

	Present	**Absent**	**Not Sampled**
Parietal epithelial inclusions			
Proximal tubular inclusions			
Distal tubular inclusions			
Peritubular capillary inclusions			
Vascular intimal inclusions			
Vascular medial inclusions			

COMMENTS:

FABRY NEPHROPATHY WORKING GROUP SCORE SHEET — DEFINITIONS

The circled section indicated by a mark should be the only section scored for *light microscopy*. In addition, the circled toluidine blue *thick section* circled section should be the only section scored for these assessments.

The "Comments" box can be used to denote lesions seen on any other sections, but should not be included in the quantitative scoring.

LIGHT MICROSCOPY

1) *Podocyte inclusions* should be scored as absent, or present on a score from 1 to 3 for each individual glomerulus. Glomeruli that cannot be scored due to for instance incomplete sections of global sclerosis should be indicated in the box *"indeterminate"* for scoring. Glomeruli with less than 25% of the tuft represented in the section are also indeterminant for inclusion scoring. The total number of *scorable glomeruli* is then entered.

The extent of podocyte inclusions is quantitated as 0) none; 1) less than a quarter of podocytes showing vacuolization/visible inclusions by light microscopy, 2) between 25 to 50%, or 3) greater than 50%.

2) *Other lesions*

The total number of *nonsclerotic glomeruli* without segmental or global sclerosis, or ischemic collapse lesions is indicated, and glomeruli with segmental sclerosis are enumerated.

Segmental sclerosis is defined as obliteration of capillary lumen with increased matrix involving a portion of the glomerulus. Mild segmental sclerosis is scored when <50% of the tuft is affected, severe segmental sclerosis if >50% is affected.

Global sclerosis is scored when the entire glomerular tuft is sclerosed.

Adhesions are scored when there is continuity of connective tissue between the glomerular tuft and Bowman's capsule. Of note, adhesion is not scored as present if cells are merely abutting each other. Of note, adhesion is not scored if it is part of a well defined segmental sclerotic lesion, but only when this is the only lesion present.

Ischemia/collapse is scored when greater than half of the glomerular tuft shows corrugation of the GBM and retraction of the tuft, indicative of ischemia/collapse.

Peri-glomerular fibrosis and/or Bowman's capsule reduplication is enumerated in terms of how many glomeruli are involved, defining an involved glomerulus when more than 25% of the circumference of Bowman's capsule is affected, in the absence of global glomerulosclerosis.

Other types of glomerular lesions should be noted in the "Comments" box. Presence of mesangial hypercellularlity should be noted here.

Total number of glomeruli is a sum of all scorable and nonscorable glomeruli. Fragments of glomeruli with <25% of the tuft represented are scored as *indeterminant*. Empty Bowman's capsules or portions thereof without any capillary loops are not scored for calculating number of glomeruli.

3) *Arteries*. *Arterial sclerosis* is scored based on lesions in vessels as an average and also as a separate score for the most severely affected as outlined in previous work by Remuzzi et al (JASN 10:2591, 1999).

A score of 0 is given if vascular lesions are absent.

1+ indicates increased wall thickness but to a degree that is less than the diameter of the lumen.

2+ is given for vessels with wall thickness that is equal or slightly greater to the diameter of the lumen, and

FABRY NEPHROPATHY WORKING GROUP SCORE SHEET — DEFINITIONS

3+ is given for wall thickness that far exceeds the diameters of the lumen with extreme lumenal narrowing or occlusion.

Note: Put comment in Comment box if the intimal fibrosis versus medial relative thickness and lumen diameter are disproportionate.

Arteriolar or arterial hyalinosis is scored as present or absent, and specified as to involving the media or subendothelial location.

4) *Tubulointerstitium*

Tubular atrophy is semi-quantitatively estimated and scored to the nearest 10%.

Interstitial fibrosis is estimated and scored to the nearest 10%, except for ≤5% which is scored as 5%. Subcupsular location of fibrosis is noted in the comments.

Interstitial inflammation is noted as present or absent. If present, it is specified as to whether it is present in scarred areas or non-scarred areas or both.

THICK SECTION

Additional scoring is done from the thick-section toluidine blue-stained slide. Comment on presence or absence of interstitial fibrosis.

Number of glomeruli available on the single section is designated.

Segmental sclerosis is listed as number involved, as is

global sclerosis, defined as above.

An inclusion score is given separately for each glomerulus, and an average score then given.

Inclusions are estimated as follows:

Non-epithelial glomerular cell involvement with inclusions includes mesangial/endothelial and/or infiltrating cells, quantitated on a score of 0 to 2. 0 is scored when there are no inclusions, 1 is scored for one or two cells per glomerulus with inclusions, and 2 is scored when greater than or equal to 3 cells per glomerulus show inclusions.

Podocyte inclusions are scored as follows:

0 is scored when there are no deposits;

1+ when there are rare small inconspicuous deposits;

2+ when there are more frequent small deposits;

3+ when less than half of the tuft is filled with large expanded deposits in the presence or absence of concurrent small deposits; and

4+ when more than half the tuft is involved with large expanded deposits.

The following are scored as present, absent or not listed:

Parietal epithelial inclusions;

Proximal tubular inclusions;

Distal tubular inclusions;

Peritubular capillary inclusions;

Vascular intimal inclusions; and

Vascular medial inclusions.

Comments: If comparing to a previous biopsy, note if changes are present not captured in above.`

9

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