NEPHROPATHOLOGY- ELECTRON MICROSCOPY SEMINAR: CASE 2

ABSTRACT
We present the case of a 32 years old woman with nephrotic range proteinuria. The proteinuria was 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. Her mother died at 52 years old due to an unknown renal disease. Two of her brothers, followed elsewhere, had received kidney transplants as young adults, for end-stage renal failure (ESRF) attributed to “focal glomerular sclerosis”. Her sCr was 0.8 mg/dl, proteinuria was initially quantified as 1.4 g/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 final diagnosis of Fabry disease was made in the electron microscopy study. Due the very low prevalence of this disease we take the opportunity to discuss the light microscopy and electron microscopy characterisation of Fabry nephropathy and the differential diagnosis of the disease.

INTRODUCTION
Fabry disease is an X-linked storage disorder of neutral glycosphingolipids caused by deficient activity of the lysosomal enzyme α-galactosidase. The α-galactosidase defect results in multi-systemic accumulation of neutral glycosphingolipids with terminal α-galactosyl residues, predominantly globotriaosylceramide (Gb3). Chronic kidney disease (CKD), cardiomyopathy and stroke are major causes of morbidity and early mortality in affected adults [Desnick 2001, Desnick 2003, Zarate 2008].

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 [Valbuena 2012a].

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. In these patients, chronic kidney disease (CKD) is an invariable complication, usually progressing to 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. Nephrotic range proteinuria develops in less than 20% of male Fabry patients with CKD. Before the availability of effective renal replacement therapies, ESRF was the main cause of death of classically affected patients [Valbuena 2008, Warnock 2010].

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, and are at higher risk of premature death. Evidence of renal involvement, including proteinuria and decreased renal function, may be found in up to 40% of known adult heterozygotes. A small proportion of female patients can be as severely affected as males with the classical phenotype, reaching ESRF approximately at the same age [Ortiz 2008, Valbuena 2008, Wilcox 2008, Zarate 2008].
The diagnosis of Fabry disease and specifically of Fabry nephropathy, is often missed or delayed, particularly if early-onset neuropathic and/or cutaneous manifestations, or a positive family history, are lacking. Performing the diagnosis of de novo cases is difficult and is often carried out more than a decade after onset of first symptoms [Valbuena 2012, Valbuena 2012a]. Identification of glycosphingolipid deposits in affected organs by light microscopy or electron microscopy has been regarded as an ancillary diagnostic method of Fabry disease, particularly useful in patients with renal involvement. But, 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 [Gubler 1978, Valbuena 2012, Valbuena 2012a].

Until recently, recognition of Fabry disease did not affect the patient’s prognosis, since no treatment was available. However, the availability of enzyme replacement therapy (ERT) since 2001 with recombinant alpha-galactosidase A offers the promise of altering the natural history of this rare disease. Currently, there are two forms of ERT available for the treatment of Fabry disease: (1) Replagal® (agalidase-alfa; Shire Human Genetic Therapies, Inc., Cambridge, MA, USA) and (2) Fabrazyme® (agalidase-beta; Genzyme Corporation, Inc., Cambridge, MA, USA). Both were approved in 2001 by the European Agency for Evaluation of Medical Products; only Fabrazyme® was approved by the FDA for use in the USA. Second-step treatment is also needed in many patients. Fabry nephropathy is treatable, even in patients with fairly advanced disease. Once present, target kidney damage is not reversed, so stopping further progression is the treatment goal [Warnock 2010].

LIGHT MICROSCOPY FEATURES OF FABRY NEPHROPATHY

Light microscopy alterations are remarkable and can elucidate the diagnosis of Fabry disease. Some reports showed that histological evidence of kidney involvement precedes clinical signs in early Fabry nephropathy [Tøndel 2008]. The most characteristic finding on routine light microscopy 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. The vacuoles generally are small and uniform and impart a honeycomb appearance to these cells. Interstitial foam or lipid-laden cells can also be seen. The vacuoles are histological artifacts resulting from removal of GSL deposits during clearing and paraffin embedding of the tissue. With the methenamine silver and PAS stains, argyrophilic and PAS-positive inclusions may be identified in some affected cells [Fogo 2010, Gubler 1978, Valbuena 2012].

The pattern of cell type involvement and the prevalence of vacuolated cells of each type are roughly dependent on the age and gender of the patient and on the clinical severity of Fabry nephropathy. Males had greater podocyte vacuolization on light microscopy than females [Fogo 2010]. Cytoplasmic vacuolization is a non-specific feature of several other, often more prevalent kidney diseases, and although the pattern of cellular involvement may be suggestive of Fabry disease, the diagnosis of Fabry nephropathy can be overlooked on routine light microscopy evaluation [Valbuena 2012].

*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. Early signs of focal segmental glomerular sclerosis have been observed even in a female teenager with normal renal function and normal urinary albumin excretion. 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 or either normal or only a slightly increased urinary albumin excretion rate [Fogo 2010, Valbuena 2008, Valbuena 2012a].

**IMMUNOHISTOCHEMISTRY IN FABRY NEPHROPATHY**
Immunohistochemistry of paraffin-embedded kidney tissue sections of patients with Fabry nephropathy using an anti-Gb3 primary antibody allows the specific identification of residual Gb3 in all types of glomerular, tubular, interstitial and vascular kidney cells, although at relatively lower scorings than the corresponding histological scorings on semi-thin plastic embedded sections [Valbuena 2012].

**IMMUNOFLUORESCENCE IN FABRY NEPHROPATHY**
Immunofluorescence is important in the differential diagnosis of other kidney diseases that may occur concurrently in patients with Fabry disease. Asymptomatic IgA deposits have been repeatedly identified in kidney biopsies of patients with Fabry disease, with a frequency that seems to be higher than in non-selected autopsy cases. In cases with advanced lesions, such as segmental sclerosis, IgM, C3 and C1q may be present in capillary walls and mesangial regions with a segmental distribution and granular pattern [Valbuena 2008, Valbuena 2012].

**ELECTRON MICROSCOPY FEATURES OF FABRY NEPHROPATHY**
In semi-thin cuts of plastic-embedded sections of osmium-fixed tissues stained with toluidine- or methylene-blue, glycosphingolipids deposits appear as fine and blue darkly-stained granular or round intracellular inclusions. This semi-thin cuts allow to evaluate the presence, distribution and quantification of glycosphingolipids depositions in all individual renal cells and in particular in peritubular capillary cells and interstitial cells [Fogo 2010, Valbuena 2008, Valbuena 2012, Valbuena 2012a].

On electron microscopy, glycosphingolipids deposits typically appear as dense osmiophilic, coarsely lamellated inclusions composed of alternating dark and clear layers (myelin figures and zebra bodies). Smaller, amorphous dense osmiophilic deposits may also be seen in some cells, but are less frequent and not as immediately reminiscent of the diagnosis of Fabry disease as the myelin figures or the zebra bodies. In lamellar and amorphous bodies, high magnification reveals a regular arrangement of alternatively light- and dark-staining bands. 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. 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 [Gubler 1978, Valbuena 2012a].

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. In podocytes, the size of glycosphingolipids deposits may reach 10 µm in diameter, but also epithelial tubular cells, may contain such large inclusions [Gubler 1978].
The glycosphingolipid 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, even when they look unaffected by conventional histology. The involvement of all types of kidney cells and the morphological features of the Gb3 deposits are similar in both genders, but in the heterozygous females the pattern of storage has been described as more irregular than in males, consistent with the expected morphological expression of X-chromosome inactivation [Gubler 1978, Valbuena 2012a].

In **glomeruli** the inclusions are present in every cell type, although the epithelial cells are affected to the greatest degree. Podocytes are the most severely affected cells. Endothelial and mesangial cells contain fewer inclusions, and these are smaller. The ultrastructure of the slit diaphragms of the foot processes may be spared despite the evidence of massive glycosphingolipid deposits in the cytoplasm of podocytes. Effacement of the podocyte foot processes correlates with the presence of moderate to nephrotic proteinuria, both in male and in female patients. Membranofibrillar and non-immunogenic deposits in subendothelial location of the glomerulus are also described in Fabry patients. They are probably the remains of ruptured lysosomal membranes which previously contained glycosphingolipid inclusions. 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. “Free-floating” myelin figures are often present in Bowman’s space and the tubular lumina [Gubler 1978, Valbuena 2008, Valbuena 2012a].

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 glycosphingolipid 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 [Valbuena 2012a].

**Endothelial cells** of peritubular capillaries, arterioles, arteries, and veins contain inclusions. These glycosphingolipid 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 [Valbuena 2012a].

**Smooth-muscle cells** of arteries and arterioles and **pericytes** also contain inclusion bodies. 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 1978].

Almost all **interstitial cells** contain lipid inclusions in hemizygous cases, although they generally sparse in distribution but quite dense in appearance. 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 [Valbuena 2012a].

Even though the ultrastructural features of the glycosphingolipid inclusions are not pathognomonic, electron microscopy is the most reliable morphological method to
identify glycosphingolipid deposits in tissue specimens of patients with Fabry disease [Valbuena 2012a].

ELECTRON-MICROSCOPY PHENOCOPIES OF FABRY NEPHROPATHY

Differential diagnosis is important in Fabry nephropathy. The ultrastructural appearance of the inclusions considerably narrows the many differential diagnostic considerations.

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 but plasma α-galactosidase activity was normal [Banks 1983].

Furthermore, cytoplasmic inclusions with ultrastructural features identical to those of Fabry disease have been also described in renal glomerular cells of patients treated with chloroquine or amiodarone and in tubular cells in patients treated with aminoglycoside antibiotics. In the case of aminoglycoside-induced renal phospholipidosis, the lamellar deposits are typically restricted to epithelial tubular cells, a pattern of distribution that does not mimic Fabry nephropathy. The pattern of cell involvement in amiodarone- and chloroquin-induced renal phospholipidosis, with lamellated inclusions visible in virtually all types of kidney cells and dominant involvement of podocytes and epithelial cells of distal tubules, may lead to the erroneous diagnosis of Fabry nephropathy, if the iatrogenic phospholipidosis is not considered in the differential diagnosis [Albay 2005, Müller-Höcker 2003, Pintavorn & Cook 2008].

In chloroquine toxicity, the light microscopic features in paraffin-embedded sections may include large histiocytes with prominent vacuoles, By electron microscopy, these cells are in capillary lumina or have infiltrated into the mesangium. This features are not reported in Fabry nephropathy [Albay 2005].

In chloroquine-induced renal phospholipidosis, Ferluga reported in his lecture at the 23rd ECP (Helsinki 2011) the presence of specific curvilinear inclusions in podocytes that have not been described until now in Fabry disease.

As Fabry disease has a specific therapy since 2001 it is important to elucidate the diagnosis of this disease. Although the ultrastructural abnormalities are quite consistent and specific of Fabry disease, assay of α-galactosidase in hemizygotes and molecular studies in heterozygotes should be always undertaken to confirm the diagnosis [Valbuena 2012a].
BIBLIOGRAPHY