The importance of electron microscopy and a study of type IV collagen alpha chains in the diagnosis of Thin Basement Membrane Glomerulopathy and Alport Syndrome

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Alport Syndrome (AS) and Thin Glomerular Basement Membrane Nephropathy (TBMN) are genetically heterogeneous conditions, characterized by structural abnormalities in the glomerular basement membrane. These diseases develop with thickening and thinning of the glomerular basement membrane, due to alterations in type IV collagen, the main component of the basement membrane, representing 50% of its dry weight. Deficiency in alpha 3, alpha 4 or alpha 5 chains of type IV collagen characterize Alport syndrome. In this chapter, we will demonstrate that ultrastructural characteristics examined by electron microscopy followed by morphometry of the basement membrane, associated with a study of type IV collagen alpha chains are crucial for the diagnosis of these nephropathies.

Key words: Electron Microscopy, Immunofluorescence, Alport Syndrome, Thin Glomerular Basement Membrane Nephropathy

1. Introduction

1.1 Glomerular diseases
Glomerular diseases can be of primary or secondary origin, and can result from a wide variety of factors: immune disorders, vascular diseases, metabolic diseases and some hereditary entities. Glomerulopathies arising isolated are classified as primary, and when associated with systemic diseases, they are classified as secondary. In this chapter we will describe two primary glomerulopathies whose changes occur in the glomerular basement membrane.

1.2 Glomerulus
The glomerulus is a structure consisted of capillaries and lined by endothelial cells. On the outside, these capillaries are covered with specialized glomerular epithelial cells, named podocytes. Podocytes are highly differentiated, forming a network of processes on the external layers of the capillaries, attaching to them through the basement membrane. An external epithelial capsule, named Bowman capsule, acts as a bag to collect the glomerular filtrate and leads it towards the proximal tubule. The capillaries are connected by a cluster of cells denominated mesangial cells [1]

The glomerular filter, through which the infiltrate passes, consists of three layers: fenestrated endothelium, intervening glomerular basement membrane (GBM) and the podocytes layer. This complex ‘membrane’ is permeable to water and small solutes, but retains most of the proteins and other large molecules, as well as whole blood cells. The size of the molecule is the main determinant of the passage across the glomerular filter. Proteinuria develops in some glomerular diseases due to loss of charge selectivity of the glomerular filtration barrier [1].

The kidney glomerular basement membrane is a thick basement membrane, 300 to 350 nm thick that provides the glomerular cells with structural support. Glomerular endothelial cells and podocytes are the cells responsible for the production of this basement membrane [2]. During glomerulogenesis, the GMB is produced as two separate layers that bind to form the mature GBM [2-8]. The GBM is functionally unique; it must facilitate constant fluid flow across the glomerular filtration barrier (GFB) while tolerating hemodynamic stresses. The composition of the GBM is also specialized, with specific macromolecules exclusively found in the glomerular basement membrane, as the α5β2δ1 laminin, referred to LM-521 [6, 9].

In the adult glomerulus, the podocyte continues to add and gather matrix molecules to GBM, consisting of type IV collagen, laminin, entactin/nidogen, agrin and perlecan [6].
For an intact glomerular filter, podocyte-podocyte interaction mediated by the slit diaphragm structure, and interactions of receptors existing in the podocyte basement membrane with extracellular matrix proteins are essential [10]. As for cell matrix contacts, it was observed that they are precisely controlled and are an essential prerequisite to maintain the highly ordered foot process architecture [10]. The basement membrane matrix molecules are constituents of this connection and act as ligands for the transmembrane adhesion receptors of the podocyte foot processes. In the intracellular side of the cell-matrix interaction, the interface molecules, responsible for the cell-matrix signaling and transmission of mechanical forces over the cytoskeleton assemble into a multi-molecular adhesion complex. [10].

Recently, the relevance of the GBM to the architecture of the podocytes was exemplified by loss or effacement of the foot processes of the podocytes and by proteinuria in laminin β2 chain deficient mice [11]. α5β2β1 laminins are assembled to form the heterotrimeric laminin -11, specific of GBM [12]. The absence of a single laminin monomer is enough to cause severe destruction of the filtration barrier, leading to a nephrotic phenotype. Interestingly, an α5-laminin knockout is not capable of forming a functional laminin, leading to a halt in the early stages of nephrogenesis [13]. Genetic modification of type IV collagen, via knockout of type IV collagen α3 chain results in a syndrome similar to Alport’s, with a compensatory upregulation of type IV collagen α1 and α2 chains. In type IV collagen α3 chain knockout mice, the composition of laminin of the GBM is also shifted to α2 e β1 laminins [14], inducing the effacement of podocyte foot processes.

Severe proteinuria and/or nephrotic syndrome are the most common manifestations of glomerulopathies, hereditary or not, such as: (a) Minimal Change Glomerulopathy; (b) Focal Segmental Glomerulosclerosis (FSGS); (c) Mesangial Proliferative Glomerulonephritis; (d) Membranoproliferative glomerulonephritis; (e) Crescentic Glomerulonephritis and (f) Glomerulopathies secondary to systemic diseases such as Systemic Lupus Erythematosus and Diabetes. This proteinuria results from a increased flow of albumin and other plasma proteins through the glomerular filtration barrier. Today, the role of GBM in glomerular permeselectivity remains unclear and is considered secondary to the role of the podocyte slit diaphragm [15, 16 2001]. Proteinuria can result from primary impairments in either podocytes or the glomerular basement membrane (GBM). However how a defect in the composition or in the structure of the molecular filtration barrier components can lead to proteinuria is a subject of debate.

Proteinuria may be associated with unexpected changes in the architecture of the podocytes, as detected by electron microscopy and comprises loss or effacement of the foot processes of the podocytes. Farquhar et al. were the first to describe, in 1957, the extensive effacement of the foot processes of the podocytes in biopsies of patients with nephrotic syndrome. The effacement of the foot processes of the podocytes can be the only mark in specimens of kidney biopsies, as in nephrotic syndrome due to minimal change glomerulopathy or it may be accompanied by other abnormalities characteristic of the underlying disease such as immune deposits, inflammation or fibrosis [17]. Whether the lesion is solitary or not, the effacement of the foot processes of the podocytes is likely to be a uniform response to podocyte injury. Nevertheless, in 2003 Smithies proposed the hypothesis that the basement membrane has an important role, not secondary to the podocytes, in the glomerular permeselectivity. GBM would act as a modified gel through which macromolecules such as albumin pass primarily by diffusion, independent of the fluid flow [18]. In this model (gel permeation diffusion hypothesis), increased protein concentration in the glomerular ultrafiltrate may result from two mechanisms: (i) an alteration in the composition of GBM, as occurring in Pierson syndrome and Alport syndrome [19]; or (ii) a reduction in the fluid flow rates through the glomerular filtration barrier, as proposed to occur in cases of effacement of podocytes, due to a decrease in the filtration slit frequency, without any changes in protein diffusion rate [18].

The importance of the GBM to glomerular permeselectivity was clearly demonstrated by Jarad et al. (2006). The authors showed that proteinuria and changes in the anionic sites of the GBM precede the flattening of podocyte foot process. These findings imply that the composition and organization of GBM affect the organization of the podocyte foot processes and the epithelial slits. They also fit together with the findings of Kojima et al. (2004), stressing that GBM organization and epithelial structure are closely intertwined. Flattening of podocyte foot processes and changes associated with the organization of the filtration slits are visualized by routine electron microscopy.

2. Alport syndrome (AS)

Alport syndrome is a genetic disease characterized by structural alterations in the glomerular basement membrane, hematuria, increase of proteinuria and progressive renal insufficiency. It can be accompanied by extrarenal alterations, such as neurosensorial hearing loss and eye abnormalities. Nephritis is the most common clinical alteration in Alport syndrome. It usually starts in adolescence with intermitent proteinuria and/or hematuria, presenting chronic progressive renal failure and affecting mainly male individuals [20, 21].

Two forms are recognized in molecular genetics: the dominant form, due to mutations in the COL4A5 genes, located on the X chromosome, which encodes the α-5 chain of type IV collagen, and the autosomal recessive form, due to mutations in the COL4A3 and COL4A4 genes that are located on chromosome 2, which encodes the α-3 and α-4 chains.
of type IV collagen. Approximately 85% of the cases of Alport syndrome present the X-linked dominant form; the remaining cases present the autosomal recessive form [22-26].

Although this syndrome was described before molecular analysis, its relation with gender was already perceptible: in 1927, A. Cecil Alport reported the association of deafness with Hereditary Familial Hemorrhagic Nephritis which occurred in subsequent generations of families previously observed. He observed that male patients tended to develop nephritis and deafness, which rapidly progressed to renal failure and death, while female patients developed hematuria, deafness and lived to old age [27]. This characterization of the impact of gender on the outcome of Alport syndrome was conventional wisdom for nearly 80 years. However, the family described by Alport included a female who presented hematuria and deafness and died at 24 years of age [22].

There are six type IV collagen alpha-chains. α1 and α2 are denominated classic chains and α3, α4, α5 e α6 are denominated novel chains. The six chains, genetically distinct, are organized in three triple-helical procollagen which are different in the composition of each chain [28]. These α chains can be divided in three domains: 7S domain, amino-terminal (NH2, with approximately 15 amino acids); the central triple-helical domain (with approximately 1,400 amino acids) and the glomerular portion carboxyl-terminal noncollagenous domain (NC1; COOH with approximately 230 amino acids) [23]. The main sequence comprises glycine (Gly), hydroxylsine (X) and hydroxyproline (Y) [29].

Type IV collagen α1 e α2 chains are present in all basement membranes. Type IV collagen α3, α4 and α5 chains are selectively expressed in the basement membrane of some tissues, including those potentially affected by Alport syndrome, such as: kidney (glomerular basement membrane and tubular basement membranes), cochlea and eye. Type IV collagen α5 and α6 chains are features of skin, smooth muscle, esophagus and kidney (Bowman capsule) [23].

Type IV collagen is the main constituent of the basement membranes. Mutations present in AS produce impairment in type IV collagen α1, α3 and α5 chains. Damage to type IV collagen due to mutations interrupts the function of epithelial attachment and leads to organ impairment. These defects in the chains result in incorrect folding or assembly of monomers, which are rapidly degraded. These mutations interrupt the normal replacement of embryonic development and result in the persistence of type IV collagen α1.α1.α2 chains in renal basement membranes, cochlea and crystalline capsule. The embryonic network α1.α1.α2 (type IV collagen) is more susceptible to proteolysis than the α3.α4.α5 (type IV collagen), which is more cross-linked [28].

2.1 Diagnosis

The combination of clinical findings with light microscopy, immunofluorescence, electron microscopy and immunohistology of α1, α3 and α5 chains of type IV collagen is essential for the diagnosis of Alport syndrome. The most characteristic histological finding through light microscopy is the presence of foam cells in the interstitial compartment; however, these cells are present in a large number of biopsies and can also be observed in other glomerular diseases characterized by persistent proteinuria.

The classic picture of Alport syndrome is characterized by alternating zones of thinning and thickening of the glomerular basement membrane, splitting and lamellation of the membrane with loss of the normal lamina dense, presence of small granules within the membrane and an irregular outer contour of the glomerular basement membrane. However, not all patients with Alport syndrome will show all of these characteristic findings and even among affected individuals of the same family there can be considerable variability. Furthermore, these alterations may be observed in other glomerulopathies [24].
Fig. 1 - Electron microscopy of a patient with Alport syndrome. The image shows irregular outer contour of the basement membrane, subepithelial and lamellation of the dense lamina of glomerular basement membrane.

The combined use of electron microscopy and immunofluorescence studies for $\alpha_3$ and $\alpha_5$ chains present 90% of sensitivity and specificity to the diagnosis of Alport syndrome with mutations in the locus COL4A5 [24]. This is considered the gold standard for diagnosing this syndrome. As shown in picture X, two cases that under common light microscopy and electron microscopy suggest Alport syndrome, are only accurately diagnosed with the use of specific immunofluorescence for type IV collagen alpha chains. The use of immunofluorescence in Alport syndrome diagnosis still shows good results in the several alterations that may trigger this disease.
Fig. 2- Immunofluorescence for: (A) \( \alpha_3 \) chain of type IV collagen in a patient with Alport syndrome. (B) \( \alpha_5 \) chain of type IV collagen in a patient with Alport syndrome. (C) Normal kidney for \( \alpha_3 \) chain of type IV collagen. (D) Normal kidney for \( \alpha_5 \) chain of type IV collagen.

Dialysis and renal transplantation are recommended to the group of patients with AS in end-stage renal disease [23, 30-33]. Prognosis appears to improve after renal transplantation, increasing longevity of patients with AS and renal failure [30]. With the improvement of the renal function after transplant, it is easier to understand the occurrence of stabilization or a slower progression of hearing loss, since it results from the alteration of the basement membranes due to damages to type IV collagen [25].

3. Thin Glomerular Basement Membrane Nephropathy (TBMN)

TBMN was first described approximately 80 years ago as a curable form of hemorrhagic nephritis. TBMN is characterized by persistent hematuria in children and adults, minimal proteinuria, normal renal function, uniformly altered glomerular basement membranes (GBM), as determined by electron microscopy [28].

Thin Glomerular Basement Membrane Nephropathy seems to be far more common than Alport Syndrome, occurring in 1% of the population in comparison with the 0.02% of the Alport syndrome. The majority of patients with Thin Glomerular Basement Membrane Nephropathy present thinning of the lamina dense and the basement membrane as a whole, [19].

The majority of patients present hematuria, with no additional symptoms of progression to renal failure. Microscopic analysis of urine samples reveals red blood cells in most patients. These cells are generally dysmorphic with irregular shape and size, indicating hematuria of glomerular origin. At least a single episode of macroscopic hematuria is observed in 5 to 22% of patients, typically manifesting after physical exercise or during infection. However, the occurrence of macroscopic hematuria seems to be more common in patients with Alport syndrome and IgA nephropathy. Despite hematuria, the individuals do not usually present proteinuria or only minimally so, indicating that the podocyte is not really affected. Proteinuria develops when there is persistent hematuria and is it hardly ever observed in children. Renal function in children with TBMN is also normal [28].
In general, development of autosomal nonprogressive TBMN involves heterozygous mutations in either COL4A3 or COL4A4 loci, whereas homozygosity or combined heterozygous mutations in these genes result in autosomal recessive Alport syndrome characterized by the deterioration of the GBM [34, 35].

Likewise, a similar TBMN phenotype may be caused in a female individual with heterozygosity for a mutation in the X chromosome in the COL4A5 gene. This could be explained by a ‘dose effect’ whereby the absence of a normal allele leads to lower production of the α3:α4:α5 trimer leading to TBMN. However, the loss of two alleles results in lack of the α3:α4:α5 trimer causing Alport syndrome [36]. However, there are reports that a single heterozygous mutation in COL4A3 or COL4A4 can cause Alport syndrome in adult life [37-39]. This could mean that some mutations are more serious than others, but it still cannot be excluded that in these cases, there is another unknown mutation somewhere else, in another allele or gene that could lead to the absence of the chain. So far, few mutations have been identified in TBMN and autosomal dominant Alport syndrome for making conclusions about the differences in the pathogenesis.

### 3.1 Diagnosis

The diagnosis of TBMN may be difficult and requires familial investigation, immunofluorescence, assessment of type IV collagen alpha chains in the renal tissue and even genetic studies. Firstly, it requires the demonstration of the diffusely thin glomerular basement membrane by electron microscopy and then, it is necessary to rule out Alport syndrome by immunofluorescence for α3 and α5 chains of type IV collagen [24].

Electron microscopy reveals the typical feature of TBMN, that is, thinning of the glomerular basement membrane [28]. However, it is of extreme importance to carry out morphometry of the glomerular basement membrane in suspected cases of TBMN (Liapis, 2004; Fogo & Kashgarian, 2005; Hass, 2009). At our center it is standard to perform morphometry in all cases of hematuria with clinical suspect of TBMN.

![Figure 3](image-url) (A) Normal Glomerular Basement Membrane (B) Thin Glomerular Basement Membrane Nephropathy. The arrows show the differences in thickness of the glomerular basement membrane.

Currently, there are no standard treatment protocols for TBMN. However, this condition usually has an excellent outcome and, in many cases, it is not considered to be a disease. In some cases, however, TBMN may worsen the clinical picture [40, 41].

The diagnosis is still a dilemma for the pathologist to recognize whether the cases are, in fact, TBMN or Alport syndrome. In any case, patients with TBMN diagnosis require constant monitoring for the onset of hypertension, proteinuria or renal insufficiency.

In 2009, Haas and colleagues differentiate these two entities: patients with thin basement membrane disease do not present alteration in collagen, that is, they have normal collagen staining in α3 and α5 type IV collagen alpha chains. As for heterozygous females with Alport syndrome, they present discontinuous staining of GBM glomerular basement membrane (GBM), Bowman capsule (BC) and epidermal basement membrane (EBM), for both, 3 and 5 alpha chains of type IV collagen. In autosomal heterozygous males there is absence of staining in α3 and 5 alpha chains of type IV collagen. There is also the autosomal recessive form, in which the patient presents absence of the 3 alpha chain, but presence of the 5 alpha chain of type IV collagen in Bowman capsule, tubular basement membrane and epidermal basement membrane [24].
Alport syndrome and thin glomerular basement membrane nephropathy are primary glomerulopathies, hereditary and genetic, which develop with alterations in the glomerular basement membrane due to a deficiency of some alpha chain of type IV collagen, resulting in abnormalities in the glomerular basement membrane in Alport syndrome or the thinning of the glomerular basement membrane in thin basement membrane disease. These diagnoses are only possible with the use of electron microscopy that enables the pathologist to evaluate ultrastructural alterations, combined with morphometry to measure this membrane thickness and immunofluorescence microscopy that enables the study of the alpha chains of type IV collagen by immunostaining, to finally diagnose differentiate these two entities.

References


