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"Unbiased next generation sequencing analysis confirms the existence of autosomal dominant Alport syndrome in a relevant fraction of cases"

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Nosology, diagnosis and recent advances on the understanding of the genetic epidemiology of Alport syndrome and related collagen type IV glomerulopathies

Nosologia, diagnóstico e avanços recentes no conhecimento da epidemiologia genética da síndrome de Alport e outras glomerulopatias do colagénio tipo IV

João Paulo F. S. Oliveira

Department of Medical Genetics, Faculty of Medicine, University of Porto & São João Hospital Centre. Porto, Portugal

Collagen IV glomerulopathies[1] are a pathogenically related but clinically and genetically heterogeneous group of familial haematuric diseases including Alport syndrome (AS) and many of the families with benign familial haematuria (BFH) or thin basement membrane disease (TBMD). These disorders have in common an ultrastructural lesion of the glomerular basement membrane (GBM) elicited by pathogenic mutations in any three – i.e., α5(IV), α4(IV) or α3(IV) –, of the six genetically distinct collagen type IV chains. The earliest GBM abnormality identified on electron microscopy (EM) examination of kidney biopsies of AS patients was diffuse thinning but, on later stages, the GBM appears characteristically thickened, with splitting and lamellation of the lamina densa, enclosing electron-lucent areas that contain round, small electron-dense bodies[2,3]. The genes encoding the six α(IV) chains, named *COL4A1* through *COL4A6*, have high degrees of structural homology and are organized in three pairs, *COL4A1/COL4A2*, *COL4A3/COL4A4* and *COL4A5/COL4A6*, with cytogenetic locations, respectively at 13q34, 2q36 and Xq22. The two genes in each pair are arranged in head-to-head orientation and share a common promoter region[1,2].

Although the prevalence of AS in the general population has been estimated as low as 1:50.000 live births, it is an important cause of chronic kidney disease (CKD) progressing to end-stage renal failure (ESRF) in adolescents and young adults[1], accounting for 1.5-3.0% of the children on renal replacement therapies (RRT) in Europe and the United States of America.

Type IV collagen is exclusively found in basement membranes (BM), where it forms a complex

supramolecular network assembled by interactions between three different types of triple-helical protomers of a(IV) chains: α1α1α2(IV), α3α4α5(IV) and α5α5α6(IV)[4]. Collagen IV networks confer mechanical stability and provide a scaffold for cell surface receptors involved in a multiplicity of biological processes that critically depend on cell interactions with the underlying BM. On the basis of their sequence similarities, the six α(IV) chains can be divided into two classes: the α1-like class, composed by the odd-numbered α(IV) chains, and the a2-like class, composed by the even-numbered α(IV) chains.

Unlike the ubiquitously expressed α1(IV) and α2(IV), all other α(IV) chains have selective tissue expressions. The selectivity of the genetic expression of *COL4A3_6* accounts for the clinical consequences and patterns of organ involvement of their pathogenic mutations[5]. Since the α3α4α5(IV) protomers constitute the major component of the collagen IV network of the mature human GBM[4], *COL4A3*, *COL4A4* or *COL4A5* mutations predominantly manifest as a GBM disease. It is also relevant for the understanding of the clinical phenotypes of AS that α3(IV), α4(IV) and α5(IV) are present in ocular and cochlear BM, whereas the α5(IV) and α6(IV) are found in BM of the skin, smooth muscle and Bowman's capsule, but not in the GBM. For this reason, pathogenic mutations in *COL4A1*, *COL4A2* or *COL4A6* do not result in GBM pathology.

As compared to the networks of α1α1α2(IV) protomers, which are found in the BM of all tissues and are the predominant collagen IV network in the human GBM during the early embryonic period, the α3α4α5(IV) networks are more resistant to higher glomerular capillary pressures and to proteolytic degradation. Therefore, the developmental switch in gene expression that occurs in the embryonic development of the human kidney is crucial for the maturation of the GBM as a specialized plasma filtration barrier in the kidney[4]. Irrespective of the specific gene involved, genetic mutations that affect the normal expression, post-translational modifications or assembly of the α3α4α5(IV) heterotrimers ultimately result in persistence of α1α1α2(IV) networks in the GBM, which is presently regarded as a major factor in the pathogenesis of the collagen IV glomerulopathies.

Pathogenic mutations in *COL4A5* are the cause of AS in the majority of the families that have been genetically evaluated. In such families, the clinical phenotype[2,3] characteristically segregates as an X-linked Mendelian disorder (XLAS). In all hemizygous males, the renal disease evolves from persistent microscopic haematuria to proteinuria and progressive CKD, the majority of these patients ultimately reaching ESRF before age 40 years. Episodes of gross haematuria are also common, particularly in the first two decades of life. Worsening sensorineural hearing loss (SNHL) is usually present by late childhood or early adolescence and by age 40 years the estimated prevalence of SNHL in males with XLAS is as high as 80-90%. Ocular lesions, most typically anterior lenticonus and/or dot-and-fleck perimacular retinopathy are present in 30-40% of the cases. Heterozygous females, instead, have a milder disease and a much better prognosis than the hemizygous males. Roughly 95% of affected females exhibit persistent or intermittent microscopic haematuria. Although the lifetime prevalence of ESRF in women carrying pathogenic *COL4A5* mutations is about 40%, only 12% need RRT before age 40 years. In addition to mutations confined to *COL4A5*, microdeletions involving *COL4A5* and extending either centromerically to the 5' exons of *COL4A6* or telomerically to several adjacent genes, are associated with variant phenotypes of XLAS, presenting respectively with diffuse leiomyomatosis or with mental retardation, midface hypoplasia and elliptocytosis[1]. Homozygosity and compound heterozygosity for pathogenic *COL4A3* or *COL4A4* mutations is the cause of autosomal recessive AS (ARAS). The clinical phenotype of ARAS is similar in severity to that of XLAS in males but with no gender differences of natural history[2,3]. Parental consanguinity is an additional family history clue to the diagnosis of ARAS. Autosomal dominant AS (ADAS), as well as BFH/TBMD in 40-50% of such families, segregate with heterozygous *COL4A3* or *COL4A4* mutations[1,2,3]. However, the distinction between these collagen IV glomerulopathies is somewhat arbitrary, based on the familial risk of ESRF and ultrastructural evidence of GBM abnormalities. The diagnosis of ADAS is typically made in families with clinical and/or pathological evidence of AS and cases of ESRF in successive generations, but the clinical phenotype is milder than that seen in XLAS or ARAS and the extrarenal manifestations are less frequent. The terms BFH and TBMD are used interchangeably, but BFH is a misleading designation because a proportion of patients develop progressive CKD, eventually needing RRT, and the formal diagnosis of TBMD requires that

diffuse thinning of the GBM (to about half its normal thickness) be demonstrated on EM examination of a kidney biopsy. Family history of father-to-son transmission of the kidney disease phenotype is unequivocal evidence of autosomal dominant inheritance. It is also of note that approximately 50% of the heterozygous individuals for pathogenic *COL4A3* or *COL4A4* mutations, from families with ARAS, do not manifest any urinary abnormalities.

According to the current data on the genetic epidemiology of AS, XLAS accounts for 80-85% of the families, while ARAS accounts for about 15% and ADAS for about 5%[1,2,3].

Taking into consideration the typical clinical manifestations, as well as the distinctive ultrastructural GBM pathology of AS, Flinter *et al.* recommended the following criteria to enable the diagnosis of AS in patients presenting with haematuria of uncertain aetiology[6]: (i) family history of macro/microscopic haematuria and/or of progressive CKD or ESRF; (ii) EM evidence of AS on renal biopsy; (iii) high-tone SNHL; (iv) characteristic ophthalmological signs: anterior lenticonus and/or white perimacular flecks. The clinical diagnosis of AS can be confidently established if the patient, or the proband and other affected family members between them, fulfil at least three of those criteria. Best use of this set of diagnostic criteria requires the obtainment of a detailed family history, covering at least three-generations; that intermittent microscopic haematuria has been excluded by repeated urinalyses before any at-risk female is considered not affected; and referral for specialized audiologic and ophthalmologic examination, to appropriately screen for the extrarenal manifestations of AS. Since different features of AS may occur in different subjects within the same family, the disease phenotype should be cumulatively defined within each family. Failure to ensure the appropriate diagnostic assessment of these patients and their families is a common error in clinical practice[7]. Patients with ARAS and the 10-15% of males with XLAS as a result of *de novo COL4A5* mutations often have uninformative family histories.

Immunohistochemical staining of kidney and/or skin biopsies to characterize the expression pattern of type IV collagen chains in the local BM may also be of help in the diagnosis of AS or differential diagnosis of the specific genetic forms of AS[3]. Most males with XLAS show complete absence of immunostaining for α5(IV), α4(IV) and α3(IV) on the GBM, as well as complete absence of α5(IV) staining on the epidermal BM (EBM), while the heterozygous females exhibit discontinuous lack of expression of the same α(IV) chains on the GBM and EBM. In the majority of patients with ARAS, the GBM α(IV) immunostaining pattern is similar to that observed in males with XLAS, but staining for α5(IV) is positive in the Bowman's capsules and tubular BM. Patients with ADAS/BFH/TBMD have normal α(IV) reactivities both in the GBM and EBM. However, a normal skin or kidney α(IV) immunostaining pattern does not exclude the diagnosis of AS.

More recently, the availability of methods for reliable molecular genetic analyses of *COL4A3*, *COL4A4* and/or *COL4A5* in the clinical setting, offered a minimally invasive approach to the diagnosis of the collagen IV glomerulopathies[8], which may supersede the biopsy studies in certain circumstances. Mutation analysis is useful for confirming a clinical diagnosis and informing the genetic counselling, and also to ascertain the genetic status of prospective donors for living-related kidney transplantation, particularly of females from families with XLAS. Furthermore, knowledge of the pathogenic mutation in a family is indispensable for preimplantation genetic diagnosis (PGD) and prenatal diagnosis (PND).

The choice of which of the three genes to test first should be based on family history, clinical findings, histopathology, and relative frequencies of AS and BFH/TBMD. However, due to the absence of mutational hot-spots and the high number of exons per gene (53 in *COL4A5*, 48 in *COL4A4* and 52 in *COL4A3*), mutation screening by conventional polymerase chain reaction (PCR)-based laboratory methods – particularly direct Sanger sequencing of exonic DNA, including flanking intronic sequences –, becomes quite expensive and time-consuming, with several months usually needed to complete the study, especially in cases with less informative pedigrees[9]. Using a conventional genetic testing approach, the *COL4A5* mutation detection rate in British families fulfilling one, two, three, or four of the clinical and EM diagnostic criteria was respectively, 18%, 64%, 89%, and 81%[7]. These data justified the recommendation for first-tier mutational screening of *COL4A5* in any patient meeting at least two clinical diagnostic criteria of AS, as long as they have no evidence of autosomal

inheritance. However, the generalization of this recommendation is appropriate only for populations where XLAS is the predominant form of the disease.

Next-generation sequencing (NGS) – also known as second generation, massive parallel or high-throughput sequencing –, is the general term used to describe a number of different modern technologies that allow the sequencing of DNA much more quickly and cheaply than the conventional Sanger method[10]. The NGS is a promising new diagnostic technique for genetically heterogeneous disorders like AS[8], particularly when their clinical features are atypical or mild, or are not specific enough to guide the choice of which gene(s) to scan first for pathogenic mutation(s), using a step-by-step approach with Sanger sequencing.

It should be additionally noted that (multi)exonic deletions or duplications, which cannot be identified by direct Sanger sequencing in the heterozygous status, have been reported in approximately 10% of females whose family history is consistent with X-linked inheritance[3]. Screening for exonic deletions/duplications requires additional laboratory methods, including quantitative PCR, long-range PCR, multiplex ligation-dependent probe amplification (MLPA), or chromosomal microarray that includes the relevant gene/chromosome segment. The prevalence of *COL4A3* and *COL4A4* deletions/duplications in patients with ARAS or ADAS is not known.

In such epidemiological and methodological contexts, the results reported by Fallerini et al., from the unbiased genetic study of 237 Italian patients with clinical suspicion of AS, gain particular relevance[11]. Those investigators used an NGS protocol to simultaneously scan the genes *COL4A3*, *COL4A4* and *COL4A5* for sequence variants and found a pathogenic mutation in one of the three genes in 48 of 87 unrelated families (55%), establishing the diagnoses of XLAS in 31 (65%), of ARAS in two (4%) and of ADAS in 15 (31%) families. Furthermore, the prevalence of pathogenic mutations was high (>50%), even in patients with only one or two of the clinical and EM diagnostic criteria of AS. It is of note that a significantly lower than expected prevalence of XLAS (and correspondingly higher than expected prevalence of ADAS) was also identified in the genetic study of 65 Portuguese families with the diagnosis of AS [Nabais Sá *et al.*, manuscript submitted]. These data indicate that, at least in some European populations, ADAS must be seriously taken into account in all pedigrees with affected individuals in each generation. In such populations, NGS may be the best option for first-level genetic analysis in patients with clinical suspicion of AS.

The major concepts, updates and recommendations for practicing nephrologists are the following: (a) AS and related collagen IV glomerulopathies are disorders of the α3α4α5(IV) molecular network of the GBM; (b) there may be significant variations in the genetic epidemiology of collagen IV glomerulopathies in different populations which are relevant for genetic counselling and should be taken into consideration when planning the laboratory strategies for their molecular diagnoses; (c) the set of diagnostic criteria of AS proposed by Flinter *et al.* warrants the most appropriate clinical evaluation of patients with microscopic haematuria and of their at-risk relatives, but when stringently applied they seem to be better predictive of XLAS and ARAS, which are phenotypically very similar; (d) less stringent criteria might be applied to the diagnosis of ADAS and even patients/families fulfilling less than three diagnostic criteria may be considered for genetic scanning; (e) whereas the identification of a pathogenic mutation in *COL4A5*, *COL4A4* or *COL4A3* confirms the diagnosis of collagen IV glomerulopathies and provides crucial information for genetic counselling, family screening and for reproductive genetic testing (e.g., PGD, PND), a negative mutation scanning does not formally exclude a well-founded clinical diagnosis; (f) however, the genetic diagnosis of AS and related collagen IV glomerulopathies is available only in a limited number of molecular genetics laboratories around the world, and remains complex and expensive; (g) NGS is the appropriate first-tier molecular approach to the genetic diagnosis of collagen IV glomerulopathies in those populations (like, apparently, the Portuguese) where none of the different genetic forms of AS is much predominant.

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Correspondence to:
Professor Dr. João Paulo F. S. Oliveira
Department of Medical Genetics, Faculty of Medicine, University of Porto & São João Hospital Centre, Alameda Hernâni Monteiro 4200-319 Porto, Portugal.
E-mail adress: jpo@med.up.pt