

Making the diagnosis of Alport syndrome and the differential diagnosis of a severe superimposed second glomerulopathy in a young boy

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In a previous issue of this journal, Neves *et al.*¹ reported the occurrence of a severe, normocomplementemic, acute nephritic syndrome at age 4½ years, in a boy with past medical history of self-limited episodes of macroscopic haematuria associated with respiratory infections, who was eventually diagnosed with Alport syndrome (AS) more than 10 years afterwards. The reported baseline laboratory data were unremarkable, except for significantly elevated serum creatinine (sCr), microscopic haematuria with nephrotic range proteinuria, and severe anaemia.

In the kidney biopsy obtained for diagnostic workup of the acute nephritic syndrome, endocapillary proliferation was observed in four of the six glomeruli available for study, with extracapillary proliferation additionally present in two of them; the remaining glomeruli were morphologically normal and the immunofluorescence study showed no evidence of immune deposits. The patient was treated with corticosteroids and cyclophosphamide, with complete recovery of renal function and resolution of proteinuria. Microscopic haematuria was noted to persist thereafter and proteinuria reappeared one year later, leading to the initiation of therapy with an angiotensin-converting enzyme (ACE) inhibitor. Approximately 5 years later the patient had another self-limited episode of macroscopic haematuria, in association with an upper respiratory tract infection. This prompted a follow-up kidney biopsy, which showed a mesangial proliferative glomerulonephritis with mild glomerular sclerosis and a few deposits of immunoglobulin M (IgM) on the immunofluorescence study. The dosage of ACE inhibitor was increased and long-term corticosteroid therapy was started. Severe

bilateral sensorineural hearing loss (SNHL), that required hearing aids, was first noticed at about age 12 years; ophthalmological assessment did not show any of the characteristic ocular features of AS and genetic testing for diagnosis of X-linked AS (XLAS) was also negative. At age 16, the sCr was still within normal range and the proteinuria remained mild. The diagnosis of autosomal recessive AS (ARAS) was finally established by identification of a homozygous nonsense mutation in the *COL4A3* gene, leading to discontinuation of corticosteroid therapy.

There are several issues worth reviewing and discussing in this case report, namely the nosology of the collagen type IV-related hereditary glomerulopathies and the role of genetic testing in their diagnosis; whether the diagnosis of AS could have been made at a younger age in this patient; and whether the acute nephritic syndrome was indeed a presenting manifestation of AS or a superimposed disorder.

■ ON THE NOSOLOGY OF ALPORT SYNDROME AND THE COLLAGEN TYPE IV-RELATED HEREDITARY GLOMERULOPATHIES

AS is a multiorgan hereditary disorder caused by pathogenic mutations in the *COL4A3*, *COL4A4* or *COL4A5* genes, respectively encoding $\alpha 3_{[IV]}$, $\alpha 4_{[IV]}$ and $\alpha 5_{[IV]}$ type IV collagen chains²⁻⁴. These chains combine with each other to form triple helical $\alpha 3\alpha 4\alpha 5_{[IV]}$ protomers which, upon secretion from podocytes, are

organized in a complex supramolecular network making up the major structural component of the mature human glomerular basement membrane (GBM)⁵⁻⁷. The presence of the $\alpha3\alpha4\alpha5$ _[IV] networks also in basement membranes (BMs) in the cochlea as well as in the lens and retinal pigment epithelium of the eye explains the extrarenal manifestations of the classical phenotype of AS. The collagen type IV gene family comprises three additional genes, designated *COL4A1*, *COL4A2* and *COL4A6*, with the corresponding alpha_[IV] chains trimerizing into two additional protomers: the $\alpha1\alpha1\alpha2$ _[IV], which are found in all human BMs^{5,7}; and the $\alpha5\alpha5\alpha6$ _[IV], which have a more limited distribution in human tissues⁷, but including the BMs of the Bowman's capsule and the tubules in the kidney, as well as the epidermal basement membrane (EBM).

The classical phenotype of AS, characterized by progressive chronic kidney disease (CKD) associated with hearing and eye defects, is the most severe expression of a spectrum of pathogenically related but clinically and genetically heterogeneous familial haematuric disorders^{3,8}, which have in common the development of structural defects of the GBM, consequent to impaired assembly of the $\alpha3\alpha4\alpha5$ _[IV] networks. Hence, "*collagen type IV-related hereditary glomerulopathies*" is an appropriate designation for this group of kidney disorders⁴, which also comprises many of the families diagnosed with benign familial haematuria (BFH) and thin basement membrane nephropathy (TBMN), as well as some families with the diagnosis of familial focal segmental glomerulosclerosis (FSGS)^{3,8}.

Classical AS, typically progressing to end-stage kidney failure (ESKF) in late adolescence or young adulthood, is inherited either as an X-linked (about 85% of the families) or autosomal recessive trait (about 15% of families): XLAS is caused by mutations in *COL4A5*, whose chromosomal location is at Xq22.3, while ARAS is due to homozygous, composite heterozygous or double heterozygous mutations in *COL4A3* and/or *COL4A4*, whose loci are at 2q36.3²⁻⁴. Overall, the AS phenotypes observed in males with XLAS and in males and females with ARAS are clinically indistinguishable, with CKD progressing to ESKF at an average age of 24-25 years⁹. The prevalence of classical AS has been estimated at 1:50,000 live births².

Heterozygosity for pathogenic *COL4A3* or *COL4A4* mutations correlates with a variety of clinical/familial phenotypes^{2-4,8}. (i) rare kindreds in whom the classical phenotype of AS segregates as an autosomal dominant trait (ADAS); (ii) kindreds in whom the renal phenotype

of AS segregates in autosomal dominant fashion, but lacking the extrarenal manifestations (thus the term Alport "*disease*" rather than "*syndrome*" might be more appropriate to refer to this phenotype), with progression to ESKF frequently delayed until later adulthood; (iii) kindreds in whom there is co-segregation of microscopic haematuria and FSGS, with mild or no extrarenal involvement; (iv) about 30-40% of the kindreds diagnosed with BFM or TBMN^{10,11}; (v) absence of any clinical or laboratory evidence of renal disease, as in approximately half of the carriers from kindreds with ARAS.

While acknowledging the ongoing nosological and semantic controversies about the most appropriate nomenclature and classification system for the collagen type IV-related hereditary glomerulopathies¹²⁻¹⁷ – namely (i) whether BFH and TBMN are just clinical and electron microscopy (EM) morphological descriptors for the same condition; (ii) whether families with BFH, i.e. segregating microscopic haematuria in autosomal dominant fashion but not exhibiting an ESKF risk above that of the background population, really exist; (iii) what is the risk for progressive renal impairment in patients diagnosed with TBMN; (iv) what criteria, if any, should be used for the differential diagnosis between autosomal dominant Alport *disease* (as opposed to *syndrome*) and TBMN in kindreds exhibiting an increased risk of ESKF – their discussion is beyond the scope of this editorial commentary.

■ ON THE DIAGNOSTIC APPROACH TO MACROSCOPIC HAEMATURIA IN YOUNG CHILDREN

For three years before being hospitalized with an acute nephritic syndrome and eventually getting comprehensive nephrological assessment and care, the child reported by Neves *et al.*¹ had experienced several episodes of recurrent gross haematuria, temporally related to upper respiratory illnesses, which were actually overlooked as the first diagnostic clue to his underlying renal disease. Reportedly, those bouts of macroscopic haematuria were otherwise asymptomatic and self-limited, but it is not clear how exhaustively their differential diagnosis was pursued at baseline evaluation.

Although asymptomatic gross haematuria in children most often has a benign aetiology¹⁸, it requires prompt evaluation in order to exclude potentially life-threatening disorders¹⁹, affecting either the

kidney or the urinary tract. Complete urinalysis is the most important diagnostic test in a child with asymptomatic gross haematuria¹⁹⁻²². Brown or tea-coloured urine, presence of significant proteinuria (i.e., more than “2+” or 100 mg/dl on dipstick test) and the observation of erythrocyte casts and/or of microcytic or dysmorphic erythrocytes in the urine sediment suggest a glomerular source for the haematuria^{19,22-24}. Of the wide variety of abnormal-appearing erythrocytes that can be seen in the urine sediment²⁵, only acanthocytes – i.e., erythrocytes with discrete spheroidal or vesicle-shaped protrusions – are highly specific for haematuria of glomerular origin^{24,26}. The specificity of erythrocyte casts for diagnosing glomerular disease is similar to that of acanthocyturia but acanthocytes are far more commonly observed than erythrocyte casts in patients with glomerular haematuria, yielding a more than two-fold higher sensitivity for acanthocyturia^{24,26}. Although acanthocytes are best detected by phase-contrast microscopy²⁶ they can also be effectively detected by standard bright field microscopy^{24,25,27}; therefore, the unavailability of phase-contrast microscopy should not be an impediment to the comprehensive assessment of the urinary sediment in haematuric patients. Glomerular haematuria may be diagnosed when 40% or more of the erythrocytes in the urine sediment are dysmorphic or 5% or more acanthocytes are present²⁷.

Serologic studies to investigate immune-mediated glomerulonephritis should be also performed, including measurement of serum complement component (C3, C4) levels^{19,22}. Hypocomplementaemic glomerulonephritides (e.g., acute postinfectious glomerulonephritis, membranoproliferative glomerulonephritis, systemic lupus erythematosus, etc.) are reported to account for 4-10%^{21,28,29} of all paediatric cases of asymptomatic gross haematuria.

In children who underwent a kidney biopsy to investigate the cause of glomerular gross haematuria^{21,29,30}, presenting either recurrently or in the context of persistent microscopic haematuria, the most frequent histological diagnosis was immunoglobulin A (IgA) nephropathy, which was detected in 50-60% of the biopsies, and 20-30% of the patients were diagnosed with AS or with TBMN, respectively at 3:1 ratio. Infectious events, particularly upper respiratory tract infections (e.g. tonsillitis, pharyngitis), are major precipitators of gross haematuria in children with IgA nephropathy³¹, as well as in children with AS or TBMN²; however, IgA nephropathy seldom occurs before age

6 years^{31,32}, whereas in the majority of children diagnosed with AS who had gross haematuria, the first episode occurred in the first 5 years of life³³. Notably, the association between respiratory illnesses and episodic gross haematuria was already recognized by Cecil Alport in his original description of “hereditary familial congenital haemorrhagic nephritis”³⁴, the disorder which, much later on, was eventually named after him³⁵. Furthermore, in children presenting with glomerular haematuria, either microscopic or macroscopic, screening the proband’s first degree relatives for microscopic haematuria may contribute critical information to the differential diagnosis, since IgA nephropathy is rarely familial, while in the child with familial haematuria the diagnostic possibilities virtually narrow down to AS or TBMN².

Yet, nonglomerular problems are more than twice as common as glomerular diseases as the cause of isolated gross haematuria in paediatric patients^{21,29}, and urologic disorders must always be ruled out by careful kidney and bladder ultrasound imaging upon presentation^{18,19,22,29}. Such a recommendation is especially relevant for males, who account for the large majority of new diagnoses of gross haematuria made in children^{18,28,29}, since the occurrence of congenital upper and lower urinary tract anomalies is almost up to ten-fold higher in boys than in girls¹⁸. Stones and tumours are additional urologic causes of gross haematuria in the paediatric population that can be readily identified by ultrasonography examination^{19,22}. Nephrolithiasis was detected in about 2% of the cases of isolated gross haematuria presenting in childhood^{21,28,29}, its prevalence increasing to about 5% among children with a definite diagnosis of nonglomerular haematuria²¹, while kidney and bladder tumours accounted for approximately 1% of all paediatric cases of gross haematuria¹⁸. Screening for hypercalciuria is indicated in children with unexplained isolated gross haematuria, as hypercalciuria is a common cause of both microscopic and macroscopic haematuria in the paediatric population, even in the absence of kidney or urinary tract stones on imaging studies^{19,22}. The mechanism whereby hypercalciuria causes haematuria remains unclear but might be secondary to calcium oxalate and phosphate crystals adhering to the uroendothelium²². The prevalence of hypercalciuria among children presenting with asymptomatic gross haematuria in the absence of kidney stones has been variably reported between 10-25%^{21,28,29}. However, despite detailed clinical, laboratory and imaging assessment, the aetiology of nonglomerular gross haematuria in paediatric patients remained elusive in nearly half of the incident

cases, but the long-term prognosis of these patients appeared to be good^{21,28}.

Hence, in the patient reported by Neves *et al.*¹, a detailed examination of the urine sediment, particularly looking for dysmorphic erythrocytes, would have been of help to confirm the glomerular source of the haematuria²⁴, as soon as the first episode of gross haematuria occurred. Furthermore, the association of recurrent gross haematuria with respiratory infections in a boy under 5 years of age, should have prompted a diagnostic workup for AS and TBMN^{2,33}, including screening for microscopic haematuria in his first-degree relatives. But even with evidence of glomerular haematuria, ultrasound examination should have been performed at the onset of the clinical manifestations to rule out a urologic disorder, particularly hydronephrosis, calculi or cancer¹⁹. In this case, urologic problems have indeed been excluded retrospectively, but delaying their diagnoses in affected children can have severe consequences.

Although microscopic haematuria was noted to persist after the patient manifested the acute nephritic syndrome, it is not clear whether, until then, the episodic gross haematuria coexisted with persistent microscopic haematuria. Because persistent microscopic haematuria following an episode of gross haematuria^{21,30}, as well as recurrent unexplained gross haematuria²⁹, are indications for obtaining a diagnostic kidney biopsy in children, neglecting the proper clinical investigation and follow up of the patient after his first bout of gross haematuria also contributed to the diagnostic delay.

The observation of red blood cell casts in the first kidney biopsy was diagnostic of glomerular haematuria, but this would be anticipated in the setting of an acute glomerulonephritis with crescentic proliferation²⁴, and does not prove the glomerular origin of the previous episodes of gross haematuria.

Since the kidney biopsy obtained for diagnosis of the acute nephritic syndrome conclusively ruled out an IgA nephropathy, AS or TBMN were left as the most plausible underlying cause for the recurring episodes of gross haematuria. However, this possibility was not given proper consideration as a mandatory indication for EM examination^{13,36} of the patient's first kidney biopsy: by failing to do so, the opportunity was missed of making the diagnosis of AS on the basis of ultrastructural pathology criteria, several years before being finally established by genetic testing.

■ ON THE IMPORTANCE OF THE KIDNEY BIOPSY FOR THE DIAGNOSIS OF ALPORT SYNDROME AND THIN BASEMENT MEMBRANE NEPHROPATHY

Examination of kidney biopsy specimens of patients with AS or TBMN by standard light microscopy (LM) methods shows only nonspecific features and is completely unremarkable in the earliest stages of AS and in most individuals with TBMN³⁷⁻³⁹. Mild glomerular changes, including mesangial proliferation and expansion, can be observed early in the course of AS, while glomerular sclerosis, tubular atrophy and interstitial fibrosis become more prominent as the renal disease progresses. Presence of lipid-laden interstitial foam cells is a distinctive LM finding in patients with AS and longstanding proteinuria^{37,38}. In the smaller subset of patients diagnosed with TBMN who evolve with progressive CKD³, glomerular sclerosis, tubular atrophy and interstitial fibrosis develop with increasing age, in association with the declining renal function. In both AS and TBMN, standard immunofluorescent or immunohistochemical staining shows no immune deposits^{38,39}. Specific immunostaining shows complete absence of $\alpha 3_{[IV]}$, $\alpha 4_{[IV]}$, and $\alpha 5_{[IV]}$ from the GBM in the majority of cases of XLAS and ARAS, while in ARAS (but not in XLAS) staining for the $\alpha 5_{[IV]}$ chain in the BM of the Bowman's capsule and distal tubules is preserved^{2,37,38,40}. However, because there is a minor subset of AS kindreds who have residual expression of $\alpha 3\alpha 4\alpha 5_{[IV]}$ protomers, normal immunostaining for the component chains cannot completely rule out either XLAS or ARAS^{37,38}.

Extensive or diffuse thinning of the GBM associated with attenuation of the lamina densa is the ultrastructural hallmark abnormality in BFH and TBMN, as well as in the early stages of AS, in the heterozygous females for XLAS, and in symptomatic heterozygous relatives of patients with ARAS³⁷⁻³⁹. The thinner GBM allows the passage of red blood cells through microruptures of the glomerular capillary wall, explaining why microscopic haematuria is the earliest clinical manifestation of all these disorders. In more advanced stages of AS, the GBM has an irregular outer contour and shows unevenly alternating thin and thickened areas; the lamina densa appears segmentally splintered and lamellated and contains irregularly interspersed lucent areas, within which small electron-dense granular inclusions may be observed^{37,38}. These ultrastructural features, described as a "basket-weave" pattern, are considered diagnostic/pathognomonic for AS^{8,37,40}. In boys with XLAS, as well as in children with ARAS, irrespective

of gender, the distinctive ultrastructural GBM abnormalities of AS may be present since very early in life⁴⁰, years before the development of any of the characteristic extrarenal manifestations of the disease.

Accordingly, and despite its invasiveness (associated with risks of bleeding and infection, and requirement for general anaesthesia in younger children), kidney biopsy with EM examination has been, for many years and until recently, the method of choice for the investigation of a child with glomerular haematuria in whom AS or TBMN was suspected¹³.

■ ON THE DIAGNOSTIC APPROACHES TO ALPORT SYNDROME

■ Clinical and histopathological criteria

Prior to the availability of molecular genetics testing for collagen type IV-related glomerulopathies, Flinter *et al.*⁴¹ recommended the following set of criteria to enable the diagnosis of AS in patients presenting with glomerular haematuria of uncertain aetiology: (i) history of haematuria and/or of progressive CKD or ESKF; (ii) EM evidence of AS on kidney biopsy; (iii) high-tone SNHL; (iv) characteristic ocular signs, particularly anterior lenticonus and retinal flecks. 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 diagnostic criteria^{4,13}. Optimal use of these criteria requires clinicians to take a detailed family history, covering at least three-generations and systematically inquiring for consanguinity between the proband's parents; to carefully avoid misclassifying individuals with intermittent microscopic haematuria as non-affected, by failing to obtain repeated urinalyses; and to refer both the proband as well as their at-risk relatives for specialized audiologic and ophthalmologic examinations, to appropriately check for the extrarenal manifestations of AS. However, it should be noted that the age-dependence of the renal histopathological features and, particularly, of the extrarenal clinical manifestations of AS, are major limitations to applying the Flinter's criteria in paediatric settings, especially in young children.

Family screening for microscopic haematuria is mandatory since more than 90% of the heterozygous females for pathogenic *COL4A5* variants and approximately 50% of the heterozygotes for pathogenic *COL4A3* or *COL4A5* variants have persistent or intermittent

microscopic haematuria². Family history of early ESKF in affected males and of milder renal phenotypes in their affected female relatives are highly suggestive of XLAS. Evidence of renal involvement in father and son excludes XLAS and when both father and son manifest a clinically severe renal phenotype they most probably have ADAS. Parental consanguinity, multiple severely affected sibs with healthy or subclinically affected parents, and equally severely affected male and female siblings, are family history clues to ARAS.

Bilateral SNHL eventually develops in up to 80-90% of the males with XLAS and males and females with ARAS; it can be detected by audiometry typically from late childhood or early adolescence; and most commonly becomes clinically obvious during the second decade of life^{2,13}. Hearing loss may be a very late clinical development in patients with ADAS and in females with XLAS². The hearing loss is progressive for frequencies over 3000 Hz but it usually plateaus in adult life, so that some hearing is retained. In patients with AS, the hearing impairment is always accompanied by evidence of renal involvement². Because high-frequency SNHL is also a feature of other inherited kidney disorders, and a relatively frequent complication among patients with ESKF undergoing chronic haemodialysis treatment, the hearing impairment is more sensitive but less specific than the ocular features for diagnosis of AS⁴².

Anterior lenticonus, the central (perimacular) and peripheral coalescing fleck retinopathies, and temporal retinal thinning are the most common ophthalmological abnormalities observed in patients with AS and are (virtually) pathognomonic of its diagnosis^{2,13,42}. These eye changes usually become apparent in late adolescence or early adulthood, and can be visualised using slit-lamp ophthalmoscopy, retinal photography, and optical coherence tomography. However, whereas anterior lenticonus usually causes progressive refractive error, with most affected patients eventually requiring surgical treatment, the retinal flecks and temporal retinal thinning do not affect vision⁴². The reported lifetime prevalences of the characteristic ocular features of AS vary widely^{13,42}, depending on how comprehensive is their screening by ophthalmological examination. In young people diagnosed with AS, screening for the eye signs is advisable from the age of 15 years¹³. Anterior lenticonus will develop in up to 50% of the males but only in rare females with XLAS, and in 75% of the patients with ARAS⁴². Retinal abnormalities are eventually observed in 55-70% of the males and 20-50% of the females with XLAS, and in 75-90% of the patients with ARAS⁴².

About 80% of the males with XLAS do not show any expression of $\alpha 5_{[IV]}$ in the EBM^{2,12}, while it is normally expressed in all individuals with ARAS. Since skin biopsy is less invasive than kidney biopsy and its results may be available sooner, immunofluorescence examination of a skin biopsy specimen immunostained for $\alpha 5_{[IV]}$ may be useful for the diagnosis of XLAS^{12,37}, but has no value for diagnosis of ARAS³⁷.

Other than reporting on the lack of parental consanguinity and on the absence of family history of renal disease or deafness, Neves *et al.*¹ did not provide any data on the patient's sibship and parental family sizes and did not go into much detail on how comprehensive was the clinical screening of the patient's first degree relatives. In an average sized family, the lack of any cases of ESKF and SNHL in the parental and grandparental generations argues against the diagnosis of XLAS and ADAS. Screening the patients' mother and father for microscopic haematuria could have been crucial to the process of diagnosis and differential diagnosis because its presence in the father would strongly suggest one of the autosomal forms of the collagen type IV-related hereditary glomerulopathies (ARAS, ADAS, TBMN) while its presence only in the mother would be more in favour of the diagnosis of XLAS. Furthermore, when the mother of an affected boy has haematuria, she should be referred to an ophthalmologist with experience in AS for detailed ocular fundus examination, since the finding of typical retinal abnormalities of AS, most commonly a peripheral fleck retinopathy, indicates not only the diagnosis of AS but also that the underlying inheritance is X-linked^{13,42}. However, family history of ESKF or haematuria may be negative in patients with ARAS, as well as in up to 15% of the males with XLAS, who have the disorder as the result of a *de novo* COL4A5 mutation². In families with AS, audiologic screening for subclinical hearing impairment is not indicated in individuals without any evidence of renal involvement.

In the patient reported by Neves *et al.*¹, bilateral SHNL was first noticed at a stage advanced enough to require hearing aids. The following two recommendations allow for earlier diagnosis of SNHL in children with AS: (i) after the age of 5 years, any child diagnosed with AS should have an audiogram performed every one to two years^{2,13}; (ii) audiologic evaluation should be recommended to any child aged 10 or more years, who exhibits persistent glomerular haematuria¹³. Because the hearing impairment was the major clue to the diagnosis of AS in this patient, complying with the latter recommendation would have allowed

recognizing SNHL at a subclinical stage, triggering the necessary diagnostic procedures at a younger age. By contrast, a negative ophthalmological screening is not unexpected at age 12 years, even in AS patients with clinically overt cochlear involvement.

■ Molecular genetics testing

Molecular genetics testing looking for pathogenic variants in the COL4A5, COL4A4 and COL4A3 genes has recently been recommended as the gold standard for the diagnosis of the collagen type IV-related hereditary glomerulopathies^{3,4,12,13,16}, instead of EM examination of a kidney biopsy. Indeed, identification of the causative mutation(s) in a proband, besides confirming the diagnosis of AS, supports more informed advice about the prognosis; enables genetic screening of other relatives; facilitates genetic counselling; and, when appropriate, allows discussing with young couples at risk the reproductive options available for primary prevention of AS in their progeny (i.e., prenatal diagnosis and preimplantation genetic diagnosis)^{12,13}, paying due attention to the complex ethical, legal and social issues involved in that decision⁴³.

Technical complexity, high cost and limited availability have been, for many years, major impediments to the routine use of molecular genetics testing for diagnosis of collagen type IV-related hereditary glomerulopathies. The large sizes of the COL4A5, COL4A4 and COL4A3 genes are a challenge for their molecular analyses on a sequential gene-by-gene and exon-by-exon approach^{3,4}, using standard polymerase chain reaction (PCR)-based laboratory techniques – e.g., dideoxy chain termination sequencing (Sanger method) of exonic DNA, including the flanking intronic sequences. Such a diagnostic strategy is quite expensive and time-consuming⁴, with several months usually needed to complete the study. This holds especially true in cases with less informative pedigrees, where the criteria to specify which gene(s) should be sequenced first are not clear-cut.

Since the introduction of next-generation sequencing (NGS) technologies^{44,45} – also known as massive parallel or high-throughput DNA sequencing – the prices of molecular genetics analyses have been falling rapidly. The decreasing cost of DNA sequencing and the increasing speed, availability and affordability of NGS platforms are making routine genetic testing more feasible in many clinical settings, including in nephrology practice⁴⁶. A major operational advantage of NGS over Sanger

sequencing is that it allows for the simultaneous analysis of all genes involved in genetically heterogeneous conditions, speeding up their diagnoses and permitting to identify patients carrying pathogenic variants in more than one of the relevant genes. These are very convenient technical features for the genetic diagnosis of familial haematuric diseases^{3,46,47}, including the collagen type IV-related hereditary glomerulopathies.

However, while point mutations, small deletions or small insertions/duplications are easily detectable by either Sanger sequencing or NGS, large deletions and large insertions/duplications are not, requiring additional laboratory work-up for their identification². Methods that can be employed to screen for gross genomic rearrangements include quantitative PCR, long-range PCR, multiplex ligation-dependent probe amplification (MLPA), and specifically designed gene-targeted microarray².

Using a two-tier molecular genetics testing approach, with MLPA as the second step if the initial mutational screening of all the exons was negative, the *COL4A5* mutation detection rate in British families fulfilling one, two, three, or four of the Flinter's clinical and EM diagnostic criteria was 18%, 64%, 89%, and 81%, respectively⁴⁸. These data justified the recommendation for mutational screening of *COL4A5* in any patient meeting at least two diagnostic criteria of AS, as long as they have no evidence of autosomal inheritance. However, that guidance may be less appropriate for populations like the Portuguese^{49,50} and others in southern European countries^{51,52}, where the relative prevalences of the autosomal forms of collagen type IV-related hereditary glomerulopathies is higher than in the British.

Overall, the current analytical sensitivity of comprehensive molecular genetics testing for the diagnosis of collagen type IV-related hereditary glomerulopathies is estimated to be over 99%⁵³. Except for a couple of major differences, the known mutational spectra of the *COL4A5*, *COL4A4* and *COL4A3* genes are broadly similar^{2,9,53} (Box 1). In comparison with missense variants, nonpoint mutations (i.e., duplications, deletions, insertions) and nonsense point mutations are associated with a younger average age at onset of ESKF in both the hemizygous males with XLAS and the homozygous or composite heterozygous patients with ARAS^{2,9}. If genetic testing is negative or inconclusive, then kidney biopsy is indicated unless the diagnosis can be confirmed on family history or by ophthalmological examination of the proband or their relatives with haematuria¹³.

In the patient reported by Neves *et al.*¹, the opportunity to diagnose AS was wasted twice, respectively at the ages of 5 and 11 years, by failing to carry out the EM examination of baseline and follow-up kidney biopsies. Genetic testing for AS was first considered at the age of 12 years, when the hearing loss became clinically evident. If a multigene NGS diagnostic panel was not available, the option to start by screening for *COL4A5* mutations was a sensible one, based on the epidemiology of classical AS²⁻⁴, although only about two-thirds of the patients fulfilling two of the Flinter's diagnostic criteria will have a diagnosis of XLAS confirmed by targeted single-gene testing⁴⁸. However, if immunofluorescence examination of a skin biopsy immunostained for $\alpha 5_{[IV]}$ had been performed before genetic testing, it would have been realized that the conditional diagnostic probabilities of XLAS and ARAS in this patient were comparable. Diagnosis of ARAS was finally established at age 16 years, when (apparent) homozygosity for a cytosine to thymine transition at cDNA nucleotide position 4,441 (c.4441C>T) in the exon 48 of *COL4A3*, generating a stop codon at amino acid position 1481 (p.Arg1481*), was identified on second molecular analysis. This is a known *COL4A3* pathogenic variant that has been already identified in other affected families [see: <https://databases.lovd.nl/shared/variants/0000149717#00005463>].

Neves *et al.*¹ did not provide any details about the genetic study of their family, namely whether the presence of gross genomic rearrangements in *COL4A3* and/or *COL4A4* was excluded in the proband by appropriate molecular testing, and whether the heterozygous condition for the *COL4A3* c.4441C>T variant was confirmed in his parents. Without these data, the possibility of compound heterozygosity for *COL4A3* mutations or double heterozygosity for *COL4A3* and *COL4A4* mutations still remains open. Indeed, homozygosity for rare recessive mutations in the offspring of nonconsanguineous couples is less likely than compound heterozygosity, and suggests that both parents may share a remote common ancestor. This is particularly more prevalent in small and relatively isolated human communities, like many of those living in inland Portugal. Unfortunately, Neves *et al.* did not comment on the geographic origin(s) of their family, making it impossible to verify whether the aforementioned hypothesis would fit the proband's parents. In addition, releasing those data would contribute to the characterization of the regional genetic epidemiology of AS in Portugal, which can be a great help to expedite the molecular diagnosis.

A major benefit of having established the genetic diagnosis of ARAS in this family (and, implicitly, excluding the alternative diagnoses of XLAS or ADAS) was the chance to deliver proper genetic and reproductive counselling², particularly advising on the recurrence risk of 25% for proband's sibs and estimating the recurrence risk in the proband's offspring as <1:1000, provided the spouse is a healthy, non-related individual from the general population.

Since most individuals with ARAS develop ESKF before age 30 years², early recognition of the genetic diagnosis allows for the timely provision of support to the patient, and to their parents and other caregivers, helping them to cope with the physical and psychosocial burdens of CKD and ESKF in adolescents and young adults, while ensuring a smooth transition from the paediatric to adult-focused healthcare settings⁵⁴.

■ ALPORT SYNDROME AND RAPIDLY PROGRESSIVE CRESCENTIC GLOMERULONEPHRITIS: PATHOGENICALLY RELATED OR JUST COINCIDENTAL?

Rapidly progressive glomerulonephritis (RPGN) is a clinical condition characterized by a nephritic syndrome evolving with loss of glomerular filtration rate (GFR) of at least 50%, over a few days to a few months⁵⁵. The pathologic hallmark of RPGN is the extensive formation of glomerular crescents. These are histological lesions characterized by multilayered accumulation in the Bowman's space predominantly of cells derived by proliferation and dedifferentiation of glomerular parietal and visceral epithelial cells, infiltrating macrophages, and fibrin precipitates⁵⁵⁻⁵⁸. Thus, crescent formation can also be described as glomerular extracapillary proliferation and RPGN is frequently designated as crescentic glomerulonephritis (CsGN).

Crescent formation represents a nonspecific response to severe injury to the glomerular capillary wall, initiated by focal ruptures of the GBM which allow entry into the Bowman's space of plasma products (including coagulation factors and inflammatory mediators) and cellular elements (such as monocytes and lymphocytes) that promote crescent formation. Glomerular extracapillary proliferation is often accompanied by necrotizing inflammation of capillaries, venules, arterioles and

small arteries^{55,56}. Circumferential as opposed to segmental crescents, as well as a higher percentage of affected glomeruli ($\geq 50\%$ as opposed to $< 50\%$) in the diagnostic kidney biopsy are histological markers of more severe, rapidly progressing renal disease^{55,56,59}. By comparison, patients exhibiting extracapillary proliferation in $< 50\%$ of the glomeruli have a more indolent clinical course, with a comparatively slower loss of GFR, and significantly higher prevalences of hypertension and severe proteinuria⁵⁹. Reflecting the longer time course of the disease process, the glomerular crescents observed in such patients show a relative prevalence of fibrotic components, while those observed in patients presenting with typical RPGN are predominantly cellular^{56,59}.

Based on the presence, specificity and distribution of immune deposits on immunomorphologic assessment of kidney biopsy specimens by direct immunofluorescence, RPGN can be classified into three major categories^{55,56}: (i) type 1 or anti-GBM antibody disease, presenting with linear deposits of immunoglobulin G (IgG) and accounting for 10-15% of the overall cases; (ii) type 2 or immune complex disease, presenting with granular deposits of immunoglobulins, accounting for 15-25% of the overall cases, but for 45% of the patients diagnosed before age 21 years; and (iii) type 3 or pauci-immune disease, presenting with few or no immune deposits and accounting for 60-80% of the overall cases. Because more than 80% of patients with pauci-immune disease have circulating antineutrophil cytoplasmic antibodies (ANCA), this form of RPGN is also termed ANCA-associated vasculitis (AAV). Type 3 ANCA-negative RPGN has also been referred to as idiopathic.

Crescent formation can complicate any glomerular disease, but only in anti-GBM disease, AAV, and the glomerulonephritides of Henoch-Schönlein purpura and systemic lupus erythematosus (histologic classes 3 and 4), they have been observed in more than half of the biopsied cases⁵⁶; however, anti-GBM disease and AAV are the only conditions where more than half of the patients show crescents in $> 50\%$ of the glomeruli⁵⁶.

An estimated 3-12% of AS patients who have received a kidney transplant develop RPGN secondary to *de novo* anti-GBM disease⁶⁰, usually manifesting within the first year after transplantation. Posttransplantation anti-GBM disease in patients with AS is due to the formation of antibodies against epitopes in the $\alpha 3_{[IV]}$, $\alpha 4_{[IV]}$ and $\alpha 5_{[IV]}$ chains, which are present in the allograft but missing in the native kidneys.

In the pretransplantation setting, reports of RPGN in patients with AS are exceptionally rare. Four cases^{33,61,62} were reported before the availability of serological testing for ANCAs, including two siblings who developed CsGN many years apart. The occurrence in siblings was taken as evidence that CsGN might be an unusual manifestation within the pathological spectrum of AS⁶², but it is now well-known that immunogenetic factors play a role in the aetiopathogenesis of both anti-GBM disease and AAV^{55,56}. In a Portuguese male diagnosed with XLAS⁶³, who developed RPGN at age 15 years, insufficient tissue sampling precluded the immunofluorescence evaluation of the kidney biopsy.

So far, only three patients with AS and necrotizing CsGN have been reported with sufficient genetic or family history information, as well as complete serologic and nephropathology data⁶⁴⁻⁶⁶: all three were adolescent males with XLAS, but only one of them, aged 17 years old, presented with typical RPGN, having finally received the diagnosis of AAV superimposed on AS nephropathy⁶⁵. The other two patients, respectively aged 11 and 17 years old, were initially referred for evaluation of nephrotic syndrome, but while in the former⁶⁶ the kidney biopsy showed extracapillary proliferation in >50% of the glomeruli, the proportion of affected glomeruli in the latter⁶⁴ was only 10%. Although in these latter two cases the clinical and kidney pathology data were interpreted as most consistent with a rare histological manifestation of AS, superimposed idiopathic renal-limited pauci-immune CsGN could not be ruled out^{64,65}.

The patient reported by Neves *et al.*¹ is exceptional in this context because of his much younger age at the manifestation of RPGN, and for being the first in whom the underlying collagen type IV-related glomerulopathy was ARAS. The absence of immune deposits on the immunofluorescence study of the kidney biopsy established the pathological diagnosis of type 3 RPGN, necessarily excluding type 2 RPGN, which is the most common cause of CsGN in children^{56,67}, as well as type 1 RPGN. Pauci-immune CsGN is the second commonest cause of RPGN in children^{56,67}, frequently presenting as a renal limited vasculitis, without any evidence of vasculitic involvement of other organs. Up to 20% of these patients do not have detectable ANCAs^{56,67}. The lack of systemic manifestations of disease in the patient reported by Neves *et al.*, as well as the negative test results for ANCAs, fit in perfectly with this diagnosis. However, severe anaemia is not a usual feature of renal limited pauci-immune RPGN, or a manifestation of the early stages of AS, and haematuria is almost never a

cause of anaemia¹⁹. The reported clinical, laboratory and pathology data allow excluding most of the differential diagnoses of RPGN that can evolve with severe or disproportionate anaemia, either caused by iron deficiency secondary to pulmonary haemorrhage^{55,67,68}, or due to haemolysis^{55,68,69}. Unfortunately, although the severe anaemia could be a confounder of the diagnosis in their patient, Neves *et al.* did not discuss its aetiology at all. Their contention that the development of extracapillary proliferation might be a rare morphologic presentation of AS, instead of a superimposed acquired disease, requires more convincing demonstration. The patient's full response to the intensive immunosuppressive therapy instituted for CsGN suggests otherwise, and the subsequent stabilization of the renal disease manifestations, with normal sCr at age 16 years, does not suggest a particularly aggressive clinical course for ARAS.

Collagen type IV, laminins, nidogens, and heparan sulphate proteoglycans are the major components of the GBM^{5,6}. Because the glomerular injury that appears most effective at initiating crescent formation is focal rupture of the GBM, agents that lyse those proteins and are released during glomerular inflammation (e.g. neutrophil elastase, matrix metalloproteinases) are important participants in the induction of CsGN^{7,56,58}.

The human GBM undergoes a process of morphogenetic maturation involving the transition from predominant expression of $\alpha1_{[IV]}$ and $\alpha2_{[IV]}$ collagen type IV chains, which is a distinctive feature of the earlier stages of glomerular development, to the predominant expression of $\alpha3\alpha4\alpha5_{[IV]}$ protomers in the fully developed glomeruli⁵⁻⁷. The resulting collagen type IV $\alpha1\alpha1\alpha2_{[IV]}$ and $\alpha3\alpha4\alpha5_{[IV]}$ GBM networks have different biomechanical properties, with the latter being more well-suited to accommodate the higher intraglomerular hydrostatic capillary pressures of the adult kidney while also providing greater resistance to proteolytic injury⁵⁻⁷.

In collagen type IV-related hereditary glomerulopathies, pathogenic mutation(s) in any one of the *COL4A3*, *COL4A4* or *COL4A5* genes may reduce or totally abolish the quantity of $\alpha3\alpha4\alpha5_{[IV]}$ protomers secreted by the podocytes, reducing the density of the $\alpha3\alpha4\alpha5_{[IV]}$ network and leading to a compensatory increase of the $\alpha1\alpha1\alpha2_{[IV]}$ network. This renders the GBM thinner and more susceptible to haemodynamic stress and to the action of proteases⁵⁻⁷. The degree of damage to the GBM architecture explains the clinical and kidney pathology spectra of these glomerulopathies, the early

appearance of haematuria, and the relatively later onset of proteinuria and progressive CKD in the more severe cases.

Theoretically, this might also make the patients with collagen type IV-related hereditary glomerulopathies more susceptible to glomerular lesions ultimately resulting in RPGN, particularly those with the most severe forms of AS. However, considering the likely publication bias of such cases⁷⁰, the number of patients reported so far is too exceptional for that hypothesis to be supported by the available epidemiological data on AS² and RPGN⁵⁵. Indeed, no case of AS was reported in a series of 632 consecutive native kidney biopsies specimens with CsGN⁵⁶ and no case of CsGN superimposed on AS was identified in a paediatric series of 294 native kidney biopsies reviewed for diagnoses of double glomerulopathy, despite 5 of the 9 patients with coexistent or superimposed glomerulopathies having AS or TBMN⁷¹.

Double glomerulopathies, involving either the coexistence of two different glomerulopathies or superimposition of a second glomerulopathy onto a first, are not uncommon in children, having been noticed in about 3% of 294 children who have undergone a diagnostic kidney biopsy in a single major paediatric hospital⁷¹. The possibility of double glomerulopathy, occurring by chance in most cases, should be considered whenever the clinical course is atypical for a single, already diagnosed primary renal disease, or if it changes abruptly during follow-up, even if both diseases are rare^{65,71}.

Key messages

- Recurrent macroscopic haematuria in the first few years of life is highly suggestive of AS.
- When the diagnosis of AS is suspected, detailed family screening for renal and extrarenal manifestations of AS is mandatory, as it may provide crucial diagnostic information.
- Electron microscopy examination of kidney biopsy is required for pathologic diagnosis of AS nephropathy.
- Genetic testing has been recently recommended as the gold standard for the diagnosis of AS and related collagen type IV-related hereditary glomerulopathies.
- Genetic diagnosis of AS helps to predict the risk of early-onset ESKF and enables preimplantation and prenatal diagnosis.
- Given the epidemiology of the collagen type IV-related hereditary glomerulopathies in Portugal, the most cost-effective first-tier approach to their genetic diagnosis is simultaneous next generation sequencing of the *COL4A3*, *COL4A4* and *COL4A5* genes.
- Patients with inherited nephropathies may develop other kidney disorders, which should be appropriately diagnosed and treated; the possibility of a superimposed glomerulopathy should be suspected if the clinical course of the primary disease changes atypically.

Box 1

Summary of the currently known genetic pathology of the *COL4A3/4/5* genes

- Overall, 40-50% of the disease-causing mutations are missense substitutions;
- Glycine substitutions in the conserved (Gly)-X-Y repeat sequence of the collagenous domain of the α [IV] chains make up 75-80% of the missense variants identified in the *COL4A5*, but only 40-45% and those identified in the *COL4A3* and *COL4A4* genes;
- About 5% of all pathogenic variants identified in any of the three genes are point mutations that result in a premature stop codon (i.e. nonsense variants);
- Mutations affecting the mRNA splicing mechanism (i.e. splice site variants) represent about 20% of the known mutations in *COL4A5* and *COL4A4*, but only about 10% of those known in *COL4A3*;
- Frameshifting mutations, resulting from small deletions/insertions/duplications, constitute 27% of the mutations described in *COL4A4*, but only 20% of those described in *COL4A5* and *COL4A3*;
- Gross genomic rearrangements are the cause of the XLAS in approximately 10-12% of the affected families, but comprise only about 1-5% of the pathogenic variants identified in the *COL4A4* and *COL4A3* genes.

This is particularly true for inherited glomerulopathies, but especially for TBMN given its high prevalence in the general population^{10,11}. Physicians caring for patients with renal disease, either in paediatric or adult health-care settings, should be aware of this possibility in order to undertake the appropriate diagnostic workup and start the indicated treatment as quickly as possible. Patients who may have superimposed RPGN require urgent medical attention, because even a few days' delay in their diagnosis and treatment can have a major negative impact on outcome^{55,67}.

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