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Expert consensus guidelines for the genetic diagnosis of Alport syndrome

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Abstract

Recent expert guidelines recommend genetic testing for the diagnosis of Alport syndrome. Here, we describe current best practice and likely future developments. In individuals with suspected Alport syndrome, all three *COL4A5*, *COL4A3* and *COL4A4* genes should be examined for pathogenic variants, probably by high throughput-targeted next generation sequencing (NGS) technologies, with a customised panel for simultaneous testing of the three Alport genes. These techniques identify up to 95% of pathogenic *COL4A* variants. Where causative pathogenic variants cannot be demonstrated, the DNA should be examined for deletions or insertions by re-examining the NGS sequencing data or with multiplex ligation-dependent probe amplification (MLPA). These techniques identify a further 5% of variants, and the remaining few changes include deep intronic splicing variants or cases of somatic mosaicism. Where no pathogenic variants are found, the basis for the clinical diagnosis should be reviewed. Genes in which mutations produce similar clinical features to Alport syndrome (resulting in focal and segmental glomerulosclerosis, complement pathway disorders, *MYH9*-related disorders, etc.) should be examined. NGS approaches have identified novel combinations of pathogenic variants in Alport syndrome. Two variants, with one in *COL4A3* and another in *COL4A4*, produce a more severe phenotype than an uncomplicated heterozygous change. NGS may also identify further coincidental pathogenic variants in genes for podocyte-expressed proteins that also modify the phenotype. Our understanding of the genetics of Alport syndrome is evolving rapidly, and both genetic and non-genetic factors are likely to contribute to the observed phenotypic variability.

Keywords Alport syndrome · Collagen IV genes · Next generation sequencing · Pathogenic variants

Introduction

Alport syndrome is an inherited glomerular disease characterised by haematuria, progressive renal failure, hearing

The following guidelines were developed by an international group of expert adult and paediatric physicians, geneticists and researchers who work on Alport syndrome, and met for discussion at the Second Alport syndrome meeting in Gottingen, Germany in 2015 and at Glasgow, UK in 2017. The guidelines have been further refined until a consensus was reached after a year of email discussions. These guidelines are complementary to the ‘Expert guidelines on the diagnosis and management of Alport syndrome and thin basement membrane nephropathy’ (JASN 2013).

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loss and ocular abnormalities [1, 2]. It is the second most common cause of monogenic kidney failure after autosomal dominant polycystic kidney disease [3]. Eighty-five percent of families have X-linked inheritance with *COL4A5* mutations, and 15% have autosomal recessive disease, with two mutations in *COL4A3* or *COL4A4* in trans, that is, on different chromosomes [4–6].

The demonstration of a pathogenic *COL4A5* variant confirms the diagnosis of X-linked Alport syndrome, and the demonstration of two *COL4A3* or *COL4A4* pathogenic variants confirms the diagnosis of autosomal recessive Alport syndrome [7]. Establishing the mode of inheritance is important for genetic counselling, for identifying other at-risk family members, for determining the affected status of potential living-related kidney donors, and for enabling prenatal and preimplantation genetic diagnosis. Some mutations (insertions, deletions, nonsense and splicing mutations, Gly

substitutions, with Arg, Glu or Asp) increase the likelihood of a severe phenotype with early-onset renal failure, lenticonus and central fleck retinopathy [8–10]. This genotype-phenotype correlation holds true for males with X-linked inheritance, and also for males and females with autosomal recessive disease [11]. In addition, the presence of a large deletion in *COL4A5* indicates a small but increased risk of antiglomerular basement membrane (GBM) disease post-transplantation [12–14]. Furthermore, the type of mutation is also likely to indicate future therapies, which will target mutation types, namely missense or nonsense variants.

The lack of a consensus on the the use of ‘autosomal dominant (AD) Alport syndrome’ for individuals with heterozygous *COL4A3* or *COL4A4* mutations is because most do not have a hearing loss, ocular abnormalities, or a lamellated glomerular basement membrane (GBM) and few develop end-stage renal failure [7, 15]. However, their prognosis is not necessarily benign and a small but unpredictable number have kidney failure [16, 17]. There is no obvious explanation for this when most other family members with the same mutations have life-long normal renal function. This discrepancy suggests that these cases of renal failure are not wholly genetic but have other causes too. It is usually not possible to exclude a second undetected mutation in the *COL4A3/COL4A4* genes in reported cases. The likelihood of renal failure itself is very small, and the diagnosis of Alport syndrome with all its implications would cause unnecessary anxiety when renal function will usually remain normal. In addition, the use of the term AD disease for carriers of a recessively inherited condition is not common practice. It was for these reasons that the group behind the Expert guidelines chose not to use the term ‘AD Alport syndrome’ until the effects of modifying mutations, coincidental renal disease and other complicating factors (smoking, hypertension, diabetes and obesity) are better understood. The Expert guidelines also continue to advocate the use of ‘Thin basement membrane nephropathy’ while recognising its limitations. Although not ideal, the term ‘Thin basement membrane nephropathy is widely understood.

The recent widespread adoption of next generation sequencing (NGS) technologies (Table 1) by routine diagnostic laboratories has demonstrated many more pathogenic and normal DNA variants in the genes affected in Alport syndrome. It has also indicated that the genetics of Alport syndrome is complicated, with different combinations of variants in the collagen IV genes, and digenic variants in the collagen IV and other podocyte genes, all potentially affecting the phenotype [16, 18, 19]. The international community involved in Alport gene testing has responded to developments by producing these consensus guidelines for renal physicians and laboratories that test for Alport mutations.

Table 1 Glossary of terms

A ‘normal DNA variant’ is a variant that occurs in many normal individuals without causing disease. This contrasts with a ‘pathogenic’ or ‘disease-causing variant’.
‘Next generation sequencing’ (NGS) refers to all forms of massively-parallel sequencing, namely, targeted gene panels, WES (where the exons and adjacent intronic regions of all genes are sequenced) and whole genome sequencing (where exons, introns and intergenic regions are sequenced).
‘Whole exome sequencing’ (WES) - all the exons in all of the genes in the genome are sequenced.
‘Clinical exome’ sequencing - the exons of an inclusive set of genes previously associated with human disease.
A ‘targeted NGS panel’ might include 30–50 genes affected in diseases causing a certain phenotype, such as proteinuria, haematuria; or just <i>COL4A5</i> , <i>COL4A3</i> and <i>COL4A4</i> in the case of Alport syndrome.
An ‘NGS panel’ might use a multiple PCR or capture array for enrichment.
‘Sanger sequencing’ -typically only the exons of one or a few genes are sequenced.
‘Cis’ and ‘trans’ - where two DNA variants are found in a gene on the same chromosome (cis) or different (trans) chromosomes. Generally the two mutations in autosomal recessive Alport syndrome are found on different chromosomes. When both mutations are found on the same chromosome the effect is less severe. The usual way to distinguish between cis and trans is to sequence the maternal and paternal chromosomes. Mutations in cis are inherited together from one parent. Mutations in trans have been inherited one from each parent.
‘Biallelic’ - pathogenic variants in both alleles of a gene (that is, in trans)
‘Digenic’ - pathogenic variants in two different genes
A ‘minigene’ is a gene fragment that includes an exon or exons and introns, together with the control regions for expression. Minigenes are useful in evaluating splicing variants.

Testing strategy for Alport syndrome

Alport syndrome is underdiagnosed. We know this because most reported series comprise mainly men and yet women are affected twice as often with X-linked disease [20]. Alport syndrome is suspected in an individual with persistent haematuria, proteinuria and/or renal impairment; with early-onset hearing loss, or peripheral retinopathy; or with a family history of Alport syndrome, or a family history of haematuria, renal impairment and no other obvious cause [7]. The presence of lenticonus, a central or peripheral retinopathy, a giant macular hole or temporal retinal thinning, are all pathognomonic [21]. The presence of any one of the diagnostic criteria (lamellated GBM, hearing loss, lenticonus, fleck retinopathy) is likely to result in a positive genetic test [22]. However, genetic testing has demonstrated that *COL4A3*, *COL4A4* or *COL4A5* mutations are also found in more than 30% of adult-onset familial focal and segmental glomerulosclerosis (FSGS) [23, 24]. In addition, Alport syndrome and thin basement membrane nephropathy commonly coexist with IgA

glomerulonephritis [25] but may be overlooked because the renal biopsy is not examined for GBM lamellation or thinning by electron microscopy.

Recommendation 1: Individuals with haematuria and a lamellated GBM or hearing loss, lenticonus or a fleck retinopathy are likely to have Alport syndrome and should be offered genetic testing for mutations in all three Alport genes (*COL4A5*, *COL4A3* and *COL4A4*). Individuals with focal and segmental glomerulosclerosis (FSGS) should also be offered genetic testing for mutations in the Alport genes in addition to podocyte-related genes. Cascade testing should be performed in at risk family members of an individual with X-linked Alport syndrome or a *COL4A5* mutation.

The diagnosis of Alport syndrome is particularly difficult when there are no extrarenal manifestations and proteinuria predominates. The best approach is to have a high index of suspicion for the diagnosis. Alport syndrome is common. What else could the diagnosis be? Testing the parents for haematuria is often rewarding.

Assessment of the patient with suspected Alport syndrome should include audiometry, ophthalmological review, retinal imaging and, possibly, retinal optical coherence tomography (OCT) [21, 26]. The ocular features are evident in the most severe cases from adolescence, so it is worthwhile examining the mother's retina when a boy presents with suspected X-linked disease. (The father of an affected girl will usually have been diagnosed by this time.) The retinal changes are asymptomatic and do not affect vision [27, 28]. Audiometry is worthwhile, both diagnostically and to confirm the need for a hearing aid, in all individuals with suspected Alport syndrome, and should be repeated as often as clinically indicated.

Individuals may undergo renal biopsy to demonstrate the lamellated GBM typical of Alport syndrome [7]. However, the GBM is often thinned in boys and females, and can be atypical with some mutations. Some laboratories also examine the GBM or skin for collagen IV α 5 staining [29, 30], but this technique may be difficult to interpret.

Genetic testing is more sensitive and specific for the diagnosis of Alport syndrome than renal biopsy [7], and provides predictive information about disease severity and prognosis. However a renal biopsy also indicates the amount of glomerular and tubular interstitial damage, and the presence of other abnormalities such as IgA glomerulonephritis or FSGS.

Usefulness of identifying genetic mutations

The demonstration of a pathogenic variant in *COL4A5* or two pathogenic variants in trans in *COL4A3* or *COL4A4* confirms the diagnosis of Alport syndrome. It

also confirms inheritance as X-linked or autosomal recessive. The mode of inheritance indicates who else in the family is at-risk and whether the disease may recur in subsequent generations. Knowing the mutation in a family facilitates cascade testing for other family members. Testing may demonstrate that a family member does not have the pathogenic mutation and could act as a kidney donor to an affected relative. Identification of the causative variant is required for prenatal and pre-implantation genetic diagnosis (PGD).

Knowledge of the causative mutation often indicates the likely clinical course. Thus 'severe' mutations in X-linked disease, including insertions/deletions, nonsense mutations, rearrangements with frameshifts or splicing mutations, result in early-onset renal failure, before the age of 30 years [8–11], as well as increased extrarenal features, such as ocular abnormalities and aortic aneurysms [31]. Substitutions of Gly with a charged residue, such as Arg, Glu or Asp, also result in early-onset renal failure and more extrarenal features [32]. In addition, some men with X-linked disease have an increased risk of developing antiGBM disease post-transplantation, especially those with large deletions. Clinical features in women with X-linked disease correlate less well with mutation severity, probably because of random X-chromosomal inactivation (lyonization), while coincidental factors such as preeclampsia, infections, hypertension and nephrotoxic agents may exacerbate renal impairment [33].

The same genotype-phenotype correlations also occur with autosomal recessive disease: the age at end-stage renal failure is younger for an individual with two severe mutations than with one, and younger for a person with one than none [11, 34] where severity is defined as for the *COL4A5* mutations. In addition, extrarenal features are more common with two severe mutations than with one or none [11]. A younger age at onset of renal failure and extra renal complications are also found with substitutions of Gly by Arg, Glu or Asp.

Another advantage of genetic testing is that generic treatments based on mutation type (missense or nonsense) may soon be available. For example, chemical chaperones may be useful in treating Alport syndrome due to missense mutations [35], and inhibitors of nonsense-mediated decay (puromycin, anisomycin etc.) in disease due to nonsense mutations [36]. These treatments will not be curative but may slow the rate of deterioration to end-stage renal failure.

Mutation testing in Alport syndrome

In general, mutations are different in each family with Alport syndrome and there are no mutation 'hot spots' in the affected

genes, except for the Gly residues in the collagenous intermediate domain [11]. Founder mutations (variants that are described more than five times in apparently unrelated families) have been reported in North American [37], British [34], Cypriot [38] and Eastern European [39] cohorts. Within a single pedigree, the mutation is the same in all affected members but some families include individuals with renal failure from a different cause. In addition, we have seen different second mutations in cousins who both had autosomal recessive Alport syndrome [34].

The *COL4A5*, *COL4A3* and *COL4A4* genes are enormous with 53, 52 and 48 exons respectively, and conventional Sanger sequencing of all the exons is very labour-intensive. Many more *COL4A5* variants have been reported ($n = 1900$) than for *COL4A3* and *COL4A4* ($n = 600$) [40], but only 10% of all possible pathogenic *COL4A5* variants are estimated to be known [1].

Evidence from testing laboratories suggests that 75% of pathogenic variants found are novel changes [17, 41]. In X-linked disease, about 40% of all mutations are missense, 10% are splicing mutations, 7% are nonsense mutations, and a further 30% result in a frameshift and downstream nonsense change, meaning that nearly 40% of all variants produce nonsense mutations [11, 42]. Similar proportions of pathogenic variants are seen in autosomal recessive disease.

The most common mutations are Gly substitutions. They are usually pathogenic if they affect a Gly in the intermediate collagenous domain, since Gly is the smallest amino acid and replacement with a larger residue disrupts the triple helical structure. Few Gly substitutions are non-pathogenic. It is much more difficult to distinguish between pathogenic and benign variants for non-Gly substitutions.

There is only one report of a mutation in the *COL4A5* promoter, and the only *COL4A6* mutations that are associated with Alport syndrome are large, contiguous deletions that extend from *COL4A6* into *COL4A5* [11]. Where deletions involve intron 2, they result in leiomyomatosis. There is no evidence that isolated *COL4A6* missense mutations produce the Alport phenotype, nor is there evidence for any other gene loci for Alport syndrome other than *COL4A5/COL4A6* and *COL4A3/COL4A4*. Nevertheless, rare mutations in other GBM or podocyte genes appear to produce a lamellated GBM in humans and in animal models [43].

Genotype-phenotype correlations

The commonly used DNA variant databases often include little clinical data, because many of their so-called normals have not been physically examined or the requests for testing include limited clinical information. In addition, most databases accept the submitters' assessments of pathogenicity and phenotype. Despite these limitations,

databases have contributed greatly to genotype-phenotype correlations [11].

Recommendation 2: All three *COL4A5*, *COL4A3* and *COL4A4* genes should be examined in individuals undergoing genetic testing for Alport syndrome. This can be by high throughput sequencing of a custom panel including *COL4A5*, *COL4A3* and *COL4A4* by WES, or by Sanger sequencing.

Three studies of NGS from different laboratories have confirmed that the mode of inheritance is difficult to predict clinically [17, 41, 44]. This is the reason that all three *COL4A5*, *COL4A3* and *COL4A4* genes are recommended for testing in suspected Alport syndrome. NGS detects nearly 95% of all missense and nonsense variants, insertions and deletions, and most splicing variants near intron-exon boundaries [16, 41]. NGS detects variants in *COL4A5*, *COL4A3* and *COL4A4*, and other genes, simultaneously, which is important with the increasing recognition of biallelic and digenic variants in Alport syndrome. Duplications, insertions, and deletions account for 5–10% of all pathogenic variants in Alport syndrome, but sequencing is less sensitive for their detection. One study has used the Integrative Genomics Viewer to retrospectively identify insertions and deletions that were previously only found with Sanger sequencing [41].

Recommendation 3: Another technique, such as multiplex ligation-dependent probe amplification (MLPA), may be required to detect duplications, insertions and deletions, but NGS approaches on different platforms may be configured to ensure sufficient read coverage to detect these.

Recommendation 4: When a *COL4* mutation cannot be demonstrated in an individual with suspected Alport syndrome, then the diagnosis and supporting features (GBM appearance, retinal photographs and retinal optical coherence tomography demonstration of temporal thinning) should be reviewed. Some of these individuals will have deep intronic splicing mutations or genetic mosaicism, which both require further specialised tests for their detection.

Deep intronic variants that introduce splicing defects often require whole genomic sequencing or a splicing assay for their detection. Deep intronic variants have been detected by sequencing [46], but splice site testing is typically performed using hair root cDNA [47–50] to confirm *COL4A5* variants, or lymphocyte cDNA [51] for *COL4A3/COL4A4* variants. The hair roots are stable for days at room temperature, but must be of sufficient number, and the nested primer design is critical.

Alternatively, the pathogenicity of a splicing *COL4* variant may be demonstrated with a ‘hybrid minigene’, a method that has a strong concordance with native RNA examination [52–54]. The low level of mRNA is sometimes problematic but the strategy is to use easily accessible tissues such as skin or peripheral blood to assess whether the wild-type transcript is produced [16]. Constructs containing DNA sequences from control and mutant genes are transiently transfected in a cell line (the HEK cells mimics the kidney splicing environment) where the minigene is transcribed. mRNA splicing patterns corresponding to wild type and to the mutant allele are then compared by RT-PCR analysis and sequencing of the amplification products [55]. These assays are relatively easy to perform but few diagnostic laboratories have the necessary experience.

Mosaicism is usually associated with milder clinical features. It occurred in nearly 10% of families (3/28) in one series [56–58], but is difficult to detect because of reduced mutant *COL4A5* expression. High-depth NGS overcomes this limitation and will indicate the real impact of this phenomenon in Alport syndrome. Mosaicism can also be demonstrated in males with X-linked Alport syndrome by skin or renal immunohistochemistry. The diagnosis of mosaicism has important consequences for patient management and genetic counselling [59, 60].

Description of DNA variants

Recommendation 5: All variants are described according to the normal reference nucleotide and amino acid sequences (Locus Reference Genomic (LRG) sequence, <http://www.irdg-sequence.org/>) which are agreed upon by an international expert panel and updated regularly. Variants are described using the nomenclature recommended by the Human Genome Variation Society (HGVS) (<http://www.hgvs.org/mutnomen/>), and confirmed with on-line tools such as Mutalyzer that check residue number and descriptions (<http://mutalyzer.nl/>).

The uniform nomenclature is informed by standardised criteria to ensure an unambiguous designation for any variant. This standard has been adopted internationally for all reference sequences. Thus, the longer sequence for *COL4A5* (LRG_232t2, NM_033380) with 53 exons is now used. The two extra exons each have 9 nucleotides or three codons and are located immediately after exon 41. The corresponding transcript is found in the kidney but only one mutation affecting this region has been reported to date. This change in the reference sequence means that variants reported before 2014 in exons 42–51 now have a new description. It is worthwhile confirming that any variants in exons 42–53 of *COL4A5* are

aligned to the correct reference sequence. For a quick conversion, for nucleotides after 3790 in *COL4A5*, 18 base pairs are added to the 51 exon nomenclature (ARUP, HGMD) to obtain the 53 exon nomenclature (LOVD). For amino acids beyond 1264, 6 amino acids are added to the 51 nomenclature.

How to distinguish between a pathogenic and a benign variant

The majority of likely pathogenic variants identified in the Alport genes by testing laboratories are novel [17, 41, 44]. Standards and guidelines are used to assess likely pathogenicity [61, 62]. Assessments should be performed by qualified and experienced staff, and multiple lines of evidence used for all Alport genes [61] (Table 2). However, variant interpretation is not static, and a variant currently determined to be of ‘unknown significance’ may be reclassified to ‘pathogenic’ or ‘normal’ as further information becomes available. Thus, assessments of pathogenicity will be corrected over time. There is no coordinating group of clinicians and scientists that confirms pathogenicity evaluations as there is for some mutations in cancer. However, principles for identifying pathogenicity for variants including in the collagen genes are slowly emerging. General guidelines have been developed in both the USA and Europe but those from the USA are probably more widely used [61] (Table 3).

Recommendation 6: Likely deleterious variants in the Alport genes

Variants in the Alport genes are more likely to be deleterious if they are:

Exonic deletions/insertions or duplications

Nonsense variants. However, caution is needed in the unusual circumstances where a predicted stop codon is in the last exon or in the last 50 bp of the penultimate exon so that nonsense-mediated decay does not occur, and the protein is more likely to be expressed;

Frameshift variants. Again caution is needed where an insertion or deletion is followed closely by a change that cancels the frameshift; or where the predicted stop codon occurs in the last exon or in the last 50 bp of the penultimate exon.

Mutations affecting the consensus splicing sites (± 1 or 2 nucleotides). However, again an inframe deletion/insertion may retain the critical domains of the protein;

Glycine missense variants in the intermediate collagenous domains (with Gly Xaa-Yaa repeats). But not if they affect Gly in one of the short non-collagenous interruptions. Thus, rarely, some Gly substitutions (p.Gly624Asp in *COL4A5*) are not pathogenic.

New DNA variants in apparently sporadic disease, that is, not present in the unaffected parents’ DNA.

Table 2 Evidence framework for interpretation of pathogenicity of variants in Alport syndrome

Genetic evidence	Tools	Benign			Pathogenic			Caveats
		Strong	Supporting	Supporting	Moderate	Strong	Very strong	
1. Variant databases	LOVD, HGMD, ARUP, Clin Var, OMIM websites		Reputable source and considered benign	Reputable source and considered pathogenic				Many databases contain errors; single reports may be duplicated from different publications
2. Prevalence of variant in normal databases	Measured by minor allele frequency (MAF), ExAc, 1000 genomes, dbSNP, dbVar, gnomAD	Variant occurs more often in normals than expected from disease penetrance			Variant is absent from normal databases	Variant occurs statistically more often in affected individuals than normals		'Normals' may not have been checked for haematuria; some normals are included twice
3. Segregation data		No segregation with disease within family		Segregates with disease in multiple family members	Segregates + with disease in multiple family members	Segregates ++ with disease in 10 family members of known status		Individuals with haematuria from other causes may be mistakenly considered affected
Computational (bioinformatics) and predictive data								
4. Predicts impact of mutation on protein structure or function. Includes effects of splicing variants.	In silico predictive algorithms include Mutation Taster, SIFT, PolyPhen-2, Condel, Panther, includes affecting a highly conserved (and thus important) amino acid		Multiple lines of evidence suggest no impact; splice variant with no predicted impact	Multiple lines of evidence support a deleterious effect	Novel missense change at a residue where other pathogenic missense variants are described	Same amino acid change as an established pathogenic variant	Nonsense variant, except those occurring in the last exon or in the last 50 bp of the penultimate exon	Some algorithms use the same lines of evidence so their results are not independent
5. Functional data	Functional assays are more difficult and less used in a routine diagnostic laboratory in Alport syndrome						Functional studies e. g. a murine model of the same mutation or in vitro mutagenesis studies	In silico modelling and simulation experiments may be useful
6. Other genetic evidence								
De novo variants					De novo variant without confirmed maternity and paternity	De novo variant with confirmed maternity and paternity		
Allelic data			For AR disorders, variant found in cis with a		For AR disorders, variant found in trans with a			

Table 2 (continued)

Genetic evidence	Tools	Benign		Pathogenic			Caveats	
		Strong	Supporting	Supporting	Moderate	Strong	Very strong	
Other data		Found in affected individuals with an alternate likely benign variant	pathogenic variant in the same gene	Phenotype or family history is highly specific for this gene	pathogenic variant in the same gene			

This is based on Fig. 1 and the text in Richards et al. [61] (used with permission) AR autosomal recessive

The assessment of other variants (non-Gly substitutions, other missense variants, deep intronic, intronic, inframe deletions/duplication/insertions/indels) may be more problematic. Laboratories typically use bioinformatics software to assess pathogenicity [63] including Alamut (v2.3 Rouen, France), Ingenuity (www.ingenuity.com) and in-house algorithms.

Genetic evidence for pathogenicity [63]

In general, there is no gold standard that defines pathogenicity. There is no single test that indicates with absolute certainty that a variant is pathogenic. Pathogenicity is defined more on probability using a number of criteria.

1. The variant has been described previously as pathogenic in a disease or variant database. Searching a curated and current locus-specific database (LSDB) that includes clinical associations, for example, the LOVD and Professional HGMD databases is considered *essential* (Table 2). Searching the relevant literature (PubMed, GoogleScholar, OMIM, Google search) for the variant is *recommended*. However, many variants have not been formally assessed and some assessments (including both the numbering and the interpretation of variants) are incorrect so these results are not definitive [63]. In addition the errors may be repeated in different databases.
2. The variant is absent or at very low frequency in large population cohorts with similar ancestry (1000 genomes; ExAC, gnomAD). *Essential*.
3. The variant is sporadic (not found in the parents, especially in X-linked Alport syndrome) but is still coincident with disease. *Acceptable*.
4. Co-segregation of variants with the disease within the family. Affected and definitely unaffected family members are equally useful for this type of assessment. Segregation is based on the haematuria in Alport syndrome being fully-penetrant from infancy which is true at least for COL4A5-associated disease. This approach is also useful in excluding pathogenicity. *Acceptable*.
5. The variant is enriched in affected individuals compared with controls. This is the least useful recommendation in Alport syndrome because some community-based individuals with Alport syndrome are undiagnosed and some normal variants are underreported, especially in non-Caucasians. This is also a problem with autosomal recessive disease where pathogenic variants may occur at a high carrier frequency in certain populations. *Acceptable*.

Bioinformatic evidence for pathogenicity

6. The variant affects a site that is evolutionarily conserved consistent with deleterious effects of sequence changes at

Table 3 ACMG classification of pathogenic and benign variants

	Applicability to Alport syndrome
Pathogenic	
Very strong evidence of pathogenicity	
PVS1 Null variant: nonsense, frameshift, canonical \pm 1 or 2 splice sites, initiation codon, single or multi-exon deletion	Yes
Strong evidence of pathogenicity	
PS1 Same aa change as a previously-demonstrated pathogenic variant but caused by a different nucleotide change	Yes
PS2 De novo variant in only affected individual with no family history and both maternity and paternity confirmed	Yes
PS3 Well-established in vitro or in vivo functional studies confirm damaging effect	Splicing mutations only
PS4 Prevalence of variant is increased in affected individuals compared with controls (say, OR > 5.0)	Yes
Moderate evidence of pathogenicity	
PM1 Variant located within mutational hotspot or functional domain that is not affected by benign variants	Gly residues in intermediate collagenous domain are critical
PM2 Variant absent from controls in Exome sequencing Project (ESP), 1000 genomes, ExAC, gnomAD	Yes
PM3 For AR AS, variant detected in trans with a pathogenic variant. This requires testing of parents or offspring	Yes
PM4 Protein length changes because of inframe deletions/insertions	Yes
PM5 Novel missense change at aa where a different pathogenic change has been noted previously	Yes
PM6 Assumed de novo, but without confirmation of maternity and paternity	Yes
Supporting evidence of pathogenicity	
PP1 Co-segregation with disease in multiple affected family members	Yes
PP2 Missense variant in gene where missense variants cause disease but benign missense variants are uncommon	Many pathogenic and benign missense variants in Alport syndrome
PP3 Evidence from different computational strategies	Yes
PP4 Phenotype is highly specific for disease	Yes
PP5 Reputable source reports variant to be pathogenic but evidence not available	Yes
Benign	
Stand-alone evidence of benign impact	
BA1 Allele frequency > 5% in ESP, 1000 Genomes, ExAC, gnomAD	Yes
Strong evidence of benign impact	
BS1 Allele frequency is more than expected	Yes
BS2 Observed in healthy adults where penetrance is expected at a younger age	Haematuria is most sensitive marker and found in nearly all adults with pathogenic variant
BS3 Well-established in vitro or in vivo functional studies indicate no damaging effect	Only studies for splicing; no other assays
BS4 Lack of segregation in an affected family	Yes
Supporting evidence of benign impact	
BP1 Missense variant in gene where all variants are truncating	Not applicable
BP2 Observed in cis with a pathogenic variant	Yes
BP3 Inframe deletions/insertions in a repetitive region without known function	Not applicable
BP4 No impact in computational studies	Yes
BP5 Variant found in individual with alternate molecular basis for disease	Yes (other podocyte genes)
BP6 Reputable source reports variant to be benign but evidence not available	Yes
BP7 A synonymous variant where splicing prediction algorithm predicts no effect AND the nucleotide is not highly conserved	Yes

Modified from Tables 3 and 4, Richards et al. [61] (used with permission)

aa amino acid

this location. Several websites can be used to examine the level of conservation of amino acids or nucleotides (GERP, PhastCons and PhyloP). *Recommended*.

- The variant affects a site in the protein that is predicted to cause a functional defect, through an effect on the collagenous sequence or by altering interactions with other membrane proteins [64].

Functional studies are the most conclusive means of confirming pathogenicity, but except for splicing mutations, are rarely used in Alport syndrome [65].

- In silico prediction of a pathogenic effect, using the different characteristics of the substituted amino acid, such as a Grantham score, or ‘biochemical difference’. Web-based classification tools are generally 65–80% accurate. Any in silico analysis should include at least three different programmes ideally based on different algorithms, such as Condel, Mutation Taster, SIFT, Polyphen-2 etc. Predictions combined from such tools are generally considered a single piece of evidence since the algorithms often include the same variables and are interdependent. *Acceptable*.

Splice site predictive algorithms are generally valid when used correctly. Again, three different prediction tools are recommended. *Acceptable*. Tools for predicting exonic/intronic splice enhancers and inhibitors have not been validated and are not currently recommended.

Experimental evidence for pathogenicity [63]

This approach is rarely used in Alport syndrome to confirm variant pathogenicity. It could take the form of gene disruption where the variant is demonstrated to alter protein levels, splicing or biochemical function, in a cell line or in vitro model system. The variant can also be introduced into a cell line or animal model to produce a similar phenotype (‘recapitulation’), or the phenotype in a mutated cell line or model organism can be rescued by a wild-type gene product or a knock-down of the variant allele.

The descriptive terms ‘polymorphisms’ and ‘mutation’ are not favoured for DNA variants because of incorrect presumptions of benign or pathogenic connotations respectively. Alternative terms are ‘variant of no known phenotype’, ‘variant of uncertain clinical significance’ and ‘pathogenic mutation’ in the UK; and ‘benign variant’, ‘variant of uncertain significance’ and ‘pathogenic variant’ in the USA.

A pathogenicity grading system is used commonly (Table 3). Assessing pathogenicity remains problematic [63]. Prior reports of a variant being pathogenic are not definitive. As far as possible, published variants should be reassessed as carefully as a laboratory’s own results. Even strong evidence

that a variant is deleterious and/or damaging does not equate to disease-causing. Pathogenicity should be assessed by multiple independent methods. Results returned for clinical use should highlight actionable findings but also clearly convey uncertain or ambiguous findings together with the rationale for these conclusions.

Strategy when only one pathogenic variant is found in suspected autosomal recessive Alport syndrome

Typically, autosomal recessive disease occurs with two pathogenic variants in trans in *COL4A3* or *COL4A4*. A heterozygous variant is unlikely to produce a lamellated GBM and renal failure but combinations of variants in *COL4A3* and *COL4A4* and in other podocyte-derived genes may do so. When a heterozygous variant is identified, the clinical characteristics range from haematuria to end-stage renal failure [60]. Some heterozygous variants may be associated with extra-renal features [66, 67], and treatment with ACE inhibitors also delays the onset of end-stage renal disease [68].

Recommendation 7: Where only one pathogenic variant is found in suspected autosomal Alport syndrome, this result should be reported using a genetic description rather than the term ‘autosomal dominant Alport syndrome’ until the significance of these single variants is better understood. Thus a report might read: ‘A heterozygous variant was found in the *COL4A3* gene and is considered pathogenic’ together with the basis for this assessment.

Most individuals with heterozygous variants do not develop renal failure. Where renal failure occurs, this may be because of an additional undetected pathogenic variant in an Alport gene, a further podocyte gene mutation, a coincidental renal disease such as IgA glomerulonephritis or FSGS, or an additional complication such as hypertension, diabetes, obesity or medication use. Alternatively, the absence of protective factors may also be responsible for renal failure in heterozygous individuals. Thus, one family member with the heterozygous mutation may develop renal failure but another with the same mutation does not [69].

Biallelic and digenic mutations affect the clinical phenotype in Alport syndrome

An allele is one of a pair of genes at a particular location on a particular chromosome. Biallelic variants refer to variants in two alleles, that is, on different chromosomes. In cis variants are located on the same allele of a gene, that is, on the same chromosome. In trans variants are found in the same gene but on opposite chromosomes.

Digenic variants are two variants in different genes, for example, in *COL4A5* and *COL4A3*. The situation is even more complicated for *COL4A3* and *COL4A4* because these genes are located back-to-back on the same chromosome, and a *COL4A3* variant will always be inherited together with a *COL4A4* variant if they are in cis. If a *COL4A3* and *COL4A4* variant are not inherited together, they are located on different chromosomes or in trans.

There are increasing reports of biallelic and digenic variants in Alport syndrome. There are also examples of variants in *COL4A3* and *COL4A4* in trans [17, 18], and a single report of a woman with two pathogenic *COL4A5* variants in trans [70]. In trans mutations are confirmed when the parents are each demonstrated to have one variant. Confirmation requires testing the parents' or siblings' DNA.

It is important to know if an individual has two *COL4A3* or *COL4A4* variants in cis or in trans because the corresponding phenotypes are different (Fig. 1). Individuals with two *COL4A3* or *COL4A4* variants in cis have a milder phenotype than those with two in trans [18].

The inheritance of mutations in cis and trans is different too. The offspring of an affected individual with two mutations in cis inherits both mutant alleles or none, whereas the offspring of individuals with mutations in trans inherit one, but not both, defective alleles. On average, half the offspring of a person with two in cis mutations inherit both mutant alleles. In contrast, all the offspring of an individual with two mutations in trans (and hence autosomal recessive Alport syndrome) inherit one mutation, and typically develop haematuria but not renal failure.

There are also reports of individuals with digenic mutations, affecting *COL4A3* and *COL4A4* [16, 18], and that the clinical features are then milder than where mutations both affect *COL4A3*, or both affect *COL4A4*, causing autosomal recessive Alport syndrome.

Other genes may modify the Alport clinical phenotype (Table 4)

Now that the Alport genes are commonly sequenced in gene panels for FSGS, further combinations of variants are being identified. Thus *COL4A5*, *COL4A3* and *COL4A4* mutations are sometimes found together with mutations in other podocyte genes including *ACTN4*, *NPHS2* and *MYO1E* [71–73].

FSGS often occurs secondary to Alport syndrome or a heterozygous *COL4A3* or *COL4A4* mutation, and mutations, especially R229Q in *NPHS2* [74], may coexist with heterozygous *COL4A3* or *COL4A4* mutations worsening proteinuria [72]. Digenic mutations also exacerbate disease severity in other forms of renal disease [73].

Recommendation 8: All likely pathogenic variants in the *COL4A5*, *COL4A3* and *COL4A4* genes should be reported. These may be biallelic, digenic or multiple. Thus, if a pathogenic variant is found in *COL4A5* and another in *COL4A3*, both should be reported since each is likely to affect the phenotype. Notwithstanding, some variants cannot be categorised and should remain as 'variants of unknown significance'.

Other genes that could be tested for pathogenic variants when none is found in the Alport genes (Table 5)

Other autosomal dominant causes of haematuria and renal failure include:

- Diseases of the complement pathway genes. *CFH* and other complement pathway mutations result in dense

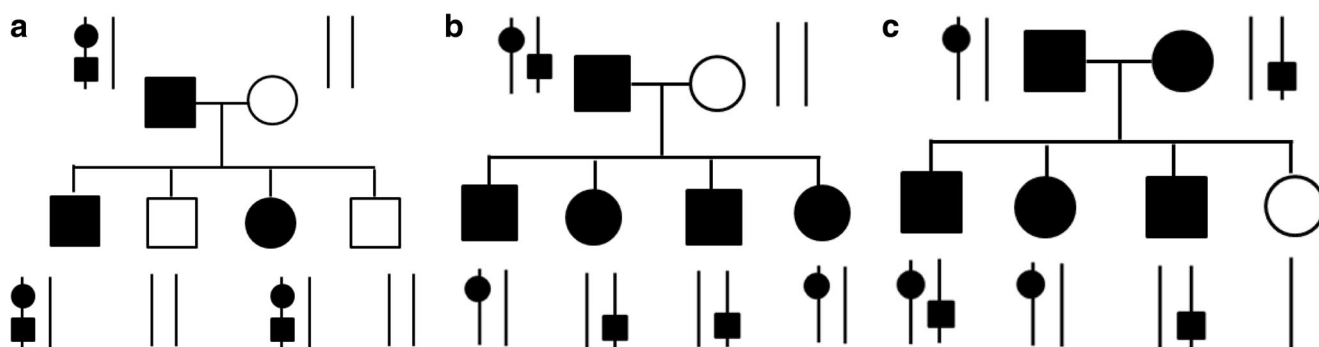


Fig. 1 Inheritance of heterozygous *COL4A3* or *COL4A4* pathogenic variants in cis or trans. **a** Inheritance of two pathogenic or mutant variants in cis, that is, in the same gene on the same chromosome, are inherited together from the one parent. This means that, on average, half the offspring inherit both mutant alleles in cis and have haematuria. **b** Inheritance of two pathogenic variants in trans, that is in the same gene, but on different chromosomes, are NOT inherited together. All offspring

then inherit one mutant variant only and have haematuria. **c** Inheritance of two pathogenic variants from different parents means that, on average, half the offspring inherited one mutant variant and have haematuria only, and one in four offspring (25%) inherits two mutant variants in trans, and have haematuria and renal failure (autosomal recessive Alport syndrome). One offspring has no mutant variant and no haematuria. Filled-in symbols have a clinical phenotype, of at least haematuria

Table 4 Potential modifiers in Alport syndrome

Gene	Protein	Function	Disease
<i>NPHS2</i>	Podocin	Podocyte foot process protein, lipid raft	FSGS (p.Arg229Gln) (R229Q), (E237Q, Glu237Gln) [72]
<i>LAMA5</i>	Laminin chain	GBM constituent	Proteinuria
<i>LAMB2</i>	Laminin chain	GBM constituent	Proteinuria
<i>APO1E</i>	Apolipoprotein 1E	Mediates binding, internalisation and catabolism of lipoproteins	FSGS, lipoprotein glomerulopathy
<i>CFHR5</i>	Complement factor H-related 5	A regulator of complement activation	HUS, DDD
<i>ACTN4</i>	α actinin 4	Cytoskeletal function	FSGS
<i>PODXL</i>	Podocalyxin-like	Found in podocytes, involved in cell adhesion and migration	Glomerulonephritis
<i>WT1</i> (also known as <i>NPHS4</i>)	Wilms tumour protein 1	Involved in normal development of the urogenital system	FSGS, Denys-Drash syndrome
<i>TRPC6</i>	Transient receptor potential cation channel subfamily C	Receptor-activated Ca channel in podocyte cell membrane	FSGS
<i>CD2AP</i>	CD2-associated protein	Scaffolding molecule that regulates the actin cytoskeleton	FSGS
<i>INF2</i>	Inverted formin 2	Polymerisation and depolymerisation of actin filaments	FSGS

See text for references

FSGS focal segmental glomerular sclerosis, DDD dense deposit disease, GBM glomerular basement membrane

deposit disease (haematuria, proteinuria, renal failure, retinal drusen [75]). Renal failure typically develops in the late teenage years. There is no hearing loss. *CFHR5* nephropathy is caused by heterozygous duplication of exons 2 and 3 of *CFHR5*, and presents with haematuria and renal impairment [76].

- Fechtner syndrome, due to mutations in *MYH9* (myosin heavy chain) gene. This is an uncommon condition, characterised by haematuria, renal failure sometimes, hearing loss, and a lamellated GBM, but also neutrophil inclusions, thrombocytopenia and large platelets [77]. The neutrophil inclusions may be overlooked and require examination of fresh blood films. The blood changes are lifelong and diagnostic. There is minimal bleeding risk.
- Fibronectin glomerulopathy. This is rare, with haematuria, but mainly proteinuria and renal failure in adulthood. It is caused by mutation in *FNI* [78].
- Hereditary Angiopathy, Nephropathy and Cramps (HANAC) syndrome. This is a rare disease caused by *COL4A1* mutations and characterised by haematuria, kidney cysts and irregularly thickened tubular and Bowman's capsular membranes, together with retinal angiopathy, neurovascular abnormalities and cramps [79].
- IgA disease. About 15% of all cases of IgA disease have another affected family member [80] and although GWAS analyses have identified chromosomal loci, relatively few genes have been identified [81].

- 'SLE is slightly more common in family members and manifests with haematuria, proteinuria or renal impairment.

Other diseases that result in kidney failure with hearing loss include Bartter syndrome, Nail-patella syndrome, Fabry disease, Branchio-Oto-Renal disease, Hypoparathyroidism, deafness and renal failure (HDR) and some mitochondrial syndromes such as MELAS [7]. Nail-patella syndrome (associated with *LMX1 β* mutations) has an irregular disrupted GBM [82] that may resemble the Alport GBM (and probably results from reduced collagen IV α 3 and α 4 expression [83]). However, this condition is distinguished by its nail and patella abnormalities, and association with proteinuria rather than haematuria.

Recommendation 9: Where Alport syndrome is suspected clinically but no pathogenic variant is found in the Alport genes, it is worthwhile reconsidering the clinical diagnosis before further genetic testing and bioinformatics analysis.

In conclusion, Alport syndrome is common but often unrecognised; some patients diagnosed with FSGS on renal biopsy have underlying mutations in the *COL4A3*, *COL4A4* or *COL4A5* genes; the mode of inheritance can be difficult to predict so all three Alport genes should be examined for variants; and biallelic and digenic mutations may affect the renal failure progression. Most pathogenic have variants in the Alport genes can be detected with sequencing; some are due to duplications, deletions or insertions and may need further,

Table 5 Other genes to test for mutations when the clinical features suggest Alport syndrome

Gene	Disease	Inheritance	Distinguishing features
<i>NPHS2</i>	FSGS	AD or AR with another podocyte gene mutation	Proteinuria, nephrotic syndrome, renal failure
<i>MYH9</i>	Fechtner syndrome	AD	Haematuria, renal failure, neutrophil inclusions, large platelets but low platelet counts, hearing loss
<i>CFH, CFHR5</i>	DDD or HUS	AD	Haematuria, renal failure, possibly with retinal drusen or lipoatrophy
<i>LMX1B</i>	Nail-Patella syndrome	AD	Haematuria, proteinuria, renal failure, dystrophic nails, absent patellae
<i>COL4A1, COL4A2</i>	HANAC	AD	
Mitochondrial <i>DNA</i>	MELAS etc.	Mitochondrial	Other cardiac and muscular weakness
<i>FNI</i>	Glomerulopathy with fibronectin deposits	AD	Proteinuria, nephrotic syndrome, hypertension, renal failure, disease recurrence post-transplant

See text for references

FSGS focal segmental glomerular sclerosis, *DDD* dense deposit disease, *AD* autosomal dominant, *AR* autosomal recessive, *HUS* hemolytic uremic syndrome, *HANAC* Hereditary Angiopathy, Nephropathy and Cramps, *MELAS* mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke

different tests; and the remaining undetected variants are usually caused by deep intronic splicing or mosaicism. Increasingly, compound heterozygous variants that include not only the Alport genes but also genes encoding other podocyte proteins.

Recommendation 10: Laboratories that test for genetic variants in Alport syndrome continue to collect and share their variants in variant databases [84]. The submission should include clinical features, an assessment of pathogenicity and the basis of this decision, as well as contact details of the submitting laboratory

Compliance with Ethical standards

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

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