Expert consensus guidelines for the genetic diagnosis of Alport syndrome

[1]Judy Savige, [2]Francesca Ariani; [2]Francesca Mari; [2]Mirella Bruttini; [2]Alessandra Renieri; [3]Oliver Gross; [4]Constantinos Deltas; [5]Frances Flinter; [6]Jie Ding; [7]Daniel P. Gale; [8]Mato Nagel; [9]Michael Yau; [10]Lev Shagam; [11]Roser Torra; [12]Elisabet Ars; [13]Julia Hoefele; [14]Guido Garosi; [9]Helen Storey,

[1]The University of Melbourne Department of Medicine, Melbourne and Northern Health, Parkville VIC 3050, AUSTRALIA; [2]Medical Genetics, University of Siena, ITALY; [3]Clinic of Nephrology and Rheumatology, University of Gottingen, GERMANY; [4]Molecular Medicine Research Centre, University of Cyprus, CYPRUS;[5]Department of Clinical Genetics, Guys' and St Thomas' NHS Foundation Trust, UK; [6]Peking University First Hospital, CHINA; [7]Centre for Nephrology, Royal Free Hospital, University College London, London, UK; [8]Centre for Nephrology and Metabolic Disorders, Weisswasser, GERMANY; [9]Genetics, Viapath, Guy's Hospital, London, UK; [10]Institute of Pediatrics, Pirogov Russian Medical University, Moscow RUSSIA; [11]Inherited Kidney Disorders, Nephrology Department, Fundacio Puigvert, Instituto de Investigaciones Biomedicas Sant Pau (IIB-Sant Pau), Universitat Autonoma de Barcelona, REDinREN, Instituto de Investigacion Carlos III, Barcelona, SPAIN; [12] Molecular Biology Laboratory, Fundacio Puigvert, Instituto de Investigaciones Biomedicas Sant Pau (IIB-Sant Pau), Universitat Autonoma de Barcelona, REDinREN, Instituto de Investigacion Carlos III, Barcelona, SPAIN; [13] Institute of Human Genetics, Technical University Munich, Munich, GERMANY; [14] Nephrology, Dialysis and transplantation, Azienda Ospedaliera Universitaria Senese, Siena, ITALY.
.

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. The guidelines have been further refined until a consensus was reached in a subsequence year of email discussion. These guidelines are complementary to the "Expert guidelines on the diagnosis and management of Alport syndrome and Thin basement membrane nephropathy' (JASN 2013).

Address for correspondence:
Prof Judy Savige
The University of Melbourne
Department of Medicine
Melbourne and Northern Health
Parkville VIC 3050
AUSTRALIA
Email: jasavige@unimelb.edu.au

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, ideally all three *COL4A5*, *COL4A3* and *COL4A4* genes should be examined for pathogenic variants, preferably 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% variants, and the remaining rare variants are 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 *in cis*, or one in *COL4A3* and another in *COL4A4,* produce a phenotype intermediate between a heterozygous (various phenotype) and compound heterozygous variants (proteinuria, renal failure). NGS may also identify further pathogenic variants in genes for podocyte-expressed proteins that 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 phenotypic variability observed.

TEXT

Alport syndrome is an inherited glomerular disease characterised by hematuria, progressive renal failure, hearing loss and ocular abnormalities [1, 2]. It is the second commonest monogenic inherited renal disease after autosomal dominant polycystic kidney disease [3]. Eighty-five % of families have X-linked inheritance and *COL4A5* mutations, and, 15% have autosomal recessive disease, with two mutations *in trans*, that is, on different chromosomes, in *COL4A3* or *COL4A4* [4-6]. There is not consensus on the use of autosomal dominant Alport syndrome where there is a heterozygous *COL4A3* or *COL4A4* variant [7].

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 [8]. 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 to enable prenatal and preimplantation genetic diagnosis. The type of mutation (insertion, deletion, nonsense and splicing mutation, and Gly substitution with Arg, Glu or Asp) determines the phenotype severity, including the risk of early-onset renal failure, lenticonus and central fleck retinopathy [9-11]. This genotype-phenotype correlation holds true for males with X-linked inheritance, and probably also for males and females with autosomal recessive disease [12]. In addition, the presence of a large deletion in *COL4A5* indicates an increased but still very small risk of antiglomerular basement membrane (GBM) disease post- transplantation [13-15]. Furthermore, future therapies are likely to target mutation types, for example, missense or nonsense variants.

The lack of consensus on the use of "autosomal dominant (AD) form of Alport syndrome"[7] for individuals with heterozygous *COL4A3* or *COL4A4* variants is because most of these individuals do not have a hearing loss or ocular abnormalities, or a lamellated glomerular basement membrane (GBM) and few develop end-stage renal failure [8, 16]. However their prognosis is not necessarily benign and a small but unpredictable number develop kidney failure [17, 18]. There is no obvious explanation why some develop kidney failure when most other family members with the same mutations have life-long normal renal function. This disparity however suggests that renal failure is not genetic but caused by other factors. It is usually not possible to exclude a second undetected mutation in the COL4A3/COL4A4 genes. The likelihood of renal failure itself is very small, and the use of Alport syndrome which implies renal failure would cause unnecessary anxiety in the majority of individuals who will have normal kidney function. Finally the diagnosis of an AD disease for carriers of a recessively inherited condition is not widespread. It was for these reasons that group behind the Expert guidelines chose not to use the term autosomal dominant Alport syndrome until the effects of modifying mutations, coincidental renal disease and other complicating factors (smoking, hypertension, diabetes, obesity) are better understood. The Expert guidelines continue to advocate the use of 'Thin basement membrane nephropathy' although recognising their limitations, until the consequences of heterozygous COL4A3 /COL4A4 mutations are better understood. Although this situation is not ideal, the term Thin basement membrane nephropathy is widely understood and used, and it was felt preferable to not use an interim term such as AD Alport syndrome.

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 [17, 19, 20]. 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.

The manuscript arose from presentations and vigorous discussion at the genetics meetings at the 2015 and 2017 International Alport syndrome workshops in Gottingen and Glasgow and further email exchanges over the subsequent months to reach consensus on the recommendations.

Testing strategy for Alport syndrome
Alport syndrome is underdiagnosed. We know this because all series comprise mainly men and yet women are affected twice as often with X-linked disease [21]. Alport syndrome is suspected in an individual with persistent hematuria,   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 hematuria, renal impairment and no obvious cause [8]. The presence of lenticonus, a central or peripheral retinopathy, a giant macular hole, or temporal retinal thinning, are all pathognomonic [22]. 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 [23].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)[24, 25]. In addition, Alport syndrome and Thin basement membrane nephropathy commonly coexist with IgA glomerulonephritis[26] but may be overlooked because the renal biopsy is not examined for GBM lamellation or thinning by electron microscopy.

Recommendation 1: Individuals with hematuria 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 hematuria 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) [22, 27]. The ocular features are evident first in the most severe cases in 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, or soon after, on examination of the urinary sediment and eGFR.) The retinal changes are asymptomatic and do not affect vision [28, 29]. 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 glomerular basement membrane (GBM) typical of Alport syndrome [8]. However the GBM is often only thinned in boys and females, and can be atypical with some mutations. Some laboratories also examine the GBM or skin for collagen IV α5 staining [30, 31], but this technique is insensitive and sometimes difficult to interpret.

Genetic testing is more sensitive and specific for the diagnosis of Alport syndrome than renal biopsy [8], and provides predictive information about disease severity and prognosis. 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 indicates whether inheritance is 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 at-risk 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(s) is required for pre-natal 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 resulting in frameshifts, and splicing mutations, result in early onset renal failure, before the age of 30 years [9-12], as well as more frequent extrarenal features, such as ocular abnormalities and aortic aneurysms [32]. Substitutions of Gly with a charged residue, such as Arg, Glu, Asp, also result in early onset renal failure and extrarenal features [33]. In addition, some men with X-linked disease are at 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 [34].

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 [12, 35] 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 [12]. A younger age at onset of renal failure and extra renal complications are also found with substitutions of Gly by charged amino acids with longer side chains such as 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, chaperones may be useful in treating Alport syndrome due to missense mutations [36], and inhibitors of nonsense-mediated decay (puromycin, anisomycin etc) in disease due to nonsense mutations [37]. These treatments will not be curative but may further 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 residue in the collagen backbone [12]. Founder mutations (variants that are described more than 5 times in apparently unrelated families) have been reported in North American[38], British [35], Cypriot [39] and Eastern European [40] cohorts. Within a single pedigree, the mutation is *usually* 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 [35].

The *COL4A5*, *COL4A3* and *COL4A4* genes are enormous with 53, 52 and 48 exons respectively, and conventional Sanger sequencing of all 153 exons is labor-intensive. Many more *COL4A5* variants have been reported (n=1900) than for *COL4A3* and *COL4A4* (n=600)[41], but only 10% of all possible pathogenic variants have been described [12].

Evidence from testing laboratories suggests that 75% of pathogenic variants found are novel changes [18] [42]. 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 a nonsense mutations [12, 43]. Similar proportions of pathogenic variants are seen in autosomal recessive disease except that large deletions may be more common, or are detected more often.

The commonest mutations are Gly substitutions. They are almost always 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 more difficult to distinguish between pathogenic and benign variants for the 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 *COL4A5* into *COL4A6* [12]. Where these extend into intron 2 they result in leiomyomoatosis. 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 in humans other than *COL4A5/COL4A6* and *COL4A3/COL4A4.* Nevertheless there are some mutations in other GBM or podocyte genes that appear to produce a lamellated GBM in human disease and animal models [44, 45].

Genotype-phenotype correlations
The commonly used DNA variant (mutation) databases often include little clinical data, because many of their so-called normal have not been physically examined or the request forms for testing include limited clinical information. In addition, many databases accept the submitters' assessments of pathogenicity and phenotype assessment. Despite these limitations, databases have contributed greatly to our understanding of genotype-phenotype correlations [12].

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 [18, 42, 46]. This is the reason that all three *COL4A5*, *COL4A3* and *COL4A4* genes are recommended for testing in suspected Alport syndrome. NGS detects missense and nonsense variants, insertions and deletions, and most splicing variants near intron-exon boundaries, with > 90% sensitivity [17, 42, 46, 47]. (Sometimes further tools are needed to detect small indels on retrospective visualisation of data.) It 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 because the available bioinformatics tools are less able to detect large rearrangements, and also because of poor DNA quality and contaminating sequencing inhibitors.

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 can sometimes be configured to ensure sufficient read coverage (usually x 200) to detect these. One study has used the Integrative Genomics Viewer to retrospectively identify insertions and deletions that were previously only found with Sanger sequencing [42].

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, 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 confirmation.

If it is critical to have a genetic diagnosis, for example, prior to kidney donation or for prenatal or preimplantation genetic diagnosis, then 'gene linkage' studies may help, especially in excluding Alport syndrome.

Deep intronic variants that introduce splicing defects often require whole genome sequencing or a splicing assay for their detection. Whole genomic sequencing has not been evaluated sufficiently for any recommendations to be made about its usefulness in detecting splicing mutations. Deep intronic variants have been detected by sequencing [48], but splice site testing is typically performed using hair root cDNA [49-52] to confirm *COL4A5* variants, or lymphocyte cDNA [53] for *COL4A3/COL4A4* variants. The hair roots are stable for days at room temperature, for example in the post, but must be of sufficient number, and the nested primer design is critical. Alternatively, the pathogenicity of a splicing *COL4* intronic or exonic variant may be demonstrated with a 'hybrid minigene', a method that has a strong concordance with native RNA examination [54-56]. 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 from the mutant allele [17]. Constructs containing DNA sequences from control and mutant gene 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 [57]. These assays are relatively easy to perform but few diagnostic laboratories have the necessary experience in cloning and cell culture.

Mosaicism is usually associated with milder clinical features. It occurred in nearly 10% of families (3/28) in one series [58-60], 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. (Depth of coverage is provided automatically with NGS reads.) 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 counseling [61, 62].

Description of DNA variants
Recommendation 5: All variants should be described according to the normal reference nucleotide and amino acid sequences (Locus Reference Genomic (LRG) sequence, http:www.irg-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 and recommended 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 was recently adopted as the reference. 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 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. However the HGMD and ARUP variant databases have not yet updated their databases. For a quick conversion, for nucleotides after 3790 in *COL4A5*, add 18 base pairs to the 51 exon nomenclature (ARUP, HGMD) to obtain the 53 exon nomenclature (LOVD). For amino acids beyond 1264, add 6 amino acids to the 51 nomenclature (ARUP, HGMD) to obtain the 53 exon nomenclature (LOVD).

How to distinguish between a pathogenic and a benign variant
Up to 75% of likely pathogenic variants identified in the Alport genes by testing laboratories are novel [18, 42, 46]. Standards and guidelines are used to assess likely pathogenicity [63, 64]. Assessments should be performed by qualified and experienced staff, and multiple lines of evidence used for all Alport genes [63] **(Table 2)**. However these variant interpretation is not static, and a variant currently determined to be of 'unknown significance' may be upgraded to 'pathogenic' or 'normal' as further information becomes available. In additions, assessments of pathogenicity and normality will be corrected over time. There is no centralised group that confirms pathogenicity evaluations as there is for some mutations in cancer. However principles for pathogenicity for the collagen genes are slowly emerging. Guidelines have been developed in both the US and Europe but those from the US are probably more widely used [65] **(Table 3)**.

Recommendation 6:
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 exon so that nonsense-mediated decay does not occur, and the protein is more likely to be expressed;
- **Frameshift variants.** However again caution is needed where an insertion or deletion is followed closely by a change that cancels the frameshift; and again 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 Glycine in one of the short non-collagenous interruptions in the intermediate collagenous domain. Thus, rarely, some Gly substitutions (p.Gly545Ala and p.Gly999Glu in *COL4A4)* are not pathogenic.
- New DNA variants in apparently sporadic disease, that is, not present in the unaffected parents' DNA.

The assessment of other variants (non-Gly substitutions, silent missense variants, deep intronic, intronic, inframe deletions/duplication/insertions/indels) may be more problematic. Laboratories typically use bioinformatics software to assess pathogenicity [66] including Alamut (v2.3 Rouen, France), Ingenuity (www.ingenuity.com) and in-house designs.

Genetic evidence for pathogenicity [66]
In general, there are no gold standards for 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. The difficulty in stating that a variant is definitely pathogenic is that Thin

basement membrane nephropathy is very common and X-linked Alport syndrome is underdiagnosed.

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 assessed and some assessments (including both the numbering and the interpretation of variants) are incorrect so these results are not definitive [66]. Often the errors are carried over from one database over to another.

2. The variant is absent or at very low frequency in large population cohorts with similar ancestry (1000 genomes; ExAC,; gnomAD, **Table 2)**. **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 hematuria 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 conditions 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 this location. Several web-sites can be used to examine the level of conservation of amino acids or nucleotides (GERP, PhastCons and PhyloP). **Recommended.**

7. 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 [67]. Functional studies are the most conclusive means of confirming pathogenicity, but, except for splicing mutations, are rarely used in Alport syndrome [68]. They are generally not available as part of a routine diagnostic service.

8. *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 3 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 [66]

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 knockdown of the variant allele.

The descriptive terms 'polymorphism' and 'mutation' for DNA variants are not favoured 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 US.

A pathogenicity grading system is used commonly **(Table 3)**. Assessing pathogenicity remains problematic [66]. 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 without other complications, but other combinations of variants in *COL4A3* and *COL4A4* and in other podocyte-derived genes, may. When a heterozygous variant is identified, the clinical characteristics range from hematuria to progressive renal failure and end-stage disease [69]. Some heterozygous variants may be associated with extrarenal features [69, 70]. Treatment with ACE inhibitors delays the onset of end-stage renal failure in individuals with heterozygous *COL4A3* or *COL4A4* mutations [71].

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 variant's significance are better understood. Thus a laboratory 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 a an additional complication such as hypertension, diabetes, obesity or nephrotoxic 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 [72].

Biallelic and digenic mutations affect the clinical phenotype in Alport syndrome
An **allele** is one of a pair of genes at a particular site on a particular chromosome. **Biallelic** variants refer to two variants in both 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 **opposite** alleles and, hence, in the same gene but on the other chromosome. **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 may 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 probably located on opposite chromosomes (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* [18, 19], and a single report of a woman with

two pathogenic *COL4A5* variants *in trans* [73]. *In trans* mutations are confirmed when the parents are each demonstrated to have one variant. This 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 **(Figure 1)**. Individuals with two *COL4A3* or *COL4A4* variants *in cis*, appear to have a milder phenotype than those with two *in trans* [19].

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. *In cis* mutations are inherited together. 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) inherits one mutation, and typically develop hematuria but not renal failure.

There are also reports of individuals with digenic mutations, affecting *COL4A3* and *COL4A4* [17, 19], 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* [74-76]. The effect on the clinical phenotype is not yet clear. It is uncommon for patients diagnosed clinically with Alport syndrome to have mutations in genes normally associated with FSGS except for the *NPHS2* p.(Arg229Gln) or R229Q variant which are common in normal European populations [77].

FSGS often occurs secondary to Alport syndrome or a heterozygous *COL4A3* or *COL4A4* mutation, and mutations, especially R229Q, may coexist with heterozygous *COL4A3* or *COL4A4* mutations worsening proteinuria [75]. Digenic mutations also exacerbate disease severity in other forms of renal disease such as FSGS [76].

In general, the clinical phenotype in a family with Alport syndrome depends on the causative mutation, but clinical variation is also common. In women with X-linked Alport syndrome this is attributed to Lyonization, that is, the variable expression of the gene on each X chromosome, in different tissues, but in affected men, the variation must be caused by modifier genes or environmental factors.

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 '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 hematuria and renal failure include:
- Diseases of the complement pathway genes. *CFH* and other complement pathway mutations result in dense deposit disease (hematuria, proteinuria), renal failure and retinal drusen [78]. Renal failure typically develops in the late teenage years to middle age. There is no hearing loss. *CFHR5* nephropathy is caused by heterozygous duplication of exons 2 and 3 of *CFHR5*,

and presents with hematuria, C3 glomerulopathy and renal impairment that is more severe in males [79].

- Fechtner syndrome, due to mutations in *MYH9* (myosin heavy chain) gene. This is an uncommon condition, characterised by hematuria, renal failure sometimes, hearing loss, and a lamellated GBM, but also neutrophil inclusions, thrombocytopenia and large platelets[45]. The neutrophil inclusions may be overlooked and must be sought using fresh blood films (less than 3 hours old), and the blood changes are life long and are diagnostic. There is a minimal bleeding risk.
- Fibronectin glomerulopathy. This is rare, with hematuria, but mainly proteinuria and renal failure in adulthood. It is caused by mutation in FN1 [80].
- Hereditary Angiopathy, Nephropathy and Cramps (HANAC) syndrome. This is a rare disease caused by *COL4A1* mutations and characterised by hematuria, kidney cysts and irregularly thickened tubular and Bowman's capsular membranes, together with retinal angiopathy, neurovascular abnormalities and cramps [81].
- IgA disease. About 15% of all cases of IgA disease have another affected family member [82] and although GWAS analyses have identified chromosomal loci, relatively few gene loci have been identified [83].
- SLE is rarely inherited but may manifest with hematuria and renal failure. The genes are not known.

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 [8]. Nail-patella syndrome (associated with *LMX1β* mutations) has an irregular disrupted GBM [84] that may resemble the Alport GBM (and probably results from reduced collagen IV α3 and α4 expression [85]). However, this condition is distinguished by its nail and patella abnormalities, and association with proteinuria rather than hematuria.

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 be common and affect the renal failure progression. Most pathogenic variants in the Alport genes can be detected with sequencing; 10% are due to duplications, deletions or insertions and may need further, different tests; and the remaining 5 – 10% of undetected variants are usually caused by deep intronic splicing or mosaicism. Increasingly, compound heterozygous variants that include not only the Alport but also genes encoding other proteins in the glomerular filtration barrier are reported.

Recommendation 10: Laboratories that test for genetic variants in Alport syndrome continue to collect and share their variants in variant databases [86]. It is most helpful if the submission includes clinical features, an assessment of pathogenicity and the basis of this decision, as well as contact details for the submitters.

LIST OF TABLES and FIGURES

Figure 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 hematuria; **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 hematuria; **c.** inheritance of two pathogenic variants from different parents means that, on average, half the offspring inherited one mutant variant and have hematuria only, and one in four offspring (25%) inherits two mutant variants *in trans*, and have hematuria and renal failure (autosomal recessive Alport syndrome). One offspring has no mutant variant and no hematuria.

Filled- in symbols have a clinical phenotype, of at least hematuria

Table 1: Glossary

A 'normal DNA variant' is a variant that occurs in many normal individuals without causing disease. In contrast a 'pathogenic' or 'disease-causing variant' results in disease.

'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 (may be 1000 or more) previously associated with human diseases.
A '**targeted NGS panel'** might include 30 – 50 genes affected in diseases causing proteinuria, or hematuria, or *COL4A5*, *COL4A3* and *COL4A4* only.
An '**NGS panel'** might use a multiple PCR or a 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.

'Bi-allelic' – 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 patterns.

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

		BENIGN		PATHOGENIC				CAVEATS
Genetic evidence	**Tools**	**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 contain errors; single reports may be duplicated from different publications
2. Prevalence of variant in normal databases	Measured by mean 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 hematuria; 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 multiple family members	Segregates ++ with disease in 10 family members of known status	Individuals with hematuria from other causes may be mistakenly considered affected
Computational (bioinformatics) and predictive data								
Predicts impact of mutation on protein structure or function. Includes effects of splicing variants.	*In silico* predictive algorithms include MutationTaster, SIFT, PolyPhen-2, Condel; Panther; includes affecting a highly conserved (and 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 lst 50 bp of the penulimte exon	Some algorithms use the same lines of evidence so their results are not independent.
Functional data	Functional assays are more difficult and less used in a routine diagnostic laboratory in Alport syndrome.						Functional studies eg a murine model of the same mutation or *in vitro* mutagenesis	*In silico* modelling and simulation experiments are in their infancy but are used for other proteins

							studies	
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 pathogenic variant in the same gene		For AR disorders, variant found _in trans_ with a pathogenic variant in the same gene			
Other data		Found in affected individuals with an alternate likely benign variant		Phenotype or family history is highly specific for this gene				

Modified from Table 1 and the text in Richards et al. Genet Med 2015: 17: 405-424.

Table 3: ACMG Classification of pathogenic and benign variants

PATHOGENIC		Applicability to Alport syndrome
Very strong evidence of pathogenicity		
PVS1	Null variant: nonsense, frameshift, canonical +/-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	Glycine residues are critical; few other hotspots
PM2	Variant absent from controls in Exome sequencing Project (ESP), 1000 genomes or ExAC	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 in-frame 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 AS
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 or ExAC	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	Hematuria 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	In frame-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

aa – amino acid
Modified from Tables 3 and 4, Richards S et al, 2015 [63]

Table 4: Potential modifiers in Alport syndrome

Gene	Protein	Function	Disease
NPHS2	Podocin	Podocyte foot process protein	FSGS (p.Arg229Gln) (R229Q), (E237Q, Glu237Gln)[75])
LAMA5	Laminin chain	GBM constituent	Proteinuria
LAMB2	Laminin chain	GBM constituent	Proteinuria
APO1E	Apolipoprotein 1E	Mediates binding, internalisation and catabolism of lipoproteins	FSGS, lipoprotein glomerulopathy
CFHR5	Complement factor H-related 5	A regulator of complement activation	HUS, DDD
ACTN4	α actinin 4	Cytoskeletal function	FSGS
PODXL	Podocalyxin-like	Found in podocytes, involved in cell adhesion and migration	Glomerulonephritis
WT1 (also known as NPHS4)	Wilm's tumour protein 1	Involved in normal development of the urogenital system	FSGS, Denys-Drash syndrome
TRPC6	Transient receptor potential cation channel subfamily C	Receptor activated Ca channel in podocyte cell membrane	FSGS
CD2AP	CD2-associated protein	Scaffolding molecular that regulates the actin cytoskeleton	FSGS
INF2	Inverted formin 2	Polymerisation and depolymerisation of actin filaments	FSGS

See text for references

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

Gene	Disease	Inheritance	Distinguishing features
NPHS2	FSGS	AD or AR with another podocyte mutation	Proteinuria, nephrotic syndrome, renal failure
MYH9	Fechtner syndrome	AD	Hematuria, renal failure, neutrophil inclusions, large platelets but low platelet counts, hearing loss
CFH, CFHR5	DDD or HUS	AD	Hematuria, renal failure, possibly with retinal drusen, lipoatrophy or haemolysis
LMX1B	Nail-Patella syndrome	AD	Hematuria, proteinuria, renal failure, dystrophic nails, absent patellae
COL4A1, COL4A2	HANAC	AD	
Mitochondrial DNA	MELAS etc	Mitochondrial	Other cardiac and muscular weakness
FN1	Glomerulopathy with fibronectin deposits	AD	Proteinuria, nephrotic syndrome, hypertension, renal failure, disease recurrence post-transplant

See text for references

REFERENCES

1. Gubler M, Levy M, Broyer M, Naizot C, Gonzales G, Perrin D, Habib R (1981) Alport's syndrome. A report of 58 cases and a review of the literature. Am J Med 70:493-505.
2. Habib R, Gubler MC, Hinglais N, Noel LH, Droz D, Levy M, Mahieu P, Foidart JM, Perrin D, Bois E, Grunfeld JP (1982) Alport's syndrome: experience at Hopital Necker. Kidney Int Suppl 11:S20-28.
3. Grunfeld JP, Joly D (1997) [Hereditary kidney diseases in adults]. Rev Prat 47:1566-1569.
4. Barker DF, Hostikka SL, Zhou J, Chow LT, Oliphant AR, Gerken SC, Gregory MC, Skolnick MH, Atkin CL, Tryggvason K (1990) Identification of mutations in the COL4A5 collagen gene in Alport syndrome. Science 248:1224-1227.
5. Mochizuki T, Lemmink HH, Mariyama M, Antignac C, Gubler MC, Pirson Y, Verellen-Dumoulin C, Chan B, Schroder CH, Smeets HJ, et al. (1994) Identification of mutations in the alpha 3(IV) and alpha 4(IV) collagen genes in autosomal recessive Alport syndrome. Nat Genet 8:77-81.
6. Feingold J, Bois E, Chompret A, Broyer M, Gubler MC, Grunfeld JP (1985) Genetic heterogeneity of Alport syndrome. Kidney Int 27:672-677.
7. Kashtan CE, Ding J, Garosi G, Heidet L, Massella L, Nakanishi K, Nozu K, Renieri A, Rheault M, Wang F, Gross O (2018) Alport syndrome: a unified classification of genetic disorders of collagen IV alpha345: a position paper of the Alport Syndrome Classification Working Group. Kidney Int 93:1045-1051.
8. Savige J, Gregory M, Gross O, Kashtan C, Ding J, Flinter F (2013) Expert guidelines for the management of Alport syndrome and thin basement membrane nephropathy. J Am Soc Nephrol 24:364-375.
9. Jais JP, Knebelmann B, Giatras I, De Marchi M, Rizzoni G, Renieri A, Weber M, Gross O, Netzer KO, Flinter F, Pirson Y, Dahan K, Wieslander J, Persson U, Tryggvason K, Martin P, Hertz JM, Schroder C, Sanak M, Carvalho MF, Saus J, Antignac C, Smeets H, Gubler MC (2003) X-linked Alport syndrome: natural history and genotype-phenotype correlations in girls and women belonging to 195 families: a "European Community Alport Syndrome Concerted Action" study. J Am Soc Nephrol 14:2603-2610.
10. Jais JP, Knebelmann B, Giatras I, De Marchi M, Rizzoni G, Renieri A, Weber M, Gross O, Netzer KO, Flinter F, Pirson Y, Verellen C, Wieslander J, Persson U, Tryggvason K, Martin P, Hertz JM, Schroder C, Sanak M, Krejcova S, Carvalho MF, Saus J, Antignac C, Smeets H, Gubler MC (2000) X-linked Alport syndrome: natural history in 195 families and genotype-phenotype correlations in males. J Am Soc Nephrol 11:649-657.
11. Gross O, Netzer KO, Lambrecht R, Seibold S, Weber M (2002) Meta-analysis of genotype-phenotype correlation in X-linked Alport syndrome: impact on clinical counselling. Nephrol Dial Transplant 17:1218-1227.
12. Savige J, Storey H, Il Cheong H, Gyung Kang H, Park E, Hilbert P, Persikov A, Torres-Fernandez C, Ars E, Torra R, Hertz JM, Thomassen M, Shagam L, Wang D, Wang Y, Flinter F, Nagel M (2016) X-Linked and Autosomal Recessive Alport Syndrome: Pathogenic Variant Features and Further Genotype-Phenotype Correlations. PLoS One 11:e0161802.
13. Zhou J, Mochizuki T, Smeets H, Antignac C, Laurila P, de Paepe A, Tryggvason K, Reeders ST (1993) Deletion of the paired alpha 5(IV) and alpha 6(IV) collagen genes in inherited smooth muscle tumors. Science 261:1167-1169.
14. Kashtan CE, Butkowski RJ, Kleppel MM, First MR, Michael AF (1990) Posttransplant anti-glomerular basement membrane nephritis in related males with Alport syndrome. J Lab Clin Med 116:508-515.
15. Ding J, Zhou J, Tryggvason K, Kashtan CE (1994) COL4A5 deletions in three patients with Alport syndrome and posttransplant antiglomerular basement membrane nephritis. J Am Soc Nephrol 5:161-168.

16. Deltas C, Pierides A, Voskarides K (2013) Molecular genetics of familial hematuric diseases. Nephrol Dial Transplant 28:2946-2960.

17. Fallerini C, Dosa L, Tita R, Del Prete D, Feriozzi S, Gai G, Clementi M, La Manna A, Miglietti N, Mancini R, Mandrile G, Ghiggeri GM, Piaggio G, Brancati F, Diano L, Frate E, Pinciaroli AR, Giani M, Castorina P, Bresin E, Giachino D, De Marchi M, Mari F, Bruttini M, Renieri A, Ariani F (2014) Unbiased next generation sequencing analysis confirms the existence of autosomal dominant Alport syndrome in a relevant fraction of cases. Clin Genet 86:252-257.

18. Fallerini C, Baldassarri M, Trevisson E, Morbidoni V, La Manna A, Lazzarin R, Pasini A, Barbano G, Pinciaroli AR, Garosi G, Frullanti E, Pinto AM, Mencarelli MA, Mari F, Renieri A, Ariani F (2016) Alport syndrome: impact of digenic inheritance in patients management. Clin Genet.

19. Mencarelli MA, Heidet L, Storey H, van Geel M, Knebelmann B, Fallerini C, Miglietti N, Antonucci MF, Cetta F, Sayer JA, van den Wijngaard A, Yau S, Mari F, Bruttini M, Ariani F, Dahan K, Smeets B, Antignac C, Flinter F, Renieri A (2015) Evidence of digenic inheritance in Alport syndrome. J Med Genet 52:163-174.

20. Voskarides K, Stefanou C, Pieri M, Demosthenous P, Felekkis K, Arsali M, Athanasiou Y, Xydakis D, Stylianou K, Daphnis E, Goulielmos G, Loizou P, Savige J, Hohne M, Volker LA, Benzing T, Maxwell PH, Gale DP, Gorski M, Boger C, Kollerits B, Kronenberg F, Paulweber B, Zavros M, Pierides A, Deltas C (2017) A functional variant in NEPH3 gene confers high risk of renal failure in primary hematuric glomerulopathies. Evidence for predisposition to microalbuminuria in the general population. PLoS One 12:e0174274.

21. Savige J, Colville D, Rheault M, Gear S, Lennon R, Lagas S, Finlay M, Flinter F (2016) Alport Syndrome in Women and Girls. Clin J Am Soc Nephrol 11:1713-1720.

22. Wang YY, Rana K, Tonna S, Lin T, Sin L, Savige J (2004) COL4A3 mutations and their clinical consequences in thin basement membrane nephropathy (TBMN). Kidney Int 65:786-790.

23. Hanson H, Storey H, Pagan J, Flinter F (2011) The value of clinical criteria in identifying patients with X-linked Alport syndrome. Clin J Am Soc Nephrol 6:198-203.

24. Gast C, Pengelly RJ, Lyon M, Bunyan DJ, Seaby EG, Graham N, Venkat-Raman G, Ennis S (2016) Collagen (COL4A) mutations are the most frequent mutations underlying adult focal segmental glomerulosclerosis. Nephrol Dial Transplant 31:961-970.

25. Voskarides K, Damianou L, Neocleous V, Zouvani I, Christodoulidou S, Hadjiconstantinou V, Ioannou K, Athanasiou Y, Patsias C, Alexopoulos E, Pierides A, Kyriacou K, Deltas C (2007) COL4A3/COL4A4 mutations producing focal segmental glomerulosclerosis and renal failure in thin basement membrane nephropathy. J Am Soc Nephrol 18:3004-3016.

26. Savige J, Rana K, Tonna S, Buzza M, Dagher H, Wang YY (2003) Thin basement membrane nephropathy. Kidney Int 64:1169-1178.

27. Usui T, Ichibe M, Hasegawa S, Miki A, Baba E, Tanimoto N, Abe H (2004) Symmetrical reduced retinal thickness in a patient with Alport syndrome. Retina 24:977-979.

28. Savige J, Sheth S, Leys A, Nicholson A, Mack HG, Colville D (2015) Ocular features in Alport syndrome: pathogenesis and clinical significance. Clin J Am Soc Nephrol 10:703-709.

29. Ahmed F, Kamae KK, Jones DJ, Deangelis MM, Hageman GS, Gregory MC, Bernstein PS (2013) Temporal macular thinning associated with X-linked Alport syndrome. JAMA Ophthalmol 131:777-782.

30. Kashtan CE (1999) Alport syndrome: is diagnosis only skin-deep? Kidney Int 55:1575-1576.

31. Yoshioka K, Hino S, Takemura T, Maki S, Wieslander J, Takekoshi Y, Makino H, Kagawa M, Sado Y, Kashtan CE (1994) Type IV collagen alpha 5 chain. Normal distribution and abnormalities in X-linked Alport syndrome revealed by monoclonal antibody. Am J Pathol 144:986-996.

32. Tan R, Colville D, Wang YY, Rigby L, Savige J (2010) Alport retinopathy results from "severe" COL4A5 mutations and predicts early renal failure. Clin J Am Soc Nephrol 5:34-38.

33. Persikov AV, Pillitteri RJ, Amin P, Schwarze U, Byers PH, Brodsky B (2004) Stability related bias in residues replacing glycines within the collagen triple helix (Gly-Xaa-Yaa) in inherited connective tissue disorders. Hum Mutat 24:330-337.
34. Rheault MN, Kren SM, Hartich LA, Wall M, Thomas W, Mesa HA, Avner P, Lees GE, Kashtan CE, Segal Y (2010) X-inactivation modifies disease severity in female carriers of murine X-linked Alport syndrome. Nephrol Dial Transplant 25:764-769.
35. Storey H, Savige J, Sivakumar V, Abbs S, Flinter FA (2013) COL4A3/COL4A4 mutations and features in individuals with autosomal recessive Alport syndrome. J Am Soc Nephrol 24:1945-1954.
36. Murray LS, Lu Y, Taggart A, Van Regemorter N, Vilain C, Abramowicz M, Kadler KE, Van Agtmael T (2014) Chemical chaperone treatment reduces intracellular accumulation of mutant collagen IV and ameliorates the cellular phenotype of a COL4A2 mutation that causes haemorrhagic stroke. Hum Mol Genet 23:283-292.
37. Bateman JF, Freddi S, Nattrass G, Savarirayan R (2003) Tissue-specific RNA surveillance? Nonsense-mediated mRNA decay causes collagen X haploinsufficiency in Schmid metaphyseal chondrodysplasia cartilage. Hum Mol Genet 12:217-225.
38. Barker DF, Pruchno CJ, Jiang X, Atkin CL, Stone EM, Denison JC, Fain PR, Gregory MC (1996) A mutation causing Alport syndrome with tardive hearing loss is common in the western United States. Am J Hum Genet 58:1157-1165.
39. Voskarides K, Patsias C, Pierides A, Deltas C (2008) COL4A3 founder mutations in Greek-Cypriot families with thin basement membrane nephropathy and focal segmental glomerulosclerosis dating from around 18th century. Genet Test 12:273-278.
40. Slajpah M, Gorinsek B, Berginc G, Vizjak A, Ferluga D, Hvala A, Meglic A, Jaksa I, Furlan P, Gregoric A, Kaplan-Pavlovcic S, Ravnik-Glavac M, Glavac D (2007) Sixteen novel mutations identified in COL4A3, COL4A4, and COL4A5 genes in Slovenian families with Alport syndrome and benign familial hematuria. Kidney Int 71:1287-1295.
41. Miner JH, Baigent C, Flinter F, Gross O, Judge P, Kashtan CE, Lagas S, Savige J, Blatt D, Ding J, Gale DP, Midgley JP, Povey S, Prunotto M, Renault D, Skelding J, Turner AN, Gear S (2014) The 2014International Workshop on Alport Syndrome. Kidney Int 86:679-684.
42. Moriniere V, Dahan K, Hilbert P, Lison M, Lebbah S, Topa A, Bole-Feysot C, Pruvost S, Nitschke P, Plaisier E, Knebelmann B, Macher MA, Noel LH, Gubler MC, Antignac C, Heidet L (2014) Improving mutation screening in familial hematuric nephropathies through next generation sequencing. J Am Soc Nephrol 25:2740-2751.
43. Hertz JM, Thomassen M, Storey H, Flinter F (2015) Clinical utility gene card for: Alport syndrome - update 2014. Eur J Hum Genet 23.
44. Baleato RM, Guthrie PL, Gubler MC, Ashman LK, Roselli S (2008) Deletion of CD151 results in a strain-dependent glomerular disease due to severe alterations of the glomerular basement membrane. Am J Pathol 173:927-937.
45. Heath KE, Campos-Barros A, Toren A, Rozenfeld-Granot G, Carlsson LE, Savige J, Denison JC, Gregory MC, White JG, Barker DF, Greinacher A, Epstein CJ, Glucksman MJ, Martignetti JA (2001) Nonmuscle myosin heavy chain IIA mutations define a spectrum of autosomal dominant macrothrombocytopenias: May-Hegglin anomaly and Fechtner, Sebastian, Epstein, and Alport-like syndromes. Am J Hum Genet 69:1033-1045.
46. Kovacs G, Kalmar T, Endreffy E, Ondrik Z, Ivanyi B, Rikker C, Haszon I, Turi S, Sinko M, Bereczki C, Maroti Z (2016) Efficient Targeted Next Generation Sequencing-Based Workflow for Differential Diagnosis of Alport-Related Disorders. PLoS One 11:e0149241.
47. Artuso R, Fallerini C, Dosa L, Scionti F, Clementi M, Garosi G, Massella L, Epistolato MC, Mancini R, Mari F, Longo I, Ariani F, Renieri A, Bruttini M (2012) Advances in Alport syndrome diagnosis using next-generation sequencing. Eur J Hum Genet 20:50-57.
48. King K, Flinter FA, Nihalani V, Green PM (2002) Unusual deep intronic mutations in the COL4A5 gene cause X linked Alport syndrome. Hum Genet 111:548-554.

49. Nakazato H, Hattori S, Ushijima T, Matsuura T, Koitabashi Y, Takada T, Yoshioka K, Endo F, Matsuda I (1994) Mutations in the COL4A5 gene in Alport syndrome: a possible mutation in primordial germ cells. Kidney Int 46:1307-1314.

50. Hertz JM, Thomassen M, Storey H, Flinter F (2012) Clinical utility gene card for: Alport syndrome. Eur J Hum Genet 20.

51. King K, Flinter FA, Green PM (2006) A two-tier approach to mutation detection in the COL4A5 gene for Alport syndrome. Hum Mutat 27:1061.

52. Tazon-Vega B, Ars E, Burset M, Santin S, Ruiz P, Fernandez-Llama P, Ballarin J, Torra R (2007) Genetic testing for X-linked Alport syndrome by direct sequencing of COL4A5 cDNA from hair root RNA samples. Am J Kidney Dis 50:257 e251-214.

53. Zhang H, Ding J, Wang F, Zhao D (2011) Mutation detection of COL4An gene based on mRNA of peripheral blood lymphocytes and prenatal diagnosis of Alport syndrome in China. Nephrology (Carlton) 16:377-380.

54. Bonnet C, Krieger S, Vezain M, Rousselin A, Tournier I, Martins A, Berthet P, Chevrier A, Dugast C, Layet V, Rossi A, Lidereau R, Frebourg T, Hardouin A, Tosi M (2008) Screening BRCA1 and BRCA2 unclassified variants for splicing mutations using reverse transcription PCR on patient RNA and an ex vivo assay based on a splicing reporter minigene. J Med Genet 45:438-446.

55. Sharma N, Sosnay PR, Ramalho AS, Douville C, Franca A, Gottschalk LB, Park J, Lee M, Vecchio-Pagan B, Raraigh KS, Amaral MD, Karchin R, Cutting GR (2014) Experimental assessment of splicing variants using expression minigenes and comparison with in silico predictions. Hum Mutat 35:1249-1259.

56. Tournier I, Vezain M, Martins A, Charbonnier F, Baert-Desurmont S, Olschwang S, Wang Q, Buisine MP, Soret J, Tazi J, Frebourg T, Tosi M (2008) A large fraction of unclassified variants of the mismatch repair genes MLH1 and MSH2 is associated with splicing defects. Hum Mutat 29:1412-1424.

57. Malone AF, Funk SD, Alhamad T, Miner JH (2017) Functional assessment of a novel COL4A5 splice region variant and immunostaining of plucked hair follicles as an alternative method of diagnosis in X-linked Alport syndrome. Pediatr Nephrol 32:997-1003.

58. Plant KE, Boye E, Green PM, Vetrie D, Flinter FA (2000) Somatic mosaicism associated with a mild Alport syndrome phenotype. J Med Genet 37:238-239.

59. Bruttini M, Vitelli F, Meloni I, Rizzari G, Volpe MD, Mazzucco G, Marchi MD, Renieri A (2000) Mosaicism in Alport syndrome with genetic counselling. J Med Genet 37:717-719.

60. Krol RP, Nozu K, Nakanishi K, Iijima K, Takeshima Y, Fu XJ, Nozu Y, Kaito H, Kanda K, Matsuo M, Yoshikawa N (2008) Somatic mosaicism for a mutation of the COL4A5 gene is a cause of mild phenotype male Alport syndrome. Nephrol Dial Transplant 23:2525-2530.

61. Beicht S, Strobl-Wildemann G, Rath S, Wachter O, Alberer M, Kaminsky E, Weber LT, Hinrichsen T, Klein HG, Hoefele J (2013) Next generation sequencing as a useful tool in the diagnostics of mosaicism in Alport syndrome. Gene 526:474-477.

62. Fu XJ, Nozu K, Kaito H, Ninchoji T, Morisada N, Nakanishi K, Yoshikawa N, Ohtsubo H, Matsunoshita N, Kamiyoshi N, Matsumura C, Takagi N, Maekawa K, Taniguchi-Ikeda M, Iijima K (2016) Somatic mosaicism and variant frequency detected by next-generation sequencing in X-linked Alport syndrome. Eur J Hum Genet 24:387-391.

63. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E, Voelkerding K, Rehm HL (2015) Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med 17:405-424.

64. . W (2013) Practice guidelines for the evaluation of pathogenicity and reporting of sequence variants in clinical molecular genetics, Association for Clinical Genetic Science, UK

65. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E, Voelkerding K, Rehm HL, Committee ALQA (2015) Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med 17:405-424.

66. MacArthur DG, Manolio TA, Dimmock DP, Rehm HL, Shendure J, Abecasis GR, Adams DR, Altman RB, Antonarakis SE, Ashley EA, Barrett JC, Biesecker LG, Conrad DF, Cooper GM, Cox NJ, Daly MJ, Gerstein MB, Goldstein DB, Hirschhorn JN, Leal SM, Pennacchio LA, Stamatoyannopoulos JA, Sunyaev SR, Valle D, Voight BF, Winckler W, Gunter C (2014) Guidelines for investigating causality of sequence variants in human disease. Nature 508:469-476.

67. Parkin JD, San Antonio JD, Pedchenko V, Hudson B, Jensen ST, Savige J (2011) Mapping structural landmarks, ligand binding sites, and missense mutations to the collagen IV heterotrimers predicts major functional domains, novel interactions, and variation in phenotypes in inherited diseases affecting basement membranes. Hum Mutat 32:127-143.

68. Malone AF, Funk SD, Alhamad T, Miner JH (2016) Functional assessment of a novel COL4A5 splice region variant and immunostaining of plucked hair follicles as an alternative method of diagnosis in X-linked Alport syndrome. Pediatr Nephrol.

69. Marcocci E, Uliana V, Bruttini M, Artuso R, Silengo MC, Zerial M, Bergesio F, Amoroso A, Savoldi S, Pennesi M, Giachino D, Rombola G, Fogazzi GB, Rosatelli C, Martinhago CD, Carmellini M, Mancini R, Di Costanzo G, Longo I, Renieri A, Mari F (2009) Autosomal dominant Alport syndrome: molecular analysis of the COL4A4 gene and clinical outcome. Nephrol Dial Transplant 24:1464-1471.

70. Rosado C, Bueno E, Felipe C, Valverde S, Gonzalez-Sarmiento R (2015) Study of the True Clinical Progression of Autosomal Dominant Alport Syndrome in a European Population. Kidney Blood Press Res 40:435-442.

71. Temme J, Peters F, Lange K, Pirson Y, Heidet L, Torra R, Grunfeld JP, Weber M, Licht C, Muller GA, Gross O (2012) Incidence of renal failure and nephroprotection by RAAS inhibition in heterozygous carriers of X-chromosomal and autosomal recessive Alport mutations. Kidney Int 81:779-783.

72. Stefanou C, Pieri M, Savva I, Georgiou G, Pierides A, Voskarides K, Deltas C (2015) Co-Inheritance of Functional Podocin Variants with Heterozygous Collagen IV Mutations Predisposes to Renal Failure. Nephron 130:200-212.

73. Mohammad M, Nanra R, Colville D, Trevillian P, Wang Y, Storey H, Flinter F, Savige J (2014) A female with X-linked Alport syndrome and compound heterozygous COL4A5 mutations. Pediatr Nephrol 29:481-485.

74. Lennon R, Stuart HM, Bierzynska A, Randles MJ, Kerr B, Hillman KA, Batra G, Campbell J, Storey H, Flinter FA, Koziell A, Welsh GI, Saleem MA, Webb NJ, Woolf AS (2015) Coinheritance of COL4A5 and MYO1E mutations accentuate the severity of kidney disease. Pediatr Nephrol 30:1459-1465.

75. Tonna S, Wang YY, Wilson D, Rigby L, Tabone T, Cotton R, Savige J (2008) The R229Q mutation in NPHS2 may predispose to proteinuria in thin-basement-membrane nephropathy. Pediatr Nephrol 23:2201-2207.

76. Bullich G, Trujillano D, Santin S, Ossowski S, Mendizabal S, Fraga G, Madrid A, Ariceta G, Ballarin J, Torra R, Estivill X, Ars E (2015) Targeted next-generation sequencing in steroid-resistant nephrotic syndrome: mutations in multiple glomerular genes may influence disease severity. Eur J Hum Genet 23:1192-1199.

77. Tsukaguchi H, Sudhakar A, Le TC, Nguyen T, Yao J, Schwimmer JA, Schachter AD, Poch E, Abreu PF, Appel GB, Pereira AB, Kalluri R, Pollak MR (2002) NPHS2 mutations in late-onset focal segmental glomerulosclerosis: R229Q is a common disease-associated allele. J Clin Invest 110:1659-1666.

78. Savige J, Amos L, Ierino F, Mack HG, Symons RC, Hughes P, Nicholls K, Colville D (2016) Retinal disease in the C3 glomerulopathies and the risk of impaired vision. Ophthalmic Genet 37:369-376.

79. Gale DP, de Jorge EG, Cook HT, Martinez-Barricarte R, Hadjisavvas A, McLean AG, Pusey CD, Pierides A, Kyriacou K, Athanasiou Y, Voskarides K, Deltas C, Palmer A, Fremeaux-Bacchi V, de Cordoba SR, Maxwell PH, Pickering MC (2010) Identification of a mutation in complement factor H-related protein 5 in patients of Cypriot origin with glomerulonephritis. Lancet 376:794-801.

80. Castelletti F, Donadelli R, Banterla F, Hildebrandt F, Zipfel PF, Bresin E, Otto E, Skerka C, Renieri A, Todeschini M, Caprioli J, Caruso RM, Artuso R, Remuzzi G, Noris M (2008) Mutations in FN1 cause glomerulopathy with fibronectin deposits. Proc Natl Acad Sci U S A 105:2538-2543.

81. Plaisier E, Gribouval O, Alamowitch S, Mougenot B, Prost C, Verpont MC, Marro B, Desmettre T, Cohen SY, Roullet E, Dracon M, Fardeau M, Van Agtmael T, Kerjaschki D, Antignac C, Ronco P (2007) COL4A1 mutations and hereditary angiopathy, nephropathy, aneurysms, and muscle cramps. N Engl J Med 357:2687-2695.

82. Scolari F, Amoroso A, Savoldi S, Mazzola G, Prati E, Valzorio B, Viola BF, Nicola B, Movilli E, Sandrini M, Campanini M, Maiorca R (1999) Familial clustering of IgA nephropathy: further evidence in an Italian population. Am J Kidney Dis 33:857-865.

83. Milillo A, La Carpia F, Costanzi S, D'Urbano V, Martini M, Lanuti P, Vischini G, Larocca LM, Marchisio M, Miscia S, Amoroso A, Gurrieri F, Sangiorgi E (2015) A SPRY2 mutation leading to MAPK/ERK pathway inhibition is associated with an autosomal dominant form of IgA nephropathy. Eur J Hum Genet 23:1673-1678.

84. Heidet L, Bongers EM, Sich M, Zhang SY, Loirat C, Meyrier A, Broyer M, Landthaler G, Faller B, Sado Y, Knoers NV, Gubler MC (2003) In vivo expression of putative LMX1B targets in nail-patella syndrome kidneys. Am J Pathol 163:145-155.

85. Morello R, Zhou G, Dreyer SD, Harvey SJ, Ninomiya Y, Thorner PS, Miner JH, Cole W, Winterpacht A, Zabel B, Oberg KC, Lee B (2001) Regulation of glomerular basement membrane collagen expression by LMX1B contributes to renal disease in nail patella syndrome. Nat Genet 27:205-208.

86. Savige J, Dalgleish R, Cotton RG, den Dunnen JT, Macrae F, Povey S (2015) The Human Variome Project: ensuring the quality of DNA variant databases in inherited renal disease. Pediatr Nephrol 30:1893-1901.