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Case Report

Trigenic *COL4A3*/*COL4A4*/*COL4A5* pathogenic variants in Alport syndrome: a case report

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Short Title: Trigenic Alport syndrome

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Abstract
Alport syndrome (AS) is a hereditary kidney disorder of type IV collagen caused by pathogenic variants in the *COL4A3*, *COL4A4* and *COL4A5* genes. Previously several cases of digenic AS, caused by two pathogenic variants in two of the three *COL4A* genes, have been reported. Patients with digenic AS may present with a more severe phenotype compared to patients with single variants, depending on the percentage affected type IV trimeric collagen chain. We report a newly discovered case of trigenic AS.
A 52-year-old female presented with hematuria at the age of 24 years and developed hypertension by the age of 30. Over the years she developed chronic kidney disease; the most recent eGFR was 44ml/min/1.73m^2. She has symmetric high-tone sensorineural hearing loss. Full genetic analysis revealed a heterozygous pathogenic variant c.2691del in *COL4A3,* a heterozygous pathogenic variant c.1663dup in *COL4A4,* and a complete heterozygous deletion of *COL4A5*.
We describe the first patient with AS caused by pathogenic variants in all three *COL4A* genes, designated trigenic AS. This case report emphasizes the importance of examining all three *COL4A* genes, even in patients with a mild Alport phenotype, for optimal follow-up of the patient and adequate genetic counseling of family members.

Introduction

Alport syndrome (AS) is an inherited disorder of type IV collagen expressed in the basement membranes of kidney glomeruli, eyes and cochleae. AS is caused by pathogenic variants in the *COL4A* genes: *COL4A3*, *COL4A4*, and *COL4A5*. Variants in the *COL4A5* gene, located on the X-chromosome, cause X-linked Alport syndrome (XLAS). Variants in *COL4A3* and *COL4A4*, located head-to-head on chromosome 2, cause autosomal recessive or dominant AS [1, 2].

Conventionally, genetic analysis in patients with AS was performed using Sanger sequencing, which was time-consuming given the extensive size of the genes (*COL4A3* 52 exons, *COL4A4* 48 exons, and *COL4A5* 53 exons, respectively). Furthermore, a sequential gene analysis was terminated when a pathogenic variant was detected. Nowadays, Next-Generation Sequencing (NGS) is the most efficient method for analyzing the *COL4A* genes collectively, detecting all variants present. Since NGS may fail to detect exon deletions and duplications, additional multiplex ligation-dependent probe amplification (MLPA) is performed [3]. Implementation of NGS resulted in the discovery of digenic AS: pathogenic variants in two of the *COL4A* genes.

In digenic AS all possible combinations have been described and the phenotype is more severe compared to patients with one heterozygous variant in *COL4A3* or *COL4A4* [4-6]. When variants are configured in cis (i.e., located on the same allele), this mimics autosomal dominant inheritance, however with a more severe phenotype. Two variants in trans (i.e., located on different alleles) mimic autosomal recessive inheritance, though with a less severe phenotype for the offspring [4-7]. Based on the population frequency of *COL4A* pathogenic variants, we expect more patients to be identified with digenic AS in the future using the current genetic analyses [8].

We present a case in which genetic analyses revealed pathogenic variants in all three *COL4A* genes. This case emphasizes the current guideline advice to analyze all three *COL4A* genes in patients suspected with Alport syndrome [7].

Case Report/Case Presentation

A 52-year-old female (shown in Fig. 1; III:2 and referred to as index case) was referred for evaluation of her kidney disease. She was diagnosed with hematuria at age 24 and developed hypertension by the age of 30. During pregnancy (at 35 years of age) she was diagnosed with pre-eclampsia and nephrotic syndrome with severe proteinuria (up to 17 grams/day) for over 6 weeks. Post pregnancy the proteinuria decreased to levels between 0.5 – 1.5 g/day with ACE inhibitor treatment.

The index case developed postpartum cardiomyopathy (with left ventricle dilatation), which recovered over the years. She underwent a gastric bypass at the age of 43 to treat obesity. She suffers from high-tone sensorineural hearing loss and has been wearing hearing aids since the age of 36.

She experienced rapid loss of kidney function in the period between 2006 and 2013 as shown in Supplemental File 1. However, the rate of kidney function decline improved after recovery from other medical conditions (obesity, pre-eclampsia, and cardiomyopathy). Currently, chronic kidney disease (CKD) is classified as stage G3bA2 with an eGFR of 44ml/min/1.73m^2 and an albumin/creatinine ratio of 46 mg/g with ACE inhibitor therapy.

X-linked Alport syndrome was suspected based on the disease symptoms (Table 1), and genetic analysis was performed.

Genetic analysis

Analysis of index case III:2's DNA with NGS enhanced with single molecule Molecular Inversion Probes (smMIP), revealed two heterozygous pathogenic variants: c.2691del in *COL4A3* and c.1663dup in *COL4A4*. These variants both predict a frameshift, possibly resulting in truncated *COL4A3* (p.(Gly898Glufs*26)) and *COL4A4* proteins (p.(Ala555Glyfs*4)) respectively. Both variants have not been described in the literature or gnomAD database. In the Dutch Leiden Open Variation Database (LOVD) the *COL4A3* variant was submitted once (by our center) and the *COL4A4* variant was not submitted before [10]. Furthermore, CNV analysis by smMIPs suggested a heterozygous deletion of the entire *COL4A5* gene expanding to at least exons 1 to 4 of *COL4A6*, which was confirmed by MLPA. A further description of the methods is given in Supplemental File 2.

Family history and segregation analysis (shown in Fig. 1: family pedigree)

The father (II:1) died suddenly at the age of 48 years, probably due to a heart attack. The father was not known with hypertension or a kidney disorder and did not suffer from hearing loss.
The 75-year-old mother (II:2) has hypertension, hematuria, and microalbuminuria. She has been treated with RAAS blockade since the age of 65. Her kidney function has remained stable for the past 10 years (CKD stage G2A1), the most recent eGFR is 84 ml/min/1.73m^2. Genetic analysis only revealed the heterozygous pathogenic variant c.1663dup in the *COL4A4* gene.
The sister (III:4) does not have any signs of a kidney disorder or hearing disorder. She denied any (genetic) evaluation.
The son of the index case (IV:1) was referred to the pediatrician for evaluation of hypertension at the age of 10. His BMI was 15.8 kg per m^2 and the hypertension was attributed to the use of methylphenidate [9]. Remarkably there was variable proteinuria (varying from absent up to 2067 mg/g creatinine) with normal serum albumin and minimal microscopic hematuria (maximum of 3-10 erythrocytes per field of view). His kidney function was normal (eGFR 120 ml/min/1.73m^2). After initiation of ACE inhibitor treatment, there was no persistent hematuria or proteinuria currently at the age of 16. This suggests a mild phenotype, with temporary pressure-dependent proteinuria during intercurrent events such as infections. Genetic analysis revealed the heterozygous pathogenic variant c.2691del in the *COL4A3* gene.

Discussion/Conclusion

We describe a female patient with Alport syndrome and pathogenic variants in all three *COL4A3*, *COL4A4*, and *COL4A5* genes. We designated this to be trigenic Alport syndrome. Based on the population frequency of a digenic predicted pathogenic *COL4A3* plus a *COL4A4* variant of 1:44,793 and 1:245,920 for combinations of *COL4A5* plus *COL4A3* or *COL4A4*, trigenic AS must be extremely rare [8].
We were unable to investigate the inheritance of the three respective variants in the index case (III:2) in full detail. Because we only detected the *COL4A3* variant in the son (IV:1), the *COL4A3* and *COL4A4* variants are inherited in *trans*. The *COL4A4* variant is maternal (II:2); the *COL4A3* variant is likely paternal (II:1). However, since the father (II:1) passed away at an early age this could not be genetically confirmed. He was not reported as hypertensive or having hematuria, proteinuria or kidney failure or deafness at the time of death, suggesting the *COL4A5* deletion in the index patient is likely *de novo*. Yet, there is also a slight possibility of paternal (or maternal) postzygotic gonadal mosaicism of the *COL4A5* deletion.
Deletions of *COL4A5* extending to *COL4A6* are associated with diffuse leiomyomatosis-Alport syndrome (DL-AS). The deletion detected in the index case extends beyond exon 4 of *COL4A6* and she does not have symptoms of DL-AS. This is in line with previously reported cases showing deletions of exon 1 and 2 of the *COL4A6* gene cause DL-AS, while further expanding deletions do not lead to DL-AS. The exact pathogenesis of this phenomenon is unclear [11, 12].
In our index patient, the kidney function and proteinuria recovered over time and the decline in kidney function has slowed down ever since (now CKD stage G3bA2 at 52 years of age). This illustrates in our view the contribution of lifestyle factors to chronic kidney disease. The renal phenotype of AS appears relatively mild. Longer follow-up of this index case is needed to determine if kidney function will deteriorate to end-stage kidney disease (ESKD) in the future.
We can speculate on the possible genotype-phenotype correlation for all three pathogenic variants. Heterogenous phenotype has been described in females with heterozygous *COL4A5* variants, heterozygous *COL4A3* or *COL4A4* variants, and in digenic AS. Disease severity spans the entire spectrum between normal phenotype and progression to ESKD [13, 14]. Asymptomatic cases are reported in 2.5% of females with XLAS [13] and 5.2% of individuals with heterozygous *COL4A3* or *COL4A4* variants[14]. In the population-based Geisinger MyCode/DiscovEHR study the percentage of asymptomatic individuals was even higher; 36% for pathogenic variants in *COL4A3* [15].
In digenic AS including a *COL4A5* variant, although all women had hematuria, proteinuria was absent in 38% of them. Similarly, proteinuria was absent in 34% of cases of digenic AS with *COL4A3* and *COL4A4* variants [6]. The reported cases of digenic AS indicate that the phenotype is, in general, more severe compared to individuals with a single variant [6, 14]. Thus, we would expect the

phenotype in trigenic AS to be even more severe than in digenic AS. However, the phenotype of our case matches the spectrum of phenotypes described for digenic AS (due to variants in *COL4A3* and *COL4A4*), except our case presented with proteinuria at an earlier age than described in the literature for digenic AS due to variants in *COL4A3* and *COL4A4* and later than the median age described for digenic AS including a variant in *COL4A5* [6].

We suggest several explanations for this mild phenotype. Firstly, we hypothesize that heterozygosity of respective *COL4A3, COL4A4* and *COL4A5* variants explains the relatively mild phenotype [6]. Based on the calculated prediction and considering a 50:50 X-inactivation, we would expect 87.5% of triple helix collagen molecules to be defective. However, the contribution of the deletion of *COL4A5* may be limited due to possible skewing of X-inactivation [16]. The percentage of residual functional triple helix collagen may therefore be > 12.5%. The contribution of the *COL4A5* variant is also potentially weak for the extra-renal phenotype: hearing loss is also described in patients with digenic AS (due to variants in *COL4A3* and *COL4A4*) and also, but less frequent, in patients with heterozygous *COL4A3* or *COL4A4* variants [6, 14].

Also, the type of mutations could contribute to a more favorable phenotype. The heterozygous complete deletion of the *COL4A5* gene results either in normal expression of the α5(IV) chain or in absent expression in cells. Jais et al described a large group of women with XLAS, including 12 families with large deletions of *COL4A5* involving the *COL4A6* gene, and 10 patients exhibiting diffuse leiomyomatosis-AS. Three of these individuals had normal urinalysis [13]. The *COL4A3* and *COL4A4* pathogenic variants in our index patient were both probable protein-truncating variants. In the Geisinger MyCode/DiscovEHR study, only 52% of the patients with a protein-truncating variant in *COL4A3* had any phenotypic feature, showing that protein-truncating variants are associated with a milder phenotype compared to Glycine substitutions. The absence of disease symptoms in patients with protein-truncating mutations may be explained by production of functional collagen heteromers by the non-truncated allele [15].

In the literature genetic modifiers in AS have been described [7]. Our patient also illustrates the role of lifestyle factors, since proteinuria was reduced with treatment of blood pressure using ACE inhibitors, and weight reduction after bariatric surgery for obesity. The history of the son illustrates that hypertension and infections could have a modifying effect. This suggests that patients with collagenopathies could have a mild phenotype, but show increased vulnerability of the glomerular basement membrane (GBM) to intercurrent events, dependent on the percentage of residual functional triple helix collagen.

Although trigenic pathogenic variants have been described before in a family with hypertrophic cardiomyopathy, we describe the first case of AS in the literature with trigenic pathogenic variants [17]. There is an earlier report by Zhang et al. describing an individual with three variants [18]. However, the described variant in *COL4A3* should be interpreted as a Variant of Unknown Significance (*VUS)* based on amino acid conservation between species and paralogous type IV collagen NC1 domains [19], allele frequency in the population (dbSNP, gnomAD) and the LOVD, so this case does not meet the criteria for trigenic AS.

Our findings are relevant for nephrologists, clinical geneticists, and patients with AS to be aware of the possibility of trigenic variants. It emphasizes the importance of examining all three *COL4A* genes in patients with suspected AS, even when a familial pathogenic variant is identified before. It is also important to identify the nature of the variants, e.g., inheritance in *cis* or *trans*, as this helps determine the risk of inheritance and prognosis for family members [5, 6].

In conclusion, this case report expands knowledge of the genetic diagnosis of AS, revealing the possibility of trigenic pathogenic variants. It is crucial to examine all three *COL4A* genes, even in patients with a relatively mild Alport phenotype, for optimal follow-up of the patient and counseling of family members.

Statements

Statement of Ethics

In accordance with Dutch law, this Case Report did not require formal approval by the medical ethics committee. This was confirmed by our hospital's human research committee.

The authors declare that they have obtained written consent from the patients reported in this Case Report. Consent was obtained from the participants for publication of the details of their medical case that appears within this Case Report and associated supplementary material.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Data collection: Dipti Rao, Marlies Cornelissen and Michel van Geel. Genetic analysis and interpretation: Michel van Geel. Clinical data interpretation: Dipti Rao, Rutger Maas, Marlies Cornelissen and Jack Wetzels. Drafting the article: Dipti Rao. Review and revisions: Dipti Rao, Rutger Maas, Marlies Cornelissen, Jack Wetzels and Michel van Geel. Reading and approving the final manuscript: Dipti Rao, Rutger Maas, Marlies Cornelissen, Jack Wetzels and Michel van Geel.

Data Availability Statement

The data used to support the findings of this case report are available from the corresponding author upon reasonable request.

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Figures

Figure 1:

Title: Family pedigree

Subtitle/explanatory text: Family members with identified variants and their clinical phenotype

Legend: Abbreviations*: del: deletion; dup: duplication; n: normal/no pathogenic variant detected; ?: individuals not genetically tested.*

The arrow indicates the index case.

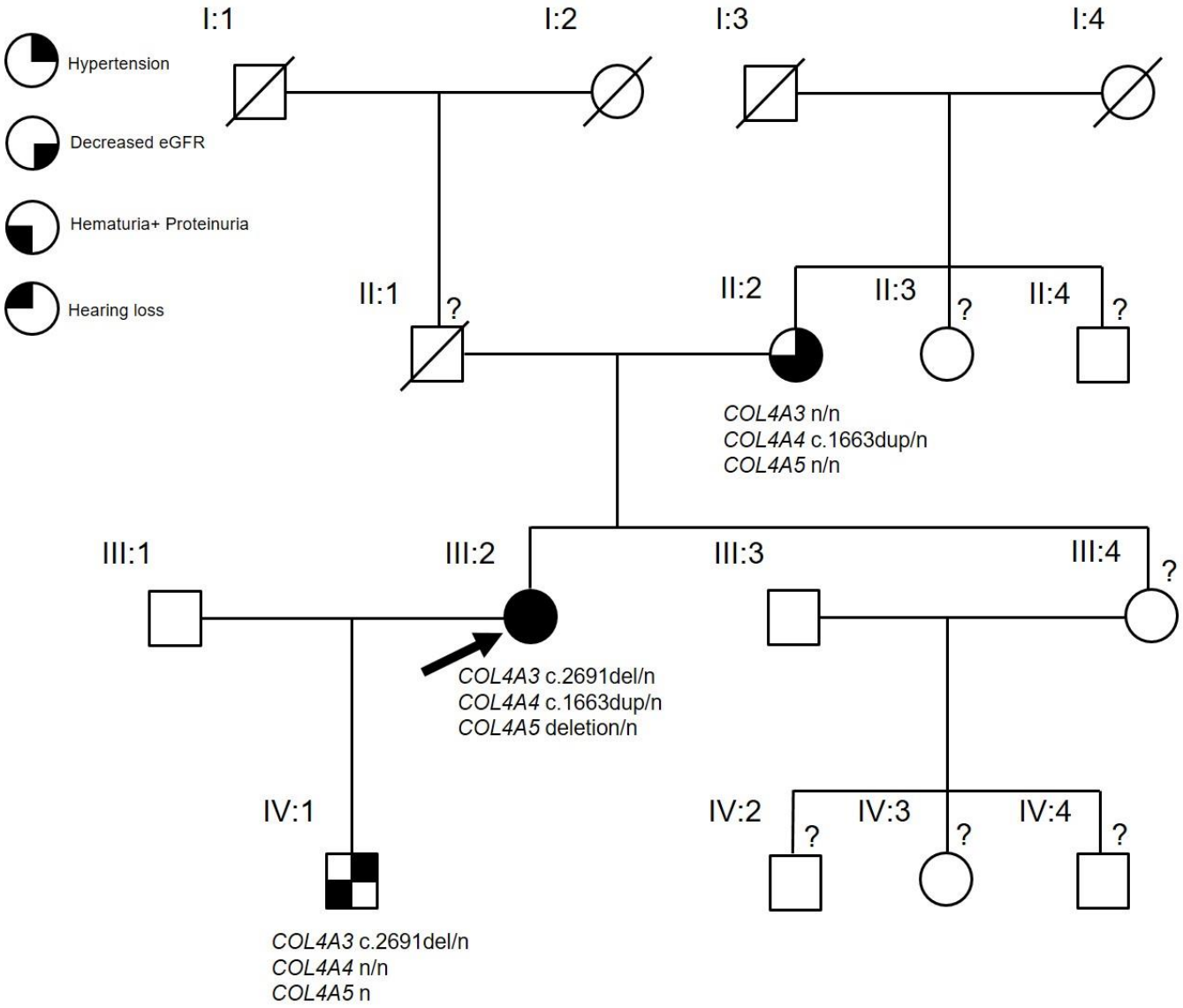

Table 1. Clinical characteristics of patients

			First presentation		Most recent follow-up					Extrarenal features		Genetics				
Patient	**Age (yrs)**	**Sex**	**Age (yrs)**	**Presenting symptoms**	**Creatinine (mg/dL)**	**eGFR[a] (ml/min/ 1.73m^2)**	**Hema-turia**	**Protei-nuria**	**Medication**	**Hearing loss**	**Ocular lesions**	**Gene**	**Exon**	**Nucleotide change**	**Amino acid change**	**Mutation type**
III:2 (index case)	52	F	24	Hematuria	1.40	44	Yes	ACR 46 mg/g	Lisinopril 1dd10mg	Yes (36 yr)	No	*COL4A3* *COL4A4* *COL4A5*	17 23 1-53[b]	c.2691del c.1663dup c.(?_-1)_(*1_?)del	p.(Gly898Glufs*26) p.(Ala555Glyfs*4) p.0	Frameshift Frameshift Deletion
IV:1	16	M	10	Hypertension	0.85	>90	No	PCR 71 mg/g	Enalapril 2dd5mg	No	NA	*COL4A3*	17	c.2691del	p. (Gly898Glufs*26)	Frameshift
II:2	75	F	63	Hypertension hematuria, proteinuria	0.71	84	Yes	ACR 25.7 mg/g	Losartan 1dd100mg	No	NA	*COL4A4*	23	c.1663dup	p. (Ala555Glyfs*4)	Frameshift
II:1	†	M	No renal phenotype, no hearing loss. Deceased at 48 yr (cardiac event)													
III:4	50	F	No renal phenotype, no hearing loss. Denied (genetic) evaluation													

[a] eGFR was estimated from CKD-EPI for age >18 years old and Bedside Schwartz formula for age <18 years old.

[b] including at least exon 1-4 of *COL4A6*

Note: Conversion factors for units: creatinine mg/dL to µmol/L, ×88.4; Albumin/creatinine ratio mg/g to mg/mmol, x 0.113; Protein/creatinine ratio in mg/g to mg/mmol, x 0.113.

Abbreviations*: yrs: years; F: female; M: male; ACR: albumin/creatinine ratio; PCR: protein/creatinine ratio; NA: ocular examination not available*