TYPE IV COLLAGEN NEPHROPATHIES: ALPORT SYNDROME AND BEYOND

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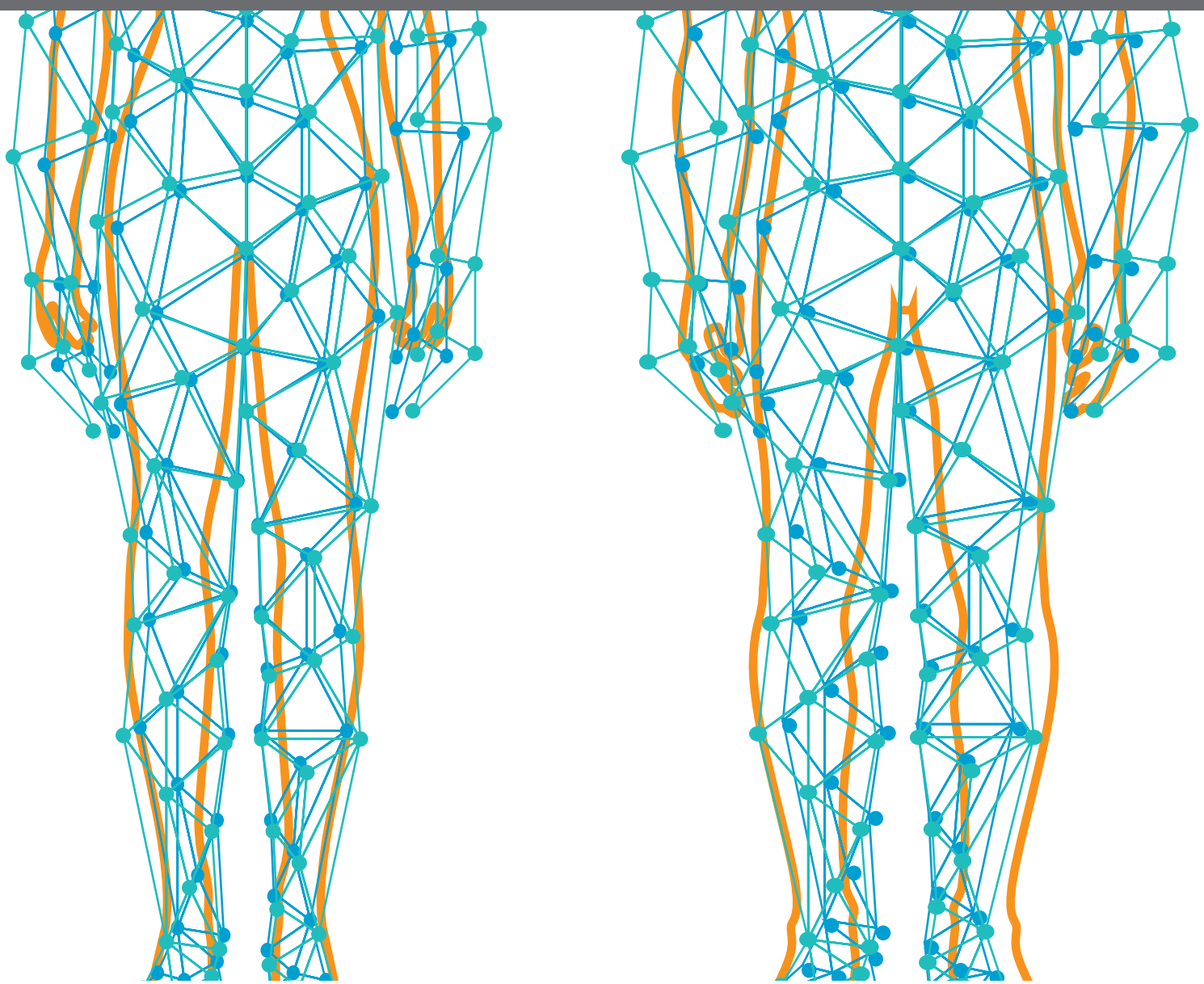
PUBLISHED IN: Frontiers in Medicine



frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-83250-503-8
DOI 10.3389/978-2-83250-503-8

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TYPE IV COLLAGEN NEPHROPATHIES: ALPORT SYNDROME AND BEYOND

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Citation: Borza, D.-B., Gross, O., Abrahamson, D., Savige, J., eds. (2022). Type IV Collagen Nephropathies: Alport Syndrome and Beyond. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-83250-503-8

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frontiers | Frontiers in Medicine

TYPE Editorial
PUBLISHED 30 September 2022
DOI 10.3389/fmed.2022.1039949

OPEN ACCESS

EDITED AND REVIEWED BY
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SPECIALTY SECTION
This article was submitted to Nephrology, a section of the journal Frontiers in Medicine

RECEIVED 08 September 2022
ACCEPTED 20 September 2022
PUBLISHED 30 September 2022

CITATION
Borza D-B, Abrahamson DR, Gross O and Savige J (2022) Editorial: Collagen IV nephropathies: Alport syndrome and beyond. *Front. Med.* 9:1039949. doi: 10.3389/fmed.2022.1039949

Editorial: Collagen IV nephropathies: Alport syndrome and beyond

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KEYWORDS

glomerular basement membrane (GBM), collagen type IV, thin glomerular basement membrane disease, collagen IV nephropathies, COL4A3 mutation, COL4A4 mutations, COL4A5 mutations, Alport syndrome

Editorial on the Research Topic
Type IV collagen nephropathies: Alport syndrome and beyond

Mammalian type IV collagen is a family of six chains that form three types of heterotrimeric molecules, which assemble into supramolecular networks in basement membranes. A network comprising α345(IV) collagen, produced by podocytes, is a major component of the glomerular basement membrane (GBM), important for maintaining the normal function of the glomerular filtration barrier (1). Pathogenic variants in the *COL4A3*, *COL4A4*, and *COL4A5* genes encoding the α3(IV), α4(IV), and α5(IV) collagen chains result in a spectrum of nephropathies with diverse clinical presentation.

The most severe forms are X-linked and autosomal recessive (AR) Alport syndrome, characterized by progressive kidney failure, hearing loss, and ocular abnormalities. Heterozygous pathogenic variants in the *COL4A3* and *COL4A4* genes result in autosomal dominant (AD) Alport syndrome, formerly known as thin membrane nephropathy, which represents the carrier state for AR Alport syndrome (2). Digenic Alport syndrome denotes pathogenic changes in two of the three Alport genes, such as *COL4A5* plus *COL4A3*; or *COL4A3* plus *COL4A4* (3). AD Alport syndrome is the commonest genetic kidney disease affecting about one in 100 individuals, while X-linked Alport syndrome affects about one in 2,000, and AR and digenic Alport syndrome are both much rarer (4).

This Research Topic presents a collection of articles describing developments in the area of collagen IV nephropathies, including but not limited to Alport syndrome.

The article by Savige et al. reviews genotype-phenotype correlations for pathogenic *COL4A3–COL4A5* variants. The variant features that determine disease severity are the same for *COL4A5, COL4A3,* and *COL4A4* in X-linked, AR, and AD Alport syndrome. Large rearrangements, truncating variants, and splice site changes generally result in more severe disease than missense variants. For missense variants, Gly substitutions result in more severe disease than non-Gly substitutions. Among these, Gly substitutions with bulkier residues (e.g., Arg, Glu, Asp, Trp, Val) are associated with more severe disease than substitutions with small residues (Ala, Ser). Understanding genotype-phenotype correlations in Alport syndrome is important because they help predict early onset kidney failure and extra-renal complications, and the need for therapy with renin-angiotensin-aldosterone blockade (5).

The article by Comić et al. also addresses the multifaceted phenotypic and genotypic spectrum of collagen IV nephropathies. This study illustrates the complex clinical and genetic picture of individuals with a type IV-collagen-related nephropathy, indicating the need for a refined nomenclature and teamwork between clinicians and geneticists.

The article by Cerkauskaite et al. reports the analysis by next generation sequencing in a cohort of Lithuanians with suspected Alport syndrome. Molecular testing of 171 individuals led to the detection of 99 individuals with 44 disease-causing variants, including 27 novel variants (nine in each of the *COL4A3, COL4A4,* and *COL4A5* genes).

Recent advances in genetic analysis highlight the importance of detecting splicing variants in *COL4A5*, which account for about 15% of all cases of X-linked Alport syndrome. Aberrant splicing results from both canonical and non-canonical splice site variants, the latter including deep intronic changes and substitution in exons. The article by Yamamura et al. reviews the contribution of *COL4A5* splicing variants to the pathogenesis of X-linked Alport syndrome, the latest diagnosis strategies, and the prospects for new therapeutic approaches.

One article by Deng et al. identifies two *COL4A3* variants initially presumed to be missense [p.(Leu1598Arg)] or synonymous [p.(Thr255Thr)], which instead were demonstrated to induce aberrant RNA splicing. These findings highlight the importance of transcript analysis of unclassified exonic sequence variants for better molecular diagnosis.

The second article by Deng et al. reports the detection of low-level somatic mosaic *COL4A5* splicing variant in an asymptomatic female, who gave birth to two boys with X-linked Alport syndrome caused by a hemizygous disease-causing *COL4A5* variant. Although the disease-causing variant was not detected in the mother's genomic DNA by Sanger sequencing, both wild type and very low-level mutant *COL4A5* were identified by droplet digital PCR. This illustrates that some cases of X-linked Alport syndrome attributed to presumed *de novo COL4A5* mutations are due to parental mosaicism.

Mutations in the *COL4A3–COL4A5* genes are the commonest cause of inherited kidney failure after polycystic kidney disease. No specific therapies for Alport syndrome exist yet. Chavez et al. provide an overview of novel therapeutic agents to arrest disease progression in Alport syndrome, currently under investigation. These include an oral Nrf2 activator, anti-miRNA-21 oligonucleotides, endothelin type A receptor inhibitors, inducers of cholesterol efflux, DDR1 inhibitors, osteopontin-blocking agents, as well as the drugs hydroxychloroquine, metformin, and paricalcitol. The review also discusses future therapeutic strategies such as chaperon therapy, genome editing, and stem cell therapy.

Cosgrove and Madison review the consequences of the altered GBM composition in Alport syndrome, with emphasis on the molecular and cellular mechanisms underlying the initiation and progression of Alport glomerular pathology. Specifically, they cite evidence for upregulation of endothelin-1 in glomerular endothelial cells, activation of endothelin A receptors and CDC42 in mesangial cells, and ectopic deposition of mesangial matrix proteins in Alport GBM.

The α345(IV) collagen is also the autoantigen targeted pathogenic anti-GBM autoantibodies, which bind to the GBM and alveolar basement membranes causing rapidly progressive glomerulonephritis and pulmonary hemorrhage (6). A case report by Sobotta et al. describes a patient with acute respiratory distress syndrome secondary to anti-GBM antibodies, who has recovered pulmonary function after treatment with eculizumab—a monoclonal antibody that binds to complement C5 and prevents its cleavage by C5 convertases, thereby inhibiting the activation of the terminal complement cascade.

In summary, the current Research Topic is a collection of nine original and review articles which describe genotype-phenotype correlations for all three Alport genes, report novel pathogenic variants (including uncommon types), review the mechanisms of initiation and progression of Alport glomerular pathology, and overview novel therapies for Alport syndrome. These articles highlight the most recent developments in these areas of research.

Author contributions

D-BB wrote the original draft. DA, OG, and JS edited the manuscript. All authors reviewed and approved the final manuscript.

Funding

D-BB was supported by a pilot grant funded by the grant U54 MD007593 from the National Institute on Minority Health and Health Disparities of the National Institutes of Health, and by award W81XWH-20-1-0698 from the Lupus

Research Program of the US Department of Defense. OG was supported by the German Research Foundation DFG (GR 1852/6-1).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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CASE REPORT
published: 23 September 2021
doi: 10.3389/fmed.2021.720949

Case Report: Eculizumab and ECMO Rescue Therapy of Severe ARDS in Goodpasture Syndrome

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Introduction: Goodpasture's syndrome is a life-threatening autoimmune type IV collagen disease characterized by the presence anti–glomerular basement membrane antibodies, rapid progressive glomerulonephritis and/or pulmonary hemorrhage.

Methods: Here, we describe new therapeutic options, which take recent advances in unraveling Goodpasture's pathogenesis into account.

Results: In a 17-year old male, severe Goodpasture's syndrome resulted in acute respiratory distress syndrome (ARDS). Within 1 day after hospital admission, the patient required extracorporeal membrane oxygenation (ECMO). Despite steroid-pulse and plasmapheresis, ARDS further deteriorated. Eleven days after admission, the patient was in a pre-final stage. At last, we decided to block the complement-driven lung damage by Eculizumab. Three days after, lung-failure has stabilized in a way allowing us to initiate Cyclophosphamide-therapy. As mechanical ventilation further triggers Goodpasture-epitope exposure, the patient was taken from pressure support - breathing spontaneously by the help of maintaining ECMO therapy. After a total of 24 days, ECMO could be stopped and pulmonary function further recovered.

Conclusions: In conclusion, our findings suggest that life-threatening organ-damage in Goodpasture's syndrome can be halted by Eculizumab as well as by lung-protective early withdrawal from pressure support by the help of ECMO. Both therapeutic options serve as new tools in otherwise hopeless situations to prevent further organ-damage and to gain time until the established immunosuppressive therapy works in otherwise lethal autoimmune-diseases.

Keywords: Goodpasture syndrome, lung failure, ARDS, ECMO-therapy, eculizumab, type IV collagen, anti-GBM disease, vasculitis

OPEN ACCESS

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Specialty section:
This article was submitted to Nephrology, a section of the journal Frontiers in Medicine

Received: *05 June 2021*
Accepted: *31 August 2021*
Published: *23 September 2021*

Citation:
Sobotta M, Moerer O and Gross O (2021) Case Report: Eculizumab and ECMO Rescue Therapy of Severe ARDS in Goodpasture Syndrome. Front. Med. 8:720949. doi: 10.3389/fmed.2021.720949

INTRODUCTION

Patients with Goodpasture syndrome develop rapid progressive glomerulonephritis and lung hemorrhage. This life-threatening autoimmune disease is caused by circulating anti-glomerular basement membrane (GBM) autoantibodies, which produce linear IgG staining of glomerular capillaries in kidney biopsy specimens (1, 2). The α3(IV) NC1 domain has been identified as the Goodpasture autoantigen (3). Involvement of the lung and GBM is caused by the presence of this target antigen in these tissues. Multigenetic loci that influence the T-cell response, autoreactive CD4+ helper T cells, and macrophage activity modulate the disease (4, 5).

Two dominant α3(IV) NC1 epitopes have been described; they do not bind native cross-linked α3/4/5 hexamers until they are dissociated (6). The epitopes are cryptic and concealed by covalent bonds of the α3/4/5 hexamers. The formation of these sulfilimine crosslinks is catalyzed by peroxidasin with hypohalous acids as intermediary products (7). The presence of aggressive hypohalous acids in this chemical process may explain why, in the case of a failure of this process, such as in Goodpasture syndrome, fulminant organ damage occurs, with GBM ruptures, plasma leakage, crescent formation and pulmonary hemorrhage.

Extracorporeal membrane oxygenation (ECMO) is used to back up and bridge blood gas exchange in patients with severe acute respiratory distress syndrome (ARDS) refractory to conventional mechanical ventilation (8–10). ECMO helps gain time for the treatment of the lung disease and de-escalate the ventilation settings (11). One very rare cause of ARDS is Goodpasture syndrome. The use of ECMO in patients with Goodpasture syndrome has been reported in only a few cases (12–15).

Eculizumab is a terminal complement inhibitor that binds to the human C5 complement protein, thus blocking the generation of proinflammatory C5a and C5b-9. It is approved for the treatment of atypical hemolytic-uremic syndrome and paroxysmal nocturnal hemoglobinuria (16, 17). Eculizumab has been used in several complement driven severe diseases, such as refractory membranoproliferative glomerulonephritis (18); however, it has not previously been applied in Goodpasture syndrome. The scientific evidence on the possible role of complement (dysregulation) specific to Goodpasture syndrome is limited. In the kidney, immunofluorescence may demonstrate the presence of complement components, in particular C3 and C1q, along the GBM (2, 7).

We describe a patient who developed the most severe ARDS due to Goodpasture syndrome, thus requiring ECMO therapy. Despite steroid pulse therapy and plasmapheresis, no significant improvement in lung function was achieved. At a pre-final stage, we blocked the complement-driven lung damage using a single dose of Eculizumab. In addition, early weaning from pressure support ventilation with the help of maximal ECMO therapy further stabilized the patient in this critical situation. With the maintenance therapy of Cyclophosphamide and Prednisolone, as well as intermittent plasmapheresis, the pulmonary function recovered within 3 weeks. These findings indicate that in addition to the early withdrawal of pressure support ventilation bridged by ECMO, Eculizumab may slow down complement-driven lung damage in life-threatening autoimmune diseases, thus gaining time until the immunosuppressive and immune-reductive therapies, including Cyclophosphamide and plasmapheresis, can take effect.

METHODOLOGY

Written informed consent was provided by the patient and both parents for the publication of his case. All methods, procedures and laboratory test described in the results section are documented in the electronic patient record of our intensive care unit and origin from the routine methods and documentation system used in our University hospital Goettingen.

RESULTS

Clinical Presentation

We report a 17-year-old male smoker in excellent physical condition (soccer player) who contacted his family physician because of mild dyspnea and cough. The family physician made the diagnosis of bacterial bronchitis and prescribed azithromycin. The patient continued his apprenticeship in the scaffolding industry, including the use of spray cans (with equipment for respiratory protection). Despite antibiotics, the symptoms became worse, and aggravating hemoptysis appeared. After 6 days, the patient contacted his family doctor again with dyspnea and hemoptysis and was admitted to a local city hospital. The patient was immediately transferred to the ICU and required intubation and mechanical ventilation within several hours. The diagnosis of ambulant acquired pneumonia was made, and antibiotic therapy was escalated to clarithromycin and meropenem. The same day, due to complete failure of the conventional mechanical ventilation, the city hospital contacted the ECMO task force of our University hospital. The team performed veno-venous cannulation on site, and the patient was transported to our University hospital by helicopter. In summary, the 17-year old patient required intubation on the first day of hospital admission and also, a few hours later, ECMO therapy needed to be initiated on the first day of hospital admission.

At the time of admission to our University hospital, the ECMO blood flow was 4.5 l/min, and the oxygen flow of 3 liters was accompanied by continued invasive ventilation at a bi-level positive airway pressure mode (BIPAP) with a positive end-expiratory pressure (PEEP) of 15 cmH2O, a positive inspiratory pressure (PIP) of 30 cmH_20 and a fraction of inspired oxygen (FiO_2) of 80%.

A recruitment CT scan for acute respiratory distress syndrome (ARDS) did not show a significant recruitment potential of the lungs (**Figures 1A,B**). Small airway disease was diagnosed with bilateral inflammatory consolidations in the lower lobes, which, at this time, appeared as a typical bilateral pneumonia in the conventional im-aging (**Figure 1C**). Bronchoscopy showed rather pale mucous membranes, no swelling and no pus; however, fresh blood and blood clots of different ages, as well as very vulnerable airways were present. Bronchoalveolar lavage was taken for microbiologic workup; however, lung biopsy was not performed because of very stringent anticoagulation under ECMO therapy. Due to the complete absence of kidney involvement besides microhematuria without acanthocytes (**Table 1**), Goodpasture syndrome was not suspected during the first days at our ICU. Antibiotic therapy was further escalated to meropenem, vancomycin and clarithromycin (**Table 1**).

Three days after admission, the microbiological examinations showed no significant results with the exception of one positive finding of adenovirus-DNA and Candida dubliniensis in a culture from the bronchoalveolar lavage (**Table 1**). Both findings were not suspected to be the cause of the severe ARDS. This finding led to further examination, including a positive

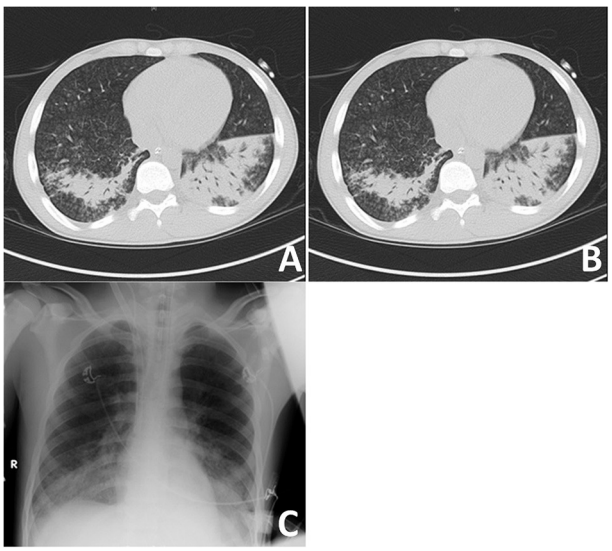

FIGURE 1 | Imaging of most severe autoimmune lung disease on admission. **(A)** Recruitment CT-scan for acute respiratory distress syndrome (ARDS) at FiO_2 of 100% in deep analgo-sedation, as well as muscle relaxation with cis-atracurium at a PEEP-level of 5 cmH_2O and PIP at 15 cmH_2O with tidal volumes at 6 ml/kg ideal body weight (IBW) in an expiratory hold after 3 min at this level; **(B)** CT-scan after 1 min of recruitment at a PEEP-level of 10 cm H_20 and a PIP of 35 cm H_2O, followed by 1 min at a PEEP-level of 20 cm H_2O and a PIP of 45 cm H_2O at an inspiratory hold; **(C)** Conventional imaging with bilateral inflammatory consolidations in lower lobes.

result for antinuclear antibodies (ANA-immunofluorescence 1:320; cANCA and pANCA negative). The patient's worsening critical condition led us to initiate Prednisolone pulse therapy at day four after admission to our ICU. The proof of anti-glomerular basement membrane (anti-GBM) antibodies and low complement levels (C3c and C4, see **Table 1**) soon led to the diagnosis of Goodpasture syndrome (without kidney involvement) as the cause of severe ARDS.

Treatment of Goodpasture Autoimmune Disease

Daily plasmapheresis was initiated; however, Cyclophosphamide therapy had to be postponed due to the high Procalcitonin levels and the very critical lung condition of the patient, which would not have tolerated worsening of the possibly ongoing bacterial pneumonia.

Despite daily plasmapheresis and steroid pulse therapy, the respiratory condition further deteriorated to a prefinal condition at the ninth day after admission. As an ultima ratio, we applied an off-label single dose rescue therapy with 900 mg eculizumab (plasmapheresis was paused for 48 h; meningococcal prophylaxis and vaccination were applied). Consequently, within the next 3 days, lung failure stabilized in a way that enabled us to initiate intravenous Cyclophosphamide therapy at bi-weekly doses of 500 mg. In parallel, Prednisolone was reduced to 1 mg/kg per day, and daily plasmapheresis was continued until anti-GBM antibodies could no longer be detected in ELISA.

As mechanical ventilation triggers Goodpasture epitope exposure, we aggressively reduced pressure support; using dexmedetomidine, an adequate awakening without significant signs of delirium could be established. No neurological failures were identified. The tidal volumes further improved under intermittent CPAP ventilation. After only 3 days, 17 days after admission, the mechanical ventilation weaning was accomplished. The patient was removed from pressure support and was breathing spontaneously bridged by maximal ECMO therapy. At this time, the ECMO conditions included a blood flow of 4.5 l/min and an oxygen flow of 5 l/min. ECMO-weaning was successful at day 24 after admission to our University hospital (**Figure 2A**). Full mobility was reached after 34 days without subjective dyspnea during normal physical stress. After the last course of Cyclophosphamide (1,000 mg), the patient was subsequently discharged from our ICU unit and transferred back home to his local hospital (**Figure 2B**). There, he received a final 500 mg dose of Cyclophosphamide, and the tracheostoma was closed. Anti-GBM antibodies were monitored on a weekly basis for 6 months and remained negative. Prednisolone was tapered to 5 mg and discontinued after 6 months. All kidney tests remained normal, and lung function completely recovered. Four months after admission to our ICU, the patient scored his first goal in a full 90 min soccer game in his youth team and now plays and trains regularly for an adult team 12 months after.

DISCUSSION

Although approved for atypical hemolytic-uremic syndrome and paroxysmal nocturnal hemoglobinuria (7, 8), the use of Eculizumab has been described in other complement driven severe diseases, such as refractory membranoproliferative glomerulonephritis (18). However, to our knowledge and according to the manufacturer (personal information by pharmacovigilance Alexion Pharmaceuticals, Munich, Germany), it has not previously been applied in Goodpasture syndrome. For that reason, both parents have been informed about the off-label use of Eculizumab prior application and have given their written consent. Our example might contribute to expand the (limited) therapeutic options in very severe Goodpasture cases.

The inhalation of hydrocarbons or tobacco may trigger Goodpasture syndrome for unknown reasons. Bromine is a required cofactor for peroxidasin, which is the only currently known essential function of bromine in animals (6, 19). In our patient, the inhalation of hydrocarbons may have affected the reactant bromide, thus indirectly influencing peroxidasin production of aggressive hypohalous acids. In addition, it may have altered the patient's extracellular chloride gradient, which is required for type IV collagen assembly (20). This toxic cocktail may have ultimately led to the uncovering of the cryptic NC1α3(IV) epitope and initiated the Goodpasture syndrome disease process. In conclusion, aggressive hypohalous acids and further ventilation pressure trauma resulted in the most severe, life-threatening lung hemorrhage. A severe bacterial lung infection was considered

TABLE 1 | Urine analysis, laboratory tests and microbiological diagnostic workup on admission.

Urine test Values	**Result**	**Reference**
Urine erythrocytes (sediment)	>20	Neg
Urine acanthocytes (sediment)	neg	Neg
Urine dysmorphic erythrocytes (sediment)	neg	Neg
Urine protein [mg/l]	225.6	10–140
Urine immunoglobulin G [mg/l]	neg	Neg
Urine Alpha1-microglobuline [mg/l]	16.40	<2.0
Urine Albumin g/g Creatinine	31.91	<30
Blood test Values	**Result**	**Reference**
Hemoglobin [g/dl]	8.7	13.5–17.5
White blood cell count [tsd/μl]	15.4	4.0–11.0
Platelet count [tsd/μl]	303	150–350
Creatinine [mg/dl]	0.77	0.62–1.08
Blood urea nitrogen [mg/dl]	18	7–21
Procalcitonin [μg/l]	17.1	< = 0.1
Interleukin 6 [pg/l]	98.5	<7.0
C3c [g/l]	0.56	0.83–1.52
C4 [g/l]	0.11	0.13–0.37
Lactate-dehydrogenase [U/l]	323	105–235
Microbiological workup	Testing for	Results
Blood cultures	*Bacteria*	Negative
Tracheal secretion cultures	Bacteria	Negative
Tracheal secretion PCR 1	Influenza A/B, RSV, EBV, CMV, HSV, VZV	Negative
Tracheal secretion PCR 1	Adenovirus-DNA	Positive
Tracheal secretion PCR 2	Influenza A/B, RSV, EBV, CMV, HSV, VZV	Negative
Tracheal secretion PCR 2	Adenovirus-DNA	Negative
Serology 1	Aspergillosis, Candidosis, Legionellosis, Chlamydia, Mycoplasma pneumoniae and Coxiella	Negative
Serology 2	Influenza A/B, Adenovirus	Negative
Serology INF-y-Tbc	M. Tuberculosis	Negative
Bronchoalveolar lavage 1	Bacteria, M. Tuberculosis	Negative
Bronchoalveolar lavage 2 PCR	Adenovirus, Influenza A/B, RSV, EBV, CMV, Pneumocystis jerovieci, Mycoplasma pneumoniae, Chlamydia trachomatis and pneumoniae, VZV	Negative
Bronchoalveolar lavage 3 culture	Candida dubliniensis	Positive
Urine culture	Bacteria	Negative

a further initial trigger. As a consequence, severe systemic bacterial infection (as indicated by high procalcitonin levels) in a very critical respiratory situation initially hindered us from applying cyclophosphamide in addition to steroid pulse therapy and plasmapheresis.

The diagnosis of Goodpasture syndrome was confirmed via its clinical presentation, the presence of anti-GBM antibodies in ELISA and immunofluorescence, complement depletion and its clinical and serological response to immune-suppressive therapy. Diagnosis was not further confirmed by kidney biopsy as the kidney involvement was minimal without proteinuria or hematuria. Moreover, despite initial bronchoscopic evaluation, severe lung hemorrhage and strict anti-coagulation therapy during ECMO therapy hindered us from performing lung (or kidney) biopsy. The response to therapy was monitored by respiratory parameters and anti-GBM titers.

Although lymphocytes and macrophages contribute generally to pulmonary and renal damage in Goodpasture syndrome, autoreactive T cells play a unique role in the progress of the disease: they not only enhance B cell function and antibody production, but a distinct T-cell epitope may have a direct causative function in glomerular and alveolar injury (2, 4). The T-cell epitope is similar to some microbial peptides; therefore, distinct pulmonary infections (as in our patient) may not only damage the basement membrane but also provoke an autoimmune response. After the initiation of Good-pasture syndrome, activation of the complement cascade and proteases further contribute to pulmonary and GBM damage. Complement in our patient is not considered to be critical for his Goodpasture's pathogenesis, but to be a very important enhancing pathway for additional lung injury. Consequently, our rationale underlying the application of the complement inhibitor Eculizumab was

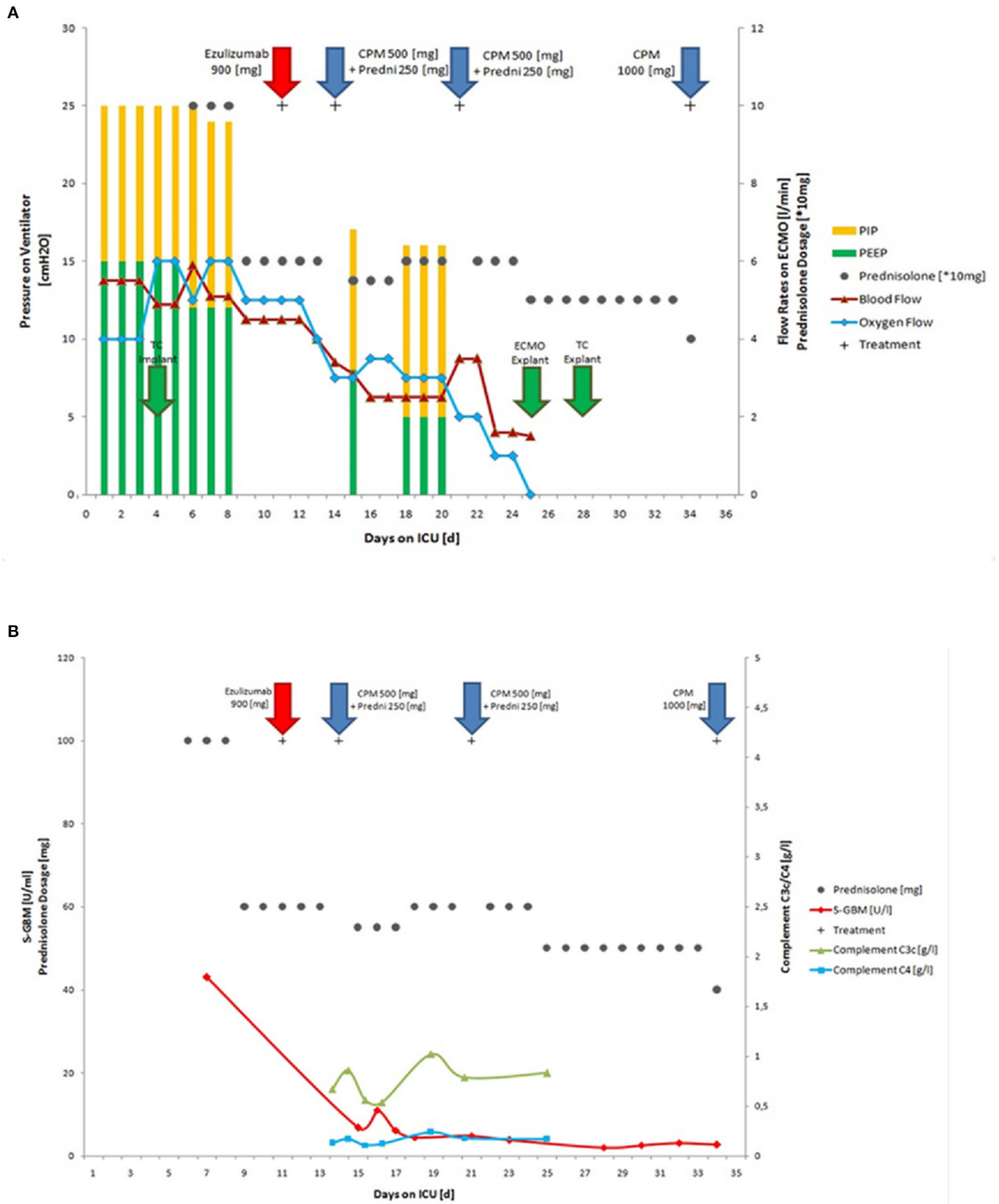

FIGURE 2 | Course of autoimmune disease during therapy. The 17-year old patient required intubation on the first day of hospital admission and also, a few hours later, ECMO therapy needed to be initiated on the first day of hospital admission. **(A)** ECMO and mechanical ventilation conditions during treatment in the ICU, including implantation and explantation of tracheal cannula (TC explant), PIP, PEEP, blood flow, oxygen flow and immunosuppressive therapy with Eculizumab, Cyclophosphamide, and Prednisolone; **(B)** Titers of anti-GBM and complement C3/C4 during therapy (normal ranges: S-GBM Elisa <7 U/ml; C3 0.83–1.52 g/l; C4 0.13–0.37 g/l) ECMO, extracorporeal membrane oxygenation; TC, tracheal cannula; CPM, Cyclophosphamide; Predni, Prednisolone; PIP, positive inspiratory pressure; PEEP, positive end expiratory pressure; Explant, explantation.

to prevent further generation of proinflammatory C5a and also the lytic membrane pore C5b-9, which are thought to cause major lung damage. Despite the novel use of Eculizumab, the clinical improvement subsequently observed may have also been a delayed response to conventional components of therapy (plasmapheresis and steroids).

In addition, the mechanical stress on the lung tissue by pressure support ventilation is thought to have further triggered antigen presentation and therefore maintained epitope exposure in this life-threating autoimmune disease. Thus, decreasing mechanical strain on the alveolar epithelium via modification of the ventilation parameters might have contributed to the patient's recovery. Here, additional damage to the epithelial cells complement autoregulation via surface bound regulators might have been prevented or reduced. In our patient, a fulminant progress led to severe ARDS according to the Berlin definition (21), thus requiring ECMO therapy, which can significantly decrease the mortality (22). In our patient, we selected a lung-protective ventilation strategy with the lowest tidal volumes. Low tidal volumes have been reported to reduce further lung injury and mortality, as well as the number of ventilator-days (in other lung diseases) (23, 24). To further support lung recovery, we performed an early wake up on ECMO. Similar strategies have previously been reported in other settings, including the bridging to lung transplantation (25). In contrast to previously reported cases (12–15), we aimed to wean the patient off of mechanical ventilation prior to weaning him from ECMO therapy, taking into account the mechanical triggers and pathogenesis of Goodpasture syndrome (26, 27). The combination of complement blockade to prevent further lung damage and early weaning from pressure support ventilation with the help of prolonged ECMO led to a rapid improvement of the previous almost desperate situation: the titer of anti-GBM antibodies decreased within days, and the weaning from the respirator was accomplished 7 days after eculizumab therapy. The ECMO weaning was accomplished after an additional 7 days, 24 days after admission and 14 days after eculizumab.

In conclusion, our findings suggest that Eculizumab may heal, irrespective of the trigger, complement-driven organ damage in life-threatening Goodpasture syndrome. Furthermore, lung-protective early withdrawal from pressure support bridged by ECMO may prevent further harm to the lung tissue in Goodpasture syndrome. Both therapeutic options take Goodpasture pathogenesis into account and may serve as an important tool in otherwise hopeless situations to prevent further organ damage and gain time until the established immunosuppressive therapy begins to take effect in otherwise fatal autoimmune diseases.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

MS, OM, and OG: conceptualization, methodology, and investigation. MS: writing—original draft preparation and visualization. OM and OG: writing—review, editing, and supervision. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

The authors thank the health care workers of the intensive care unit.

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frontiers in Medicine

REVIEW
published: 08 February 2022
doi: 10.3389/fmed.2022.841391

The Contribution of *COL4A5* Splicing Variants to the Pathogenesis of X-Linked Alport Syndrome

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X-linked Alport syndrome (XLAS) is caused by pathogenic variants in *COL4A5* and is characterized by progressive kidney disease, hearing loss, and ocular abnormalities. Recent advances in genetic analysis and further understanding of genotype-phenotype correlations in affected male patients raises the importance of detecting splicing variants in *COL4A5*. Aberrant splicing of *COL4A5* is caused not only by canonical splice site variants but also non-canonical splice site variants such as deep intronic changes or even substitutions in exons. Patients with splicing variants account for ∼15% of all cases in XLAS. In addition, it has been shown that there is a significant difference in kidney survival depending on the aberrant splicing patterns of transcripts- in particular in-frame or out-of-frame nucleotide changes in transcripts. Therefore, cDNA analysis of patient mRNA is necessary to determine the impact of splice site variants and to confirm a diagnosis of XLAS and to predict the kidney prognosis. However, it is usually difficult to amplify *COL4A5* transcripts extracted from peripheral blood leukocytes. For these cases, *in vitro* minigene assays or RNA sequence extracted from urine derived cells can confirm aberrant splicing patterns. Moreover, controlling aberrant splicing by nucleic acids or small molecular compounds in genetic diseases are attracting attention as a potential therapeutic strategy. Here, we review the frequency of splicing variants in *COL4A5*, the latest diagnostic strategies, and the prospects for new therapeutic approaches.

Keywords: alport syndrome, *COL4A5*, splicing, genotype phenotype correlation, minigene

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Nephrology, a section of the journal Frontiers in Medicine

Received: *22 December 2021*
Accepted: *10 January 2022*
Published: *08 February 2022*

Citation:
Yamamura T, Horinouchi T, Aoto Y, Lennon R and Nozu K (2022) The Contribution of COL4A5 Splicing Variants to the Pathogenesis of X-Linked Alport Syndrome. Front. Med. 9:841391. doi: 10.3389/fmed.2022.841391

INTRODUCTION

Alport syndrome (AS) is an inherited disorder with progressive kidney disease, frequently accompanied by sensorineural hearing loss and specific ocular abnormalities (1–4). AS is caused by defects of in the type IV collagen network, a major structural component of basement membranes in the kidney, inner ear, and eye. Six distinct type IV collagen α-chains (α1–α6) have been identified and are encoded by the genes *COL4A1–COL4A6*. Pathogenic variants in the *COL4A5*, which encodes the type IV collagen α5 chain, are known to cause X-linked Alport syndrome (XLAS); XLAS is the most common inherited form of AS with ∼80% of all AS patients (5). Our group confirmed this distribution in our cohort of all genetically diagnosed Japanese AS families (n = 397) where we found 74% (n = 295) had XLAS (6).

Regarding the genotype of XLAS, all types of variant category have been registered as causative variants to clinical genetic databases similar to other human inherited diseases. In addition, it is already known that strong genotype and kidney phenotype correlation exists in affected male patients with XLAS; patients with missense or small in-frame variants show less severe phenotypes compared to patients with truncating variants (e.g., nonsense, a small insertion/deletion leading to a premature stop codon) (7–10). In addition, we recently focused on the difference in transcripts of splicing variants in *COL4A5* based on whether the abnormal transcript has in-frame deletion (the total number of nucleotides is multiple of 3) or out-of-frame deletion (not multiple of 3). According to this analysis, we revealed that male patients with splicing variants leading to in-frame transcripts had less severe phenotypes than those with out of frame transcripts (10, 11).

Recent advances in genetic analysis have enabled comprehensive and efficient screening of multiple genes including *COL4A3, COL4A4,* and *COL4A5* for patients suspected as having Alport syndrome. However, specific variants causing abnormal splicing such as deep intronic variants cannot be detected by (targeted) exome sequencing and a consensus approach for detecting deep intronic variants has not been established (12–15). Moreover, transcript analysis targeting genomic DNA variants, which are suspected to causing aberrant splicing is challenging because of the stability of mRNA, and the extremely low expression level of *COL4A5* transcripts in accessible cells such as peripheral blood leukocytes.

In this review, we provide a comprehensive overview of the investigation and functional analysis of splicing variants in the *COL4A5* gene; including the frequency of these variants, the latest diagnostic strategies, and the prospects for new therapeutic approaches to regulate splicing patterns.

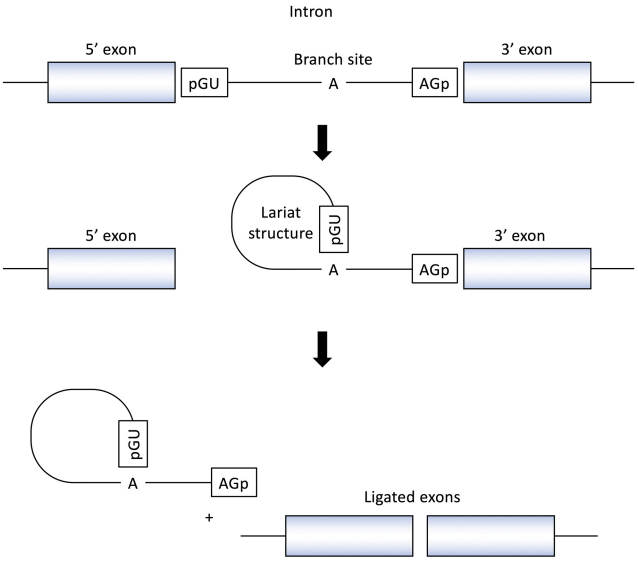

FIGURE 1 | Splicing reactions and important splicing elements. There are several essential splicing motifs located in boundary region between exons and introns. In addition to highly conserved dinucleotides of both 5′ and 3′ side of intron (GU/AG), adenosine (A) at the branch site and polypyrimidine tract (not shown in figure) are located in intron. pre-mRNA splicing process take place in two transesterification steps. In the first step, the 2′-OH group of the adenosine (A) at the branch site performs a nucleophilic attack on a phosphate (p) at the 5′ splice site. This leads to cleavage of the 5′ exon from the intron and the formation of a lariat structure. In the following step, a second transesterification reaction, which involves the phosphate at the 3′ end of the intron, detach the intron from exon and ligates the two exons.

SPLICING ABNORMALITIES AND HUMAN GENETIC DISEASES

RNA splicing is a form of RNA processing in which precursor messenger RNA (pre-mRNA) is transformed into mature messenger RNA (mRNA) in the sequence of protein biosynthesis. In higher eukaryotes, the nucleotide sequence of genomic DNA (gDNA) is divided into the protein coding region (exon) and non-coding region (intron). Pre-mRNA newly transferred from gDNA include both exons and introns. The process of pre-mRNA splicing removes introns from pre-mRNA, and the remaining exons are combined to form mature mRNA (**Figure 1**).

In the process of pre-mRNA splicing, specific sequences located in intron play an important role. Dinucleotides of both the 5' and 3' side of intron are highly conserved (GU/AG) and called the splice donor site and the splice acceptor site, respectively. In addition, other conserved motifs such as the branch site and the polypyrimidine tract, located upstream of the 3′ splice site is also important to determine the location of splicing. Pre-mRNA splicing takes place in two transesterification steps. In the first step, the 2′-OH group of the adenosine (A) at the branch site performs a nucleophilic attack on a phosphate (p) at the 5' splice site. This leads to cleavage of the 5′ exon from the intron and the formation of a lariat structure. In the following step, a second transesterification reaction, which involves the phosphate at the 3′ end of the intron, detaches the intron from exon and ligates the two exons (16) (**Figure 1**).

In addition to above essential splicing motifs, additional sequence elements known as enhancers or silencers are needed for accurate splicing. These regulatory sequences are located both in exons and introns and called exonic/intronic splicing enhancers/silencers. Furthermore, the splicing process, including the precise recognition of the splice site, is catalyzed by the huge complex of proteins and enzymes termed the spliceosome (17).

These splicing motifs or elements are known as a target of variation in genetic diseases. Variants in this region often cause aberrant splicing and result in pathology. The importance of splicing variants is illustrated by the fact that nearly 15% of human genetic diseases are estimated to be caused by variants located in the 5' or 3' consensus splice sites (18). In addition, comprehensive studies of cDNA analysis for all detected pathogenic variants in patients with ataxia-telangiectasia (OMIM#208900) and neurofibromatosis type I (OMIM#162200) revealed that nearly 50% of variants resulted in aberrant splicing patterns (19, 20). Surprisingly, among the splicing variants in these reports, a minority were detected in the conserved dinucleotide of 5' and 3' side (GU/AG) and

TABLE 1 | Mutational characteristics of reported cohort of X-linked Alport syndrome.

Characteristics	Jais et al.		Bekheirnia et al.		Yamamura et al.	
	N with data	*n* (%)	*N* with data	*n* (%)	*N* with data	*n* (%)
Mutation types in families	195		175		269	
Missense mutation		74 (38.9)		89 (50.9)		144 (53.5)
Nonsense mutation		14 (7.2)		N/A[a]		19 (7.1)
Splicing variant		**29 (14.9)**		**24 (13.7)**		**49 (18.2)**
Small rearrangement		40 (20.5)		N/A[a]		44 (16.4)
Large rearrangement		38 (19.5)		14 (8.0)		13 (4.8)

[a]*It is unable to specify the numbers of patients with these two types of variants because this study classified nonsense mutations and truncating small rearrangements into "truncating mutation."*

most of other variants located in exons or other intronic regions and caused abnormal splicing. The mechanism of how variants located in exon region causes aberrant splicing is mainly explained by the disruption of splicing regulatory elements such as exonic splicing enhancer (ESE) or silencer (ESS) (21). If a nucleotide substitution is in the sequence of these important motifs, the signal to be recognized as an exon is weakened and this can cause exon skipping in process of splicing.

SPLICING VARIANTS IN *COL4A5* GENE

Frequency of Splicing Variants in *COL4A5*

To date, several retrospective studies reported the proportion of patients with splicing variants in XLAS. Jais et al. (7) investigated genetic and clinical characteristics in 195 families with XLAS and revealed that 29 families (14.9%) had splice site variants. Bekheirnia et al. (8) also reported the proportion of each variant in 175 families with male XLAS patient and it revealed 13.7% of all families possessed splice site variants. Recently, our group investigated 269 Japanese families with XLAS and we found that splicing variants were detected in 18.2% of all families (**Table 1**) (10). The higher proportion of splicing variants in our study was thought to be the effect of active transcriptional analysis to detect deep intronic variants and exonic splicing variants other than canonical dinucleotides splice site (GU/AG) variants. Interestingly, among 71 male XLAS patients from 49 families with splicing variants in our study, only 35 patients (26 families) had variants in canonical dinucleotides splice site of *COL4A5* gene. These findings highlight the importance of splicing variants in *COL4A5* and demonstrate similarity to the other inherited diseases mentioned in previous section.

Genotype-Phenotype Correlation in Splicing Variants With XLAS

As described above there are strong genotype-phenotype correlations in males with XLAS; in particular, patients with missense or small in-frame variants (so-called non-truncating variants) have less severe phenotypes compared to patients with truncating variants (e.g., nonsense, small insertion, or deletion leading to out-of-frame sequences) (7–9). In addition, patients with splice site variants have shown intermediate severity, between the phenotypes associated with non-truncating and truncating variants.

Although splicing variants can be classified depending on their transcript pattern (i.e., in-frame or out-of-frame transcript), genotype-phenotype correlation analysis based on transcriptional analysis had not been conducted. Recently, we focused on this transcriptional difference in splicing variants and analyzed the kidney survival of patients with splicing variants. We found a significant difference between patients with in-frame splicing variants and those with out-of-frame splicing variants; the median kidney survival of patients with the in-frame splicing variants ($n = 33$) was 28 years, whereas it was 23 years for patients with the out-of-frame splicing variants ($n = 32$; $P < 0.05$) (10). This result demonstrates the value of transcriptional analysis for splicing variants in XLAS to estimate their kidney prognosis and enable genetic counseling.

Exonic Variants Causing Abnormal Splicing in *COL4A5*

Some exonic variants, which can be considered as missense or nonsense if transcriptional analysis is not conducted, affect splicing and may cause disease. In particular, single-base substitutions at the last nucleotide position in each exon are reported to likely affect splicing patterns (19, 20). However, no studies have addressed the characteristic of exonic variants in the *COL4A5* gene which are likely to affect splicing. Therefore, we focused on the variants affecting the last nucleotide of exons in *COL4A5* and conducted a comprehensive *in vitro* transcript analysis (22). We found 14 reported variants located in last nucleotide of any *COL4A5* exon from the Human Gene Mutation Database (HGMD) and six novel variants from our cohort. All 14 variants in HGMD are reported as missense and most of them are glycine substitution, which is most common type of missense variant in *COL4A5* (3). Furthermore, using an *in vitro* functional splicing analysis, 17 out of the total 20 variants showed aberrant splicing. In this study, we also conducted the genotype-phenotype correlation analysis of the splicing variants caused by substitution of last nucleotide in exons comparing to our previous report of missense variants. We found that the median age of developing end-stage kidney disease (ESKD) in cases with splicing variants was significantly worse than those with missense variants (27 vs. 40 years old, $P < 0.01$). From this result, we

concluded that variants located in last nucleotide position of exons in *COL4A5*, even if they are glycine missense substitution, should be considered likely splicing variants and examined by transcriptional analysis.

In addition, the importance of synonymous or silent variants in abnormal splicing in *COL4A5* should be considered. We analyzed *COL4A5* transcripts in three patients clinically diagnosed XLAS with synonymous variants by using the *in vitro* functional splicing assay and analysis of patient mRNA. This revealed that all three cases showed aberrant splicing patterns (23, 24). Interestingly, among these three cases, one patient had both aberrant and normal transcripts by mRNA analysis, and they exhibited a milder phenotype. This finding suggested that synonymous variants in *COL4A5* can affect splicing pattern and might show milder phenotype via producing mixture of both normal transcripts and aberrant splicing.

Intronic Variants Outside the Canonical Splice Site Causing Abnormal Splicing in *COL4A5*

Although the canonical dinucleotides splice site (GU/AG) is important for correct splicing and variants in this region cause aberrant splicing, intronic variants outside this region may also influence splicing. Indeed, there are several non-canonical intronic variants around exon-intron boundaries that have been reported in genomic databases such as HGMD (14). However, the pathogenicity of those intronic variants were not all proven by transcript analysis. Recently, our group reported the results of transcript analysis for seven non-canonical intronic variants in *COL4A5* (6 reported variants and one from our cohort) by using *in vitro* splicing analysis with or without *in vivo* RNA sequencing. Consequently, five variants were expected to cause aberrant splicing (by skipping the respective exon) while one variant was found less likely to alter the splicing pattern (15). From the above, we should carefully judge the pathogenicity of non-canonical intronic variants and transcript analysis is recommended to assess their influence on splicing.

It has been known that deep intronic variants in *COL4A5* also can cause aberrant splicing. Although this type of variant can be confirmed by only mRNA analysis because vast majority of intronic substitutions are polymorphisms, several pathogenic variants in deep introns of *COL4A5* have been reported (14, 25). While variants close to exon-intron boundary frequently cause exon skipping, deep intronic variants in *COL4A5* show the pathogenicity by the creation of cryptic exon (26, 27).

DIAGNOSTIC STRATEGY OF *COL4A5* SPLICING VARIANTS

Genetic Analysis for XLAS

Previously, Sanger sequencing was widely used for the genetic diagnosis of Alport syndrome. However, screening of all three Alport genes (*COL4A3/COL4A4/COL4A5*) by conventional Sanger sequencing is time-consuming because each gene contains ∼50 exons with no hotspots. Therefore, targeted exome sequencing with Next Generation Sequencing (NGS) has become the first line screening method for the genetic analysis. However, it should be noted that targeted exome sequencing, which screen exons and exon–intron boundaries, cannot detect all types of variant.

For example, large deletion across exons and copy number variations (CNVs) could not be screened by direct sequence and multiplex ligation-dependent probe amplification (MLPA) is the only way to detect this type of variant. However, a paired analysis approach, comparing NGS data of patients and normal controls, recently enabled us to screen CNVs and we detected *COL4A5* CNVs successfully with this method (28). Similarly, deep intronic variants causing aberrant splicing also could not be detected by standard sequencing for exons and exon-intron boundaries. Although whole genome sequencing (WGS) approach can screen deep intronic variants, it is difficult to detect pathogenic variants located in introns among the vast majority of non-pathogenic polymorphisms. Therefore, it is essential to carry out transcriptional analysis such as RNA sequencing to detect deep intronic variants.

Transcriptional analysis is important to assess the pathogenicity of intronic variants located outside canonical dinucleotides splice site (GU/AG). Variants located in canonical dinucleotide splice site can be diagnosed as pathogenic because of its critical effect on splicing (29), other intronic variants need to be assessed by transcriptional analysis to determine whether they cause aberrant splicing or not. In addition, it should be noted that there is a rare exception (<1%) of the type of canonical dinucleotide splice site (i.e., GC/AG etc.) and variants in this site do not always lead to exon skipping but sometimes partial deletion of exons or exonization of introns, which is important information to estimate renal prognosis for the evaluation of in-frame or out-of-frame deletions at the transcript levels (11, 14).

To detect pathogenic variants including splicing variants effectively, we conduct the genetic analysis for patients suspected as having XLAS in a stepwise manner, as shown in **Figure 2**. Briefly, targeted exome sequencing including all three Alport genes is performed first and then screening for copy number variations (CNVs) in the *COL4A3/ COL4A4/COL4A5* genes is performed using paired analysis for patients in whom no pathogenic variants are detected by targeted exome sequencing. When paired analysis detects the possibility of CNVs in cases, MLPA is used to confirm CNVs. In addition, for patients with no obvious pathogenic variants, RNA sequencing using RT-PCR will be performed to detect aberrant splicing by intronic variants.

Although the frequency of patients with CNVs is lower than those with splicing variants, our screening for CNVs can be conducted using the data of NGS analysis therefore this step is placed earlier than RNA sequencing. In addition, as the screening for CNVs using NGS data may not sufficiently detect CNVs of small size (smaller than 1,000 bp), we add MLPA analysis for the patients who are strongly suspected of having Alport syndrome from their clinical findings even if any pathogenic variants in *COL4A3/COL4A4/COL4A5* genes were not detected by NGS and RNA sequencing.

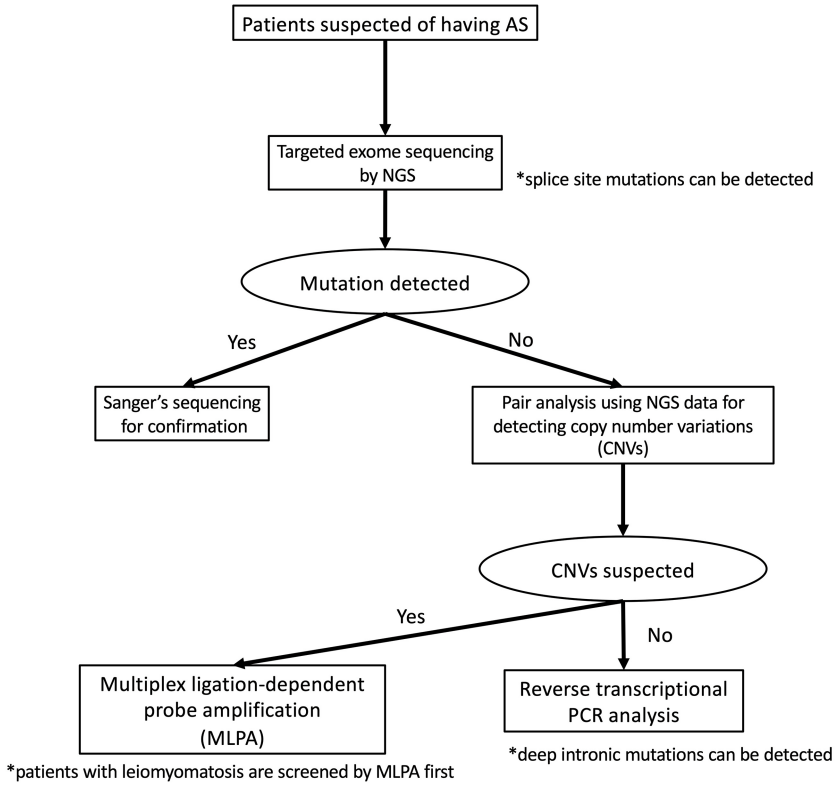

FIGURE 2 | Mutational analysis approach for patients suspected as having Alport syndrome in our laboratory. In our laboratory, patients suspected as having Alport syndrome are first screened targeted exome sequencing by using NGS. If this first screening does not detect any pathogenic variants, screening for copy number variations (CNVs) in the *COL4A4/COL4A4/COL4A5* genes is performed using paired analysis. When paired analysis detects the possibility of CNVs in cases, multiplex ligation-dependent probe amplification (MLPA) is used to confirm. In addition, for patients with no obvious pathogenic variants, RNA sequencing using reverse transcription PCR is performed.

Source of Transcriptional Analysis (Blood, Kidney, Hair Root, and Urine Derived Cell)

Peripheral leukocytes or hair roots have been traditionally used as a common source of mRNA for *COL4A5* transcriptional analysis because of its accessibility. However, the expression level of *COL4A5* mRNA in these samples is low and the nested PCR technique is required to amplify the targeted regions (14, 30). Although mRNA from patient kidney biopsies has abundant *COL4A5* expression, the cDNA analysis for *COL4A5* by using mRNA from kidney is not a common procedure since we now conduct a genetic analysis first approach for the diagnosis of Alport syndrome without performing a kidney biopsy. In contrast, patient urine samples have good accessibility and mRNA directly extracted from urine sediments is also abundant in *COL4A5* expression and can be used for RT-PCR (10, 11, 14, 23).

Urine-derived cells have been shown to express podocyte markers, and podocytes are the main source of type IV collagen α3α4α5 in the glomerular basement membrane (31, 32). Sergio et al. reported that urine derived "podocyte-lineage" cells from a patient with Alport syndrome expressed all three Alport gene mRNA and could be used for RT PCR analysis (32). Our group also use this cultured urine derived cells as a main source of mRNA for RNA sequencing of Alport genes. Comparing to direct extraction of mRNA from urine sediment, mRNA from urine derived cultured cells is easy to handle because of its larger and stable amount of RNA.

In vitro Splicing Assay (Minigene Analysis)

In addition to transcript analysis using mRNA from patient derived samples, an *in vitro* functional splicing assay (minigene analysis) using an expression vector is also useful and can be applied for possible splicing variants in *COL4A5*. We routinely examine novel intronic variants or variants suspected of causing aberrant splicing by using a minigene assay, which is constructed to encode two cassette exons (A and B), an intervening sequence containing a multiple cloning site and a promoter region (**Figure 3**).

The analytical method of this assay is shown in **Figure 3**. Briefly, hybrid minigene constructs are created by inserting a test sequence fragment consisting of target gDNA region (exons) and the flanking introns into the multiple cloning site within an intervening intron between two exons (exon A and B) of the minigene construct. If a patient sample (or gDNA) is not available, the variant is introduced by site-directed mutagenesis using PCR. Then, hybrid minigenes are transfected into cultured

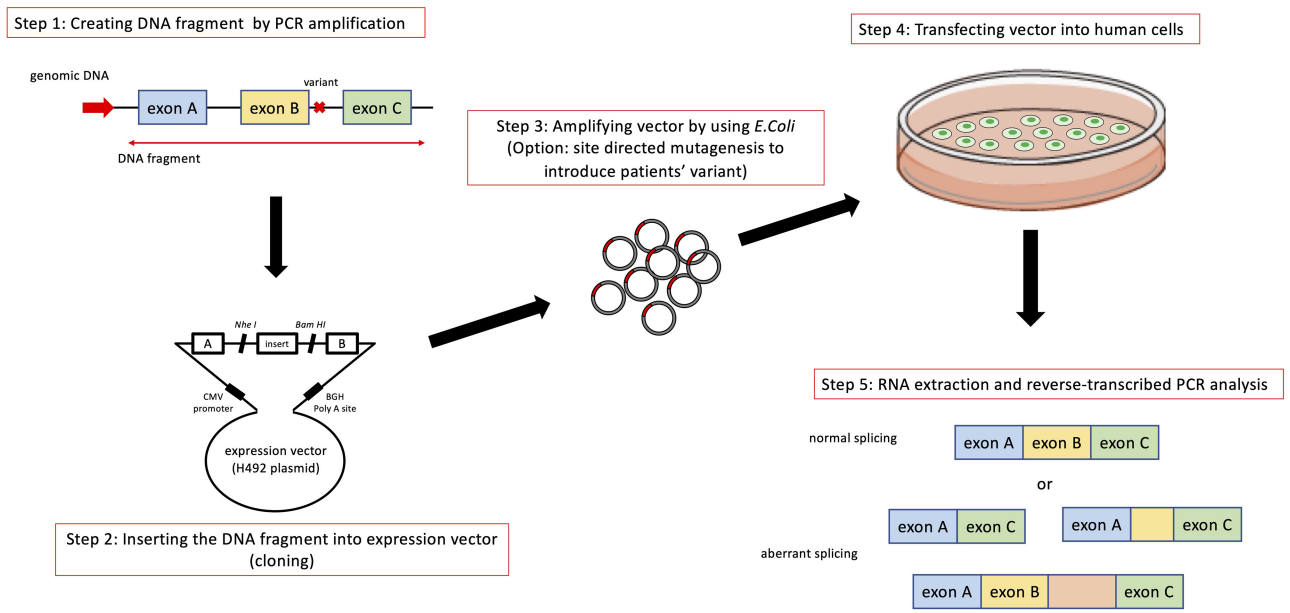

FIGURE 3 | Schematic diagram of minigene analysis to test possible splicing variants. Step 1: Cleaving DNA fragment including the position of mutation by PCR from wild type and patient's gDNA. In addition to the exon close to the mutation being analyzed, we routinely include prier and following exons in DNA fragment. Step 2: Hybrid minigene constructs are created by inserting a test sequence fragment consisting of target exons and its flanking introns into the multicloning site within an intervening intron between two exons (exon A and B) of the minigene construct. Step 3: The hybrid minigene (cloning reaction) is amplified using *E. coli*. If patient's gDNA is not available, we introduce test mutation by using mutagenesis PCR. Step 4 and 5: These hybrid minigenes (both wild type and mutant) are transfected into human derived cells and culture them to express minigene derived mRNA. Step 5: Total RNA is reverse-transcribed into cDNA and the PCR is performed using specific. PCR products are analyzed by means of electrophoresis and direct sequencing.

human cells and total RNA was reverse- transcribed into cDNA and the PCR will be performed. Finally, PCR products are analyzed by means of electrophoresis and direct sequencing (**Figure 3**).

As described earlier, it is often hard to obtain samples for transcript extraction with high expression of target genes for inherited kidney diseases. However, the minigene assay has high flexibility because it does not require any mRNA or even gDNA samples from patients. Variants can be introduced to the minigene by using site directed mutagenesis allowing transcript analysis even if patient sample is not available (33, 34). We have analyzed several novel intronic variants in various inherited kidney diseases using this assay (35–40). As for intronic variants of *COL4A5*, several studies report using the minigene assay including our studies (11, 15, 41). In addition, most recently, our group applied this *in vitro* splicing assay for variants located in exons of *COL4A5* to clarify the characteristic of exonic splicing variants (22, 24).

THERAPEUTIC APPROACHES FOR TARGETING SPLICING VARIANTS IN *COL4A5*

As described elsewhere (42, 43), there are currently no curative therapies for Alport syndrome including XLAS. Current standard of care is with nephroprotective drugs such as renin-angiotensin-aldosterone system (RAS) blockade. Additional agents including bardoxolone methyl, the mi-RNA 21 inhibitor, endothelin receptor blocker, and sodium-glucose transport protein 2 (SGLT2) inhibitors are being trialed in Alport syndrome. Therapy is important and there is a difference in the treatment effect of RAS blockade for male XLAS patients between genotypes, truncating and non-truncating variants (10). However, non-specific nephroprotective therapy is currently not enough to prevent ESKD in their early age for the patients with truncating variants and the development of disease targeted new therapy is required.

As an inherited disease, the causative gene *COL4A5* can be a target of future therapy in addition to non-specific kidney protective therapies. Although one potential gene therapy is direct editing of variants by CRISPR-Cas based system or supplying complete *COL4A5* cDNA by using AAV vector, these approaches have not been successfully applied to XLAS so far. In addition, the process of splicing also attracts considerable attention as its potential therapeutic target in various inherited diseases in recent years. Specifically, antisense oligonucleotides (ASOs) therapy has been approved for several inherited diseases such as spinal muscular atrophy (SMA) and Duchene muscular dystrophy (DMD) and its application for Alport syndrome is also expected (44–49).

Our laboratory has already developed the ASO-mediated exon skipping therapy for the male XLAS model mice (with specific *Col4a5* nonsense variant) and reported its effects in

2020 (50). As described earlier, male XLAS patients have strong genotype-phenotype correlation that patients with truncating variants show severer kidney phenotype than those with non-truncating variants including in-frame deletion variants even at the transcript level. We aimed to amend the truncating transcript caused by the nonsense variant in *COL4A5* to an in-frame transcript by using ASO. Our ASO was designed to combine to the splicing regulatory motif on *COL4A5* pre-mRNA and to introduce exon skipping. As a result, we successfully proved its treatment effectiveness in XLAS model mice treated with ASO by showing a significant improvement compared to vehicle treated mice both clinically and pathologically (50).

Exon skipping therapy using ASO is potentially applied for some of splicing variants in *COL4A5*. About half of these variants result in truncating transcript due to skipping of exon where the nucleotide number is not a multiple of three (out-of-frame deletion). For these variants, additional exon(s) skipping introduced by ASO may be applied as treatment. If the total nucleotide number of skipped exons is a multiple of three, final transcript is in-frame and may lead milder phenotypes. This multiple exon skipping approach has been already considered as a promising treatment for DMD (51–53). In addition, splicing variants which lead to cryptic exon activation are a potential target of this exon skipping approach. A cryptic exon skipped transcript is normal and would result in a much milder phenotype.

In addition to ASO, cell-permeable RNA-targeting small molecules are also attract attention as novel candidates of splicing modifying treatment. A part of the small molecules binds RNA and has an influence on the splicing process. Most recently, risdiplam was approved by FDA as the first small molecule splicing modifier for SMA (54). It was shown that risdiplam analogs directly bind to pre-mRNA of SMN2 to introduce the inclusion of exon 7, which is usually skipped, thereby restoring the production of the SMN2 protein (55). This exon inclusion approach by a small molecule has also been tried for familial dysautonomia caused by intronic variants (IVS20 + 6T>C) in *IKBKAP* resulting in exon 20 skipping. Two different small molecules have also been reported to increase the normal splicing with inclusion of exon 20 in patients or patient derived cells (56, 57). Small molecules have advantages in terms of the administration route-ASOs are typically administered via intravenous, percutaneous or intrathecal routes, whereas small molecules can be administered orally.

Major problems of developing splicing modifying ASOs or small molecules for XLAS are the specificity of mechanism of action of these drugs and rarity of the treatment-amenable patient population. Each ASO or small molecule can bind a specific region of RNA and shows the action of splicing regulator and one drug may applicable for a few variants. As shown in previously, XLAS has no hotspot region and drugs therefore need to individual exons. However, there has been much interest in the development of individualized treatments for inherited diseases with small numbers of patients, and the FDA has issued a recommendation for the development of individualized ASOs to facilitate progress to individualized therapy.

CONCLUSION

Splicing variants account for significant proportion of the total variants in XLAS and this proportion might be increasing accompany with advances of gene analysis in future. Thus, it is recommended to conduct RNA sequencing for the patients suspected as having XLAS in whom standard exome sequencing did not detect any variants (6). In addition, even for the cases with obvious splicing variants caused by canonical dinucleotides splice site (GU/AG) variants, transcript analysis by RNA sequencing or *in vitro* functional splicing assays will clarify the transcript pattern. This is important due to the difference in kidney prognosis of male XLAS patients between transcript types (in-frame vs. out-of-frame). Moreover, it should be noted that even exonic nucleotide substitutions can cause aberrant splicing and we should consider transcript analysis of those who have variants in specific regions (end of each exons) or show an atypical phenotype for their genotype; patients with severe phenotypes accompanied by missense variants or mild phenotype by nonsense mutation. Development of novel disease specific therapy targeting splicing mechanisms for XLAS is expected in the future.

AUTHOR CONTRIBUTIONS

TH wrote the Section of intronic variants outside the canonical splice site causing abnormal splicing in *COL4A5*. YA wrote the Section of exonic variants causing abnormal splicing in *COL4A5*. TY wrote the remaining sections. RL and KN critically reviewed the manuscript. All authors read and approved the final version of the manuscript.

FUNDING

This work was supported by grant from the Uehara Memorial Foundation (No. 202040073 to TY).

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frontiers in Medicine

REVIEW
published: 09 February 2022
doi: 10.3389/fmed.2022.846152

Molecular and Cellular Mechanisms Underlying the Initiation and Progression of Alport Glomerular Pathology

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Alport syndrome results from a myriad of variants in the COL4A3, COL4A4, or COL4A5 genes that encode type IV (basement membrane) collagens. Unlike type IV collagen $\alpha1(IV)_2\alpha2(IV)_1$ heterotrimers, which are ubiquitous in basement membranes, α3/α4/α5 have a limited tissue distribution. The absence of these basement membrane networks causes pathologies in some, but not all these tissues. Primarily the kidney glomerulus, the stria vascularis of the inner ear, the lens, and the retina as well as a rare link with aortic aneurisms. Defects in the glomerular basement membranes results in delayed onset and progressive focal segmental glomerulosclerosis ultimately requiring the patient to undergo dialysis and if accessible, kidney transplant. The lifespan of patients with Alport syndrome is ultimately significantly shortened. This review addresses the consequences of the altered glomerular basement membrane composition in Alport syndrome with specific emphasis on the mechanisms underlying initiation and progression of glomerular pathology.

Keywords: glomerulus, glomerular basement membrane, podocyte, mesangial cell, pathology

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Edited by:
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Reviewed by:
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Dale Abrahamson, University of Kansas Medical Center, United States

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Specialty section:
This article was submitted to Nephrology, a section of the journal Frontiers in Medicine

Received: *30 December 2021*
Accepted: *17 January 2022*
Published: *09 February 2022*

Citation:
Cosgrove D and Madison J (2022) Molecular and Cellular Mechanisms Underlying the Initiation and Progression of Alport Glomerular Pathology. Front. Med. 9:846152. doi: 10.3389/fmed.2022.846152

INTRODUCTION

In terms of rare diseases, Alport syndrome is relatively common with recent genetic evidence suggesting a frequency approaching 1 in 2,000 people (1). Most affected individuals (80-85%) harbor variants in the X-linked COL4A5 gene, and thus are hemizygous with mosaicism in female carriers associated with a milder form of the disease who pass the variants to half of their sons. The remaining cases are caused by variants in COL4A3 and COL4A4 genes and are inherited in an autosomal recessive manner (2, 3). There is a strong genotype/phenotype association with the severity of kidney disease, with about 40% of the cases caused by Gly-X-Y missense variants, which result in a milder form of the disease and more severe variants (nonsense variants deletions, etc.) accounting for most cases (4). In most, but not all cases, the variants lead to the absence of all three type IV collagen chains in the glomerular basement membrane (GBM). This change in basement membrane composition results in a GBM that is notably thinner and contains fewer interchain disulfide crosslinks which weakens the elastic integrity of the glomerular capillary tufts making them susceptible to the mechanical forces of normal capillary blood flow (5). Consistent with this, glomeruli from Col4A3-/- Alport mice have a 30% reduction in Young's modulus, a measure of biophysical stiffness (6). When the capillary tufts are subjected to excessive forces under hypertensive conditions, the rate of disease progression is accelerated in the mouse (7). It was recently shown using intravital microscopy that the glomerular capillaries in Alport mice have a

significantly enlarged diameter compared to wild type mice likely owing to the enhanced elasticity of the thinner and less crosslinked GBM collagen network (8). This may contribute to why ACE inhibitors, which reduce intraglomerular pressure, slow the progression of Alport kidney disease in both mice and human (9–11). A recent mass-spectroscopy-based proteomics study demonstrated aan overall reduction in basement membrane proteins in glomerular matrix from Alprot mice relative to wild type mice with an overall increase in interstitial matrix proteins (12). These changes are likely to contribute to the biomechanical prpoperties of the GBM in Alport syndrome. Of note, there are some variants that change the biochemical properties of the GBM without eliminating α3/α4/α5 protomers. For example, a variant that causes in frame skipping of exon 30 results in a GBM with normal type IV collagen composition (albeit less α3/α4/α5 networks than wild type) results in a robust Alport kidney phenotype in both mice and men (13). This underscores the importance of the structural integrity of the GBM in maintaining normal GBM function and supports the idea that biomechanical strain is a major contributor to disease onset.

The mature glomerular basement membrane is two separate networks of type IV collagen; a subendothelial network comprised of type IV collagen $\alpha1_2/\alpha2$ protomers and a thicker subepithelial network comprised of type IV collagen α3/α4/α5 protomers (14). These networks contribute to both the structural integrity and the assembly of the GBM (15, 16). It has been suggested based on super resolution analysis of GBM protein architecture that the type IV collagen $\alpha1_2/\alpha2$ network might be close enough to activate podocyte collagen receptors discoidin domain receptor 1 (DDR1) and integrin α2β1 while the α3/α4/α5 network in wild type mice is central to the GBM and thus too distant to engage collagen receptors on podocytes (17). Double knockouts of COL4A3 and either DDR1 or integrin α2 show attenuated kidney disease progression, implicating these receptors as contributors to the pathobiology of Alport glomerular disease (18, 19). Definitive proof of collagen receptor activation in Alport podocytes is lacking, however.

ALPORT GLOMERULAR DISEASE ONSET

Even though the GBM type IV collagen network is congenitally structurally different in Alport syndrome, the GBM functions to prevent proteinuria and maintain a healthy glomerular filtration rate for several weeks in mice and several years in humans. If this functional competence could be maintained through therapeutic intervention, end stage kidney disease (ESRD) can be delayed, and lifespan increased. After proteinuria is established the current standard of care, angiotensin converting enzyme inhibitors (ACEi), have reduced efficacy (10, 11). This is also true in the mouse models (9). Thus, the driver(s) of glomerular disease onset should reveal therapeutic target(s) with maximum efficacy for blocking or slowing the Alport kidney disease progression.

One of the earliest events documented to date is significantly elevated expression of endothelin-1 (ET-1) in the endothelial cells of Alport glomeruli (20). This is observed as early as 2 weeks in the 129 Sv ARAS model and 5 weeks in the Bl/6 XLAS model; before proteinuria is observed and before notable ultrastructural defects in the GBM are observed. Expression of ET-1 is further elevated when mice are made hypertensive, suggesting that the induction of ET-1 is a consequence of elevated biomechanical strain on the glomerular capillary tufts. One consequence of elevated ET-1 expression is the activation of endothelin A receptors (ET_ARs) on mesangial cells. Activated ET_ARs lead to downstream activation of CDC42 resulting in the formation of mesangial filopodia. The filopodia progressively invade the subendothelial aspect of the glomerular basement membrane and deposit mesangial matrix proteins including laminin 211 in the GBM (21). Filopodial invasion was validated by 3-dimensional electron microscopy (22). In this same work, podocyte process invation of the GBM was also reported. Laminin 211 has been shown to activate focal adhesion kinase(FAK) on glomerular podocytes resulting in NF-kappaB activation which results in elevated expression of pro-inflammatory cytokines as well as matrix metalloproteinases (23). Blocking FAK or ET_ARs with small molecule inhibitors ameliorates Alport GBM disease (20, 23, 24). Laminin 211 is deposited in the GBM in Alport dog models as well as humans (20, 25) confirming this process as a relevant pathological mediator in patients with Alport syndrome. In addition to aberrant cell signaling, laminin 211 in the GBM contributes to the GBM permeability defects (26), suggesting its progressive accumulation in the GBM is relevant to the progressive increase in proteinuria. Thus, disease onset involves three glomerular cell types: cross-talk between the glomerular endothelial cells and mesangial cells resulting in the deposition of mesangial ECM in the GBM, which causes podocyte injury.

Over 20 years ago, a connection between integrin α1β1 and the deposition of laminin 211 in Alport GBM was described where deletion of *ITGA1* significantly slowed the rate of laminin 211 deposition in the GBM and increased lifespan by 50% (27). Since then, we noted that α1β1 integrin in glomerular mesangial cells is required for lipopolysaccharide-mediated activation of CDC42 and the formation of mesangial filopodia (21). Thus, there appears to be a convergence between blocking integrin α1β1 receptors and blocking ET_ARs in the activation pathway for CDC42 in glomerular mesangial cells and thus α1 integrin itself may be a relevant target for blocking events related to glomerular disease initiation in Alport syndrome. Key aspects of Alport glomerular disease initiation are summarized in **Figure 1**.

ALPORT GLOMERULAR DISEASE PROGRESSION

Once the mesangial filopodia begin to invade the subendothelial space of the glomerular capillaries, we begin to observe the deposition of mesangial matrix proteins in the GBM. This includes laminin 211, which has been implicated in directly injuring podocytes (23). While podocyte injury by laminin α2 was shown to be mediated by FAK activation leading to NFkappaB activation and the induction of MMPs and pro-inflammatory cytokines, the specific receptors on podocytes that mediate FAK activation have not yet been identified. Collagen-mediated podocyte damage was shown to be the result of DDR1

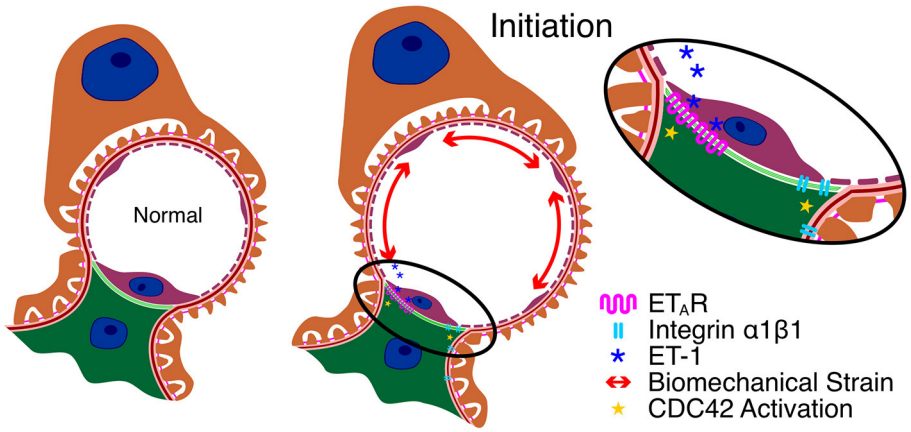

FIGURE 1 | Key aspects of Alport glomerular disease initiation. Due to the change in basement membrane composition, the GBM in Alport syndrome is thinner and more elastic, resulting in increased capillary diameter and biomechanical strain on the cells that adhere to the capillary. This results in elevated expression of ET-1 in the endothelial cells which activates ET_ARs on mesangial cells resulting in CDC42 activation. Blockade of integrin α1β1 on mesangial cells prevents ET_AR-mediated CDC42 activation and thus prevents initiation through an unknown mechanism.

and integrin α2β1 activation (18, 19). The downstream effector functions of this co-receptor signaling includes both previously recognized Alport glomerular disease mediators as well as several genes that have been implicated in podocyte injury and/or CKD in other disease models but not yet implicated in Alport glomerular pathogenesis. Previous work showed that double knockouts of COL4A3 and either DDR1 or integrin α2β1 resulted in attenuated kidney disease progression in the autosomal Alport mouse model, clearly implicating aberrant DDR1 and/or α2β1 signaling in Alport glomerular pathogenesis (18, 19).

One of the earliest cytokines implicated in Alport kidney and glomerular pathogenesis was TGF-β1 (28, 29). In the glomerulus, TGF-β1 blockade prevented GBM dysmorphology but did not improve lifespan (27) in the interstitium, TGF-β1 blockade prevented the genesis of interstitial myofibroblasts and interstitial fibrosis (30). TGF-β1 signaling has been directly implicated in studies using STAT3 inhibitors which slowed the progression of Alport kidney pathogenesis in a mouse model (31). Blockade of the Activin type II receptor, a member of the TGF-β receptor family, attenuated Alport glomerular and interstitial disease progression as well (32). BMP-7 is an inhibitor of the TGF-β signaling cascade that induces fibrosis. It has been shown in animal models, including an Alport mouse model, that recombinant BMP-7 ameliorates kidney disease (33). Along these same lines, deletion of the natural BMP-7 antagonist uterine sensitization-associated gene 1 (USAG1) in an Alport mouse model ameliorated progression of glomerular and interstitial disease (34). TGF-β1 might be an early indicator of Alport kidney pathogenesis as both serum and urinary TGF-β1 were significantly higher in pre-proteinuric Alport patients compared to controls (35). Collectively this work makes a definitive connection between TGF-β and Alport kidney disease progression.

Cholesterol accumulates in the podocytes in models of focal segmental glomerulosclerosis (FSGS) as well as Alport syndrome (36). Treatment with cyclodextrins which promote cholesterol efflux ameliorates the progression of Alport kidney disease. These compounds cause significant hearing loss in humans (37) necessitating the identification of alternate means to stimulate cholesterol efflux. Recently small molecules have been identified that activate ATP-binding cassette transporter (ABCA1)-mediated cholesterol efflux via targeting Oxysterol Binding Protein Like 7 (OSBPL7) (38). This approach also ameliorated Alport kidney disease progression and increased lifespan by about 20%. Interestingly DDR1 activation is linked to lipotoxicity (39), which may partially explain why deletion of DDR1 in Alport mice slows kidney disease progression (18).

Capillary endothelial cell damage also contributes to Alport glomerular disease progression. It was shown that injection of amniotic fluid stem cells would slow the progression of Alport kidney disease (40). It was later shown that vesicles derived from these stem cells protect against VEGF-induced endothelial cell damage, showing that VEGF plays an important role in Alport glomerular pathology and that trapping of VEGF by these vesicles represents a viable targeted approach for slowing the disease progression (41).

Podocytopenia is observed in Alport syndrome. Tumor necrosis factor-α has been implicated in podocyte apoptosis that might contribute to reduced podocyte numbers (42). More recently it was shown that Alport podocytes during progression of the glomerular disease re-enter the cell cycle, passing the G1/S checkpoint which can lead to podocyte detachment, contributing to reduced podocyte numbers (43). Podocytopenia has been directly linked to glomerulosclerosis in models of FSGS, suggesting that TNF-α may be a prominent effector of glomerular disease progression in Alport syndrome (44). Key aspects of Alport glomerular disease progression are summarized in **Figure 2**.

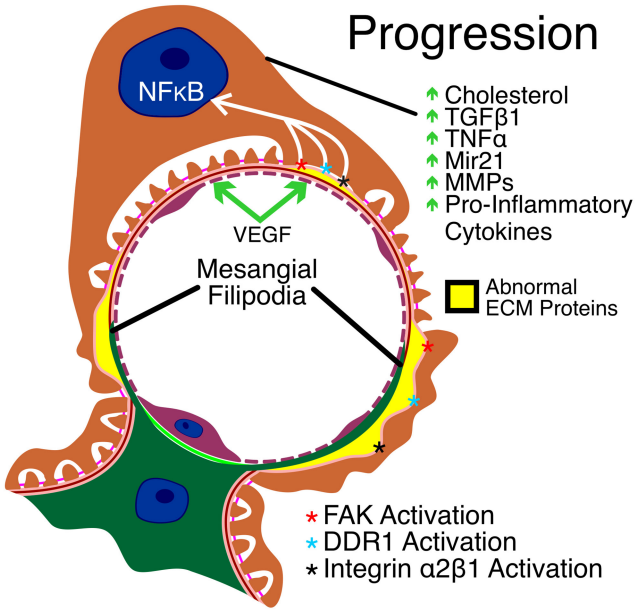

FIGURE 2 | Key aspects of Alport glomerular disease progression. As a result of CDC42 activation, mesangial filopodia invade the space between the endothelial cells and the GBM. The abnormal ECM accumulates in the apical aspects of the GBM, contributing to the irregular thickening of the GBM (in yellow) and activating yet unknown laminin α2 receptors resulting in FAK activation and downstream NF-kappaB activation. In addition, collagen α1(III) in the GBM activates DDR1 and α2β1 receptors on podocytes leading to further podocyte injury. The resulting injury to podocytes in mediated by cholesterol retention and elevated expression of TGF-β1, TNF-α, mir21, MMPs and several pro-inflammatory cytokines. Endothelial cell injury is mediated by elevated VEGF.

CLINICAL TRIALS: WHERE ARE WE NOW?

Many potential therapeutic targets have been inferred from pre-clinical studies of the mouse model, but never made it to clinical trials. These include key cell signaling targets including the BMP antagonist USAG1 (34), α1β1 integrin (27) α2β1 integrin (19), DDR1 (18), αvβ6 integrin (45), STAT3 (31), lysyl oxidase like-2 (46), focal adhesion kinase (23) and osteopontin (47). Other targets include epigenetic modification by histone deacetylases (48), and matrix metalloproteinases including MMP 12 (49). Recently the diabetes drug metformin was shown to ameliorate the severity of Alport kidney disease in the XLAS mouse model (50). While metformin had no effect on extending lifespan in the 129 Sv ARAS model it did moderately increase lifespan in the Bl/6 XLAS model. All of these approaches act on elements of disease progression, which likely accounts for their limited capacity to slow disease progression and increase lifespan.

Other pre-clinical studies have been conducted that attempt to replace the defective type IV collagen gene product with a fully functional one. Since these approaches actually aim to repair the GBM composition, they have the capacity to arrest glomerular disease initiation and thus provide maximum therapeutic benefit. The first attempt was using gene therapy to introduce a full-length type IV collagen α5 mRNA into dog podocytes by direct effusion of viral particles into the kidney artery. The work showed some promise but was ultimately unsuccessful (51). Heidet et al. (52) showed that introduction of the human α3(IV)/α4(IV) genes by way of transgenesis using a yeast artificial chromosome rescued the phenotype, albeit small defects in the GBM were observed. Related to this, Lin et al. (53) showed using a tamoxifen-inducible transgene, that introduction of full-length collagen α3(IV) would fully rescue when the transgene was induced at post-natal day 1, and to a lesser extent when introduced at post-natal day 21 (pre-proteinuric mice), providing proof of concept that gene therapy (or gene editing) may be a feasible approach for improving kidney function in Alport patients. Stem cell therapy in Alport mice has also been attempted using bone marrow-derived stem cells (54, 55) and stem cells from amniotic fluid (40) with very limited success.

Several therapeutic approaches are in either the planning or active stages of clinical trials in Alport patients. These include Bardoxylone methyl, a nuclear factor erythroid 2–related factor 2 (NRF2) agonist that regulates reduction/oxidation reactions and NF-kappa B activation, which successfully filed an NDA. Recent setbacks suggest that this approach might not meet FDA expectations. Concerns included a lack of pre-clinical work in animal models and a poorly understood increase in proteinuria that may reflect elevated intraglomerular pressure which itself could be pathologic. The endothelin A receptor antagonist (ET_AR) atrasentan is currently in Phase 2 clinical trials for Alport syndrome. Of note, a similar ET_AR antagonist, sitaxentan, improved kidney function in Alport mice, but increased lifespan by only 20% (20). Recruitment of Alport patients has begun for a planned trial using the anti-microRNA-21 Lademirsen, which was shown in Alport mice studies to improve kidney function and lifespan by 50% (56). Preliminary work in humans using sodium-glucose cotransporter-2 inhibitors showed some promise in Alport patients and other forms of CKD, however larger numbers will be needed to see if the data is indeed significant (57). Importantly, none of these therapeutic approaches has been shown in animal models to provide any benefit beyond the standard of care (ACEi or ARBs) except for metformin, and for this only in the 129 Sv ARAS model (50).

SUMMARY

The initiation and progression of Alport glomerular pathogenesis involves extensive cellular crosstalk between the endothelial cells, mesangial cells, and podocytes. Mesangial cell-derived ECM deposition in the GBM clearly plays an important role in aberrant podocyte cell signaling resulting in podocyte injury which is a major of glomerular disease progression. Given that Alport syndrome is the result of variants in COL4A3, COL4A4 or COL4A5 genes, the ultimate therapy would correct the variant(s) using a gene editing approach, replace the defective network using recombinant collagen α3/α4/α5 protomers, or introduce a correct copy of the defective gene to enough podocytes to be therapeutically beneficial. Such procedures would likely need to be administered before the onset of

proteinuria. Until such therapies are available the best approach would be to delay the glomerular disease onset for as long as possible and then use approaches that slow progression additively or synergistically with the standard of care (ACEi or ARBs).

AUTHOR CONTRIBUTIONS

DC wrote and edited the manuscript. JM produced the figures and edited the manuscript. Both authors contributed to the article and approved the submitted version.

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frontiers in Medicine

ORIGINAL RESEARCH
published: 07 March 2022
doi: 10.3389/fmed.2022.847056

Detection of Very Low-Level Somatic Mosaic *COL4A5* Splicing Variant in Asymptomatic Female Using Droplet Digital PCR

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Background: Alport syndrome is a hereditary glomerulopathy featured by haematuria, proteinuria, and progressive renal failure. X-linked Alport syndrome (XLAS) due to *COL4A5* disease-causing variants is the most common form. In the case of XLAS resulting from 10–18% presumed *de novo COL4A5* disease-causing variants, there are only a few studies for mosaicism in the probands or parents. Very low-level (<1.0%) somatic mosaicism for *COL4A5* disease-causing variants has not been published.

Materials and Methods: Chinese XLAS families with suspected parental mosaicism were enrolled in the present study to evaluate the forms of mosaicism, to offer more appropriate genetic counseling. PCR and direct sequencing were used to detect *COL4A5* disease-causing variants harbored by the affected probands in parental multi-tissue DNAs (peripheral blood, urine sediments, saliva, hair), and droplet digital PCR (ddPCR) was used to quantify the mutant *COL4A5* allelic fractions in parental different samples such as peripheral blood, saliva, and urine sediments.

Results: A Chinese asymptomatic female with suspected somatic and germline mosaicism was enrolled in the present study. She gave birth to two boys with XLAS caused by a hemizygous disease-causing variant c. 2245-1G>A in *COL4A5* (NM_033380) intron 28, whereas this disease-causing variant was not detected in genomic DNA extracted from peripheral blood leukocytes in the woman using Sanger sequencing. She had multiple normal urine test results, and continuous linear immunofluorescence staining of α2 (IV) and α5 (IV) chains of skin tissue. Sanger sequencing demonstrated that *COL4A5* disease-causing variant c. 2245-1G>A was not detected in her genomic DNAs isolated from urine sediments, saliva, and hair roots. Using ddPCR, the wild-type and mutant-type (c.2245-1G>A) *COL4A5* was identified in the female's genomic DNAs isolated from peripheral blood, saliva, and urine sediments. The mutant allelic fractions in these tissues were 0.26% (peripheral blood), 0.73% (saliva), and 1.39% (urine), respectively.

OPEN ACCESS

Edited by:
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The University of Melbourne, Australia

Reviewed by:
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Specialty section:
This article was submitted to Nephrology, a section of the journal Frontiers in Medicine

Received: *01 January 2022*
Accepted: *09 February 2022*
Published: *07 March 2022*

Citation:
Deng H, Zhang Y, Ding J and Wang F (2022) Detection of Very Low-Level Somatic Mosaic COL4A5 Splicing Variant in Asymptomatic Female Using Droplet Digital PCR. Front. Med. 9:847056. doi: 10.3389/fmed.2022.847056

Conclusions: Germline and very low-level somatic mosaicism for a *COL4A5* splicing variant was detected in an asymptomatic female, which highlights that parental mosaicism should be excluded when a *COL4A5* presumed *de novo* disease-causing variant is detected.

Keywords: Alport syndrome, COL4A5, somatic mosaicism, germline mosaicism, *de novo* disease-causing variant

INTRODUCTION

Alport syndrome is a hereditary glomerulopathy featured by haematuria, proteinuria, and progressive renal failure. Extrarenal manifestations such as sensorineural hearing loss and ocular abnormalities are often detected. This disease occurs in less than 1:2000 (1) and is caused by disease-causing variants in *COL4A3/A4/A5*, which encode the glomerular basement membrane protein type IV collagen α3–α5 chains, respectively. X-linked Alport syndrome (XLAS) due to *COL4A5* disease-causing variants is the most common form and accounts for 85% (2).

In comparison to male patients with XLAS, female patients have a lower probability of progression to kidney failure at the same age, there is no correlation between their renal outcomes and extrarenal manifestations with COL4A5 genotypes (3). The random X-chromosome inactivation is considered as the reason for the heterogeneity of disease severity in female XLAS cases (4). It is worth noting that low-level (variant allele fraction >1.0%) mosaicism for *COL4A5* disease-causing variants has been observed in rare females with mild phenotypes or even normal urinalysis (5–8), demonstrating that mosaicism influences the disorder manifestations.

Mosaicism, in a diverse range of monogenic disorders, is a biological phenomenon which delineates an individual possessing at least two populations of genetically distinct cells arising from a single zygote (9, 10), and somatic mosaicism is caused by postzygotic *de novo* pathogenic variants (11). Recurrence risk counseling is challenging for a patient with a monogenic disease due to a presumed *de novo* disease-causing variant (12). If this disease-causing variant was inherited from mosaicism in one of the healthy parents, the mosaic parent is at an increased risk of passing on the same disease-causing variant to additional offspring with the risk depending on the proportion of mosaicism within the germ cell progenitors. If this disease-causing variant occurred post-zygotically in the proband, then sibling recurrence risk is very low.

In the case of XLAS resulting from 10–18% presumed *de novo COL4A5* disease-causing variants (13), there are only a few studies for mosaicism in the probands or parents (6, 14–18). To our knowledge, very low-level (variant allele fraction <1.0%) (19) somatic mosaicism for *COL4A5* disease-causing variants has not been published. Here, we describe an asymptomatic female with mutant *COL4A5* allelic fraction (MAF) of 0.26–1.39% in somatic tissues detected by droplet digital PCR (ddPCR).

MATERIALS AND METHODS

The clinical data and biological samples of Alport syndrome family in this study were enrolled from a multicentre online hereditary renal diseases registry database and biological sample bank established by Peking University First Hospital in 2012. This study was performed in accordance with the Declaration of Helsinki and approved by the Ethical Committee of Peking University First Hospital (approval number: 2020[72]).

Patients

Chinese XLAS families with suspected parental mosaicism, who were registered in a multicentre online registry of hereditary kidney diseases, admitted to our hospital during the period of March 2005 to August 2019, and met the inclusion and exclusion criteria were screened in our study. The inclusion criteria were as follows: (1) the probands with XLAS; (2) Sanger sequencing showed *COL4A5* disease-causing variants harbored by the probands did not be detected in their parental DNA samples extracted from peripheral blood leukocytes (3) the probands had at least one sibling with the same *COL4A5* disease-causing variant. Monozygotic twins or patients with unavailable medical records were excluded.

The diagnostic criteria of XLAS were as follows (20): (1) glomerular haematuria with or without proteinuria; (2) negative or intermittent positive immunofluorescence staining for the α5 (IV) chain in the epithelial basement membrane (EBM); (3) *COL4A5* pathogenic variants. XLAS patients were required to meet the criteria (1) along with (2) or (3).

Research Methods

Clinical Data

Patient information, including sex, age, weight, height, family history, clinical findings (age at onset of disease, renal and extrarenal features), and skin and renal biopsy results and genotypes, were reviewed.

Genetic Analysis

Genomic DNA from peripheral blood was isolated using FlexiGene DNA Kit (QIAGEN, 51206). Genomic DNA from saliva, hair, and urine sediments were isolated using the QIAamp DNA Micro Kit (Qiagen, 56304).

Multi-tissue DNAs were analyzed by PCR and direct sequencing to detect a *COL4A5* mutation. A 453 bp fragment including *COL4A5* exon 29 was amplified by PCR using a pair of primers (F: 5'-ACCCTGTTTCCAATCCTTCC-3', R: 5'- ATGGCAGCATAGGGTTTCC-3'), which were designed according to the published sequence [UCSC Genome Browser

and human GRCh37/hg19 (http://genome.ucsc.edu/)], and analyzed by direct sequencing. The PCR mixture contained 1 μl DNA template, 12.5 μl of 2×PCR Solution (Takara, RR030A), 1 μl forward and reverse primer (10 pmol/ml), respectively, and 9.5 μl deionized water. Touch-down PCR was used with the following thermal cycling conditions: 94°C for 5 min, 94°C for 30 s, 64°C for 30 s, and 72°C for 45 s. The annealing temperature reduced 1°C every 2 cycles (from 64 to 58°C), 26 cycles at the final annealing temperature of 58°C, with a final extension at 72°C for 10 min, and termination at 4°C.

Detection and Quantification of Mosaicism

Droplet digital PCR was used to test parental peripheral blood, saliva, and urine sediments DNA to detect mosaicism *via* TD-1^{TM} Digital Droplet^{TM} PCR system (TargetingOne, licensed in China, registration number: 20170025; 20190065; 20192220517). The ddPCR mixture was prepared for each sample with 15 μl 2×SuperMix, 3 μl probes (final concentration 200 nM each) and primers (final concentration 400 nM each) reaction mix, 2 μl DNA templates, and 10 μl deionized water. The ddPCR thermal profile was as follows: 95°C for 10 min, 40 cycles of 95°C for 30 s and 60°C for 1 min, and 1 cycle of 12°C for 5 min. Data analysis procedure was same as described in previous study (21, 22).

RESULTS

According to the inclusion and exclusion criteria, one of 24 patients with *COL4A5* presumed *de novo* disease-causing variants was enrolled. Familial pedigree of the male proband (III1) is shown in **Figure 1A**. He was found to have haematuria and proteinuria [urinary total protein (UTP): 1.2 g/24h] at the age of 11 years. Renal biopsy, performed at the age of 12 years, indicated mesangial proliferative glomerulonephritis under light microscope, and irregular thickness, lamellated glomerular basement membrane (GBM) under electron microscope. Immunofluorescence staining of skin tissue showed positive for α2 (IV) chain (positive control) and negative for α5 (IV) chain. At the age of 13 years, his serum creatinine was 122 μmol/l. He was diagnosed with chronic kidney disease G5 stage (estimated glomerular filtration rate: 5 ml/min/1.73 m^2) at the age of 14 years, and died of chronic renal failure at the age of 18 years. The proband's half young brother (III2) was found to have haematuria (110–120/high power field) and proteinuria (UTP: 0.25 g/24h) at the age of 8 years. Immunofluorescence staining of his skin sample showed positive for α2 (IV) chain and negative for α5 (IV) chain. The proband's mother (II2) had multiple normal urine test results,

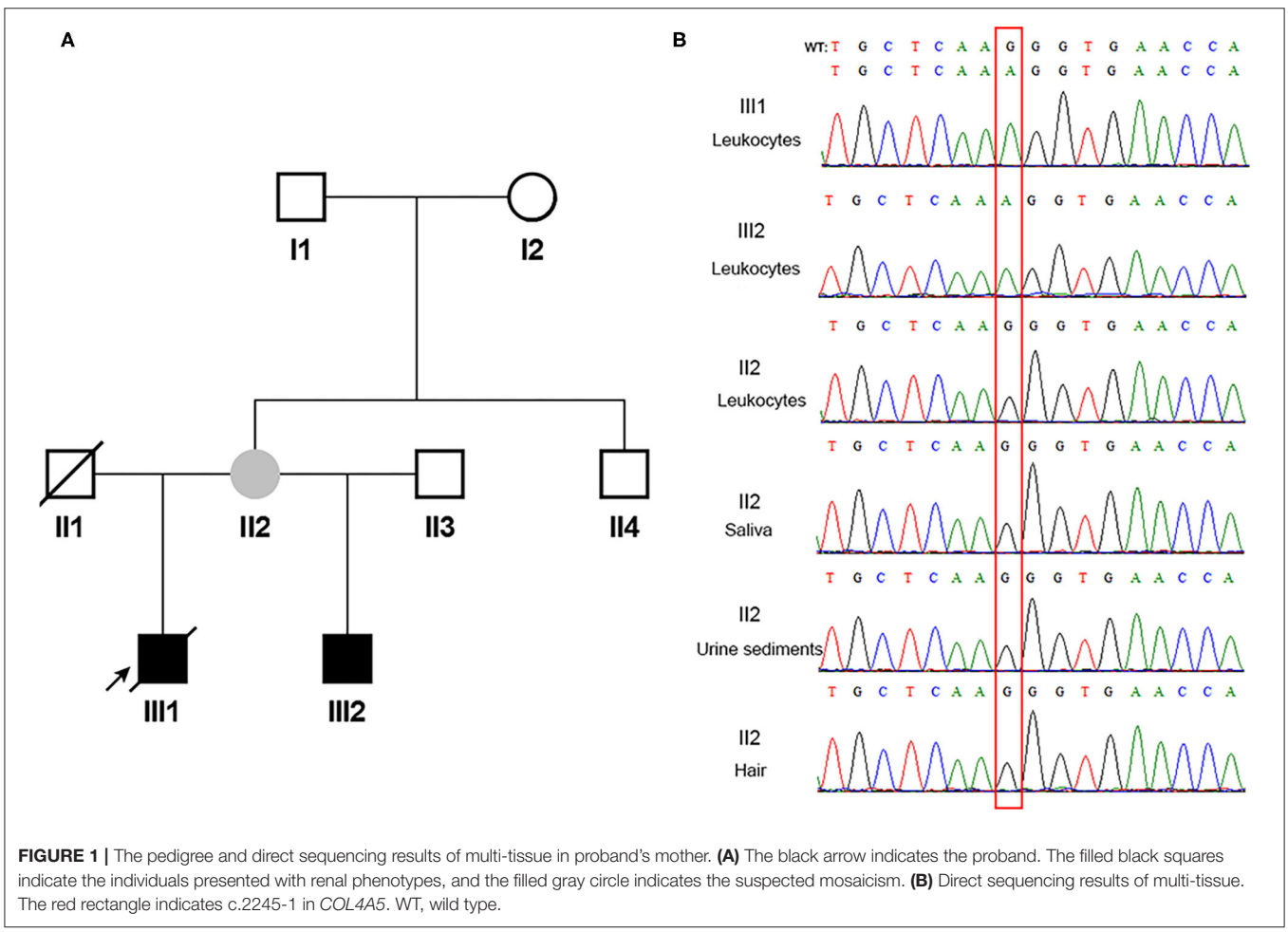

FIGURE 1 | The pedigree and direct sequencing results of multi-tissue in proband's mother. **(A)** The black arrow indicates the proband. The filled black squares indicate the individuals presented with renal phenotypes, and the filled gray circle indicates the suspected mosaicism. **(B)** Direct sequencing results of multi-tissue. The red rectangle indicates c.2245-1 in *COL4A5*. WT, wild type.

and continuous linear immunofluorescence staining of α2 (IV) and α5 (IV) chains of skin specimen. A splicing variant [*COL4A5* (NM_033380) IVS28: c.2245-1G>A] in a hemizygous state was detected in genomic DNA extracted from peripheral blood leukocytes in the proband and his half young brother by Sanger sequencing, whereas their mother was wild-type (**Figure 1B**).

Since the asymptomatic mother gave birth to two boys with XLAS, both maternal germline *de novo COL4A5* mutation and somatic and germline postzygotic *COL4A5* mosaicism were highly suspected. Her multi-tissue DNA (saliva, urine sediments, and hair) were extracted and analyzed using PCR and Sanger sequencing. **Figure 1B** shows that the sites were all wild-type in these tissues.

Droplet digital PCR, which was carried out since Sanger sequencing, failed to detect mosaic disease-causing variants. It was observed that the mother harbored the wild-type and mutant-type (c.2245-1G>A) *COL4A5* genes, and the MAFs in different tissues were 0.26% (peripheral blood), 0.73% (saliva), and 1.39% (urine sediments), respectively (**Figure 2**). Therefore, a somatic mosaicism was diagnosed. In addition, germline mosaicism was also diagnosed since she successively gave birth to two affected sons.

DISCUSSION

In this study, we described a germline and very low-level somatic mosaicism for a *COL4A5* pathogenic splicing variant in an asymptomatic female. This phenomenon was easily overlooked by analyzing Sanger sequencing results, whereas it was indicated through ddPCR method. It highlights that parental mosaicism should be excluded when a *COL4A5 de novo* disease-causing variant is detected.

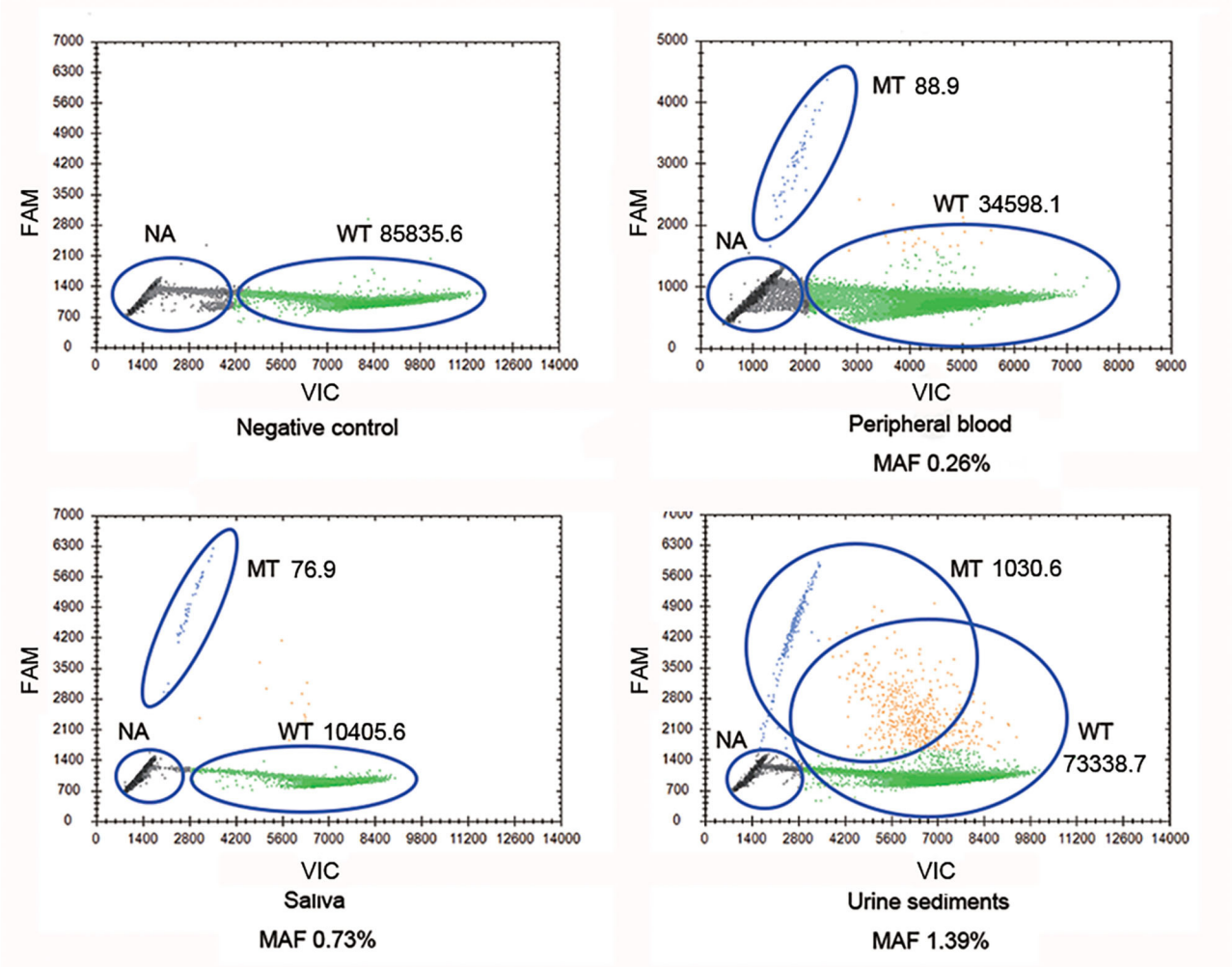

FIGURE 2 | *COL4A5* wild-type and mutant-type (c.2245-1G>A) alleles were identified in multiple samples (blood, saliva, and urine sediments) from proband's mother by droplet digital PCR (ddPCR). WT signals are from droplets containing wild-type alleles, MT signals are from droplets containing mutant alleles, NA signals are from droplets that did not contain target sequences, thus could not be amplified. WT, wild type; MT, mutant type; NA, not available.

Mosaicism is a condition where an individual is composed of at least two populations of cells with distinct genotypes. Based on the occurring time and distribution of the mosaic mutation, there are three types of mosaicism (23, 24): somatic (disease-causing variant occurs only in the cells of the body), germline (disease-causing variant occurs only in the germ cells), and somatic combined with germline (disease-causing variant occurs in both germ and body cells). The latter two types of mosaic parents can share the pathogenic variant with their offspring, and the recurrence risk depends on the germline mosaic ratio. As far as XLAS is concerned, parental germline mosaicism or somatic and gonadal mosaicism of *COL4A5* disease-causing variants can transmit the genotypes to the affected offspring. Therefore, it is very essential to identify parental mosaicism earlier to avoid faulty genetic counseling in Alport syndrome families.

At present, a variety of single-gene diseases have been reported that causative genes disease-causing variants mutations could be inherited from mosaic parents (25–27). If a disease-causing variant occurs in the early stage of embryogenesis, a high ratio of disease-causing variant in body and germline cells such as peripheral blood leukocytes could be detected by Sanger sequencing (28, 29). However, Sanger sequencing has limited sensitivity and precision, as only mosaicism with MAF higher than 10% can be effectively identified (30). If a disease-causing variant occurs in the late stage of embryonic cell differentiation, which leads to a low-level of somatic mosaicism, the recognition of mosaicism may be dependent on methods with high analytical sensitivity and precision. Targeted next-generation sequencing can detect a low (MAF in the range of 1–2%) level mosaicism (18). On the other hand, ddPCR can absolutely quantify nucleic acids, the sensitivity can reach 10^{-4} (31, 32), and the detect resolution can be single DNA template. In this study, the proband's mother was not found to be mosaic at the known pathogenic variant site in multi-tissue DNA by Sanger sequencing. Nevertheless, the very low-level MAFs ranging from 0.26 to 1.39% was demonstrated by ddPCR in different tissues. Taking account of her two affected sons, the diagnosis of somatic and germline mosaicism was confirmed. In addition, the fact that she successively gave birth to two affected sons demonstrated her eggs might have a higher fraction of *COL4A5* splicing variant. Since parental germline mosaicism with or without somatic mosaicism can transmit a pathogenic variant from an unaffected parent to their affected offspring, clinicians must consider the possibility of parental mosaicism when facing XLAS patients with presumed de novo disease causing variants, and prenatal or preimplantation genetic testing should be recommended in order to prevent their parents from giving birth to another affected child.

There are few reports about relationship between MAF and phenotypes in XLAS mosaicism patients. For male mosaicism, the lower the MAF, the milder the symptoms. When MAF $<$50%, the patient may only present with haematuria or even be asymptomatic (18). However, the phenotypes in female mosaicism may be influenced by MAF in kidney, skewed X-chromosome inactivation (33), and modifier genes (34). A previous study showed that an 11-year-old girl had a *COL4A5* disease-causing variant frequency of 22.1% in peripheral blood leukocytes, but she showed moderate proteinuria, which was a severe phenotype for a female. The heterozygous disease-causing variant in modifier gene *COL4A3* may affect disease severity (7). In this study, the proband's mother had multiple normal urinary test results, which could relate to low-level of MAF in the kidney.

In conclusion, we reported a germline and very low-level somatic mosaicism for a *COL4A5* splicing variant in an asymptomatic female, which highlights that parental mosaicism should be excluded when a *COL4A5 de novo* disease-causing variant is identified.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Committee of Peking University First Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

HD and FW designed the research study and wrote the manuscript. HD performed the research. HD and YZ analysed the data. FW edited the manuscript. JD provided patient's clinical data, offered advice on designing the research and edited the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

FUNDING

This work was supported by National Key Research and Development Program of China, the registry study of rare diseases in children (No. 2016YFC0901505), National Nature Science Foundation (81070545), Beijing Nature Science Foundation (7102148), and Beijing key laboratory of molecular diagnosis and study on paediatric genetic diseases (BZ0317).

ACKNOWLEDGMENTS

We thank all patients and their families for participating in this study.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

ORIGINAL RESEARCH
published: 21 March 2022
doi: 10.3389/fmed.2022.838983

Presumed *COL4A3/COL4A4* Missense/Synonymous Variants Induce Aberrant Splicing

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Background: The incorrect interpretation of missense and synonymous variants can lead to improper molecular diagnosis and subsequent faulty genetic counselling. The aim of this study was to evaluate the pathogenicity of presumed *COL4A3/COL4A4* missense and synonymous variants detected by next-generation sequencing to provide evidence for diagnosis and genetic counselling.

Methods: Patients' clinical findings and genetic data were analysed retrospectively. An *in vitro* minigene assay was conducted to assess the effect of presumed *COL4A3/COL4A4* missense and synonymous variants on RNA splicing.

Results: Five unclassified *COL4A3/COL4A4* variants, which were detected in five of 343 patients with hereditary kidney diseases, were analysed. All of them were predicted to affect splicing by Human Splicing Finder. The presumed *COL4A3* missense variant c.4793T > G [p. (Leu1598Arg)] resulted in a loss of alternative full-length transcript during the splicing process. The *COL4A3* transcript carried synonymous variant c.765G > A [p. (Thr255Thr)], led to an in-frame deletion of exon 13. Nevertheless, variants c.3566G > A [p. (Gly1189Glu)] in *COL4A3* and c.3990G > A [p. (Pro1330Pro)], c.4766C > T [p. (Pro1589Leu)] in *COL4A4* exhibited no deleterious effect on splicing. Among the five patients harbouring the abovementioned *COL4A3/COL4A4* variants, three patients were genetically diagnosed with autosomal recessive Alport syndrome, one patient was highly suspected of having thin basement membrane nephropathy, and the other patient was clinically diagnosed with Alport syndrome.

Conclusions: *COL4A3* presumed missense variant p. (Leu1598Arg) and synonymous variant p. (Thr255Thr) affect RNA splicing, which highlights the prime importance of transcript analysis of unclassified exonic sequence variants for better molecular diagnosis and genetic counselling. Meanwhile, the reliability of splicing predictions by predictive tools for exonic substitutions needs to be improved.

Keywords: *COL4A3*, *COL4A4*, splicing variant, minigene, genetic counselling

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Nephrology, a section of the journal Frontiers in Medicine

Received: *19 December 2021*
Accepted: *07 February 2022*
Published: *21 March 2022*

Citation:
Deng H, Zhang Y, Ding J and Wang F (2022) Presumed COL4A3/COL4A4 Missense/Synonymous Variants Induce Aberrant Splicing. Front. Med. 9:838983. doi: 10.3389/fmed.2022.838983

INTRODUCTION

The widespread application of next-generation sequencing (NGS) in clinical settings is leading to incredible progress in the molecular diagnostics of human inherited disorders. However, a large number of unknown significance or unclassified variants in disease genes pose a challenge for genetic diagnosis and subsequent genetic counselling. For example, *COL4A3/COL4A4* are the causative genes of both autosomal inherited Alport syndrome and thin basement membrane nephropathy (1), which are chronic haematuria nephropathies with significantly different prognoses. Currently, 22% of the reported *COL4A3/COL4A4* variants are classified as uncertain significance, and most of them are missense variants (refer to ClinVar database, https://www.ncbi.nlm.nih.gov/clinvar). It is difficult to assess such variants as pathogenic or neutral based solely on genomic analysis.

A study showed (2) that 5–10% of human genes contain at least one region where synonymous variants could be harmful. Some missense and synonymous variants could interfere with protein expression, conformation or function by affecting precursor mRNA (pre-mRNA) splicing, although they may only change one amino acid or not at genomic DNA level (3, 4). Our previous study demonstrated that 6 presumed missense variants caused splicing defects by analysing *COL4A5* mRNA in skin fibroblasts (5). Therefore, transcript analysis to demonstrate the effect of unknown significance variants on pre-mRNA splicing is crucial during the assessment of their pathogenicity.

In recent years, a variety of bioinformatics tools have been developed and applied to predict their effects on pre-mRNA splicing. Nevertheless, the predicted *in silico* results may not necessarily match the transcription process *in vivo* (6), and functional experiments are still needed. Our previous study increased the detection rate of *COL4A5* splicing mutations to 25% by analysing the cDNA of skin fibroblasts (17.6% on average) (7). However, since *COL4A3/COL4A4*-encoding proteins are expressed in the kidney, cochlea, and eyes, it is difficult to obtain appropriate material from patients for transcript analysis. A minigene assay *in vitro*, an attractive alternative method to assess the impact of unclassified variants on splicing, is a simple and useful solution to overcome the unavailability of patient-derived specimens.

In this study, we used a minigene assay to evaluate the pathogenicity of five presumed missense and synonymous variants in *COL4A3/COL4A4* detected by NGS to provide evidence for diagnosis and genetic counselling.

MATERIALS AND METHODS

The clinical data and gene testing results of all patients in this study were retrospectively collected from the online hereditary renal diseases registry database established in 2012 by Peking University First Hospital, the procedures were approved by the Ethical Committee of Peking University First Hospital (approval number: 2016[1179]), and informed consent was obtained from the participants or their parents.

Patients

Patients who were registered in the online registry of hereditary kidney diseases, admitted to our hospital from February 2015 to September 2018 and met the inclusion and exclusion criteria were screened in our study. The inclusion criteria were as follows: (1) haematuria; (2) missense or synonymous variants in *COL4A3/COL4A4* detected by NGS; (3) extremely rare variants with minor allele frequency <0.01% (8); (4) variants in a highly conserved amino acid of the protein; (5) harmful variants predicted by bioinformatics tools (Mutation Taster/SIFT/Polyphen-2/GERP++_RS/CADD_phred); and (6) Human Splicing Finder predicted that the variants affected splicing. We excluded patients detected with *COL4A3/COL4A4* pathogenic/likely pathogenic variants evaluated by American College of Medical Genetics and Genomics (ACMG) guidelines or those with unavailable medical records.

Research Methods

Clinical Data and Genotypes

Patient information, including sex, age, height, weight, family history, clinical findings (age at onset of disease, renal and extrarenal features), skin and renal biopsy results, and genotypes, was reviewed.

All *COL4A3/COL4A4* variants were verified by Sanger sequencing, and direct sequencing of corresponding variants was performed if parental genomic DNA samples were available. Meanwhile, the effect on RNA splicing of these variants was assessed using another three available splicing prediction tools including NNSPLICE (http://www.fruitfly.org/seq_tools/splice.html), NetGene2 (http://www.cbs.dtu.dk/services/NetGene2/), and MaxEntScan (http://hollywood.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html). NNSPLICE and NetGene2 present scores of 0–1 for the predicted site; the higher the score the more likely a variant is a splicing site.

Nomenclature

All genetic sequences involved in this study were referred to the UCSC Genome Browser and human GRCh37/hg19 (http://genome.ucsc.edu/). The DNA sequence numbering was based on the cDNA sequence for *COL4A3* (NM_000091.5)/*COL4A4* (NM_000092.5), following the recommendations of the Human Genome Variation Society (HGVS, c.1 represented the first position of the translation initiation codon).

Minigene Assay

Generation of Minigene Constructs

The assay relied on the use of a minigene vector, which contained a fragment of *COL4A3*/*COL4A4*. The genomic segment encompassing the variant exon sequence and at least one exon nearby it, along with 150 bp of the corresponding flanking intronic sequence, were inserted into the minigene expression plasmid pcDNA3.1 (ViGene Biosciences, Shandong, China). Two minigene constructs (wild-type and mutant-type) were built, and the sequence was verified by direct sequencing after synthesis.

TABLE 1 | PCR primer sequence.

Variants	Forward primer(5′-3′)	Reverse primer(5′-3′)
A	CCTGCAGCGATTTACCACAA	TGTGGGGAAACAAATTGAAACTT
B	GGAACCCTGGTGCTCAAG	TCCCATGTCTCCTGCAGTTC
C	CTGGCTAGCGTTTAAACTTAAGC	TTGCTCCCTTGTCTCCCTTT
D	TCCCTGGGAGTGTTGACCT	GGCCCTTTTCTCCCTGGA
E	GTTCTCCACAGTCAGACGGA	CTGGAGCAGAGGAAAACTGC

Cell Culture and Transfection

HEK293T cells were cultured in DMEM–GlutaMAX (Gibco, New York, USA) containing 10% foetal bovine serum (Gibco, New York, USA) and 1% penicillin–streptomycin amphotericin (Gibco, New York, USA). The wild-type and mutant minigene constructs were transiently transfected into HEK293T cells using jetPRIME (Polyplus, Strasbourg, France) according to the manufacturer's instructions. Cells were harvested 24 h post-transfection.

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT–PCR) Analysis

Total RNA was extracted from the transfected cells using TRIzol reagent (Invitrogen, California, USA) according to the manufacturer's instructions. The RT experiment was performed using TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China). The RT–PCR thermal profile was as follows: 25°C for 10 min, 42°C for 30 min, and 85°C for 5 s.

PCR Amplification of cDNA

Five pairs of primers were designed to amplify the resulting cDNA by PCR (**Table 1**). The PCR parameters were optimised as follows: 94°C for 5 min, 35 cycles of denaturation (94°C for 30 s), annealing (58°C for 30 s), and elongation (72°C for 45 s), and a final elongation at 72°C for 7 min. The amplification products were separated by 2% agarose gel electrophoresis and sequenced using a 3730XL automatic sequencer (Applied Biosystems).

Splicing defects were determined according to agarose gel image patterns of the amplification products containing the wild-type and variant alleles. The criteria were based on those proposed by Aoto et al. (9).

RESULTS

According to the inclusion and exclusion criteria, our attention was caught by five *COL4A3/COL4A4* variants detected in five patients from 343 patients with hereditary kidney diseases. These variants were evaluated as uncertain significance by ACMG guidelines. *COL4A3* gene variants c.4793T>G [p. (Leu1598Arg)] (109) and p. (Thr255Thr) (10) have been previously reported, and *COL4A4* variant c.3990G > A [p. (Pro1330Pro)] has been reported in ClinVar, whereas the effect of these three variants on pre-mRNA splicing has not been reported. The splicing effects predicted by *in silico* splice tools are shown in **Table 2**. Aberrant RNA splicing effects for these five variants were predicted by Human Splicing Finder, and 4 out of the 5 variants were predicted as deleterious by at least another tool.

Characterisation of Phenotypes and Genotypes

Patient 1 (III1 of family 1) was a 1.4-year-old boy who visited the hospital due to a darkening urine colour. The laboratory investigation showed haematuria [urinary erythrocytes were full of view under urinary sediment microscopy] and proteinuria [protein-to-creatinine ratio (PCR): 1.55 g/gcr; albumin-to-creatinine ratio (ACR): 295.74 mg/g]. His non-consanguineous parents both had haematuria (**Figure 1A**). NGS and Sanger sequencing revealed that Patient 1 had compound heterozygous *COL4A3* missense variants [exon 51: c.4793T > G, p. (Leu1598Arg), maternal; exon 37: c.3143G > A, p. (Gly1048Asp), paternal] (**Figure 1A**). These two variants were both evaluated as uncertain significance by ACMG guidelines. Among them, the allele frequency of c.4793T > G was 0 in the 1000 Genomes Project, Exome Sequencing Project (ESP) database, and the gnomAD South Asian, African/African-American, Lation/Admixed American, Ashkenazi Jewish, European and other database, 0.0007678 in the gnomAD East Asian database, and 0.00005341 in the gnomAD database. Amino acid Leucine (Leu)-1598 is highly conserved in vertebrates. *In silico* predictors such as MutationTaster, SIFT, PolyPhen-2 and CADD_phred all predicted it to be deleterious. Only Human Splicing Finder indicated that it may have an effect on splicing.

Patient 2 (III1 of family 2) presented with oedema, haematuria [20–30 red blood cells per high-power field (RBCs/HPF)], and proteinuria (1+, detected by the dipstick test) when she was 7 years old. Prednisone at a standard dose and mycophenolate mofetil treatment did not improve the proteinuria. Subsequently, renal biopsy performed in the local hospital indicated focal segmental glomerular sclerosis (FSGS) under a light microscope and segmental thickening and a lamellated glomerular basement membrane (GBM) under an electron microscope. The uncle of patient 2 had a history of haematuria for 4–5 years, but his urine protein was negative. Renal biopsy indicated thin basement membrane nephropathy (TBMN). Neither ocular nor hearing abnormalities were found (**Figure 1B**). Two *COL4A3* missense variants [exon 42: c.3566G > A, p. (Gly1189Glu), paternal; exon 51: c.4793T > G, p. (Leu1598Arg), maternal] (**Figure 1B**) in a compound heterozygous state were detected in genomic DNA extracted from the peripheral blood leukocytes of patient 2. They were all evaluated as uncertain significance by ACMG guidelines. Among them, the allele frequency of c.3566G > A was 0 in the 1000 Genomes Project, and ESP databases. Amino acid Glycine (Gly)-1189 is highly conserved in vertebrates. MutationTaster, SIFT, PolyPhen-2 and CADD_phred all predicted it to be deleterious. Human Splicing Finder, NetGene2 and MaxEntScan indicated that it may have an effect on splicing.

Patient 3 (III1 of family 3) was a 25-year-old man. Haematuria (10–20 RBCs/HPF) and proteinuria (1+) were detected when he

TABLE 2 | Description of splicing effects predicted by *in silico* splice tools.

Variant	Predictors of *in silico* splice tools						
	Human splicing finder		NNSPLICE		NetGene2		MaxEntScan
	Acceptor	Donor	Acceptor	Donor	Acceptor	Donor	
A: *COL4A3* Exon 51-c.4793T > G, p. (Leu1598Arg)	Alteration of an exonic ESE site		Same score between wild type and variant sequences		Same score between wild type and variant sequences		Same score between wild type and variant sequences
B: *COL4A3* Exon 42-c.3566G > A, p. (Gly1189Glu)	Creation of an exonic ESS site; Alteration of an exonic ESE site		Same score between wild type and variant sequences		WT: 0.07 Mut: 0.22		WT: 0.98 Mut: 0.42
C: *COL4A3* Exon 13-c.765G > A, p. (Thr255Thr)		WT: 83.68 Mut: 73.59		WT: 0.98 Mut: 0.42		WT: 0.51 Mut: 0	WT: −24.93 Mut:−30.37
D: *COL4A4* Exon 42-c.3990G > A, p. (Pro1330Pro)	Activation of an exonic cryptic acceptor site; Creation of an exonic ESS site		Same score between wild type and variant sequences		WT: 0 Mut: 0.42		Same score between wild type and variant sequences
E: *COL4A4* Exon 47-c.4766C > T, p. (Pro1589Leu)	WT: 53.65 Mut: 81.52		WT: 0.63 Mut: 0.66		WT: 0.94 Mut: 0.82		WT: 9.85 Mut: 8.04

The reference sequence of COL4A3 and COL4A4 transcripts was NM_000091.5 and NM_000092.5, respectively. WT, wild-type sequence; Mut, variant sequence; ESE, exonic splicing enhancer; ESS, exonic splicing silencer.

was 3 years old. His renal function was normal, whereas at the age of 23 years old, he developed gout, and an elevated level of serum creatinine (150 μmol/L) was detected. Renal biopsy revealed mild mesangial proliferative glomerulonephritis under a light microscope and segmental thickening and thinning and lamellated GBM under an electron microscope. He had no ocular or hearing abnormalities. Laboratory findings showed his serum creatinine was 163 μmol/L, urinary protein was 0.76 g/24 h and estimated glomerular filtration rate was 49.6 ml/min/1.73 m^2 when he was 25 years old. His multiple maternal members (including his mother, aunt, uncle, and grandfather) only presented with haematuria (**Figure 1C**). *COL4A3* synonymous and missense variants [exon 13: c.765G > A, p. (Thr255Thr) maternal; exon 50: c.4664C > T, p. (Ala1555Val), paternal] (**Figure 1C**) in a compound heterozygous state were detected in patient 3's peripheral blood leukocytes genomic DNA. They were all evaluated as uncertain significance by ACMG guidelines. Among them, the allele frequency of c.765G > A was 0 in the 1000 Genomes Project, ESP databases, and gnomAD East Asian, South Asian, Lation/Admixed American, Ashkenazi Jewish, European (Finnish) and other database, 0.00004136 in the gnomAD African/African-American database, 0.000007801 in the gnomAD European (non-Finnish) database, and 0.000007133 in the gnomAD database. This variant was in the last nucleotide of exon 13. *In silico* MutationTaster predicted it to be deleterious. Human Splicing Finder, NNSPLICE, NetGene2 and MaxEntScan indicated that it may have an effect on splicing.

Patient 4 (IV1 of family 4) was an 11.3-year-old boy. From the age of 3, multiple laboratory tests showed haematuria, no proteinuria, and normal renal function. Renal biopsy was performed on his grandmother at the local hospital due to positive urine occult blood when she was 53 years old. Her GBM was observed to have irregular thickening under electron microscopy. Her urinary protein was negative, and her renal function was normal. At the age of 50, she was found to have hearing loss and needed to wear a hearing aid. Haematuria without proteinuria was detected in the grandmother's sister when she was 55 years old. She had normal renal function without hearing loss or ocular abnormalities. Patient 4's mother had increased numbers of urinary red blood cells without proteinuria or hearing loss (**Figure 1D**). *COL4A4* synonymous and splicing variants [exon 42: c.3990G > A, p. (Pro1330Pro), paternal; intron 15: c.930 + 1G > A, maternal] (**Figure 1D**) in a compound heterozygous state were detected in patient 4's peripheral blood leukocytes genomic DNA. They were evaluated as uncertain significance and pathogenic by ACMG guidelines. Among them, the allele frequency of c.3990G > A was 0 in the 1000 Genomes Project, ESP databases and the gnomAD African/African-American, Lation/Admixed American, Ashkenazi Jewish, European and other database, 0.0006783 in the gnomAD East Asian database, 0.00009902 in the gnomAD South Asian database, and 0.00006097 in the gnomAD database. *In silico* MutationTaster predicted it to be deleterious. Human Splicing Finder and NetGene2 indicated that it may have an effect on splicing.

Patient 5 (II2 of family 5) was a 34-year-old woman who manifested haematuria and proteinuria for seven years.

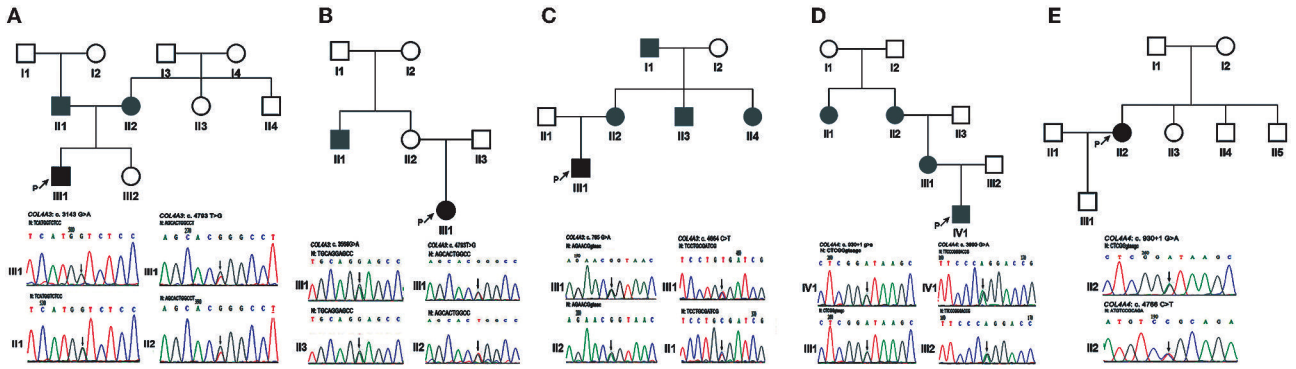

FIGURE 1 | The pedigree of five families and Sanger sequencing of PCR amplified products containing the variants for probands 1–5 and their parents. **(A)** Family 1. **(B)** Family 2. **(C)** Family 3. **(D)** Family 4. **(E)** Family 5. Black arrows indicate the probands and the changed nucleotides. The filled black squares and circles indicate the individuals who presented with haematuria and proteinuria, and the filled grey squares and circles indicate the individuals who featured only haematuria. N, normal sequence. Intron and exon sequences are depicted by lower and capital case letters, respectively. The reference sequence of *COL4A3* and *COL4A4* transcripts was NM_000091.5 and NM_000092.5, respectively.

Laboratory investigation showed haematuria (5–10 RBCs/HPF) and proteinuria (ACR: 570.22 mg/g; urinary total protein: 1.04 g/24 h), and the estimated glomerular filtration rate was 81.9 ml/min/1.73 m^2. Renal biopsy under electron microscopy suggested that the GBM ultrastructural changes were in accordance with Alport syndrome. Haematuria and/or proteinuria were not observed in her family members (**Figure 1E**). *COL4A4* heterozygous missense and splicing variants [exon 47: c.4766C > T, p. (Pro1589Leu); intron 15: c.930 + 1G > A] (**Figure 1E**) were detected in patient 5's peripheral blood leukocytes genomic DNA. Lacking DNA samples from her parents, the evaluation of the *cis* vs. *trans* nature of these two variants was not done. They were evaluated as uncertain significance (PM2, PM3, PP3) and pathogenic (PVS1, PM2, PP3) by ACMG guidelines. Among them, the allele frequency of c.4766C > T was 0 in the 1000 Genomes Project, ESP databases and the gnomAD African/African-American, Lation/Admixed American, Ashkenazi Jewish and other database, 0.00005595 in the gnomAD East Asian database, 0.00003272 in the gnomAD South Asian database, 0.00005332 in the gnomAD European (non-Finnish) database, 0.00004716 in the gnomAD European (Finnish) database, and 0.00003629 in the gnomAD database. Amino acid Proline (Pro)-1589 is highly conserved in vertebrates. MutationTaster, SIFT, PolyPhen-2 and CADD_phred all predicted it to be deleterious. Human Splicing Finder and MaxEntScan indicated that it may have an effect on splicing.

The Splicing Effect of Two Variants in *COL4A3*: c.4793T > G and c.765G > A

Agarose gel electrophoresis showed that the wild-type minigene transcription product had two bands, suggesting alternative splicing, while the variant transcription product (c.4793T > G) exhibited only one band after PCR amplification (**Figure 2A**). Direct sequencing of the PCR products showed that there were two wild-type transcripts (679 bp and 506 bp long) and a variant transcript (506 bp). The longer wild-type transcript contained exons 49, 50, 51 and 52, while the shorter transcript skipped exon 51 (173 bp); the variant transcript also skipped exon 51 during the splicing process (**Figure 2B**). These results indicated that the variant c.4793T > G could affect splicing at the mRNA level, which was consistent with the prediction of Human Splicing Finder.

The variant (c.4793T > G) was reclassified according to the ACMG guidelines and the minigene assay results. It could result in a deletion of the normal transcript, which belonged to the null variant (PVS1), and was re-evaluated as "pathogenic" based on the existing evidence (PVS1, PM2, PP3, and PP5). The variant *in trans* c.3143G > A was reclassified as "likely pathogenic" (in line with PM2, PM3, PP3 and PP4). Autosomal recessive Alport syndrome was diagnosed in patient 1 according to his clinical manifestation, family history of haematuria, and genetic testing results. The recurrence risk of his siblings was 25%.

If primers were located at the inserted exons, the variant transcript (c.765G > A) would be <100 bp and could not be sequenced, so we set the forward primer on the plasmid. The wild-type minigene transcription products were all longer than those of the variants (c.765G > A) after PCR amplification and agarose gel electrophoresis (**Figure 2C**). Direct sequencing of the PCR products showed that the two variant transcripts all skipped exon 13 during the splicing process (**Figure 2D**), which is predicted to cause an in-frame deletion of 26 amino acids during translation. The results indicated that it affected splicing at the mRNA level, consistent with the prediction of four splice site prediction tools.

The variant c.765G > A was reclassified as "pathogenic" (PVS1, PS3, PM2, PP3) according to the ACMG guidelines and minigene assay results. The variant c.4664C > T *in trans* was classified as likely pathogenic (PM2, PM3, PP3, PP4). Autosomal recessive Alport syndrome could be

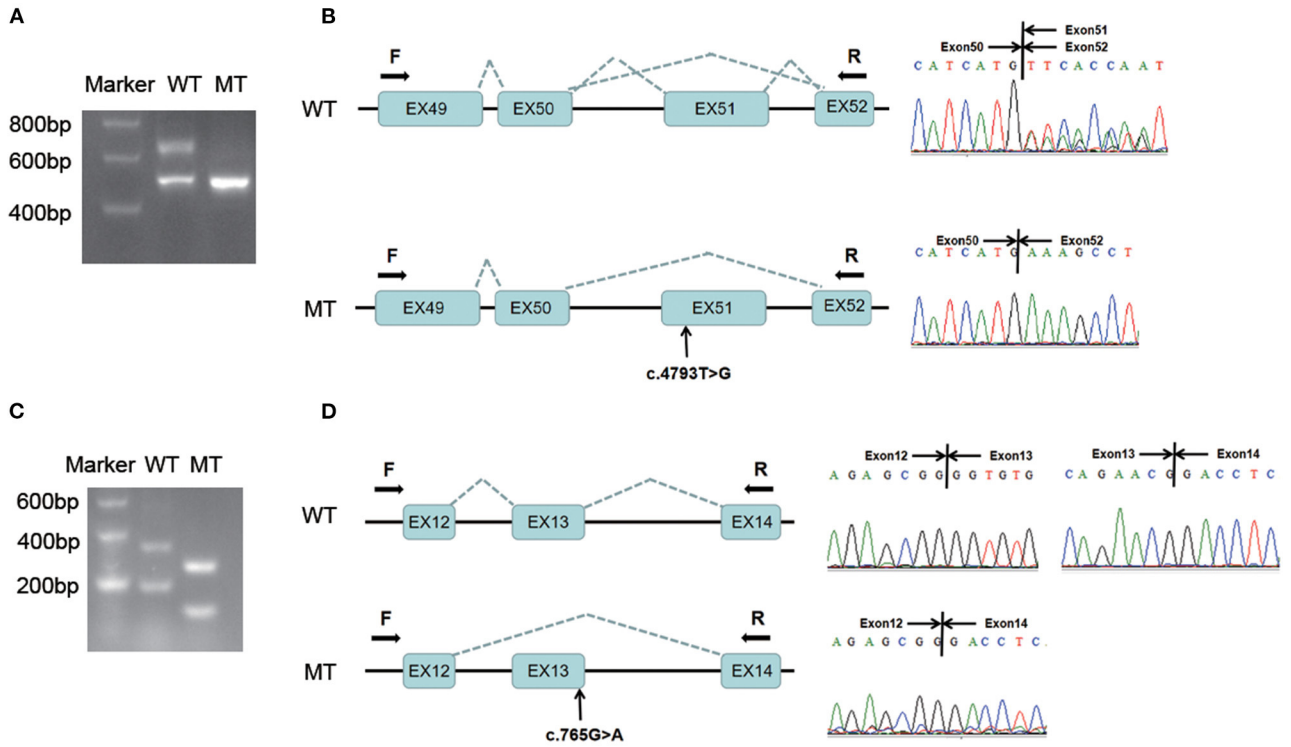

FIGURE 2 | Minigene assay analysis of two variants in COL4A3: c.4793T > G and c.765G > A. **(A)** The amplification products of the wild-type and variant minigene constructs (*COL4A3*: c.4793T > G) were separated by 2% agarose gel electrophoresis. The wild-type transcription product had two bands, while the variant transcription product exhibited only one band after PCR amplification. **(B)** Direct sequencing of the PCR products showed that there were two alternative splicing transcripts exon51+/exon51− in the wild-type minigene constructs, while the variant transcript skipped exon 51 during the splicing process. **(C)** The amplification products of the wild-type and variant minigene constructs (c.765G > A) were separated by 2% agarose gel electrophoresis. Although the wild-type and variant transcription products exhibited two bands after PCR amplification, the variant transcripts were all shorter than those of the wild-type. **(D)** Direct sequencing of the PCR products revealed that the two variant transcripts all skipped exon 13 during the splicing process. WT, wild-type; MT, mutant-type. F, forward primer; R, reverse primer.

diagnosed in patient 3. The recurrence risk of his siblings was 25%.

Variants c.3566G > A in *COL4A3* and c.3990G > A and c.4766C > T in *COL4A4* Exhibited no Deleterious Effect on Splicing

Figure 3A shows that the length of the wild-type minigene transcription product was equal to that of the variant (c.3566G > A in *COL4A3*) after PCR amplification and agarose gel electrophoresis. Direct sequencing indicated that the two transcripts were all 320 bp and made up of partial exon 41, exon 42 and partial exon 43 (**Figure 3B**). Only the first nucleotide of exon 42 was discrepant between the wild-type and mutant-type transcripts, which suggested that the variant was missense, not compatible with the prediction of Human Splicing Finder and MaxEntScan. The variant c.4793T > G was the same as patient 1 and reclassified as "pathogenic"; thus, the variant c.3566G > A *in trans* was re-evaluated as "likely pathogenic" (PM2, PM3, PP3 and PP4). Autosomal recessive Alport syndrome was diagnosed in patient 2 according to the clinical manifestations, renal biopsy results, family history of haematuria, and genetic testing results. The recurrence risk of her siblings was 25%.

The length of the wild-type minigene transcription product was equal to that of the variant (c.3990G > A in *COL4A4*) after PCR amplification and agarose gel electrophoresis (**Figure 3C**). Direct sequencing indicated that the two transcripts were all 229 bp and contained part of exons 41 and 42 (**Figure 3D**). Only the mutant-type transcript harboured the variant c.3990G > A, which suggested that the variant was synonymous and not compatible with the prediction of Human Splicing Finder and NetGene2. Considering the clinical manifestations and minigene assay results, it was believed that c.3990G > A was not pathogenic, and TBMN was highly suspected in patient 4, but long-term follow-up was needed to confirm the diagnosis.

The length of the wild-type minigene transcription product was equal to that of the variant (c.4766C > T in *COL4A4*) after PCR amplification and agarose gel electrophoresis (**Figure 3E**). Direct sequencing showed that the two transcripts were all 592 bp and contained partial exon 46, exon 47 and partial 48 (**Figure 3F**). Only the mutant-type transcript harboured the variant c.4766C > T, which suggested that the variant was missense and not compatible with the prediction of Human Splicing Finder and

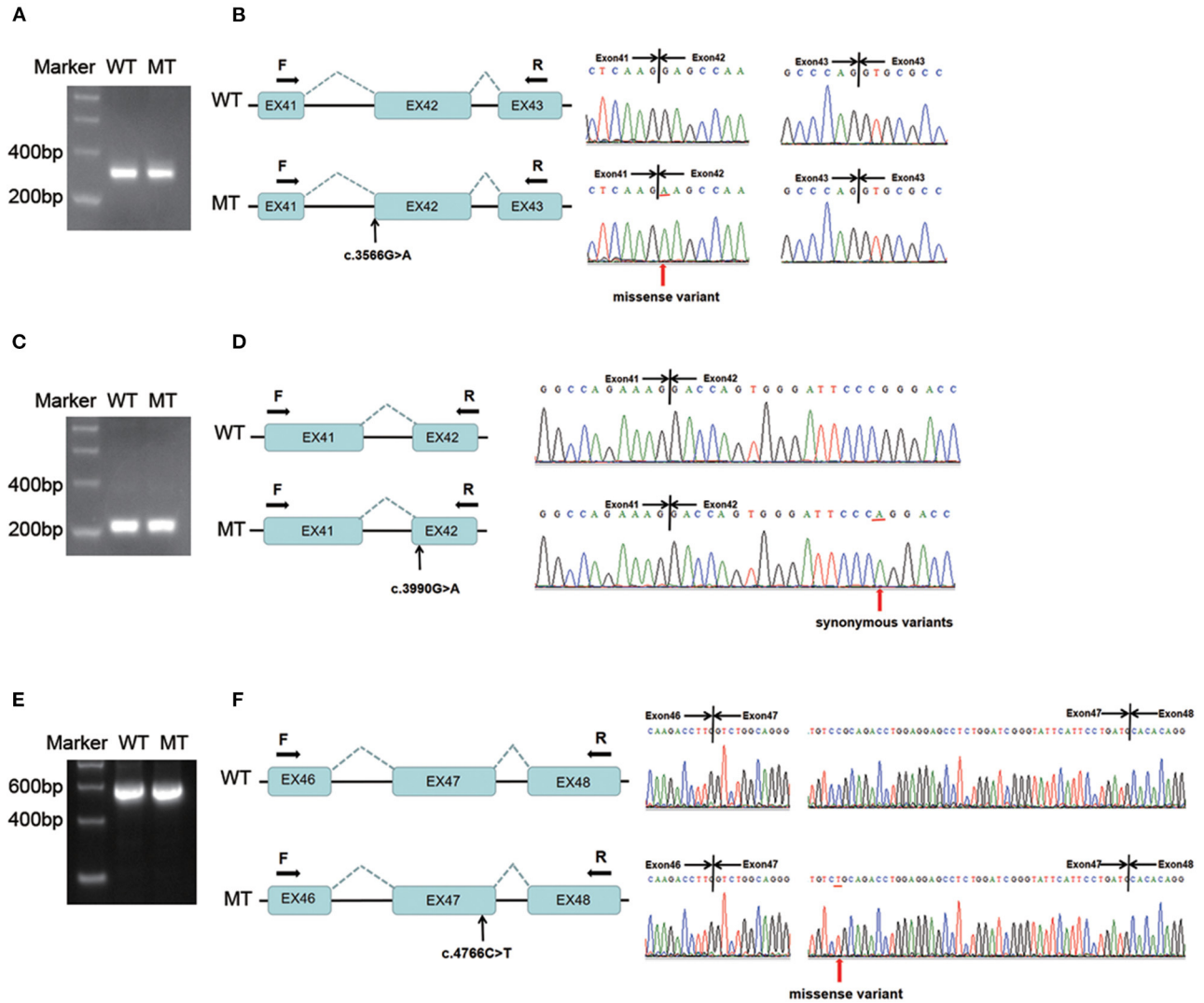

FIGURE 3 | Minigene assay analysis of variants c.3566G > A in *COL4A3* and c.3990G > A, c.4766C > T in *COL4A4*. **(A)** The amplification products of the wild-type and variant minigene constructs (c.3566G > A) were separated by 2% agarose gel electrophoresis. The lengths of the two bands were the same. **(B)** Direct sequencing showed that the two transcripts were identical except for the mutant nucleotide (c.3566G > A). **(C)** The amplification products of the wild-type and variant minigene constructs (c.3990G > A) were separated by 2% agarose gel electrophoresis. The lengths of the two bands were the same. **(D)** Direct sequencing showed that the two transcripts were identical except for the mutant nucleotide (c.3990G > A). **(E)** The amplification products of the wild-type and variant minigene constructs (c.4766C > T) were separated by 2% agarose gel electrophoresis. The lengths of the two bands were the same. **(F)** Direct sequencing showed that the two transcripts were identical except for the mutant nucleotide position (c.4766C > T). WT, wild-type; MT, mutant-type; F, forward primer; R, reverse primer.

MaxEntScan. The pathogenicity re-evaluation had not been changed. Considering the clinical manifestations, renal biopsy and minigene assay results, Alport syndrome could be diagnosed in patient 5. However, the inheritance pattern could not be determined since her family members had no haematuria and we lacked parental DNA samples.

DISCUSSION

In this study, we assessed the pathogenicity of five *COL4A3/COL4A4* unclassified presumed missense and synonymous variants that may affect splicing as predicted by the *in silico* tool Human Splicing Finder. It was confirmed by the minigene assay that the *COL4A3* missense variant p. (Leu1598Arg) and synonymous variant p. (Thr255Thr) theoretically speculated based on the nucleotide substitutions at the genomic DNA level may be splicing variants. Similar phenomena had been observed in *COL4A5* gene using *in vitro* minigene analysis combined with or without analysis of patient-derived RNA from urine sediments and peripheral blood leukocytes (9, 11). These pathogenic variants cannot be accurately evaluated based on the NGS results of genomic DNA alone if mRNA analysis is not performed. In addition,

it has not yet been reported that reclassification of single base variants in the *COL4A3* coding region based on functional experimental results.

Splicing is catalysed by a dynamic spliceosome, which is comprised of five different small nuclear ribonucleoproteins (snRNPs). snRNPs are mainly synthesised by five small nuclear RNAs (snRNAs) named U1, U2, U4, U5, and U6 due to their uracil richness. They can accurately recognise5′ splice sites, 3′ splice sites and branch points, thereby catalysing the premRNA splicing process (12). The *cis*-regulatory elements comprise exon splicing enhancers (ESEs), intron splicing enhancers (ISEs), exon splicing silencers (ESSs) and intron splicing silencers (ISSs) located in either exons or introns. These *cis*-splicing regulatory elements work by recruiting *trans*-acting factors to activate or suppress splice site recognition or spliceosome assembly (13, 14). Therefore, variants in the genome could affect the splicing process by repressing 5′ or 3′ splice sites, thus generating new splice sites, or having an impact on recruiting *trans*-acting factors.

At present, 619 *COL4A3/COL4A4* variants have been reported, of which splicing variants account for ∼13.6% (refer to the human gene mutation database, HGMD), and most of these variants are located at typical splice sites, namely highly conserved 5′ donor or 3′ acceptor sites (↓GT or AG↓). Studies have shown that 15–60% of human genetic diseases are caused by splicing variants (15), and it has been speculated that the true proportion of splicing variants in *COL4A3/COL4A4* may be underestimated. Daga et al. found that a deep intronic variant in *COL4A4* could alter splicing products by analysing RNA extracted from Alport patient's urine-derived podocyte lineage cells (16). In our previous study, atypical intronic variants were detected in 10.7% (3/28) of patients with X-linked Alport syndrome by analysing the cDNA of skin fibroblasts (7), and deep intronic splicing variants in Alport syndrome causative genes could be detected by the analysis of patient-derived RNA (17). Jourdy et al. found that synonymous variants in *F8* could lead to new transcripts by creating new splicing sites (18). RNA analysis of peripheral blood leukocytes demonstrated that missense variant p. (R919G) in *ATP7B* causing Wilson's disease could lead to exon skipping (3). Given that deep intronic, missense and synonymous variations may be cryptic splice sites, it is important to re-evaluate the pathogenicity of such variants using transcript analysis. In this study, using an *in vitro* minigene assay, it was shown that *COL4A3* variants c.4793T > G [p. (Leu1598Arg)] and c.765G > A [p. (Thr255Thr)] had effects on pre-mRNA splicing, providing supporting evidence for reclassification of their pathogenicity. Meanwhile, two other variants *in trans* with these variants were re-evaluated as likely pathogenic, allowing for accurate genetic diagnosis and genetic counselling for patients 1 and 3.

Many bioinformatics tools have been used to predict splicing variants. Among them, Human Splicing Finder has been widely used for evaluating variant effects on 5′ splice sites, 3′ splice sites, enhancers, silencers, and branch points (19). However, no splice site prediction tool can be 100% accurate. It was suggested to using multiple *in silico* tools to pinpoint unclassified variants worthy of transcript analyses (20). Nonetheless, except *COL4A3* variant c.765G > A [p. (Thr255Thr)], no splicing abnormalities were observed for the variants (c.3566G > A in COL4A3 and c.3990G > A and c.4766C > T in COL4A4) with a splicing defect predicted using at least two splice site prediction tools. Additionally, *in vitro* minigene assay analysis of *DYSF* missense and intronic variants showed that 41% of the tested variants were correctly predicted by Human Splicing Finder (6). In the present study, a false-positive prediction was observed in three of five variants, in accordance with the previous report (6). Therefore, in a molecular diagnostic setting, it is essential to compare *in silico* splicing predictions and transcript analysis results. The minigene assay is a popular approach to examine whether a splicing variant affects splicing of the neighbouring exon (21, 22), and it provides good agreement with experiments using patient-derived RNA (23, 24). Given the unavailability of specimens from our patients, such as urine or kidney tissue, and our previous study showed that *COL4A3/COL4A4* variants detected in mRNA isolated from Epstein Barr virus-transfected lymphocytes were not always confirmed at the genomic DNA level (data not reported), we used a minigene assay to analyse the consequences of the selected variants. Our study showed that the corresponding transcripts were obtained successfully, and as described above, two predictions were confirmed, which indicated that it was possible and feasible to investigate the effect of *COL4A3/COL4A4* variants on splicing by the minigene assay.

It is worth discussing the interpretation of the results of the minigene assay. The alternative transcripts, which are revealed by using different vectors and transfecting cell lines, are slightly different from those found by analysing RNA extracted from the peripheral blood (25, 26). This phenomenon may be due to differences in the expression of splicing factors or splicing regulatory factors in different cells. In addition, it may be difficult to evaluate genotype-phenotype correlations when the splicing variants change the relative proportion of different transcripts, and the minigene assay cannot precisely simulate the tissue/cell environment in which the gene is normally expressed. For example, minigene assay analysis demonstrated that the variant c.388 + 5G > A in haemophilia A causative gene *F8* leads to two transcripts: one to an abnormal transcript with skipping of the associated exon and one corresponding to the wild-type transcript, which was inconsistent with the patient's severe phenotype (18). In this study, we showed that *COL4A3* variant c. 4793T > G led to the production of a transcript lacking exon 51, whereas the wild-type minigene recombinant clone caused the expression of two transcripts: one containing exon 51 and another without exon 51. It has been previously shown that there are three distinct *COL4A3* transcripts in foetal (12 weeks) and adult kidney tissues: alternative transcript I containing exons 48–52; alternative transcript II without exon 49; and alternative transcript III without exon 51 (27). Variations could be seen in the expression of these isoforms: alternative transcript I was dominant and accounted for more than 70%, and the relative expression of alternative transcript III in foetal kidney (5.5%) was higher than that in the adult kidney (4.5%), which suggested a slight change in alternative

splicing along with organ development. Therefore, due to the loss of the full-length transcript, the *COL4A3* variant c.4793T > G was considered a splicing variant. Three patients harbouring this heterozygous variant plus another heterozygous *COL4A3* nonsense variant p. (Gly709Ter), intronic splicing variant c. 3752-511_3955 + 576 del, or missense variant p. (Gly1155Asp) demonstrated the typical ultrastructural basket-weave change in the GBM or a thin GBM and an absence of type IV collagen α5 chain in the GBM and only normal immunostaining of Bowman's capsule (typical immunofluorescence staining pattern for autosomal recessive Alport syndrome) (28), suggesting these *COL4A3* variants affect type IV collagen α3 chain production.

There were some limitations in this study. First, only unclassified missense and synonymous variants in *COL4A3/COL4A4* were screened for splicing analysis. Some frameshifts (29) or nonsense variants (30) may also interfere with splicing and need to be identified. Second, only HEK293T cells were employed to assess the impact on splicing of selected exonic substitutions detected in the patients without immunostaining for type IV collagen α5 chain in renal tissues, and the samples from our patients such as urine or kidney specimen which specific expresses type IV collagen α3 and α4 chains are unavailable, to avoid possible false-negative results, at least one other human cell line, such as HeLa cells, should be used to verify the results from HEK293T cells. Third, Horinouchi et al. showed different nucleotide changes affecting the same codon may or may not affect splicing of an exon using minigenes (11), whereas there were no previously published pathogenic variants at the same codons for the variants in this study, we could not compare the effect on RNA splicing.

CONCLUSION

For the first time, we demonstrated that the presumed missense variant p. Leu1598Arg and synonymous variant p. Thr255Thr in *COL4A3* affected pre-mRNA splicing, which extends this gene mutational spectrum and highlights the importance of transcript analysis of exonic sequence variants in the gene to assist with molecular diagnosis and genetic counselling. Meanwhile, we showed that the accuracy of *in silico* predictive tools for splicing variant prediction was limited.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/genbank/, OL998453-OL998462.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Committee of Peking University First Hospital. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

HD and FW designed the research study and wrote the manuscript. HD performed the research. HD and YZ analysed the data. JD provided help and advice on designing the research and writing the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

FUNDING

This work was supported by National Nature Science Foundation (81070545), Beijing Nature Science Foundation (7102148), National Key Research and Development Program of China, the registry study of rare diseases in children (No. 2016YFC0901505), and Beijing Key Laboratory of Molecular Diagnosis and Study on Pediatric Genetic Diseases (BZ0317).

ACKNOWLEDGMENTS

We thank all patients and their families for participating this study.

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frontiers in Medicine

ORIGINAL RESEARCH
published: 28 March 2022
doi: 10.3389/fmed.2022.859521

Identification of 27 Novel Variants in Genes *COL4A3, COL4A4,* and *COL4A5* in Lithuanian Families With Alport Syndrome

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OPEN ACCESS

Edited by:
Robert P. Woroniecki, Stony Brook Children's Hospital, United States

Reviewed by:
Gianluca Caridi, Giannina Gaslini Institute (IRCCS), Italy
Daw-Yang Hwang, National Health Research Institutes, Taiwan

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Specialty section:
This article was submitted to Nephrology, a section of the journal Frontiers in Medicine

Received: *21 January 2022*
Accepted: *02 March 2022*
Published: *28 March 2022*

Citation:
Cerkauskaite A, Savige J, Janonyte K, Jeremiciute I, Miglinas M, Kazenaite E, Laurinavicius A, Strupaite-Sileikiene R, Vainutiene V, Burnyte B, Jankauskiene A, Rolfs A, Bauer P, Schröder S and Cerkauskiene R (2022) Identification of 27 Novel Variants in Genes COL4A3, COL4A4, and COL4A5 in Lithuanian Families With Alport Syndrome. Front. Med. 9:859521. doi: 10.3389/fmed.2022.859521

Introduction: Alport syndrome (AS) is an inherited disorder characterized by hematuria, proteinuria, and kidney function impairment, and frequently associated with extrarenal manifestations. Pathogenic variants in *COL4A5* usually cause X-linked Alport syndrome (XLAS), whereas those in the *COL4A3* or *COL4A4* genes are associated with autosomal dominant (AD) or recessive (AR) inheritance. To date, more than 3000 different disease-causing variants in *COL4A5, COL4A3*, and *COL4A4* have been identified. The aim of this study was to evaluate the clinical and genetic spectrum of individuals with novel, pathogenic or likely pathogenic variants in the *COL4A3-A5* genes in a previously unstudied cohort.

Methods: In this study molecular analysis by next generation sequencing (NGS) was performed on individuals from a Lithuanian cohort, with suspected AS. The presence of AS was assessed by reviewing clinical evidence of hematuria, proteinuria, chronic kidney disease (CKD), kidney failure (KF), a family history of AS or persistent hematuria, and specific histological lesions in the kidney biopsy such as thinning or lamellation of the glomerular basement membrane (GBM). Clinical, genetic, laboratory, and pathology data were reviewed. The novelty of the *COL4A3-A5* variants was confirmed in the genetic variant databases (Centogene, Franklin, ClinVar, Varsome, InterVar). Only undescribed variants were included in this study.

Results: Molecular testing of 171 suspected individuals led to the detection of 99 individuals with 44 disease causing variants including 27, previously undescribed changes, with the frequency of 9/27 (33,3%) in genes *COL4A5, COL4A3* and *COL4A4* equally. Three individuals were determined as having digenic AS causing variants: one in *COL4A3* and *COL4A4*, two in *COL4A4* and *COL4A5*. The most prevalent alterations in genes *COL4A3-5* were missense variants ($n = 19$), while splice site, frameshift, unknown variant and stop codon changes were detected more in genes *COL4A4* and *COL4A5* and accounted for 3, 3, 1 and 1 of all novel variants, respectively.

Conclusion: Genotype-phenotype correlation analysis suggested that some variants demonstrated intra-familial phenotypic variability. These novel variants represented more than half of all the variants found in a cohort of 171 individuals from 109 unrelated families who underwent testing. Our study expands the knowledge of the genetic and phenotypic spectrum for AS.

Keywords: Alport syndrome, *COL4A3*, *COL4A4*, *COL4A5* variants, novel, digenic inheritance, genotype-phenotype correlation

INTRODUCTION

Alport syndrome (AS) is a progressive hereditary glomerular disease characterized by hematuria, proteinuria and progressive kidney function impairment with the prevalence of at least one in 2000 for the X-linked form and one in 100 for autosomal dominant (AD) inheritance (1). It is responsible for about 1–2% of all cases with kidney failure (KF) (2). AS can also be associated with extrarenal manifestations, including bilateral sensorineural hearing loss and specific ocular lesions (3).

AS is caused by pathogenic variants in the *COL4A3*, *COL4A4*, and *COL4A5* genes encoding type IV collagen α3, α4, and α5 chains, respectively. Pathogenic variants in *COL4A5* usually cause X-linked Alport syndrome (XLAS), while alterations in *COL4A3* and *COL4A4* genes are associated with autosomal dominant AS (ADAS) or autosomal recessive AS (ARAS) (4–6). There is also evidence of digenic inheritance in AS due to the transmission of variants in two of the three genes in addition to the classic Mendelian inheritance (7).

The type and position of AS pathogenic variants such as missense, nonsense, splice site or frameshift rearrangements in *COL4A3-A5* may lead to a variable kidney disease course with a different age at KF onset and manifestation of ocular and hearing abnormalities (8–13). Several studies have demonstrated a significant improvement in kidney function in AS individuals after early initiation and long-term treatment with angiotensin converting enzyme inhibitors (ACEi) (14–16). Therefore, early diagnosis of AS in individuals with persistent hematuria and proteinuria has a significant impact on prognosis. Molecular genetic testing of type IV collagen genes might hence provide information essential for diagnosis, prognosis, genetic counseling and treatment options (17). Previous papers have declared that a pathogenic variant was the best evidence for the diagnosis of AS (1). However, interpretation of these variants may be complicated due to the incomplete or conflicting evidence of pathogenicity. A recent consensus statement recently indicated that clinical features and family history of kidney failure or persistent hematuria, together with the identified genetic variant, are the strongest evidence for the diagnosis of AS (17).

To date, more than 3,000 different pathogenic variants in the *COL4A3*, *COL4A4*, and *COL4A5* genes are recorded (4). Many phenotypes of AS have been identified in a high number of individuals, but it appears that many possible variants of these genes are still unknown. A genetic diagnosis is essential to treatment and prognosis, and therefore the number of identified disease-causing variants and their clinical phenotype must be increased. In this study we report the results of an extensive mutational analysis and the genotype-phenotype correlations in a sizable Lithuanian cohort of 171 individuals that led to the identification of 27 novel variants in the type IV collagen genes.

STUDY PARTICIPANTS AND METHODS

The study was approved by the Vilnius Regional Biomedical Research Ethics Committee of Lithuania (BioAlport, No 158200-16-857-367). All participants in this study signed a consent form regarding the interview and likely uses of their data. Consent from individuals under 18 years old was obtained from their parents.

All individuals were clinically examined by adult or pediatric nephrologists. The study cohort included the individuals treated at Vilnius University Hospital Santaros Klinikos (VUH SK) in <city>Vilnius</city>, Lithuania from 2016 to 2021. Their age ranged from 2 months to 82 years old. Clinical diagnoses of individuals were made by a nephrologist based on clinical manifestations, laboratory analysis, such as hematuria, proteinuria and creatinine levels, and a family history of persistent hematuria or AS. Kidney histopathological data collection included light microscopy, immunofluorescence, and electron microscopy examination of biopsy tissues. All individuals with high-grade suspicion for AS that met the

TABLE 1 | Diagnostic features and criteria for suspicion of AS in our study.

Clinical features (persistent permanent microscopic hematuria, proteinuria, stage of chronic kidney disease based on eGFR (estimated glomerular filtration rate, using EPI-CKD formula) as follows: stage I (eGFR >90 mL/min/1.73 m^2); stage II (eGFR 60–89 mL/min/1.73 m^2); stage IIIa (eGFR 45–59 mL/min/1.73 m^2); stage IIIb (eGFR 30–44 mL/min/1.73 m^2); stage IV (eGFR 15–29 mL/min/1.73 m^2); stage V (eGFR < 15 mL/min/1.73 m^2), bilateral sensorineural hearing loss, anterior lenticonus etc.)
Kidney biopsy (thinning, thickening, splitting or lamination of GBM, foot process effacement, FSGS)
Positive family history for clinical features of specific clinical criteria (positive for individuals with the symptoms above)
Positive family history of AS (currently diagnosed AS in family)
Incidental genetic diagnosis of AS searching the different diseases (ex. ADPKD, IgA nephropathy, nephronophthisis and etc.)

GBM, glomerular basement membrane; FSGS, focal segmental glomerulosclerosis; ADPKD, autosomal dominant polycystic kidney disease.

eligibility criteria (**Table 1**) provided a blood sample DNA extraction. This was applied onto a Dried Blood Spot DBS-filtercard, called CentoCard® and sent to Centogene laboratory, in Rostock, Germany.

The genetic testing was performed by next generation sequencing (NGS) technologies in this laboratory. Genomic DNA was enzymatically fragmented, and regions of interest were enriched using DNA capture probes, targeted against the coding regions, and known pathogenic/likely pathogenic variants of panel genes. The libraries were then sequenced on an Illumina platform. The entire coding region of the *COL4A3*, *COL4A4*, and *COL4A5* genes, including 10 bp of flanking intronic sequences, were amplified and sequenced. Missing fragments were completed using classical Sanger sequencing to achieve 100% coverage of all genes of this panel. Data analysis, including alignment to the hg19 human reference genome (Genome Reference Consortium GRCh37), variant calling, and annotation was performed using validated software from Centogene. Data screening and bioinformatics analysis were performed depending on the targeted gene capture and high-throughput sequencing from DNA samples. The *in silico* prediction of the damaging effects of each identified variant was assessed using MutationTaster, PolyPhen-2 and SIFT. Splicing variants were analyzed by Human Splicing Finder (http://www.umd.be/HSF3/index.html) in addition to MutationTaster. Pathogenic variants which could not be classified with either of the *in silico* tools were declared as not applicable (N/a). Only previously undescribed in the literature variants, were included in this study. Genetic variants were checked and compared in five databases—Centogene, Franklin, ClinVar, InterVar and Varsome (last review in December 2021), and therefore classified into four categories due to their pathogenicity using The American College of Medical Genetics and Genomics (ACMG) criteria—pathogenic (class 1), likely pathogenic (class 2), variant with unknown significance (VUS, class 3) and likely benign variant/polymorphism (class 4). Most of the novel variants were submitted into ClinVar Database (accession SUB11112191 (SCV002098105 - SCV002098129), however in this study there was a partial non-availability of raw data due to the time period restriction imposed by Centogene laboratory on stored data (please see Data availability section).

Individuals with hetero/hemizygous pathogenic variants in *COL4A5 (1)* compound heterozygous or homozygous variants in *COL4A3* or *COL4A4 (2)*, singular heterozygous variants in *COL4A3* or *COL4A4 (3)*, or digenic variants in two of the following genes *COL4A3/A4/A5 (4)* were named as having XLAS, ARAS, ADAS, or digenic AS, respectively. Although there is still some debate about naming the individuals with heterozygous *COL4A3/A4* variants with ADAS, thin basement membrane nephropathy (TBMN) or carriers of autosomal recessive AS, in this study we chose to use the term ADAS following the Kashtan et al. proposed unified classification of AS in 2018 (13).

Individuals with a molecular diagnosis of AS were referred to an ophthalmologist where specific evaluation of the eyes was performed, including slit-lamp examination for corneal abnormalities, ophthalmoscopy for perimacular fleck retinopathy, retinoscopy for lenticonus and Optical Coherence Tomography (OCT) for temporal retinal thinning. In cases of ocular changes anterior segment and fundus photography were performed. Audiograms for screening bilateral hearing abnormalities were performed in most of the individuals. Family members with high index of suspicion for AS were referred for molecular analysis and consultation with a geneticist for further evaluation.

RESULTS

Molecular analysis of all exons in the *COL4A3*, *COL4A4*, and *COL4A5* genes of 171 individuals from 109 different families led to the detection of 44 genetic variants (in 51 families, 99 individuals), including 27 novel changes in 51 individuals (41% males and 59% females) from 26 unrelated families. To be noted, one *COL4A3* (c.520G>A (p.Gly174Arg) VCV000447175) and four *COL4A4* variants (c.594+1G>A VCV000438704.2; c.2756A>G (p.Glu919Gly) VCV000599099; c.3044G>A (Gly1015Glu) VCV000557146; c.2347G>A (p.Gly783Arg) VCV000252464) were reported in the ClinVar database by other laboratories, however no clinical data of the individuals with these variants were available. Our main aim was to describe genotype—phenotype correlations in previously undescribed variants.

Age at diagnosis ranged from 1 to 65 years (median age was 26.1 years) of the individuals with novel variants. Clinical data of screened individuals with an established genetic diagnosis are listed in detail in **Supplementary Tables 1–3**.

The frequency of the novel variants in each group of three *COL4A5*, *COL4A3*, and *COL4A4* genes was 33.3% (9/27) equally. Three individuals had compound heterozygous pathogenic variants in *COL4A3* or *COL4A4* genes, while no homozygous variants in these genes were detected. Three individuals were determined as having digenic AS causing variants: one in *COL4A3* and *COL4A4*, and two in *COL4A4* and *COL4A5*. In this study, 35% (9/26) of the families had XLAS, 8% (2/26) had ARAS, 8% (3/26) had digenic disease and the majority 54% (14/26) had one heterozygous variant in *COL4A3* or *COL4A4*, thus ADAS. Notably, some individuals with digenic or autosomal recessive AS were from the same families with XLAS or ADAS.

All individuals had microscopic hematuria with or without kidney failure. KF appeared in two males with likely pathogenic (according to ACMG, Varsome) variants in COL4A5, while ADAS in ARAS manifest to KF for one person in each group. A kidney biopsy had been performed on 22 individuals with AS, and all of them had particular histological abnormalities of the glomerular basement membrane (GBM).

Bilateral neurosensory hearing abnormalities and specific ophthalmologic lesions were seen in 12 and 9 individuals, respectively. Four out of 24 individuals (16.7%) had hearing abnormalities, while ocular abnormalities, including retinal flecks and retinal thinning, were found in six individuals (25%) with heterozygous COL4A3 and COL4A4 mutations. Bilateral hearing loss was seen in all three individuals with ARAS, while

TABLE 2 | Summary of demographic and clinical data of individuals with novel *COL4A3*, *COL4A4,* and *COL4A5* variants found in study.

	COL4A3		*COL4A4*		*COL4A5*	Digenic inheritance
	ADAS	**ARAS**	**ADAS**	**ARAS (or biallelic heterozygous)**		
Total number of individuals	8	2	16	2	14	3
Gender	F-4	F-2	F-8	F-1	F-13	F-2
	M-4	M-0	M-8	M-1	M-7	M-1
Age at diagnosis (range)	1–63	33	2-65	6–32	4–51	14–41
Number of individuals with KF (age at KF)	None	1	1	N/a	2	None
Number of individuals with ocular abnormalities	1	None	4	None	4	None
Number of individuals with hearing abnormalities	1	2	3	None	6	None
Number of individuals with kidney biopsy	5	2	3	2	7	2

F, female; M, male; diff, different; KF, kidney failure; Ind, individual; ab., abnormalities; N/a, not applicable; N/d, no data; DIG, digenic; CKD, chronic kidney disease; FSGS, focal segmental glomerulosclerosis; pro, proteinuria; P, pathogenic; LP, likely pathogenic; VUS, variant with unknown significance; XLAS, X-linked Alport syndrome, AS, Alport syndrome, ARAS, autosomal recessive Alport syndrome; ADAS, autosomal dominant Alport syndrome.
Individuals with digenic Alport syndrome are included into digenic section despite the single novel variants in a particular gene.

TABLE 3 | Summary of novel *COL4A3*, *COL4A4,* and *COL4A5* variants found in study.

Type of pathogenic variant	***COL4A3***	***COL4A4***	***COL4A5***
Total missense	9	6	4
Glycine substitution	7	3	4
Other missense variants	2	3	0
Stop codon	0	1	0
Splice site	0	1	2
Frame shift	0	0	3
Unknown variant	0	1	0

none had ocular abnormalities. Ocular changes of individuals with XLAS were found in four out of seven males (57.14%) and included anterior lenticonus, posterior subcapsular cataract and temporal retinal thinning. Hearing loss occurred in six males and one female with XLAS. Please see **Table 2** and **Supplementary Tables 1–3**.

The most prevalent variants in genes *COL4A3-5* were missense changes ($n = 19$), 14 of which (74%) were glycine substitution. The splice site, frameshift, stop codon and unknown variants were more common in genes *COL4A4* and *COL4A5* and represented or 3, 3, 1 and 1 of all novel variants, respectively (**Table 3**).

COL4A5

Twenty-two individuals from 9 different families carried one variant in *COL4A5*. Summary of novel *COL4A5* variants found in a study can be seen in **Table 4** and in more details in **Supplementary Table 1**. Seven males were diagnosed with XLAS. Fifteen individuals with heterozygous variants were females. Two females had digenic variants in genes *COL4A4* and *COL4A5*. Forty-one percent of the individuals with *COL4A5* changes were younger than 18 years old at the time at diagnosis. Nine novel variants were identified in the *COL4A5* gene (**Table 4**; **Supplementary Table 1**). According to the Centogene laboratory, three changes were classified as likely pathogenic, all three variants were described as disease causing or probably damaging by PolyPhen-2, while the rest of the variants were N/a. Two changes were judged as VUS while three of the variants were classified as disease causing. Five variants were considered as pathogenic using ACMG criteria in Varsome database, while InterVar four of these variants considered to be likely pathogenic and one as VUS.

Four *COL4A5* variants were identified as glycine substitutions. Two sequence variants were observed in *COL4A5* located at the splice donor site. The analysis of the pathogenic potential of these discovered variants using MutationTaster and Human Splicing Finder, showed functional loss of the affected protein caused by aberrant splicing. Three frame shift changes in *COL4A5* were also discovered (**Table 4**; **Supplementary Table 1**).

The clinical features are seen in **Table 4** and **Supplementary Table 1**, while two individuals with digenic changes are described in digenic section. Proteinuria was discovered in 17 of 20 individuals. Two individuals with the same variant *COL4A5* p.Gly926Val reached KF at the age of 24 and 32, while eGFR < 60 ml/min/1.73 m^2 was detected in 25% of individuals with *COL4A5* variants. Bilateral neurosensory hearing loss and ocular lesions were found in six and four individuals, respectively.

Our clinical interpretation of novel *COL4A5* variants was based on clinical course, family history, results of kidney biopsy, sex and age; and was listed in **Table 4**.

COL4A3

Eleven individuals from eight different families had previously unreported variants in *COL4A3*. Summary of novel *COL4A3* variants found in a study can be seen in **Table 5** and in more details in **Supplementary Table 2**. Eight with clinically diagnosed AS had one heterozygous variant, two individuals (twin sisters) both had compound heterozygous variants in *COL4A3,* and one had digenic variants in genes *COL4A3* and *COL4A4*. Altogether, nine novel variants were identified in *COL4A3* (**Table 5**; **Supplementary Table 2**). According to the

TABLE 4 | Summary of novel *COL4A5* variants found in a study (only heterozygous and hemizygous variants with X linked AS).

Variant	No of ind.	No of M/F	No of diff. families	Age at diagnosis (range)	Pro	KF (age at KF)	No of ind. with ocular ab.	No of ind. with hearing ab.	Ind. with kidney biopsy	ACMG (ClinVar, Varsome, InterVar, Centogene, Franklin DB)	Our clinical interpretation on disease course
One hemizygous or heterozygous variant in *COL4A5* (XLAS)											
c.3508G>C (p.Gly1170Arg)	3	M-1; F-2	1	11–43	+	N/a (but male has CKD IIIb)	None	None	1 (male with FSGS)	3-LP; 1-P; 1-N/A	Pathogenic
c.3106+1G>A	3	M-1; F-2	1	4–37	+	N/a	1	1	N/a	2-LP; 2-P; 1-N/A	Likely pathogenic
c.1417_1418del (p.Val473Glufs*3)	3	F-3	1	28–36	+	N/a (but female has CKD IIIa)	None	1	N/a	3-LP; 1-P; 1-N/A	Pathogenic (KF in family males)
c.347delC (p.Pro116Glnfs*39)	2	M-1; F-1	1	6–35	+	N/a	None	1	1 (lamellation of GBM)	2-LP; 2-P; 1-N/A	Likely pathogenic
c.883G>A (p.Gly295Ser)	1	M	1	22	+	N/a	None	None	1 (lamellation of GBM)	1-LP; 1-P; 1-N/A; 2-VUS	Likely benign course or late onset (normal eGFR)
c.2777G>T (p.Gly926Val)	3	M-2; F-1	1	24–47	+	2 males	2	2	1	1-LP; 1-P; 1-N/A; 2-VUS	Pathogenic
c.1374delinsTT (p.Pro459Serfs*6)	2	M-1; F-1	1	20–51	+	N/a	1	1	N/a	3-LP; 1-P; 1-N/A	Likely pathogenic or late onset form (eGFR for male is normal)
c.3554-2A>G	2	F-2	1	9–14	+	N/a	N/d	N/d	2 (FSGS)	3-LP; 1-P; 1-N/A	Likely pathogenic
c.466G>C (p.Gly156Arg)	1	F	1	15	+	N/a (CKD IIIb)	None	None	1 (FSGS)	1-P; 1-N/A; 3-VUS	Pathogenic

No, number; F, female; M, male; diff, different; KF, kidney failure; Ind, individual; ab., abnormalities; N/a, not applicable; N/d, no data; DIG, digenic; CKD, chronic kidney disease; FSGS, focal segmental glomerulosclerosis; pro, proteinuria; P, pathogenic; LP, likely pathogenic; VUS, variant with unknown significance; XLAS, X-linked Alport syndrome, AS, Alport syndrome, ARAS, autosomal recessive Alport syndrome; ADAS, autosomal dominant Alport syndrome.

TABLE 5 | Summary of novel *COL4A3* variants found in a study (ARAS and ADAS).

Variant	No of ind.	M/F	No of diff. families	Age at diagnosis (range)	Pro	KF (age at KF)	No of ind. with ocular ab.	No of ind. with hearing ab.	Ind. with kidney biopsy	ACMG (ClinVar, Varsome, InterVar, Centogene, Franklin DB)	Our clinical interpretation on disease course
One heterozygous novel variant in *COL4A3* (ADAS)											
c.520G>A (p.Gly174Arg)	2	M-1; F-1	1	6–35	+	N/a	None	None	1 (lamellation of GBM)	3-P; 2-VUS	Likely pathogenic
c.2711G>T (p.Gly904Val)	1	M	1	31	+	N/a	None	None	1 (TBM)	1-N/A; 4-VUS	Likely benign
c.416G>A (p.Gly139Glu)	1	F	1	63	+	N/a	N/d	None	1 FSGS	1-N/A; 4-VUS	Likely pathogenic
c.4717G>A (p.Gly1573Ser)	2	M-1; F-1	1	1–30	+	N/a	1	N/d	N/a	1-N/A; 4-VUS	Likely benign or late onset
c.593G>T (p.Gly198Val)	1	M	1	53	+	N/a	None	1	1 (FSGS)	1-N/A; 4-VUS	Likely pathogenic
c.2188G>C (p.Gly730Arg)	1	F	1	42	+	N/a	None	None	1 (TBM)	1-N/A; 4-VUS	Likely benign
Compound heterozygous variants in *COL4A3* (ARAS)											
c.4702C>T (p.Pro1568Ser)	2	F-2	1 (twin sisters)	33	+	1	None	2	2 (FSGS)	1-N/A; 4-VUS 1-LP	Pathogenic
c.3247G>C (p.Gly1083Arg)									1-N/A; 3-VUS		

No, number; F, female; M, male; diff, different; KF, kidney failure; Ind, individual; ab., abnormalities; N/a, not applicable; N/d, no data; DIG, digenic; CKD, chronic kidney disease; FSGS, focal segmental glomerulosclerosis; pro, proteinuria; P, pathogenic; LP, likely pathogenic; VUS, variant with unknown significance; XLAS, X-linked Alport syndrome, AS, Alport syndrome, ARAS, autosomal recessive Alport syndrome; ADAS, autosomal dominant Alport syndrome.

Centogene laboratory, all nine novel variants were judged as variants with unknown significance by prediction tools (seven changes as probably damaging by PolyPhen-2 and eight as disease causing variants by MutationTaster, however one variant was described as polymorphism). To compare, Franklin genetic databases described two variants as pathogenic and eight variants as VUS (**Table 5**; **Supplementary Table 2**). One variant was considered as pathogenic and one as likely pathogenic using ACMG criteria in Varsome database, however other variants were debatable and described as likely pathogenic or VUS, similar to InterVar judgment.

Of the nine different *COL4A3* variants found in the study cohort, two (78%) were glycine substitutions and two (22 %) were other missense changes (**Table 5**; **Supplementary Table 2**).

Their clinical features are seen in **Table 5** and **Supplementary Table 2**, while one person with a digenic change is described in digenic section. Proteinuria was present in eight individuals with *COL4A3* variants. One person had reached KF due to compound heterozygous *COL4A3* variants at the age of 33. Bilateral neurosensory hearing loss and ocular lesions were found in three and one individuals, respectively.

Our clinical interpretation of novel *COL4A3* variants was based on clinical course, family history, results of kidney biopsy, sex and age; and was listed in **Table 5**.

COL4A4

Twenty individuals from 11 different families had novel variants in *COL4A4*. Summary of novel *COL4A3* variants found in a study can be seen in **Table 6** and in more details in **Supplementary Table 3**. Sixteen with AS (80 %) were carriers of only one heterozygous variant, two individuals had two heterozygous variants in *COL4A4,* and two had digenic variants in genes *COL4A4* and *COL4A5*. In total, nine novel or previously undescribed variants were identified in *COL4A4* (see **Table 6**; **Supplementary Table 3**). According to Centogene laboratory, four of these variants were classified as likely pathogenic (two of these changes were determined as probably damaging by PolyPhen-2 or disease-causing variants by MutationTester). Two novel VUS sequence variants were classified as polymorphism by MutationTaster and benign by PolyPhen-2, while one VUS was described as disease causing by both prediction programs. One variant was described as pathogenic. To compare, Franklin genetic databases described one variant as pathogenic, two variants as likely pathogenic and five variants as VUS (see **Table 6**; **Supplementary Table 3**). Two variants were considered as pathogenic and three as VUS using ACMG criteria in Varsome database, however other variants were debatable and described as likely pathogenic or VUS, although InterVar judged these variants as VUS.

Of the nine identified different *COL4A4* variants, six variants were missense changes of which 3 (33%) were glycine substitutions (**Table 6**; **Supplementary Table 3**), while the remaining three variants were classified as other missense variants. One splice site, and one stop codon changes detected in the *COL4A4* gene are presented in **Table 6** and **Supplementary Table 3**. The analysis of the pathogenic potential of these sequence variants by Human Splicing Finder MutationTaster, suggested functional loss of the damaged protein caused by aberrant splicing or stop codon.

The clinical features are seen in **Table 6**, while two individuals with digenic variants are described in digenic section. Proteinuria was discovered in eight of 18 individuals. One person with heterozygous *COL4A4* variant reached KF at the age 55, while eGFR (estimated glomerular filtration rate, using CKD-EPI, Chronic Kidney Disease Epidemiology Collaboration formula) <30 ml/min/1.73 m^2 was revealed in 11% (2/18) of individuals with *COL4A4* changes. Bilateral neurosensory hearing loss and ocular lesions were found in 3 and 4 individuals, respectively.

Our clinical interpretation of novel *COL4A4* variants was based on clinical course, family history, results of kidney biopsy, sex and age; and was listed in **Table 6**.

Digenic

Three individuals from three different families carried digenic variants. Genetic analysis indicated a frameshift change in the *COL4A5* gene combined with a missense variant in the *COL4A4* gene in 2 individuals (**Table 7**; **Supplementary Tables 1, 3**). One person had one novel missense change in *COL4A3* and a previously reported in the literature splice site variant in *COL4A4* (**Table 7**; **Supplementary Table 2**). Altogether, five novel digenic variants were identified. Two novel changes in *COL4A4* (33%) were classified as VUS. These two variants p.Arg1637Gln and p.Ala1384Val in *COL4A4* were described as probably damaging and benign, respectively, by both prediction tools (PolyPhen-2, and MutationTaster). Two identified variants in *COL4A5*, according to Centogene, were classified as pathogenic, whereas N/a by PolyPhen-2, and MutationTaster. One novel variant in *COL4A3* p.Arg341Cys was judged as disease causing by one program and polymorphism by the other.

Both identified variants in *COL4A4* were missense variants, while variants in *COL4A5* were associated with frame shift changes (**Table 7**; **Supplementary Tables 1, 3**). One glycine substitution was discovered in *COL4A3* (**Table 7**; **Supplementary Table 2**). All individuals had proteinuria, neither of them had a neurosensory hearing loss nor ocular lesion. None of the individuals reached KF.

Our clinical interpretation of novel digenic variants was based on clinical course, family history, results of kidney biopsy, sex and age; and was listed in **Table 7**.

DISCUSSION

Heterozygous disease causing variants in *COL4A3* or *COL4A4* demonstrate wide phenotypic spectrum of manifestations, ranging from asymptomatic isolated hematuria to progressive kidney disease and extrarenal lesions (13). In cases with isolated hematuria, the terms "thin basement membrane disease," "familial benign hematuria," and "carriers of ARAS" have been used (17–19). However, many individuals with heterozygous *COL4A3* or *COL4A4* disease causing variants eventually develop FSGS and severe proteinuria and some progress to KF (20, 21). The aim of redefining nomenclature from TBMN to ADAS is to simplify the diagnostic terminology and to improve the early diagnosis of AS and increase the chances of affected

TABLE 6 | Summary of novel *COL4A4* variants found in a study (ARAS and ADAS).

Variant	No of ind.	M/F	No of diff. families	Age at diagnosis (range)	Pro	KF (age at KF)	No of ind. with ocular ab.	No of ind. with hearing ab.	Ind. with kidney biopsy	ACMG (ClinVar, Varsome, InterVar, Centogene, Franklin DB)	Our clinical interpretation on disease course
One heterozygous novel variant in *COL4A4* (ADAS)											
c.4151C>T (p.Ala1384Val)	1	F	1	31	N/d	N/a	None	None	N/a	1-N/A; 4-VUS	Likely benign
c.594+1G>A	5	M-2; F-3	2	11–64	+	1	2	1	1 (FSGS)	1-LP; 3-P	Likely pathogenic
c.2756A>G (p.Glu919Gly)	2	M-1; F-1	1	2–40	+	N/a	None	None	1 (FSGS)	5-VUS	Likely pathogenic or late onset
c.4315G>A (p.Gly1439Ser)	3	M-2; F-1	1	10–46	+	N/a	None	1	N/a	1-LP; 1-N/A; 3-VUS	Likely benign or late onset
c.3044G>A (Gly1015Glu)	3	M-3	1	2–39	None	N/a	2	N/d	N/a	2-LP; 1-N/A; 2-VUS	Likely benign
c.657+2dup	1	F	1	65	None	N/a	None	1	1 (TBM)	1-LP; 2-N/A; 2-VUS	Likely benign
c.2347G>A (p.Gly783Arg)	1	F	1	4	None	N/a	None	None	N/a	1-LP; 4-VUS	Likely benign Difficult to interpret due to young age of individual
Compound heterozygous variants in *COL4A4* (ARAS)											
c.594+1G>A (novel)	1	M	1	6	+	N/a	None	None	1	LP; 3-P	Pathogenic
p.Gly527Cys (described variant)									LP		
c.4720C>T (p.Gln1574*) (novel)	1	F	1	32	+	N/a	None	None	1 (TBM)	LP; 3-P; 1-N/A	Likely benign (eGFR normal)
c.3307G>A (p.Gly1103Arg) (described variant)									LP		

No, number; F, female; M, male; diff, different; KF, kidney failure; Ind, individual; ab., abnormalities; N/a, not applicable; N/d, no data; DIG, digenic; CKD, chronic kidney disease; FSGS, focal segmental glomerulosclerosis; pro, proteinuria; P, pathogenic; LP, likely pathogenic; VUS, variant with unknown significance; XLAS, X-linked Alport syndrome, AS, Alport syndrome, ARAS, autosomal recessive Alport syndrome; ADAS, autosomal dominant Alport syndrome.

TABLE 7 | Summary of novel digenic variants in *COL4A3-A5* (digenic AS).

Variant	No of ind.	M/F	No of diff. families	Age at diagnosis (range)	Pro	KF (age at KF)	No of ind. with ocular ab.	No of ind. with hearing ab.	Ind. with kidney biopsy	ACMG (ClinVar, Varsome, InterVar, Centogene, Franklin DB)	Our clinical interpretation on disease course
Digenic variants in *COL4A5* and *COL4A4*											
COL4A5 c.1417_1418del (p.Val473Glufs*3)	1	F	1	14	+	N/a	None	None	1 (FSGS)	3-LP; 1-P; 1-N/A	Likely pathogenic
COL4A4 c.4151C>T (p.Ala1384Val)									1-N/A; 4-VUS		
COL4A5 c.1374delinsTT(p.Pro459Serfs*6)	1	F	1	26	+	N/a	None	None	N/a	3-LP; 1-P; 1-N/A	Likely benign
COL4A4 c.4910G>A (p.Arg1637Gln)									1-N/A; 4-VUS		
Digenic variants in *COL4A3* and *COL4A4*											
COL4A3 c.1021C>T (p.Arg341Cys)	1	M	1	41	+	N/a	None	None	1 (TBM)	1-LP; 1-P; 1-N/A; 2-VUS	Likely benign
COL4A4 c.-101-4A>G									Polymorphism		

No, number; F, female; M, male; diff, different; KF, kidney failure; Ind, individual; ab., abnormalities; N/a, not applicable; N/d, no data; DIG, digenic; CKD, chronic kidney disease; FSGS, focal segmental glomerulosclerosis; pro, proteinuria; P, pathogenic; LP, likely pathogenic; VUS, variant with unknown significance; XLAS, X-linked Alport syndrome, AS, Alport syndrome, ARAS, autosomal recessive Alport syndrome; ADAS, autosomal dominant Alport syndrome.

individuals receiving proper monitoring and well-timed therapy (13). Therefore, in this study we used ADAS terminology for the heterozygous *COL4A3* and *COL4A4* variants. According to Kashtan, 1% of individuals with ADAS reach KF, while this increases in those with risk factors such as proteinuria, FSGS, GBM thickening and lamellation, family history of progressive kidney disease, up to 20% of cases (13). In our study, one person out of 24 developed KF at the age of 55 years (A49737, COL4A4 c.594+1G>A; NM_000092.5). In a similar study, individuals with ADAS reached KF at a median age of 70 years (22). The ADAS burden depends on the type of pathogenic change. Nonsense and stop codon variants are more often associated with KF compared with missense variants (9). In our study, the person who developed KF had a splice site variant in *COL4A4*. There were also other risk factors for KF such as FSGS in the kidney biopsy, severe proteinuria, and late diagnosis of AS, with no early initiation of renoprotective treatment. However, the individual's offspring, only had persistent hematuria and minimal proteinuria even as adults. Interestingly, one person's grandson (A4/49716^{3*bi}) was diagnosed with ARAS due to compound heterozygous variants in the *COL4A4* gene, inherited from his mother (the person's daughter) and his father. The same variant (*COL4A4* c.594+1G>A; NM_000092.5) was also found in 64-year-old male (A494487) from a different, unrelated family, who at the time of diagnosis, had chronic kidney disease (CKD) stage IV and his kidney histology revealed FSGS. Despite severe kidney function impairment, the proteinuria level was low (<1 g/d). Therefore, the same variant can be associated with interfamilial and extrafamilial variation and emphasizes the importance of careful evaluation and monitoring of individuals with heterozygous *COL4A3* or *COL4A4* variants. Although extrarenal manifestations in ADAS are rare, one study reported that 13.3% of individuals with ADAS developed a sensorineural hearing loss (23). However, these results are consistent with our study. Another study reported that 23% of individuals had abnormal ocular findings, but only two individuals (<1%) had anomalies possibly related to AS (24). In our study, ocular abnormalities, including retinal flecks and retinal thinning, were found in 25% of individuals with heterozygous *COL4A3* and *COL4A4* variants. These results indicate that phenotypic manifestations in individuals with ADAS vary significantly. However, extrarenal expression is usually milder and appears less frequently than in XLAS males and ARAS individuals. Nevertheless, the development of risk factors may have a significant impact on the disease burden (3, 25). Alternatively our individuals with hearing loss and ocular abnormalities could have had a second undetected pathogenic variant and hence ARAS or digenic AS.

Genotype-phenotype correlations are only now emerging in ARAS individuals (26). One study reported that ARAS individuals with nonsense variants reach KF earlier in life and frequently have more nephrotic range proteinuria with a higher prevalence of ocular and sensorineural hearing changes, than with compound or biallelic missense variants (27). In our study, KF was found in only one woman (A3/3232145^{5*bi}) at the age of 33 with compound novel heterozygous missense variants c.4702C>T (p.Pro1568Ser) and c.3247G>C (p.Gly1083Arg) in *COL4A3*. Her biopsy demonstrated FSGS and a thinned and lamellated glomerulus basement membrane. Interestingly, the person's heterozygous twin sister (A3/3232146^{5*bi}) with the same variants, had a milder clinical phenotype but similar kidney biopsy features including FSGS. Different phenotypes might be explained by the dizygotic form of the twins and modifying environmental factors, and more Alport twin studies might be helpful. One study reported that hematuria and proteinuria were found in all individuals with ARAS and 43% of them developed KF (26). Both twins in our study had nephrotic range proteinuria which is a prognostic factor for KF. The third person (A4/49716^{3*bi}) with ARAS, diagnosed at the age of 6, was from a different family and had two compound heterozygous *COL4A4* variants gene. A novel pathogenic splice site variant c.594+1G>A (NM_000092.5) was detected in *COL4A4* and shared its phenotype with the already described likely pathogenic variant in *COL4A4* c.1579G>T (p.Gly527Cys) (22). This boy (A4/49716^{3*bi}) at the age of 12 had both hematuria and proteinuria (detected from the age of 1), without any deterioration of kidney function. Many cases of ARAS are overlooked because of the lack of a positive family history and different genotype-phenotype correlations among family members (28).

Large fragment deletions, nonsense variants or frameshift variants are associated with a higher risk of KF than splicing or missense variants in *COL4A5* gene (12), but in this study the most severe phenotype including KF was associated with a missense *COL4A5* variant c.2777G>T (p.Gly926Val, NM_033380.3). This novel *COL4A5* variant causes an amino acid change from Gly to Val at position 926 and is classified as a variant of uncertain significance in Centogene and Franklin's databases, while Varsome database describes this variant as likely pathogenic (PM2, MM1, PP2, PP3). Two brothers from the same family with this variant developed KF by 24 and 32 years of age. Interestingly the younger individual developed KF 8 years earlier than his older brother, suggesting that the same gene variants might result in significantly different phenotypes. In individuals with AS, the variant type determines the age at KF in general (29). In our study, only two of seven males (29%) with XLAS developed KF, which is consistent with a better prognosis than noted previously (29% compared with 90%) (10). The mean age of KF in individuals with missense variants, splicing site variants, and truncation variants have been reported as 37, 28, and 25 years, respectively (8). Males studied here were relatively young (median age of 24 years), at the time of diagnosis and evaluation of the phenotype, which might explain the lower rate of KF than in other studies. However, four individuals with *COL4A5* variants had significant nephrotic range proteinuria (≥3, 5 g/d) which can also be associated with a future decline in kidney function and the main predictor of KF (30–32).

The phenotype in women is usually milder (10, 33), however it is known that heterozygous females with XLAS still have a lifetime risk of KF and other extrarenal manifestations (34). Although in our study most of the females had

only hematuria, three women A5338, A56511, A5343159 with heterozygous variants p.Gly1170Arg, Val473Glufs*3, p.Gly156Arg in *COL4A5* had decline of eGFR < 60 ml/min/1.73 m^2. A genotype - phenotype correlations demonstrate a high intrafamilial heterogeneity due to random X-chromosomal inactivation thus prediction of the disease course is complicated (12). Bilateral sensorineural hearing loss appeared in 71.4% individuals with XLAS, which consists with other studies (10, 35). According to several studies, lenticonus may have a prognostic significance on eGFR decline (10). In our study, two males with anterior lenticonus presented with KF. However, further studies are required to address the exact relationship between kidney and extrarenal manifestations.

Digenic inheritance still raises many questions, and a more complex inheritance should be considered when reviewing prognosis and at-risk family members (36). In our study three individuals aged 14–41, from three different families, had digenic variants in these genes. According to the recent guidelines for Genetic Testing and Management of Alport Syndrome, digenic inheritance may be associated with more severe phenotypes including kidney impairment (37). However, in our study none of individuals with digenic variants regardless of the variant type (missense and splicing changes) reached KF. Variants in the *COL4A5* gene in combination with *COL4A3* or *COL4A4* variants have on-Mendelian inheritance and therefore, family-specific risk assessment is critical.

CONCLUSION

In conclusion, 27 novel pathogenic variants were identified by NGS testing of all three *COL4A3-A5* genes in individuals with suspected AS from a single center over a 5-year period. All index cases had hematuria and four (7.8%) had KF. About half the affected individuals had ADAS but this cohort also included ARAS, XLAS and digenic disease. These novel variants represented more than half of all the variants found in a cohort of 171 individuals from 109 unrelated families who underwent testing. We identified pathogenic or likely pathogenic variants in all the individuals with KF or with ocular abnormalities consistent with a high detection rate in XL and AR Alport syndrome. The detection rate of pathogenic variants was less in those with persistent hematuria alone. These results increase the number of known *COL4A3*, *COL4A4,* and *COL4A5* variants and our understanding of genotype-phenotype correlations in AS. The genetic variants in *COL4A3-5* have a significant impact on the diagnosis, treatment, and prognosis for individuals with AS.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: ClinVar Database, accession SUB11112191 (SCV002098105 - SCV002098129). However, authors declare that in this study there is a partial non-availability of raw data due to the time period restriction imposed by Centogene laboratory on stored data (older than 5 years variants and its raw data are not stored in this laboratory). Data non-availability could potentially be a major limitation of the study.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Vilnius Regional Biomedical Research Ethics Committee of Lithuania (BioAlport, No 158200-16-857-367). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

AC, RC, and JS contributed to conception and design of the study. AC, RS-S, VV, RC, BB, AJ, AL, MM, PB, SS, and AR organized the database. AC performed the statistical analysis and wrote the first draft of the manuscript. AC, KJ, IJ, RC, and JS wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.859521/full#supplementary-material

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Conflict of Interest: AR, PB, and SS were employed by CENTOGENE. The genetic testing for the individuals of this research was made by CENTOGENE free of charge by participating in other research - BioAlport study.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
published: 25 April 2022
doi: 10.3389/fmed.2022.848389

Novel Therapies for Alport Syndrome

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Alport syndrome (AS) is a hereditary kidney disease associated with proteinuria, hematuria and progressive kidney failure. It is characterized by a defective glomerular basement membrane caused by mutations in type IV collagen genes *COL4A3/A4/A5* which result in defective type IV collagen α3, α4, or α5 chains, respectively. Alport syndrome has three different patterns of inheritance: X-linked, autosomal and digenic. In a study of CKD of unknown etiology type IV collagen gene mutations accounted for the majority of the cases of hereditary glomerulopathies which suggests that AS is often underrecognized. The natural history and prognosis in patients with AS is variable and is determined by genetics and environmental factors. At present, no preventive or curative therapies exist for AS. Current treatment includes the use of renin-angiotensin-aldosterone system inhibitors which slow progression of kidney disease and prolong life expectancy. Ramipril was found in retrospective studies to delay the onset of ESKD and was recently demonstrated to be safe and effective in children and adolescents, supporting that early initiation of Renin Angiotensin Aldosterone System (RAAS) blockade is very important. Mineralocorticoid receptor blockers might be favorable for patients who develop "aldosterone breakthrough." While the DAPA-CKD trial suggests a beneficial effect of SGLT2 inhibitors in CKD of non-metabolic origin, only a handful of patients had Alport in this cohort, and therefore conclusions can't be extrapolated for the treatment of AS with SGLT2 inhibitors. Advances in our understanding on the pathogenesis of Alport syndrome has culminated in the development of innovative therapeutic approaches that are currently under investigation. We will provide a brief overview of novel therapeutic targets to prevent progression of kidney disease in AS. Our review will include bardoxolone methyl, an oral NRf2 activator; lademirsen, an anti-miRNA-21 molecule; sparsentan, dual endothelin type A receptor (ETAR) and angiotensin 1 receptor inhibitor; atrasentan, oral selective ETAR inhibitor; lipid-modifying agents, including cholesterol efflux transporter ATP-binding cassette A1 (ABCA1) inducers, discoidin domain receptor 1 (DDR1) inhibitors and osteopontin blocking agents; the antimalarial drug hydroxychloroquine; the antiglycemic drug metformin and the active vitamin D analog paricalcitol. Future genomic therapeutic strategies such as chaperone therapy, genome editing and stem cell therapy will also be discussed.

Keywords: Alport, Alport disease, Alport nephritis, Alport hereditary nephritis, COL4, type IV collagen, collagen 4, treatment

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Nephrology, a section of the journal Frontiers in Medicine

Received: *04 January 2022*
Accepted: *14 March 2022*
Published: *25 April 2022*

Citation:
Chavez E, Rodriguez J, Drexler Y and Fornoni A (2022) Novel Therapies for Alport Syndrome. Front. Med. 9:848389. doi: 10.3389/fmed.2022.848389

INTRODUCTION

Alport syndrome (AS), also called hereditary nephritis, is an inheritable progressive glomerular disease that is generally associated with sensorineural hearing loss and ocular abnormalities. It presents with structural anomalies and dysfunction of the glomerular basement membrane (GBM) caused by genetic mutations affecting the type IV collagen α3/α4/α5 chains (1). Type IV collagen is essential for GBM stability and constitutes the majority of total GBM protein mass. The α1 to α6 type IV collagen chains are genetically different and they assemble to form 3 distinct heterotrimers: α1α1α2, α3α4α5 and α5α5α6 (2). In AS, pathogenic variants in the genes *COL4A3* and *COL4A4* (which are located on chromosome 2) and *COL4A5* (which is located on the X chromosome) produce defective type IV collagen α3, α4 or α5 chains respectively, which hampers the appropriate assembly of the GBM (3, 4).

CLASSIFICATION

The new classification scheme categorizes AS into 3 different types based on the affected type IV collagen genes involved and mode of inheritance: X-linked AS, autosomal or digenic (**Table 1**).

According to this classification system it is not necessary to have a positive family history, extrarenal manifestations or evidence of progressive kidney disease to make a diagnosis of AS. Females with heterozygous *COL4A5* variants are classified as having AS instead of being labeled as "carriers" of the disease. Patients with isolated microscopic hematuria and heterozygous variants in *COL4A3* or *COL4A4* are considered to have autosomal dominant Alport syndrome (ADAS), which therefore eliminates the diagnosis of thin basement membrane nephropathy (TBMN) (3). It must be recognized that this latter point is controversial among experts on this condition and this is not a universal agreement (5).

Genetic testing in AS is proposed for patients who have persistent dysmorphic hematuria for longer than 6 months; persistent proteinuria >0.5 g/g, family history of hematuria or kidney disease; sensorineural hearing loss with hematuria; lenticonus, fleck retinopathy or temporal retinal thinning; biopsy-proven focal segmental glomerulosclerosis (FSGS) or steroid-resistant nephrotic syndrome; GBM lamellation; chronic kidney disease of unknown etiology with hematuria and familial IgA glomerulonephritis. Genetic testing is also recommended in first-degree relatives of people with known pathogenic variants in *COL4A3-COL4A5* genes (6).

TABLE 1 | New classification system of Alport syndrome.

Inheritance	Affected gene (s)	Allelic state	Mutation phenotype
X-linked	*COL4A5*	Heterozygous (males)	NA
		Heterozygous (females)	NA
Autosomal	*COL4A3* or *COL4A4*	Homozygous or compound heterozygous	Recessive
		Heterozygous	Dominant
Digenic	*COL4A3*, *COL4A4* and *COL4A5*	Variable	

NA, not applicable Adapted data from Kashtan et al. (3).

PREVALENCE

The prevalence of Alport syndrome varies greatly in different reports with estimates ranging from one in 5,000 to one in 53,000 patients with kidney disease. X-linked Alport syndrome (XLAS) is caused by *COL4A5* gene mutations and accounts for 70–80% of patients with AS. Autosomal recessive Alport syndrome (ARAS) is caused by homozygous or compound heterozygous mutations in *COL4A3* and *COL4A4* genes and accounts for ∼5% of patients with AS. A recent study estimated the prevalence of predicted pathogenic *COL4A3-COL4A5* variants in populations without kidney disease. Predicted pathogenic *COL4A5* variants were found in one in 2,320 persons. Predicted pathogenic heterozygous *COL4A3* and *COL4A4* variants affected one in 106 individuals, consistent with the finding of TBMN in kidney biopsies in donors without known kidney disease. Predicted pathogenic compound heterozygous variants appeared in one in 88,866 persons and digenic variants in at least one in 44,793 persons. However, these population frequencies of predicted *COL4A3-COL4A5* pathogenic variants must be modified according to the disease penetrance of individual variants (7).

The true prevalence of AS is likely underestimated. A recent study from Columbia University using genetic testing to evaluate cases of chronic kidney disease of unknown etiology showed that Mendelian nephropathies accounted for 21% of the cases. Monogenic glomerulopathies were the most frequent and this was mainly driven by mutations in collagen IV genes which accounted for nearly one third of all hereditary kidney diseases (8).

NATURAL HISTORY AND PROGNOSIS

AS encompasses a diverse phenotypic spectrum with a broad range of clinical manifestations. The typical kidney histological lesion in a patient with established AS is described on electron microscopy as irregular thinning and thickening GBM with lamellated appearance which has a characteristic "basket-weave" pattern (9). When a kidney biopsy performed in a patient with isolated hematuria shows exclusively a thin GBM it has been proposed to use the terms "benign familial hematuria" or "thin basement membrane nephropathy." However, this is an erroneous assumption since there is growing evidence that some of these patients are at risk of developing progressive kidney disease. In patients with AS, a thin GBM can also be identified and therefore this histologic finding should not be considered a separate disorder. A thin GBM by itself is not sufficient to estimate prognosis in the absence of additional clinical, pathologic, pedigree and genetic data.

The likelihood of kidney disease progression in patients affected with AS is variable and is mainly determined by the type of gene mutation. Large deletions, nonsense mutations and small mutations affecting reading frames that cause a decreased or absent functional protein represent a high risk of progression to end stage kidney disease (ESKD) by age 30. The risk is minor with missense or splice site mutations (10). Also, environmental factors may contribute to kidney disease progression.

Men with XLAS and all patients with ARAS typically develop "classic" AS features which include progressive kidney disease with hematuria and proteinuria, sensorineural deafness and ocular abnormalities (eg, lenticonus or maculopathy). While less recognized as a typical syndromic feature, aortic aneurisms are often recognized in males with XLAS (11).

In cases of XLAS, sex is an important prognostic factor regarding CKD progression. Among untreated men with XLAS, around 50 and 90% progress to ESKD by ages 25 and 40, respectively. Women with XLAS exhibit a variable phenotype due to the process of X chromosome inactivation. Among women with XLAS, about 12% reach ESKD before age 40 and this proportion increases to about 30% by age 60 and 40% by age 80 (10). Patients with ARAS frequently progress to ESKD by age 40 regardless of sex (12).

Patients with ADAS exhibit a wide variety of manifestations which range from asymptomatic to kidney-limited disease with microscopic hematuria or proteinuria and in some cases progression to ESKD. The phenotype associated with a heterozygous mutation in *COL4A3* or *COL4A4* can vary significantly even within members of the same family, and this variability of expression is likely multifactorial. This incomplete penetrance may be related to the presence of modifier genes that ameliorate or exacerbate the effects of type IV collagen gene mutations and other factors such as high blood pressure, high sodium diet, obesity and smoking which may independently contribute to kidney disease (3, 13).

In patients with ADAS, the estimated risk for ESKD is $\geq$20% for those with risk factors for progression (proteinuria, FSGS, GBM thickening and lamellation, sensorineural hearing loss, genetic modifiers) and <1% in the absence of these risk factors (3).

The estimated risk for progression to ESKD in patients with digenic AS is variable and depends on the affected genes: for *COL4A3* and *COL4A4* mutations in trans simulating autosomal recessive inheritance the risk is up to 100%; for *COL4A3* and *COL4A4* mutations in cis simulating autosomal dominant inheritance the risk is up to 20% and for affected men with mutations in *COL4A5* and either *COL4A3* and *COL4A4* the risk is up to 100% (3).

Type IV collagen gene mutations have also been associated with FSGS and genetic testing should be considered particularly in those patients with positive family history of FSGS and younger age at presentation. Type IV collagen gene mutations were discovered in 38% of patients with familial FSGS and 3% with sporadic FSGS with more than half of the mutations appearing in *COL4A5*. The presence of hematuria, hearing loss and GBM abnormalities might indicate the possibility of an underlying *COL4* mutation (14). In a cohort of 193 individuals of the Toronto GN registry with predominantly sporadic FSGS who underwent whole-exome sequencing, the genetic diagnostic rate was 11% and from these patients 55% had definitely pathogenic variants in *COL4 (A3/A4/A5)* genes (15). Individuals with biopsy-proven FSGS who have pathogenic variants in the type IV collagen genes should be classified as Alport syndrome patients. It is important to avoid the use of immunosuppressive therapy on these patients because this would be ineffective and potentially harmful. Consistent with these observations, genetic testing should be obtained in all patients with biopsy-proven FSGS or steroid resistant nephrotic syndrome (6).

KIDNEY TRANSPLANTATION

Patient with Alport syndrome who progress to ESKD are generally excellent candidates for kidney transplantation. Preemptive kidney transplantation should be pursued when possible. In patients with AS, graft survival rates are equal or better compared to those seen in patients with other causes of ESKD. It is recommended that family members who are *COL4A3* and *COL4A4* heterozygotes not be considered for kidney donation (6). There is evidence from individuals with hematuria or heterozygous pathogenic *COL4A3* or *COL4A4* variants who acted as kidney donors who developed worsening kidney function or proteinuria over time (16–18). While the development of anti-GBM antibodies without clinical manifestation is quite common, posttransplant anti-GBM nephritis is an unusual but possibly catastrophic complication of kidney transplantation in AS patients (19).

TREATMENT

At present, there is no curative treatment for Alport syndrome. The use of renin-angiotensin-aldosterone system (RAAS) inhibition (RAASi) is the current standard of care to slow progression of kidney disease.

RAAS Inhibition

Gross and colleagues published in 2012 that use of angiotensin converting enzyme inhibitors (ACEi) in a cohort of 174 patients with AS (predominantly males with XLAS) was associated with a later onset of renal replacement therapy and longer life expectancy compared to a cohort of 109 untreated AS relatives over a mean follow up of more than two decades. This benefit was greater in those patients who started therapy at an earlier stage, especially in those patients who had isolated hematuria or microalbuminuria at the time of treatment initiation (20). Data from the European Alport Registry showed that the use of RAAS blockade in heterozygous Alport carriers is associated with a slower kidney disease progression. Mean age at onset of therapy was 28 years and average time on therapy was 5.8 years on this analysis. Onset of renal replacement therapy (RRT) occurred less frequently and significantly later in patients who were treated with RAAS blockade (21). Yamamura and colleagues examined a group of Japanese male patients with XLAS with proven *COL4A5* variants and showed that the renal protective effect of RAAS

blockade was present regardless of the type of *COL4A5* gene variant (truncating vs. non-truncating) (22).

Most ACE Inhibitors are currently authorized by the Food and Drug Administration (FDA) for treatment of hypertension in adults or children ≥6 years old (23). However, the EARLY PROTECT trial showed that use of ramipril in children with AS aged ≥2 years when there is either isolated microscopic hematuria or microalbuminuria (defined as 30–300 mg albumin/g creatinine) is safe and effective at slowing kidney disease progression compared to placebo. Ramipril was initiated at a mean age of 8.8 ± 4.2 years. In this study, ramipril therapy reduced the risk of disease progression by almost 50%. Disease progression was defined as progression to the next disease stage according to the extent of renal damage and loss of function. These stages were defined as (0) microhematuria without microalbuminuria, (I) microalbuminuria [30–300 mg albumin/g creatinine], (II) proteinuria [>300 mg albumin/g creatinine], (III) >25% decline of normal kidney function (CrCl), (IV) end stage kidney disease. A hazard ratio of 0.51 translates to 5.4 children who need to be treated with ramipril for 3 years to prevent one progression of the disease in one child (24).

New clinical practice recommendations support earlier initiation of ACEi in patients affected with AS. Therapy should be started at the time of diagnosis in men with XLAS and in all patients with ARAS, if age ≥24 months. In females with XLAS and in males and females with ADAS, therapy can be started at the onset of microalbuminuria (25).

Mineralocorticoid Receptor Blockers

Serum aldosterone levels remain elevated in some patients treated with ACEi and/or ARBs, a phenomenon known as "aldosterone breakthrough" which has been correlated with left ventricular hypertrophy and higher rates of albuminuria (26). Rubel and colleagues evaluated if the concomitant use of spironolactone on top of ramipril in *COL4α3*$^{-/-}$ mice offered any additional benefit compared to ramipril monotherapy. Dual therapy resulted in a slower kidney disease progression and decreased proteinuria and fibrosis. However, survival did not change. This was possibly due to premature death from side effects of dual therapy such as hyperkalemia (27). Spironolactone effects in human subjects are being evaluated in an observational clinical trial [NCT02378805] (28). The observed favorable outcomes of the non-steroidal mineralocorticoid receptor blocker finerenone on delaying CKD progression in patients with diabetic kidney disease (DKD) (29, 30) suggest the need to test its efficacy in non-diabetic CKD.

SODIUM-GLUCOSE COTRANSPORTER-2 INHIBITORS (SGLT2I)

SGLT2i block renal glucose absorption in the proximal convoluted tubule which promotes glucosuria and lowers blood glucose. These drugs have nephroprotective properties which are independent of glycemic control and are thought to be mediated by glucose-induced osmotic diuresis and concomitant natriuresis leading to afferent arteriole vasoconstriction and a reduction in intraglomerular pressure and reduction in albuminuria (31). There is extensive evidence about the benefit of these drugs to slow the progression of CKD of diabetic and non-diabetic origin (32, 33). In a cohort of 5 pediatric patients with Alport syndrome (mean age 10.4 years) with an eGFR >60 ml/min/1.73 m^2 the use of dapaglifozin was well tolerated and resulted in a 22% reduction in proteinuria by 12 weeks (34).

In diabetic rats, SGLT2i also reduced cardiac accumulation of toxic lipids and free fatty acid uptake (35). In patients with non-alcoholic fatty liver disease and type 2 diabetes mellitus (T2DM) the use of SGLT2i reduced liver fat and improved ALT levels (36). Therefore, it is conceivable that the nephroprotective effect of SGLT2i is also mediated by reducing renal lipotoxicity, which we have found to be pathogenetic in experimental AS (37). While the DAPA-CKD trial suggests a beneficial effect of SGLT2 inhibitors in CKD of non-metabolic origin, the number of patients with Alport syndrome included on this cohort was low and therefore conclusions cannot be extrapolated for this population at this time. However, the efficacy of SGTL2 inhibitors in the treatment of AS should be explored (38).

NEW THERAPIES FOR ALPORT SYNDROME

AS has become a very appealing disease for drug companies to target for multiple reasons: (i) AS is a great example of progressive CKD with proteinuria and fibrosis that can be a model of other more common causes of kidney disease, (ii) there are no curative therapies for AS, (iii) patients with AS are young and often have no other comorbidities which makes them excellent candidates for clinical trials, (iv) there is a large population affected which would benefit from treatment and (v) any new medication approved for this condition will receive orphan drug designation with might have associated benefits, such as shortened time to approval, monetary benefits and a period of market exclusivity (39). Therefore, the search for a curative treatment for Alport syndrome continues (**Table 2**).

Bardoxolone

Bardoxolone methyl is a semisynthetic triterpenoid that activates nuclear factor erythroid 2-related factor 2 (Nrf2), a transcription factor that modulates the expression of multiple genes involved in inflammation, oxidative stress, and cellular energy metabolism (40, 41). By activating the Keap1-Nrf2 pathway, bardoxolone methyl also reduces nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB), the primary transcription factor producing proteins causative of inflammation and the generation of reactive oxygen species (42, 43). Bardoxolone increased GFR in patients with T2DM and CKD stage 3 over a 52-week period (44) which led to the creation of the BEACON trial, a randomized clinical trial (RCT) that evaluated the efficacy and safety of bardoxolone in patients with T2DM and CKD stage 4. Unfortunately, there was a high number of heart failure hospitalizations in the patients treated with bardoxolone which resulted in early trial discontinuation. There was also an increase in blood pressure and an increase in proteinuria in the bardoxolone group (45).

TABLE 2 | Clinical trials for Alport Syndrome listed on clinicaltrials.gov.

Drug name and sponsor	Mechanism of action	Status	Phase and study name	Number of patients	Intervention	ClinicalTrials.gov Identifier
Bardoxolone methyl Reata Pharma-ceuticals	Nrf2 activator NF-κB inhibitor	Completed Recruiting	Phase 3 CARDINAL Phase 3 EAGLE	157 480	Oral bardoxolone vs placebo for 100 weeks Oral bardoxolone for up to 5 years (single arm)	NCT03019185 NCT03749447
Lademirsen (RG-012, SAR339375) Genzyme, a Sanofi Company	Anti-microRNA-21 drug. Reduces P42/P44 MAPK pathway activation	Active, not recruiting	Phase 2 HERA	43	Weekly subcutaneous injection of anti-microRNA-21 drug vs. placebo for 110 weeks	NCT02855268
Sparsentan Travere Therapeutics	Dual endothelin type A receptor and angiotensin II type 1 receptor antagonist	Recruiting	Phase 2 EPPIK	57	Oral sparsentan for 108 weeks (single arm) Includes pediatric patients with AS and other proteinuric kidney diseases	NCT05003986
Atrasentan Chinook Therapeutics	Endothelin type A receptor antagonist	Recruiting	Phase 2 AFFINITY	80	Oral atrasentan for 52 weeks (single arm) Includes adult patients with AS and other proteinuric kidney diseases	NCT04573920
Hydroxychloroquine (HCQ) Shanghai Children's Hospital	Immunomodulatory drug	Recruiting	Phase 2 CHXLAS	50	Oral HCQ + benazepril vs. benazepril monotherapy for 6 months	NCT04937907
Benazepril, valsartan and Fluvastatin Mario Negri Institute for pharmacologic research	Drug combination inhibits ACE, ARB and HMG CoA reductase	Completed	Phase 2. Effects of an Intensified treatment with ACE-I, ATA II and statins in AS	9	Drugs: benazepril, valsartan and Fluvastatin (single arm)	NCT00309257
Ramipril Institut fuer anwendumgsorientierte Forschung und klinische Studien GmbH	Angiotensin converting enzyme inhibitor	Completed	Phase 3. Early prospective therapy trial to delay renal failure in Children with AS (EARLY PROTECT)	66	Ramipril (blinded) Placebo to ramipril Ramipril (open-label)	NCT01485978
R3R01 & River 3 Renal Corp	Lipid-modifying drug	Not yet recruiting	Phase 2. Study to evaluate R3R01 in patients with Alport syndrome and patients with Focal Segmental Glomerulosclerosis	50	Oral R3R01 for 12 weeks	NCT05267262

More recently, the CARDINAL phase 3 study evaluated the use of bardoxolone methyl in a group of patients with AS. 157 patients were randomly assigned to receive once-daily oral bardoxolone ($n = 77$) or placebo ($n = 80$). The primary endpoint was the change in eGFR after 100 weeks of treatment compared to baseline. Average age at screening was 39.2 years. The mean baseline eGFR was 62.7 ml/min/1.73 m^2 and mean UACR was 141 mg/g (46).

The FDA has analyzed data from CARDINAL and has concluded that there is no evidence that bardoxolone is effective at slowing kidney disease progression in patients with AS. The FDA also raised major efficacy and safety concerns related to bardoxolone use. It has been speculated that bardoxolone leads to an increase in intraglomerular pressure, which over time could have detrimental effects that might lead to an accelerated progression to kidney failure. It has also been suggested that the 4-week washout period was not long enough to resolve the reversible pharmacodynamic effect of bardoxolone and that the time to resolution of such effect is predicted to be at least 60 days (based on an elimination half-life of 15 days of bardoxolone). Major concerns regarding drug safety are worsening albuminuria, hypertension and a decrease in body weight. There is a lack of data using bardoxolone in animal models of Alport syndrome or other adequately controlled clinical trials in AS or CKD to show that bardoxolone is effective at delaying kidney function decline.

Anti-microRNA-21

MicroRNAs (miRNAs) are short non-coding RNAs which can regulate gene expression by inhibiting the translation or increasing the degradation of their target messenger RNAs (47). The ability of a single miRNA to regulate multiple downstream target mRNAs altered in disease conditions makes miRNAs attractive therapeutic targets with the potential to impact a

variety of molecular pathways (48). miRNA-21 has been found to be dysregulated in multiple kidney disorders, including AS. It was shown that renal miRNA-21 is upregulated in *Col4α3*$^{-/-}$ mice and the use of anti-miRNA-21 oligonucleotides significantly slows kidney disease progression and improves survival in Alport mice (49). In patients with AS, the expression of miRNA-21 in kidney samples was found to be significantly higher compared to controls and it was correlated with severity of kidney disease as evidenced by proteinuria, kidney function biomarkers and renal pathology scores (50). A phase 2 RCT of lademirsen (previously known as RG-012) which is a subcutaneous injection of an anti-miRNA-21 molecule is underway (HERA clinical trial [NCT02855268]). This trial, sponsored by Sanofi, will enroll a total of 45 patients with AS and results are anticipated to be accesible by 2023 (51).

Endothelin Type A Receptor (ETAR) and Angiotensin II Type 1 Receptor (AT1R) Inhibitors

The activation of ETAR has an important role in renal and inner ear pathologies in patients with AS. Despite being standard of care in patients with AS, the use of RAASi does not mitigate the impact on hearing.

Sparsentan, a dual ETAR/AT1R inhibitor, was able to extend lifespan in AS mice and lead to greater reductions in proteinuria compared to a selective AT1R inhibitor (losartan) or selective ETAR inhibitor (atrasentan) when treatment was initiated at 4 weeks. Preventive use of sparsentan was also able to mitigate the structural and functional auditory changes in AS mice. This auditory benefit was not observed with losartan (52).

The clinical trial EPPIK (NCT05003986) is evaluating use of sparsentan oral suspension in a pediatric population with AS with UPCR $\geq$1.0 g/g and other proteinuric glomerular diseases (53).

Sparsentan showed greater reduction in proteinuria compared to irbesartan after 8 weeks of treatment in patients with FSGS in a phase 2 RCT. In the patients treated with sparsentan, 16.4% of patients developed hypotension and 12.3% reported edema, but none of these adverse events were serious and there were no patients who dropped out from the study (54). The phase 3 DUPLEX study is ongoing and this will evaluate extended antiproteinuric efficacy and kidney protective potential of dual ETAR and AT1R blockade in patients with FSGS (55).

Atrasentan is an oral selective ETAR inhibitor that reduced albuminuria and risk of renal events in patients with diabetes and CKD. Edema and anemia were more frequent in the atrasentan group compared to placebo (56, 57).

AFFINITY (NCT04573920) is a phase 2 open-label trial of atrasentan which is currently recruiting patients and it will include a cohort of 80 patients with AS as well as other proteinuric kidney diseases (58).

Lipid-Modifying Drugs

The frequency of cardiovascular disease (CVD) is much higher in patients with CKD compared to those without CKD. The majority of deaths in patients with CKD are attributed to CVD, mainly atherosclerotic heart disease, cardiac arrhythmias and cardiac arrest. Individuals with familial hypercholesterolemia are at increased risk of developing CKD (59). Irrespectively of systemic hyperlipidemia accompanying CKD, research from us and others has shown that renal lipotoxicity rather than systemic dyslipidemia is the main contributor to the pathogenesis and progression of proteinuric kidney diseases (60–67). The accumulation of excessive lipids in non-adipose tissue is described as lipotoxicity and this is associated with cell dysfunction and apoptosis (68). We have recently described an important accumulation of triglycerides (69) and esterified cholesterol (37, 70, 71) in the kidney cortex of experimental AS. In fact, lipid droplets, foamy podocytes and foamy interstitial cells are frequently seen in kidney biopsies of patients with AS, but their origin remains unknown.

An evolving research field is identifying key enzymes or transporters in intrarenal lipid homeostasis as potential therapeutic targets to treat proteinuric kidney diseases.

When there is cholesterol deficit, cholesterol can be synthesized *de novo via* 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) or cholesterol attached to low-density lipoprotein (LDL) can be released into the cells through the LDL receptor (LDLR). Conversely, excessive cholesterol can be extracted from the cell through ATP-binding cassette A1 (ABCA1) and ATP-binding cassette subfamily G member 1 (ABCG1). Accumulation of cellular cholesterol stimulates the cholesterol-esterifying enzyme sterol O-acyltransferase (SOAT1), which transforms free cholesterol to cholesterol esters that are deposited in lipid droplets. In podocytes, the uptake of free fatty acids for triglyceride synthesis occurs through cluster of differentiation 36 (CD36) (65).

Excessive aggregation of free cholesterol results in cellular toxicity. Maintenance of adequate levels of free cholesterol is expedited by cholesterol esterification and reverse cholesterol transport (RCT) pathways which induce the efflux of cholesterol to HDL. Impaired RCT can promote glomerulosclerosis and tubulointerstitial damage (72). Glomerular and tubular lipid accumulation has been described in experimental models of kidney disease of metabolic and non-metabolic origin, including DKD (61, 63, 73), FSGS and AS (69–71, 74) and this is attributable to abnormalities in lipid metabolism. Among different lipid species, esterified cholesterol accumulation in kidney cortex correlates best with kidney outcomes compared to accumulation of other lipids (75).

Statins and Ezetimibe

The use of statins in CKD patients is essential to reduce cardiovascular risk. KDIGO 2013 guidelines recommend use of a statin in adults aged $\geq$50 years with eGFR <60 ml/min/1.73m^2. This does not apply for end stage kidney disease patients (1A) (76). It has been speculated that statin treatment may be associated with slower kidney disease progression (77). However, there is no convincing evidence for such statement. The beneficial effects of statins observed in *Col4a3*$^{-/-}$ mice (78) have not been confirmed in patients with AS. The use of ezetimibe was found to partially protect from albuminuria and from CKD progression in *Col4a3*-/- mice in association with reduced triglyceride content. In these studies, ezetimibe was found to reduce CD36 dependent fatty acid uptake (69). Whether this effect may be observed in patients affected with AS remains to be established.

Inducers of Cholesterol Efflux

The expression of cholesterol efflux transporter ABCA1 is reduced in glomeruli of individuals with T2DKD (61) as well as in experimental models of AS and FSGS (62, 70). There is a causative directional link between TNF expression in glomerular cells and an altered cholesterol efflux *via* ABCA1 and decreased cholesterol esterification by SOAT1 resulting in cholesterol aggregation and increased risk of proteinuria (71, 75). Studies have shown that either genetic or drug-induced activation of ABCA1 or use of a cholesterol sequestrant is effective in reducing proteinuria in AS, DKD and FSGS.

We previously proved that in the *COL4α3* knockout (KO) mice (AS mice) there is glomerular accumulation of cholesterol esters which suggests that CKD in AS may be a disorder of fatty kidney disease. Administration of hydroxypropyl-β-cyclodextrin (HPβCD), a cholesterol-chelating sugar, was found to protect AS mice from developing proteinuria, progressive kidney disease and renal fibrosis and this was associated with a lower concentration of kidney cholesterol esters, lipid droplets and cholesterol crystals, but also with increased serum HDL levels. Moreover, HPβCD treatment resulted in prolonged lifespan (70).

Unfortunately, the cholesterol removal effect of cyclodextrin is not selective, and its parenteral route of administration is impractical. Concerns have been raised about potential ototoxicity of HPβCD due to loss of cochlear outer hair cells which would be of particular concern in patients with AS who are already susceptible to hearing loss (79).

More recently, we developed a series of small molecule oral agents belonging to the class of 5-arylnicotinamide compounds (Cpds). These Cpds increase ABCA1-dependent cholesterol efflux by targeting Oxysterol Binding Protein Like 7 (OSBPL7). OSBPL7 is present in the kidney, including the glomeruli and podocytes. Cpds were able to induce ABCA1 and cholesterol efflux from cholesterol-repleted podocytes *in vitro* and decreased proteinuria, attenuated kidney function decline and prolonged lifespan in mouse models of AS and FSGS, even when the Cpds were administered in mice with established CKD and proteinuria. Despite reducing the amount of cholesterol esters in the kidney in the *COL4α3* KO mice the serum cholesterol levels did not change, which suggests that these Cpds may be protective mainly by reducing the concentration of cholesterol and lipids in target organs (37). These Cpds have a greater antiproteinuric effect compared to the one of ramipril previously reported in a syngeneic model of AS (69). Unlike the Cpds, the administration of RAAS blockade did not prolong lifespan in older *COL4α3* mice, despite being the most effective therapy for early intervention in this model (80–82). An oral molecule that increases the functional activity of the ABCA1 transporter is currently under development. However, this information is not publicly available at this time.

Discoidin Domain Receptor 1 (DRR1) Inhibitors

DDR1 is a receptor tyrosine kinase that is stimulated by collagens. It is involved in the development of fibrotic disorders. DDR1 deficient *Col4a3*-/- mice were reported to be protected from proteinuria and renal failure, suggesting DDR1 as an amenable therapeutic target (82). Pharmacological inhibition of DDR1 in *COL4α3*$^{-/-}$ mice caused mild reductions in albuminuria and renal fibrosis (83). More recently, we have described a new mechanism linking DDR1 activation to renal lipotoxicity. *De novo* production of the collagen type 1 has been observed in *Col4α3* knockout mice (AS mice). Collagen 1 activates DDR1 and induces CD36-mediated podocyte lipotoxic injury and disruption of podocyte-GBM interaction with subsequent development of proteinuria. CD36 induces oxidative stress and apoptosis in podocytes by increasing intake of free fatty acids and accumulation of intracellular triglycerides. Therefore, the clinical development of DDR1 inhibitors for the treatment of AS remains an interesting opportunity.

Osteopontin Blocking Agents

Osteopontin (OPN) is a protein with an important role as a regulator of inflammation, heart failure and tumor metastases (84, 85). In animal models of albuminuria, kidney OPN mRNA and protein levels were elevated and in children with nephrotic syndrome, urinary OPN levels were elevated (86). Ding and colleagues demonstrated that OPN expression is increased in the renal tubules of the *Col4α3*$^{-/-}$ AS mouse where it regulates dynamin-3-mediated (DNM3) increased LDL receptor (LDLR) expression, increases LDL-cholesterol influx and leads to defective mitochondrial bioenergetics.

OPN genetic deletion caused a significant reduction in the expression of DNM3 and LDLR in the AS mouse and reduced albuminuria, tubulointerstitial inflammation, apoptosis, hypertension, hearing loss and visual deficits (74). OPN blocking agents such as antibody or RNA aptamer might be a potential therapeutic target in patients with AS.

Hydroxychloroquine

The antimalarial drug hydroxychloroquine (HCQ) is an immunomodulatory drug commonly used to treat rheumatologic diseases such as systemic lupus erythematosus and rheumatoid arthritis. It blocks the immune system by inhibiting Toll-like receptor signaling and suppressing cytokine production and T cells by decreasing the expression of CD154 (87).

A phase 2 RCT of patients with IgA nephropathy (IgAN) who were receiving optimized RAAS inhibitor therapy and had a mean eGFR 54 ml/min/1.73 m^2 and median proteinuria of 1.7 g/d showed that the addition of HCQ compared to placebo significantly reduced proteinuria at 6 months (0.9 vs. 1.9 g/d; P 0.002) (88). A case control study compared the safety and efficacy of HCQ and glucocorticoids in individuals with IgAN with proteinuria >1 g/d despite optimization of RAASi treatment. The reduction in proteinuria achieved by HCQ was only slightly lower to glucocorticoids over a period of 6 months. However, HCQ was safer than glucocorticoid treatment (89).

A phase 2 RCT to assess the safety and effectiveness of HCQ in patients with X-linked Alport syndrome (NCT04937907) is under way in China. Enrollment includes AS patients who have been on a stable dose of enalapril for 6 months (90).

Metformin

Metformin is an oral hypoglycemic agent which effectively reduces HbA1c in patients with T2DM with a low risk for hypoglycemia. In a mouse model of AS, use of metformin reduced proteinuria and ameliorated progression of kidney disease. Metformin suppressed renal inflammation, fibrosis and glomerular injury. The dual administration of metformin and losartan prolonged survival of AS mice when compared to losartan monotherapy. Both metformin and losartan modified podocyte-related molecular pathways associated to inflammation and metabolism according to transcriptome analysis. In addition, metformin was found to regulate multiple genes also related to metabolism which were not affected by losartan (91). This study raises the possibility that combination of RAASi and metformin may slow CKD progression and prolong survival also in non-diabetic chronic kidney disease. Considering the low cost and wide availability of metformin this might be explored further in research trials. It is important to mention that metformin is excreted unchanged in the urine since it is not metabolized and the FDA applied a box warning to this drug and recommends to avoid its use in advanced kidney disease (eGFR $<$30 ml/min/1.73 m^2) due to its potential association with lactic acidosis.

Paricalcitol

Paricalcitol is an active vitamin D analog. An elevated parathyroid hormone (PTH) and a low 1,25-dihydroxyvitamin D are associated with lower bone density, heart disease, blunted immune system and higher mortality in patients with ESKD. Recent studies in hemodialysis patients suggest that paricalcitol is associated with a better survival and tolerability compared to calcitriol (92). It is possible that activation of vitamin D receptors has beneficial effects on the cardiovascular system that reduce mortality in CKD and that this is independent of calcium, phosphorus and PTH. Paricalcitol suppresses renin production at higher than conventional doses (93, 94). Downregulation of the RAAS may be the mechanism by which paricalcitol has some nephroprotective and antifibrotic effects.

Chaperones

Protein misfolding in the endoplasmic reticulum (ER) leads to loss of function or toxic effect designated as ER stress in genetic diseases caused by genetic mutations. Inappropriately folded proteins are generally recognized by the cell quality-control mechanisms and are retained or destroyed in the ER. Chaperones are ubiquitous molecules that enable correct protein assembly by attaching to and stabilizing unfolded proteins allowing them to become functional and correctly routed to the location where they will reside.

Use of chaperone therapies as a potential therapeutic approach to protein misfolding diseases has become one of the major targets of clinical research. Migalastat is an oral chaperone which stabilizes mutant α-galactosidase and facilitates adequate enzyme routing to lysosomes and is used to treat patients affected with Fabry's disease (95). Chaperones are useless when there is absence of protein which is the case in diseases caused by truncating mutations (deletions, frameshift and nonsense mutations). However, estimates show that around 40–50% of males with XLAS (96, 97) and around 40% in male and female patients with ARAS (98) exhibit missense mutations as the cause of their AS.

A study in fibroblast cell lines of men with XLAS showed that the use of chaperone sodium 4-phenyl butyric acid (PBA) increased levels of collagen IV α5 mRNA and decreased ER stress and autophagy (99). When oral treatment with the chaperone PBA was administered to *COL4α1* mutant mice, either as a preventative treatment or for those with established disease, there were fewer intracranial hemorrhage cases. However, PBA did not prevent from developing eye and kidney defects (100).

Genome Editing Therapy

Genome editing therapy is an experimental technique that aims to correct defective genes in order to cure a disease. It can be executed *via* different methodologies which include rendering deleterious mutations inactive, adding protective mutations or therapeutic transgenes, or distortion of viral DNA (101, 102). Replacement of a mutant allele by a corrected copy of the gene requires the effective delivery of the latter *via* a vehicle, for instance a virus or nanoparticle, to an approachable tissue compartment (103). If the normal gene substitutes the abnormal allele there will be proliferation of these new modified cells which may generate the desired protein in sufficient amounts to restore a normal phenotype (104). Here we describe some of the therapeutic concepts currently under investigation.

The Clustered Regularly-Interspaced-Short Palindromic Repeat (CRISPR/Cas9) system has became a promising gene editing therapy for many rare genetic disorders, with successful *in vitro* results in cases of homozygous β-thalassemia generating functional red blood cells precursors (102), but with less success in the manipulation of podocytes and their regeneration.

The CRISPR/Cas9 system consists of two components: a single-guide RNA (sgRNA) and an endonuclease Cas9. The sgRNA can guide Cas9 to the genomic site of action where a highly precise double strand break (DSB) should occur following a Watson-Crick base pairing recognition. DSBs caused by CRISPR/Cas9 are generally corrected through non-homologous end joining which may generate deletions and insertions. However, the precision of the DSB correction can be augmented by using a "repair template" donor DNA compatible with the genomic region of interest which can be used to synthesize de-novo wild type DNA (105).

A recent study by Daga et al. (106), demonstrated that it is feasible to obtain podocyte-lineage cells derived from urine recreating the physiological conditions seen in these particular cells, allowing to effectively define the *COL4* variants correction index after experimental interventions. This novel tool was applied for two different forms of hereditary transmission in AS, X-linked and autosomal dominant variants, using a self-inactivating dual-plasmid approach where a homologous repair is mainly induced by a self-cleaving streptococcus pyogenes Cas9 (SpCas9) coupled with a sgRNA from CMV inducing DNA damage, while the other plasmid carries a double stranded DNA (dsDNA) donor fragment. This approach resulted in very high correction rates which ranged from 44% in the *COL4A3* gene to

58% in the *COL4A5* gene and it led to a reduced percentage of indels (10.4% for *COL4A3* and 8.8% for *COL4A5*).

The number of podocytes decreases significantly over time in patients with AS as a consequence of structural defects which lead to apoptosis and cell death. CRISPR/Cas9 gene therapy is expected to be more effective in the early phase of the disease since at this time a functional GBM can still be potentially restored (106). Despite these promising results, there is a long way ahead of us until this proof of concept can be transitioned to *in vivo* experiments due to the difficulty in manipulation of podocytes.

Lin et al. used a *COL4A3*$^{-/-}$ mouse model of AS and an inducible transgene system and they discovered that the podocyte secretion of $\alpha 3\alpha 4\alpha 5$(IV) heterotrimers into a defective GBM was effective at restoring the absent collagen IV network which slowed the course of renal disease and prolonged survival (107). Funk et al. increased the expression of *COL4*α*3* transgene in endothelial cells of *COL4A3*$^{-/-}$ Alport mice through adenovirus-mediated gene transfer. Unfortunately, these AS mice did not exhibit *COL4A3/A4* or assembled $\alpha 3\alpha 4\alpha 5$(IV) heterotrimer staining and subsequent resolution of the disease specific phenotype was not achieved (108).

Reports of succesful delivery of *COL4A5* gene into swine kidney with an adenovirus vector resulted in increased deposition of α5 (IV) collagen into the GBM. However, this technique is impractical since it requires to administer the vector directly in the renal artery which may not be possible in human trials (109).

Another concept being explored in AS is the X-chromosome (Xc) reactivation approach. It is known that during the early embryological development of females, an Xc on each cell is randomly inactivated (lyonization), ensuring an even expression of cells of female XX compared to male XY (110). Based on this premise, the idea of reactivating the healthy copy of *COL4A5* gene on the inactivated Xc has been contemplated in cases of X-linked AS in women. Unfortunately, this concept continues to be in the early experimental phase, with the major limiting factor being concerns regarding off-target effects.

Stem Cell Therapy

The use of induced pluripotent stem cell (iPSC) lines is a valuable tool to study AS pathologic mechanisms and evaluate the efficacy of experimental therapies. The rationale of the potential use of iPSCs as a therapy in AS is based on the concept that these cells will eventually migrate and engraft into the renal glomeruli, differentiating into functional podocytes and generating a new healthy GBM (111). One study proved the efficacy of stem cell transplantation of wild-type bone marrow into irradiated *COL4A3*$^{-/-}$ mice which partially restored the expression of the type IV collagen α3 chain, reducing proteinuria and overall improving kidney histology (112). Another study demonstrated that transplanting first trimester human fetal chorionic stem cells into a mouse model of AS delays kidney disease progression (113). Despite the promising results, other studies have failed to demonstrate the same effect in cases of *COL4A3* deficient mice (114). Bone marrow transplantation is considered as a last resource measure for life threatening diseases, given its high mortality risk along with the uncertainty of the available studies using this method, hence is not considered as a potential treatment of AS.

DISCUSSION

Alport syndrome is a frequent inherited kidney disease for which currently there is no curative treatment available. Conventional treatment aims to slow progression to kidney failure using RAASi. Drug development in AS is currently under way with drug targets at different stages of the disease. Experimental drugs may target the altered function of the GBM or the interaction between GBM with podocytes and endothelial cells. Other new drugs target tubular cell injury or the inflammation and fibrosis seen in late stages of the disease. Targeting defective collagen chains early in the disease through gene therapy might be the therapeutic approach with the highest potential to reverse this disorder. Research advances on the innovative therapeutic approaches for this condition are exciting and may bring hope to the millions of patients affected.

AUTHOR CONTRIBUTIONS

EC prepared manuscript draft and created the tables. JR prepared manuscript draft. YD and AF revised the manuscript. All authors contributed to the article, reviewed, and approved the final version of the manuscript.

FUNDING

Research in AF laboratory is supported by the National Institute of Health [Grant Numbers R01DK117599, R01DK104753, R01CA227493, U54DK083912, UM1DK100846, and U01DK116101] and by the Miami Clinical Translational Science Institute [Grant Number UL1TR000460]. He also received support from the Alport Syndrome Foundation, Boehringer Ingelheim, and Hoffman La Roche. YD is supported by the KL2 program of the Miami Clinical Translational Science Institute [Grant Number UL1TR000460].

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Conflict of Interest: AF is Chief Scientific Officer of L&F Health LLC, holds equity interests in L&F Research and is the inventor of assets developed by ZyVersa Therapeutics. ZyVersa has licensed worldwide rights to develop and commercialize hydroxypropyl-beta-cyclodextrin for treatment of kidney disease from L&F Research. AF is the scientific founder and holds equity in River 3 Renal Corporation.

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frontiers | Frontiers in Medicine

REVIEW
published: 06 May 2022
doi: 10.3389/fmed.2022.865034

Genotype-Phenotype Correlations for Pathogenic *COL4A3–COL4A5* Variants in X-Linked, Autosomal Recessive, and Autosomal Dominant Alport Syndrome

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OPEN ACCESS

Edited by:
Roberta Fenoglio, Ospedale San Giovanni Bosco, Italy

Reviewed by:
Daw-Yang Hwang, National Health Research Institutes, Taiwan
Florian Grahammer, University Medical Center Hamburg-Eppendorf, Germany

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Specialty section:
This article was submitted to Nephrology, a section of the journal Frontiers in Medicine

Received: *29 January 2022*
Accepted: *24 March 2022*
Published: *06 May 2022*

Citation:
Savige J, Huang M, Croos Dabrera MS, Shukla K and Gibson J (2022) Genotype-Phenotype Correlations for Pathogenic COL4A3–COL4A5 Variants in X-Linked, Autosomal Recessive, and Autosomal Dominant Alport Syndrome. Front. Med. 9:865034. doi: 10.3389/fmed.2022.865034

Alport syndrome is inherited as an X-linked (XL), autosomal recessive (AR), or autosomal dominant (AD) disease, where pathogenic *COL4A3* – *COL4A5* variants affect the basement membrane collagen IV α3α4α5 network. About 50% of pathogenic variants in each gene (major rearrangements and large deletions in 15%, truncating variants in 20%, splicing changes in 15%) are associated with "severe" disease with earlier onset kidney failure, and hearing loss and ocular abnormalities in males with XL inheritance and in males and females with AR disease. Severe variants are also associated with early proteinuria which is itself a risk factor for kidney failure. The other half of pathogenic variants are missense changes which are mainly Gly substitutions. These are generally associated with later onset kidney failure, hearing loss, and less often with major ocular abnormalities. Further determinants of severity for missense variants for XL disease in males, and in AD disease, include Gly versus non-Gly substitutions; increased distance from a non-collagenous interruption or terminus; and Gly substitutions with a more (Arg, Glu, Asp, Val, and Trp) or less disruptive (Ala, Ser, and Cys) residue. Understanding genotype-phenotype correlations in Alport syndrome is important because they help predict the likely age at kidney failure, and the need for early and aggressive management with renin-angiotensin system blockade and other therapies. Genotype-phenotype correlations also help standardize patients with Alport syndrome undergoing trials of clinical treatment. It is unclear whether severe variants predispose more often to kidney cysts or coincidental IgA glomerulonephritis which are recognized increasingly in *COL4A3-, COL4A4* - and *COL4A5*-associated disease.

Keywords: genotype-phenotype correlation, Alport syndrome, *COL4A3*, *COL4A4*, *COL4A5*, XL Alport syndrome, AR Alport syndrome, AD Alport syndrome

INTRODUCTION

Alport syndrome is the commonest inherited kidney disease and the second commonest cause of inherited kidney failure after polycystic kidney disease (1). It is characterized by persistent haematuria and a family history of haematuria or kidney failure (2). The typical clinical features are seen in males with X-linked (XL) disease due to pathogenic *COL4A5* variants and in males and females with autosomal recessive (AR) disease and two pathogenic variants in *COL4A3* or *COL4A4*. These result in haematuria, kidney failure, hearing loss and ocular abnormalities. Affected basement membranes including the glomerular membrane (GBM) are thinned, lamellated, and moth-eaten. Autosomal dominant (AD) Alport syndrome is caused by a heterozygous pathogenic variant in *COL4A3* or *COL4A4* and associated with isolated haematuria and a thinned rather than lamellated membrane (3, 4). With AD Alport syndrome the risk of kidney failure is small, and hearing loss and ocular abnormalities are rare (5). AD Alport syndrome is also sometimes known as thin basement membrane nephropathy and represents the carrier state for autosomal recessive Alport syndrome (3).

STRUCTURE OF THE COLLAGEN IV NETWORK

The six *COL4A1–COL4A6* genes code for the collagen IV α1–α6 chains (6). These genes belong to two families, *COL4A1*-like (*COL4A1, COL4A3,* and *COL4A5*) and *COL4A2*-like (*COL4A2, COL4A4,* and *COL4A6*) which have all arisen from reduplication of *COL4A1*. These six genes are highly homologous and the corresponding collagen IV α chains resemble each other structurally.

Each collagen IV α chain comprises a non-collagenous amino and carboxy terminus and an intermediate collagenous sequence with Gly-Xaa-Yaa repeats (**Figure 1**) as well as multiple interruptions that confer flexibility (6). All collagen chains include Gly-Xaa-Yaa repeats but the collagen IV α chains differ in that they retain the non-collagenous amino and carboxy termini through which they interact to form the α1α1α2, α3α4α5, or α5α5α6 heterotrimers and the chicken wire networks that predominate in membranes.

The α1α1α2 network is widely distributed in basement membranes in infancy and is found in adults in the blood vessels, nerves and muscles (6). The α3α4α5 trimer is the major constituent of membranes in the glomerulus (GBM), cochlea and eye having replaced the α1α1α2 trimer at about 2 years of age. The α5α5α6 trimer is found in Bowman's capsule in the kidney and in skin.

To date, more than 5,000 pathogenic variants have been described in the *COL4A3–COL4A5* genes in the two main variant databases LOVD (https://www.lovd.nl/) and Clinvar (https://www.ncbi.nlm.nih.gov/clinvar/). These are both open access databases that accept submissions from diagnostic laboratories and from the literature. LOVD also includes clinical features for many variants which allows genotype-phenotype correlations. Many more variants have been reported for *COL4A5* than for *COL4A3* and *COL4A4* because genetic testing has been performed more often for individuals suspected of having X-linked Alport syndrome. Although the definitions of variant types differ in different databases, in general, pathogenic variants in each of the *COL4A3–COL4A5* genes include 15% major rearrangements or large deletions, 20% truncating variants, 15% splicing changes, and 45% missense variants (**Table 1**).

"Severe" variants in *COL4A5* are more likely to be rearrangements or deletions, truncating, or splicing changes than missense variants (7). This means that overall severe changes account for about 50% of all pathogenic variants in each of these genes, and thus that the likelihood of finding a severe variant in a person with Alport syndrome is currently also 50%.

Deletions, frameshift variants, and termination codons all result in loss of the corresponding α chain from nonsense-mediated decay and subsequently of the collagen IV α3α4α5 triple helix from affected membranes. This is replaced with the α1α1α2 network (8) which is more susceptible to proteolysis (9). Initially the GBM is thinned but repeated episodes of damage and repair result in a lamellated, moth-eaten appearance. Splicing variants have different effects depending on whether they produce a truncating change or exon skipping (10).

In contrast, the α chains resulting from a missense variant are often associated with a disrupted trimer that is retained within the podocyte ER (11, 12), increasing ER stress and podocyte loss (13, 14). Of all the missense variants, the majority (30%) are Gly substitutions in the intermediate collagenous domain. Gly is the smallest amino acid that fits within the interior of the triple helix but can be replaced by 8 different amino acids or a termination codon (7). Most Gly substitutions are pathogenic because substitution with a larger amino acid distorts triple helix formation.

ADVANTAGES OF PREDICTING SEVERITY OF PATHOGENIC VARIANTS

It is useful for the clinician to be able to predict whether a pathogenic *COL4A3–COL4A5* variant is likely associated with severe or mild features in Alport syndrome. The typical severe form is seen in males with X-linked disease who have recurrent macroscopic haematuria, early onset proteinuria and kidney failure, that generally requires dialysis or a kidney transplant well before the age of 30 years (15–17). The GBM may be thinned or lamellated, with the collagen IV α5 chain absent from a kidney or skin biopsy. Lenticonus, central fleck retinopathy, and temporal retinal atrophy are common, and a maculopathy or macular hole may occur (18, 19). In contrast "mild" disease is associated with haematuria, later onset proteinuria and kidney failure often in middle age, together with a thinned, possibly partly lamellated, GBM (20). There may be a late onset hearing loss, and a peripheral retinopathy is common (21).

The severe and mild phenotypes are subtly different in other forms of inheritance. Females with a severe pathogenic *COL4A5* variant have an increased risk of proteinuria, and hearing loss is common but lenticonus does not occur

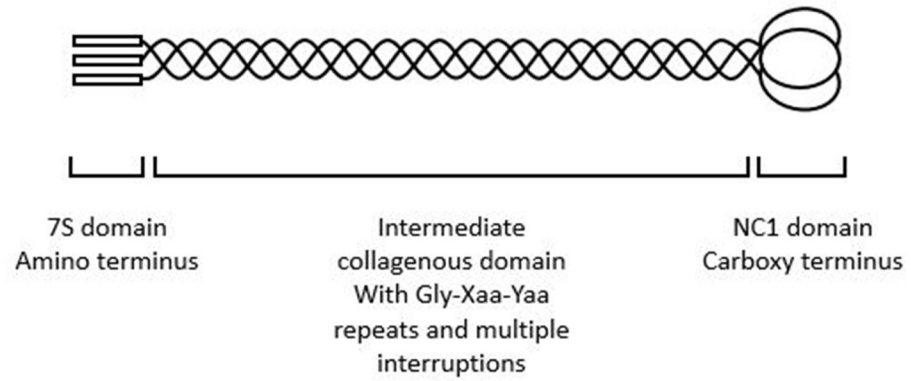

FIGURE 1 | Collagen IV α3α4α5 heterotrimer demonstrating amino terminus, intermediate collagenous domain, and carboxy terminus. The intermediate collagenous domain comprises Gly-Xaa-Yaa repeats with multiple interruptions.

TABLE 1 | Types of all pathogenic variants in *COL4A5, COL4A3,* or *COL4A4*.

	Large rearrangements/deletions/indels	Truncating variants (nonsense and frameshift)	Splice site	Missense
LOVD				
COL4A5 ($n = 1{,}967$)	20%	23%	14%	49%
COL4A3 ($n = 383$)	16%	21%	6%	60%
COL4A4 ($n = 335$)	21%	35%	8%	44%
ClinVar				
COL4A5 ($n = 1{,}031$)	99 (10%)	321 (31%)	137 (13%)	514 (50%)
COL4A3 ($n = 259$)	27(10%)	106 (41%)	44 (17%)	82 (32%)
COL4A4 ($n = 249$)	23 (9%)	116 (47%)	41 (16%)	69 (28%)

Definitions of large rearrangements etc. differ for LOVD and ClinVar. https://databases.lovd.nl/shared/genes/COL4A5/graphs; https://simple-clinvar.broadinstitute.org/ filtered for Pathogenic and Likely pathogenic, expressed as % of all Pathogenic and Likely Pathogenic variants found. The numbers include occasional variants twice because of features in overlapping categories.

(22). With AR Alport syndrome, two severe variants in *COL4A3* or *COL4A4* have a more damaging effect than one or none (23). The relationship is less clear for severe heterozygous *COL4A3* or *COL4A4* variants in AD Alport syndrome since kidney failure is not common and may be due to coincidental disease.

The advantages of being able to predict severe variants include being able to institute treatment to delay kidney failure onset. Renin-angiotensin-aldosterone system blockade delays kidney failure for years in males with X-linked disease, and the delay may be sufficiently long in women that they do not require dialysis or a kidney transplant (24, 25). SGLT2 inhibitors have demonstrated promise in Alport syndrome and are currently undergoing further investigation (26, 27). Severe variants may also indicate the need for earlier, and more aggressive, or more expensive interventions as they become available (28, 29). Some treatments are specific for severe disease. Demonstrating a severe variant may also represent a persuasive argument for dissuading an affected family member from acting as a kidney donor. In addition, recognizing severe variants is important in clinical trials where standardizing the risk of kidney failure helps compare treatment outcomes.

X-LINKED ALPORT SYNDROME IN MALES

The location where a pathogenic variant affects the collagen α chain (amino terminus, intermediate collagenous domain, interruptions, or carboxy terminus) and the variant type (large rearrangements, truncating, splicing, or missense variants) are the major determinants of variant severity and age at kidney failure, and likelihood of hearing loss and ocular abnormalities. Because pathogenic *COL4A5* variants result in kidney failure in most males the genotype-phenotype relationship is understood best in males with X-linked disease.

Many studies of *COL4A5* variants in males have found that severe changes (large rearrangements, nonsense and frameshift, and splicing variants) are associated with an earlier age at kidney failure than missense changes (15–17, 30). Splice site variants often have an effect intermediate between truncating and missense variants (16).

In the *COL4A5* variants in the LOVD database, the mean age at kidney failure for all males was 25.1 ± 10.6 years ($n = 326$). Some studies have suggested that pathogenic changes in the first 20 exons of *COL4A5* and hence the amino terminus of the collagen IV α5 chain is associated with milder disease (17) and others have found more severe disease with earlier onset

kidney failure (16). In LOVD, the average age at kidney failure for variants in the amino terminus was 20.3 ± 5.4 (n = 14), 25.6 ± 11.1 years for variants in the intermediate domain (n = 262) and 24.0 ± 8.2 (n = 50) for those in the carboxy terminus suggesting a more severe effect for the amino terminus compared with the intermediate domain (p = 0.08) but not the carboxy terminus (p = 0.12). However 11 of the 14 amino terminus changes were deletions which have a more severe outcome and were likely to have biased the results. Thus variant types must also be taken into account before assessing the severity associated with a variant location.

In the LOVD database, the average age at kidney failure for men with deletions was 21.1 ± 6.8 years (n = 75) which was earlier than the overall mean age (p = 0.002) and for those with nonsense variants was 20.4 ± 5.0 years (n = 33, p = 0.01). The mean age for men with canonical splice site variants was 25.2 ± 10.7 years (n = 45, p = 0.95). Donor splice site changes have been associated with earlier onset kidney failure than acceptor variants (17) but the mean age for splice site variants at −1 or −2 was 25.2 ± 11.6 years (n = 21) and 25.1 ± 10.2 (n = 24) for variants at +1 or + 2 (p = 0.98 compared with each other, and p = 0.95 compared with the overall mean). Duplications were associated with a mean age of 23.9 ± 6.1 years (n = 15, p = 0.66 compared with overall mean).

In the LOVD database, Gly substitutions in the intermediate collagenous domain [mean age at kidney failure of 28.4 ± 12.4 years (n = 52)] had a worse outcome than non-Gly substitutions [40.7 ± 17.6 years (n = 5), p = 0.03] (16).

Gly substitutions represent the commonest missense variants in *COL4A5*, currently accounting for most unique pathogenic variants in males (124/147, 84%).

Gly substitutions distant from a non-collagenous sequence (interruption, amino, or carboxy terminus) had a more severe phenotype with earlier onset kidney failure than Gly substitutions adjacent to a non-collagenous sequence (31). In addition substitutions with highly destabilizing residues such as Glu, Asp, Arg, Val, or Trp were associated with earlier onset kidney failure than substitutions with the less-destabilizing, Ala, Ser, or Cys (31). In LOVD, the mean age at kidney failure was 24.6 ± 8.6 for severe Gly substitutions (n = 83) and 31.1 ± 14.2 for non-severe substitutions (n = 68, p = 0.0007).

Treatment also affects the age at kidney failure for truncating variants and non-truncating variants differently. The age at kidney failure for both truncating and non-truncating variants is delayed by RAAS blockade but treatment delayed kidney failure more for non- truncating variants (30).

Severe variants are also associated with hearing loss (15–17), and ocular abnormalities, in particular, lenticonus, and fleck retinopathy (15–17). No effect of variant severity has been demonstrated on macroscopic haematuria, hypertension, or GBM thickness (15, 16).

X-LINKED ALPORT SYNDROME IN FEMALES

The correlation between *COL4A5* variant location, type and phenotype has been less clear for females with X-linked Alport syndrome. Indeed an early large study found no genotype-phenotype correlation in women with X-linked disease (32). They also found no correlation between earlier age at kidney failure in men and women from the same family (32).

Part of the lack of correlation in women has been attributed to random X chromosome inactivation (33). However it is also likely to be partly due to so few women developing kidney failure and, of those who do, this occurring in later life. Women develop hearing loss and ocular abnormalities less often too. Thus recent studies have focused instead on the relationship between genotype and proteinuria since proteinuria typically precedes kidney failure (34).

Thus a recent large study of 336 females with genetically-proven X-linked Alport syndrome from 179 families where about half the pathogenic variants were severe found that 175 (73%) had proteinuria at a median age of 7 years (34). Fifty-two of the 336 (15%) had kidney failure by the age of 65 years.

Another smaller cohort found that severe variants (all those other than missense changes) were more commonly associated with proteinuria and impaired kidney function than missense variants (35). While this cohort also included occasional individuals with digenic inheritance these were unlikely to have had a significant effect.

No studies in women with pathogenic *COL4A5* variants have demonstrated any genotype-phenotype correlations for hearing loss or ocular abnormalities.

In summary there may be a similar genotype-phenotype correlation in women with pathogenic *COL4A5* variants and proteinuria as occurs in men with kidney failure but with a smaller effect that requires a larger cohort for its demonstration. Other factors contributing to the age at kidney failure as well as X chromosome activation, include coincidental causes including hypertension, diabetes and obesity.

AR ALPORT SYNDROME

Individuals with AR Alport syndrome have two pathogenic variants in *COL4A3* or *COL4A4*, and the *COL4A3* and *COL4A4* genes are affected equally often. In the LOVD database, the age at kidney failure was not different for AR and for X-linked Alport syndrome (24.4 ± 7.8 years, n = 237, p = 0.39), and in the case of AR disease, was not different for *COL4A3* (23.2 ± 9.3, n = 35, p = 0.45) or *COL4A4* (25.4 ± 10.3, n = 26, p = 0.55) variants (7).

Individuals with two truncating variants were more likely to develop kidney failure before 30 years of age than those with only one truncating variant, who were in turn more likely than those with no truncating variant (23). This correlation has now been confirmed in a small but unrelated cohort where both age at kidney failure and age at hearing loss were earlier with at least one truncating variant than with none (36). A further study confirmed that the presence of at least one milder (missense) variant delayed kidney failure onset (p = 0.024), and that kidney survival was further increased with two missense variants [p = 0.016; (37)]. In this study, individuals with missense variants had a later onset of kidney failure, hearing loss and ocular abnormalities (37).

AD ALPORT SYNDROME

There are now several large studies and a meta-analysis of genotype-phenotype analyses in individuals with AD Alport syndrome (5, 31, 38, 39), but again the difficulty is that kidney failure is uncommon in these individuals, a large cohort might be needed to demonstrate a small effect, and kidney failure may result from coincidental causes. However again proteinuria may represent a surrogate marker for kidney failure (5).

A recent analysis of the UK 100K Genomes Project database demonstrated that certain variant types were associated with a higher penetrance of haematuria (31). It examined *COL4A3* and *COL4A4* variants that resulted in a Gly substitution and found that substitutions with a highly destabilizing residue (Arg, Glu, Asp, Val, and Trp) were associated with an increased risk of haematuria [$p = 0.018$; (31)], and that substitutions adjacent to a non-collagenous interruption or amino or carboxy terminus were associated less often with haematuria ($p < 0.001$). There was no association between haematuria and proximity to the amino terminus for missense variants that were Gly substitutions.

A further study of 240 individuals from 78 families with genetically-proven *COL4A3* or *COL4A4* variants, included 61 who developed kidney failure (24%) at a median of 67 years (58–73) (5). Fifty-seven percent of their variants were missense and the others were "severe." In this study there was no difference in the age at kidney failure for truncating variants or for missense variants that were or were not Gly substitutions ($p = 0.3$); nor for Gly substitutions, splicing or missense variants ($p = 0.90$); nor Gly substitutions with Arg, Glu, or Asp or other severe non-missense variant types compared with other Gly substitutions [$p = 0.5$; (5)]. There was however a large intrafamilial variability in age at kidney failure.

A further study found no difference in the age at developing proteinuria or kidney failure for missense and non-missense variants (38).

However a meta-analysis of family members with 74 variants where 20 were not missense found the median age at kidney failure was 55 years for missense variants and 47 years for non-missense changes [$p = 0.02$; (39)].

At least two of these studies had a very wide variation in age at kidney failure (5, 39).

HYPOMORPHIC VARIANTS

Hypomorphic or variants associated with milder disease are recognized increasingly in the *COL4A3–COL4A5* genes (**Table 2**). They are often clinically unrecognised and are commonly found in normal variant databases such as gnomAD (40). They may have features that are the opposite of severe variants such as occurring in women (for *COL4A5* changes); and for all *COL4A3–COL4A5* variants, missense variants, especially Gly substitution with Ala, Ser, or Cys; or Gly substitutions adjacent to a non-collagenous terminus or interruption; or non-Gly substitutions.

Thus, two of the commonest hypomorphic variants are p.Gly624Asp in *COL4A5* and p.Leu1474Pro in *COL4A3*. p.Gly624Asp in *COL4A5* is found in about one in 3,000 of the European population or about one quarter of all Europeans with X-linked Alport syndrome (20, 41). This variant is located immediately adjacent to a non-collagenous interruption in the intermediate collagenous domain which contributes to its mild phenotype. It is associated with late onset kidney failure, hearing loss, but a normal ocular examination, and often a thinned rather than lamellated GBM.

The p.Leu1474Pro variant in *COL4A3* occurs in up to one in 200 people. The substitution occurs in the carboxy non-collagenous domain. On its own it may be associated with a normal urinary sediment, proteinuria and FSGS (42) or in association with another *COL4A3* variant, with AR Alport syndrome, and kidney failure.

TABLE 2 | Clinical phenotype of severe and hypomorphic variants.

Mode of inheritance	Severe	Hypomorphic
X-linked Alport syndrome in males	Episodes of macroscopic haematuria with intercurrent infections; persistent haematuria, proteinuria; early onset kidney failure, hearing loss; central fleck retinopathy; lamellated GBM; more severe FSGS	Haematuria, intermittent haematuria or none; less and later onset proteinuria and FSGS; late onset kidney failure; late onset hearing loss; no lenticonus, less central retinopathy; thinned GBM or patchy lamellation
X-linked Alport syndrome in females	Haematuria, proteinuria; possibly late onset kidney failure; more widespread lamellation; FSGS	Haematuria, intermittent haematuria or none; late onset proteinuria if at all
Autosomal recessive Alport syndrome	Haematuria, proteinuria; early onset kidney failure, hearing loss; central fleck retinopathy	One or two hypomorphic variants delays age at kidney failure
Autosomal dominant Alport syndrome	Haematuria, possibly proteinuria; FSGS	Haematuria, intermittent haematuria or none

GBM, glomerular basement membrane; FSGS, focal and segmental glomerulosclerosis.

PREDICTING SEVERE VARIANTS

In summary, the determinants for variant severity appear to be the same for all the *COL4A3–COL4A5* genes (**Table 3**). The renal physician may be assisted in deciding on the variant severity in their patient after consultation with a clinical geneticist and a multidisciplinary team. About half the pathogenic variants in each gene (major rearrangements, large deletions, truncating variants, some splicing variants) are associated with more severe disease, with earlier onset proteinuria and kidney failure, hearing loss, and ocular abnormalities in males with XL and in males and females with AR disease. For AD Alport syndrome severe changes may only increase the penetrance of haematuria. Interestingly a correlation between severe variants and proteinuria may be emerging for women with XL disease.

There are also two objective methods for predicting the clinical effects of *COL4A3–COL4A5* variants but these are not widely available. One is immunohistochemical staining

TABLE 3 | Factors determining severity of genetic variants in the *COL4A3–COL4A5* genes.

Variant feature	Detail	Severity
Location	Amino terminus	Severity probably depends more on type than location
	Collagenous domain	
	Carboxy terminus	
Type	Rearrangements, large deletions	Severe
	Truncating variants	Severe
	Splice site variants	Severe if they result in truncation
	Missense variants	
Gly substitution	Gly substitutions	More severe generally than non-Gly substitutions
	Position 1 Gly substitutions	More severe than non-position 1 substitutions
	Position 1 Gly substitutions not adjacent to interruptions	More severe than variants adjacent to interruptions
	Gly substitutions with Arg, Asp, Glu, Trp, or Val	More severe than Gly substitutions with Ala, Ser, or Cys

More severe variants are associated with a younger age at kidney failure or proteinuria.

TABLE 4 | Average age at kidney failure in males with pathogenic *COL4A5* variants in LOVD in 2021 compared with 2016.

	Up to 2016 (n = 1,168) (7)	2021 (n = 35)	OR (95%CI), P-value
Missense	504 (43%)	20 (57%)	$OR = 0.57$ (0.29–1.12), $p = 0.10$
Non-missense (severe)	664 (57%)	15 (43%)	
Ave age at kidney failure	24.4 ± 7.8 years	29.2 ± 14.6 years	$p = 0.0026$

Relatively more missense (non-severe) variants were detected in 2021 which was consistent with the later age at onset of kidney failure. This may be because families with very severe disease had already been identified.

for the collagen IV α5 chain in the kidney or skin (43). Males with absent staining have a more abnormal GBM and a worse prognosis than those with positive staining (44, 45). The other method is still a research tool but uses an *in vitro* expression system to examine collagen IV α3α4α5 heterotrimer formation and secretion (46). Reduced formation and secretion correlate with proteinuria development and early onset kidney failure (46).

CAVEATS TO PREDICTING CLINICAL FEATURES FROM GENOTYPE

However there are also caveats to predicting the clinical features from the genotype based on previously reported studies.

Firstly, there is a bias to reporting more severe disease. Most publications are of hospital—based series where affected individuals have the typical Alport features with kidney failure.

Secondly, sometimes the phenotype varies even in family members with the same pathogenic variant. Twenty-three families with XL Alport syndrome, each with at least 3 males with kidney failure, included 17 families (74%) where men had a consistent age at kidney failure and six with varying ages (26%) (15). Missense and splice site variants are especially associated with divergent ages. Other studies have found more consistency (15–17). Women with XL disease and males and females with AD Alport syndrome are the populations with the most variation in the age at kidney failure. This is possibly because they have a smaller genetic risk of kidney failure and their likelihood and age may be distorted by coincidental diseases such as hypertension, obesity, diabetes, or modifying variants in other filtration barrier genes (47).

Thirdly, the mode of inheritance must be considered. It has been more difficult to correlate *COL4A5* variants in women with age at kidney failure because of random X chromosome inactivation. Women and girls have heterozygous *COL4A5* variants twice as often as men but typically have milder disease (22). Only 20% have kidney failure by the age of 60 years but hearing loss and peripheral fleck retinopathy are common (32). Milder disease occurs because, overall with balanced X inactivation, only half the female podocytes are affected and produce defective trimers. When X inactivation is skewed, a higher or lower proportion of podocytes are affected, and the phenotype varies correspondingly. With autosomal recessive inheritance, the clinical severity depends on the consequences of both variants.

Fourthly, genetic testing techniques have changed over the past 20 years of reporting genotype-phenotype studies. Many families with typical disease underwent testing when it first became available and those tested now are often demonstrated to have milder variants (**Table 4**). In addition, the currently-used technique of Whole Exome Sequencing is less sensitive for detecting large deletions, and hypomorphic variants are recognized increasingly (48).

Fifthly, commonly-used treatments such as renin-angiotensin-aldosterone inhibitors modify the age at kidney failure in Alport syndrome (24, 25). Treatments may also modify the hearing loss and ocular abnormalities.

FUTURE STUDIES

Future studies will focus on confirming more closely a genotype-phenotype correlation using proteinuria rather than kidney failure in women with X-linked Alport syndrome and in individuals with heterozygous *COL4A3* or *COL4A4* pathogenic variants. It may be possible to develop a more precise algorithm for predicting the age at kidney failure. More information will be available about hypomorphic variants, and further studies may explain whether severe

COL4A3–COL4A5 variants predispose to IgA nephropathy and cystic kidney disease, or reduce the risk of diabetic nephropathy (49).

The detection of a severely-damaging genetic variant represents a powerful argument for starting treatment with ACE inhibitors, and possibly other more specific treatments much earlier in order to delay the onset of end-stage kidney failure.

AUTHOR CONTRIBUTIONS

JS designed the project and wrote the first draft. MH, MC, and KS undertook the analysis of all the variants in the LOVD database. JG read the manuscript, provided the figure, added to the discussion, and corrected the draft. All authors reviewed and approved the final manuscript.

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frontiers | Frontiers in Medicine

TYPE Original Research
PUBLISHED 31 August 2022
DOI 10.3389/fmed.2022.957733

OPEN ACCESS

EDITED BY
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SPECIALTY SECTION
This article was submitted to Nephrology, a section of the journal Frontiers in Medicine

RECEIVED 31 May 2022
ACCEPTED 29 July 2022
PUBLISHED 31 August 2022

CITATION
Ćomić J, Riedhammer KM, Günthner R, Schaaf CW, Richthammer P, Simmendinger H, Kieffer D, Berutti R, Tasic V, Abazi-Emini N, Nushi-Stavileci V, Putnik J, Stajic N, Lungu A, Gross O, Renders L, Heemann U, Braunisch MC, Meitinger T and Hoefele J (2022) The multifaceted phenotypic and genotypic spectrum of type-IV-collagen-related nephropathy—A human genetics department experience. *Front. Med.* 9:957733. doi: 10.3389/fmed.2022.957733

The multifaceted phenotypic and genotypic spectrum of type-IV-collagen-related nephropathy—A human genetics department experience

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Disease-causing variants in *COL4A3*-5 are associated with type-IV-collagen-related nephropathy, a genetically and phenotypically multifaceted disorder comprising Alport syndrome (AS) and thin basement membrane nephropathy (TBMN) and autosomal, X-linked and a proposed digenic inheritance. Initial symptoms of individuals with AS are microscopic hematuria followed by proteinuria leading to kidney failure (90% on dialysis < age 40 years). In contrast, individuals with TBMN, an outdated histology-derived term, present with microscopic hematuria, only some of them develop kidney failure (>50 years of age). An early diagnosis of type-IV-collagen-related nephropathy is essential for optimized therapy and slowing of the disease. Sixty index cases, in whom exome sequencing had been performed and with disease-causing variant(s) in *COL4A3-5*, were evaluated concerning their clinical tentative diagnosis and their genotype. Of 60 reevaluated individuals with type-IV-collagen-related nephropathy, 72% had AS, 23% TBMN and 5% focal segmental glomerulosclerosis (FSGS) as clinical tentative diagnosis. The FSGS cases had to be re-classified as having type-IV-collagen-related nephropathy. Twelve percent of cases had AS as clinical tentative diagnosis and a monoallelic disease-causing variant in *COL4A3/4* but could not be classified as autosomal dominant AS because of limited or conflicting clinical data. This study illustrates

the complex clinical and genetic picture of individuals with a type IV-collagen-related nephropathy indicating the need of a refined nomenclature and the more interdisciplinary teamwork of clinicians and geneticists as the key to optimized patient care.

KEYWORDS

type-IV-collagen-related nephropathy, Alport syndrome, *COL4A3*, *COL4A4*, *COL4A5*

Introduction

The α3, α4, and α5 chains of the type IV collagen are an essential component of the glomerular basement membrane (GBM) and are encoded by the three genes *COL4A3*, *COL4A4*, and *COL4A5* (1). Disease-causing variants [(likely) pathogenic and pathogenic variants as per the guideline for sequence variant interpretation of the American College of Medical Genetics and Genomics and current amendments; see Material and Methods] in one of these genes are associated with type-IV-collagen-related nephropathy, comprising Alport syndrome (AS) and thin basement membrane nephropathy (TBMN) (2–8).

AS is characterized by microscopic hematuria and proteinuria leading to progressive loss of kidney function. Additionally, sensorineural hearing impairment, and eye abnormalities can be observed. AS is the second most common monogenic cause for kidney failure (1). It can be inherited in an X-linked [XLAS; hemizygous (male) or heterozygous (female) disease-causing variant in *COL4A5*] or autosomal recessive (ARAS; biallelic pathogenic variants in *COL4A3*/*COL4A4*) form (1, 9–11). The often used designation autosomal dominant AS in carriers of monoallelic pathogenic variants in *COL4A3* and *COL4A4* is differently used in the literature. In one recent publication by Furlano et al., the authors propose that any case harboring one heterozygous disease-causing variant in *COL4A3* or *COL4A4* should be designated as autosomal dominant AS independently from the clinical phenotype which ranges from microscopic hematuria to chronic kidney disease (12). In contrast, Savige et al. classify individuals with a heterozygous disease-causing variant in *COL4A3* or *COL4A4* as having autosomal dominant inherited TBMN or AS depending on the clinical phenotype and a potential positive familial history (13, 14). Furthermore, digenic inheritance has also been discussed as a possible cause in individuals with AS (15–18).

TBMN is a histopathology-derived term defined as uniform thinning of the GBM and phenotypically characterized by persistent microscopic hematuria, minimal if any proteinuria, and normal renal function (19, 20). Solely thinning of the GBM can also be found in early stages of AS (19, 21). The frequency of TBMN has been estimated to be as high as 1% of the world population (22). In up to 20% of the individuals with TBMN, disease progression to late-onset—compared to AS—kidney failure (>50 years of age) has been reported (23). This disease progress seems to be related in part to the development of focal segmental glomerulosclerosis (FSGS) (9, 24). Hence, in some cases with suspicion of a hereditary podocytopathy (hereditary FSGS), disease-causing variants in *COL4A3-COL4A5* can be found (25, 26).

The focus of this study was the reevaluation of the clinical phenotype and the reanalysis of exome sequencing data of 60 individuals with disease-causing variants in *COL4A3-5* in order to evaluate and highlight the shortcomings of the current nomenclature of AS/TBMN.

Methods

Study population

For this study, a cohort of 60 index cases of unrelated families with disease-causing variants in *COL4A3*, *COL4A4*, or *COL4A5* was investigated. These families have been recruited between October 2015 and August 2020 according to their appearance at our institute. In all individuals exome sequencing was already performed and genetic data were available. The exome data were reanalyzed in *COL4A3-5* within this study. This study was carried out according to standards of the 2013 Helsinki Declaration and authorized by the local Ethics Committee of the Technical University of Munich. Informed and written consents were obtained from all individuals or their legal guardians.

Clinical case information

Clinical and phenotypic information were obtained from clinical reports and medical history. Additionally, a standardized questionnaire was used to evaluate clinical information. The individuals were assigned to one of the following groups according to the clinical tentative diagnoses/kidney biopsy results as assigned by the referring clinician (nephrologists or pediatric nephrologists): AS, TBMN or FSGS. Age of onset of kidney failure in individuals was determined as the beginning of renal replacement therapy (hemodialysis or peritoneal dialysis) or pre-emptive kidney transplantation.

Genetic testing

For extraction of DNA from peripheral blood the automated nucleic acid purification instrument Chemagic™ 360 (PerkinElmer, Waltham, MA, USA) according to the manufacturer's protocol was used.

Exome sequencing

Exome sequencing was performed with Sure Select Human All Exon 60Mb V6 Kit (Agilent) and on a HiSeq4000 platform (Illumina) in the index cases (27). Mitochondrial DNA was derived from off-target exome reads as previously described (28). Reads were aligned to the human reference genome (UCSC Genome Browser build hg19) using Burrows-Wheeler Aligner (v.0.7.5a). Using SAMtools (version 0.1.19), detection of single-nucleotide variants (SNVs) and small insertions and deletions (indels) was accomplished. For investigation of copy number variants (CNVs) (including exon-spanning intronic regions) ExomeDepth was used. A noise threshold of 2.5 was accepted (29). The called CNVs were visualized by the Integrative Genomics Viewer (IGV, https://software.broadinstitute.org/software/igv/) to check if there was enough coverage of the examined regions and for plausibility of the CNVs. CNVs were then compared with publicly available control databases like the Genome Aggregation Database (gnomAD, https://gnomad.broadinstitute.org/about), the Database of Genomic Variants (DGV, http://dgv.tcag.ca/dgv/app/home) the databases for pathogenic CNVs like DECIPHER (https://decipher.sanger.ac.uk/) and ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/). For the analysis of *de novo,* autosomal dominant and mitochondrial SNVs and indels, only variants with a minor allele frequency (MAF) of <0.1% (Munich Exome Server with over 22,000 exomes) were considered. For the analysis of autosomal recessive and X-linked SNVs and indels [homozygous, hemizygous or (putatively) compound heterozygous], only variants with a MAF of <1.0% were considered.

Sanger sequencing

Using Sanger sequencing, segregation analysis was conducted. Oligonucleotide primer sequences are available upon request.

Variant interpretation

Publicly available databases for (likely) pathogenic variants were used for comparison of all variants found and described in this study. These databases are ClinVar, the Human Gene Mutation Database (HGMD®, http://www.hgmd.cf.ac.uk), and the Leiden Open Variation Database (LOVD, https://www.lovd.nl). The variants were rated in accordance to American College of medical Genetics and Genomics (ACMG) guidelines and current amendments (4–7). Likely pathogenic and pathogenic variants are summarized as "disease-causing variant" in the text.

Biallelic disease-causing variants in *COL4A3* and *COL4A4* in male and female individuals, a hemizygous disease-causing variant in *COL4A5* in a male individual and a heterozygous disease-causing variant in *COL4A5* in a female individual were in accordance with the clinical tentative diagnosis of AS. In females with the clinical tentative diagnosis of TBMN and a heterozygous disease-causing variant in *COL4A5*, the genotype was also fitting to the clinical tentative diagnosis, as females with heterozygous disease-causing variants in *COL4A5* can show a broad phenotypic spectrum ranging from TBMN to AS. In contrast to that, individuals with a heterozygous (likely) pathogenic variant in *COL4A3* or *COL4A4* and a clinical tentative diagnosis of AS were not automatically seen in accordance with autosomal dominant AS but further pedigree and phenotypic information were scrutinized (see Results). In turn, if cases had the clinical tentative diagnosis of TBMN and carried a heterozygous (likely) pathogenic variant in *COL4A3* or *COL4A4* (female and male individuals), genotype and phenotype were in agreement. Carriers of a hemizygous (likely) pathogenic variant in *COL4A5* and the clinical tentative diagnosis of TBMN were classified as genetically solved AS due to the unquestionable genotype of hemizygous disease-causing variants in *COL4A5* leading to AS (which can be mistaken as TBMN in early stages of disease). Furthermore, individuals with the non-specific phenotype of FSGS on kidney biopsy and disease-causing variants in *COL4A3*, *COL4A4*, or *COL4A5* were reclassified as type-IV-collagen-nephropathies (two cases ARAS, one case XLAS—female carrier; see Supplementary Table 1).

Results

Study population

The total study cohort consisted of 60 unrelated index cases (30 females and 30 males) with disease-causing variants in *COL4A3-5*. 43/60 (72%) individuals had AS phenotypically, 14/60 (23%) TBMN and 3/60 (5%) FSGS on biopsy (Figure 1). 55/60 individuals (92%) were of non-Finnish European descent. The median age of disease-onset was 7 years (range: 0–35 years of age). In nine cases, the age of disease-onset was not available. Clinical findings were as follows (cases with reported phenotype information): 25/25 (100%) individuals presented with microscopic hematuria (no data available from 35 individuals), 12/25 (48%) with the clinical phenotype of AS, 11/25 (44%) with TBMN, and 2/25 (8%) with FSGS. Proteinuria

TABLE 1 Exon coverage of genes *COL4A3-5* in exome sequencing.

Gene	Chromosomal location	Transcript number	Inheritance	Phenotype MIM number	Covered exons (>20x)
COL4A3	2q36.3	NM_000091.4	AD, AR	104200, 203780	98%
COL4A4	2q36.3	NM_000092.4	AD, AR	104200, 203780	100%
COL4A5	Xq22.3	NM_033380.3	XL	301050	88%

AD, autosomal dominant; AR, autosomal recessive; AS, Alport syndrome; XL, X-linked.

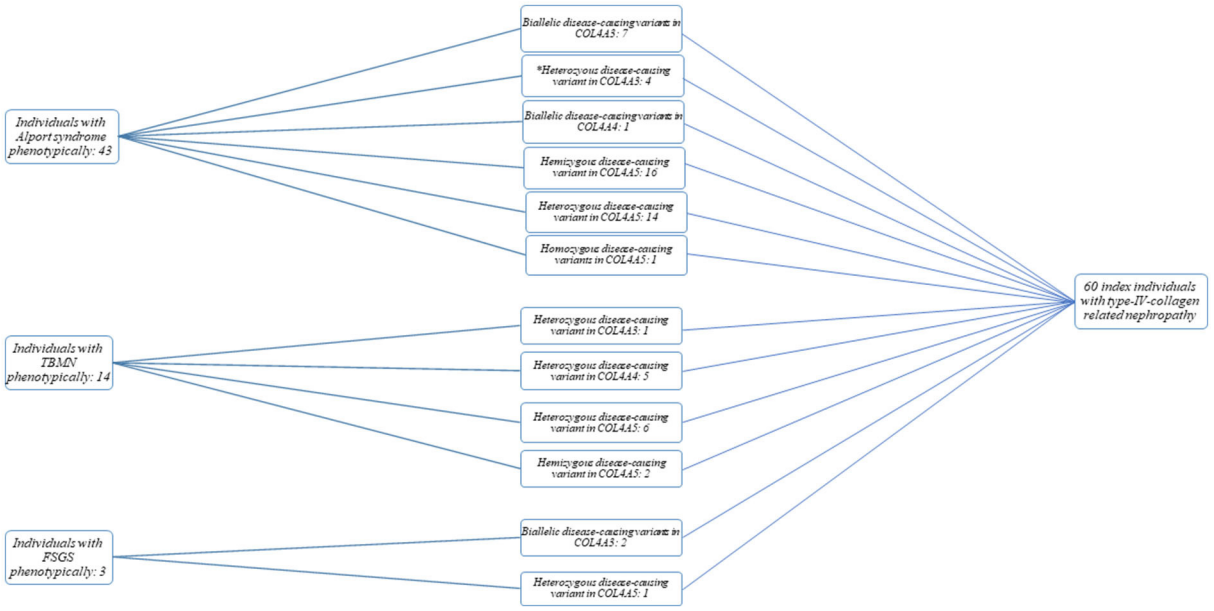

FIGURE 1
Flow chart and cohort overview. Categorization of individuals into Alport syndrome (AS), thin basement membrane nephropathy (TBMN), and focal segmental glomerulosclerosis (FSGS) phenotypic subgroups was based on clinical tentative diagnosis/kidney biopsy result. *, Individuals with a questionable phenotype.

was seen in 15/25 (60%) individuals [no data available from 45 individuals; 10/15 (67%) with AS, 3/15 (20%) with TBMN, 2/15 (13%) with FSGS]. End-stage kidney failure (ESKF) was seen in 3/17 (18%) individuals, 2/3 (67%) with AS (23 and 24 years of age), and 1/3 (33%) with TBMN (68 years of age). No data were available from 43 individuals. Eye anomalies could be observed in 8/53 (15%) individuals (no data available from 7 individuals); all of them had AS as clinical tentative diagnosis. 18/53 (34%) individuals had hearing impairment (no data available from 7 individuals), 16/18 (89%) had AS, 2/18 (11%) TBMN.

Coverage of genes *COL4A3-5*

The median coverage > 20x of all exons of the three genes *COL4A3-5* was > 95% (range: 88–100%; see Table 1). All exons were covered at least 10x.

Identification of different disease-causing variants

Within this study, 69 disease-causing variants could be identified, 61/69 (88%) of them were different. 26/61 (43%) were already described in the literature (*COL4A3*: 11, *COL4A4*: 1, *COL4A5*: 14), 35/61 (57%) were novel (i.e., not previously reported as disease-causing) (*COL4A3*: 9, *COL4A4*: 5, *COL4A5*: 21) (Supplementary Tables 1, 2).

Distribution of identified (likely) pathogenic variants in *COL4A3-5*

From the 43 individuals with AS phenotypically, 39 (91%) cases had either an autosomal recessive (biallelic disease-causing variants in *COL4A3* or *COL4A4*) or X-linked AS

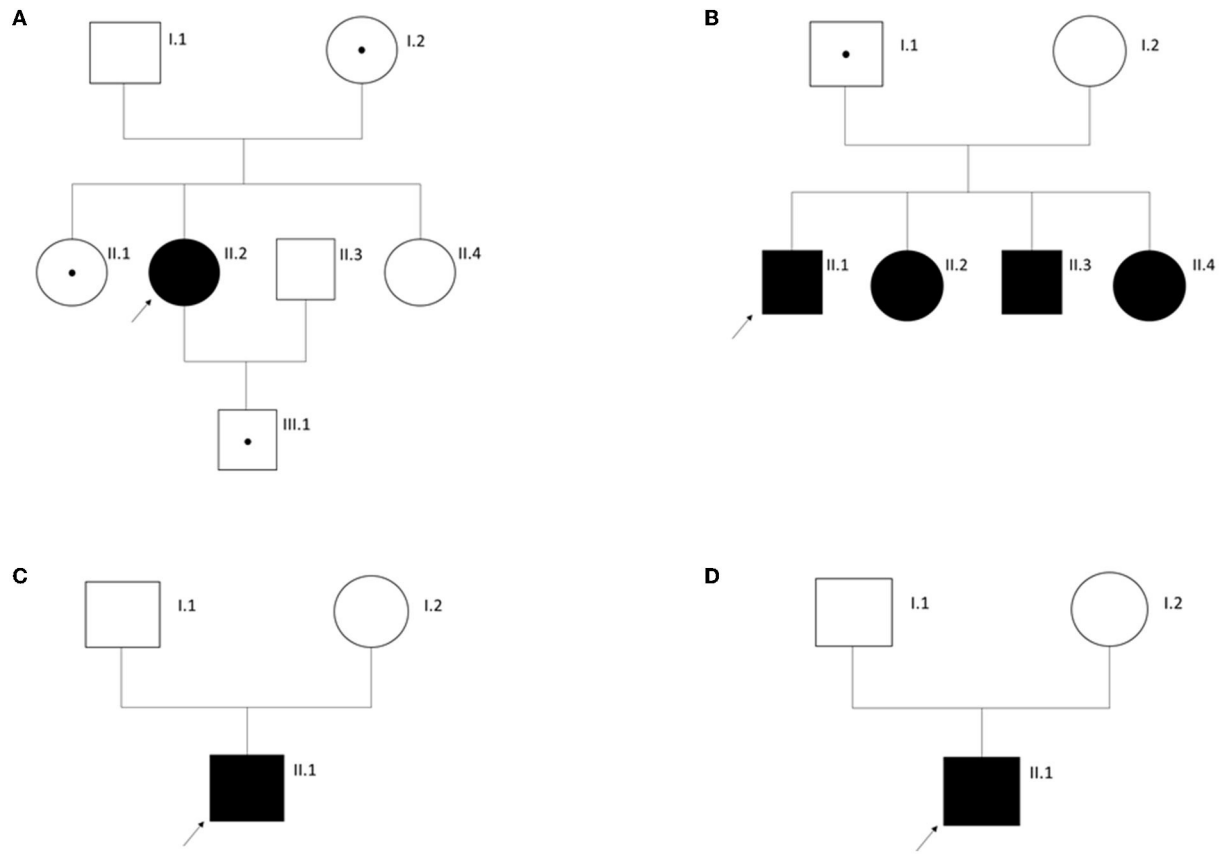

FIGURE 2
Pedigrees of families with Alport syndrome as clinical tentative diagnosis in the index individual (arrow) and only a monoallelic disease-causing variant in *COL4A3*. See Results for more information. **(A)** Family ATS-F521; **(B)** Family ATS-F663; **(C)** Family ATS-F787; **(D)** Family ATS-F788. Circles, females. Squares, males. A dot within a symbol signifies an unaffected carrier of the respective disease-causing variant.

[hemizygous (male individuals) or heterozygous/homozygous (female individuals) variants in *COL4A5*] (Figure 1; Supplementary Table 1). Of these 39 cases, 7 (18%) had (likely) pathogenic compound heterozygous/homozygous variants in *COL4A3*, 1 (3%) had a likely pathogenic homozygous variant in *COL4A4*, and 31 (79%) had a heterozygous (females) (14/31), homozygous (female; 1/31) or hemizygous (16/31) (likely) pathogenic variant in *COL4A5*.

Of the 14 individuals with TBMN phenotypically, one (7%) case had a (likely) pathogenic heterozygous variant in *COL4A3*, five (36%) in *COL4A4* and 8 (57%) in *COL4A5* (hemizygous variant: 2; heterozygous variant: 6).

Two individuals with the histopathological picture of FSGS had compound heterozygous likely pathogenic variants in *COL4A3*, and one female individual carried a heterozygous likely pathogenic variant in *COL4A5* (Supplementary Table 1).

In 4 (12%) individuals with AS phenotypically, only one heterozygous (likely) pathogenic variant in *COL4A3* gene could be identified (Supplementary Table 1). A genetic diagnosis of autosomal dominant AS was not made in these individuals because of limited or conflicting clinical data questioning their submitted diagnosis of AS. The healthy mother (62 years of age), one healthy sister (33 years of age), and the healthy son of ATS-F521-II-2 (16 years of age) also carry the variant in *COL4A3*. One additional sister of ATS-F521-II-2 also suffers from hearing impairment but has no renal phenotype. She does not carry the variant in *COL4A3* (Figure 2A). ATS-F663-II-1 inherited the variant from his healthy father but has three further affected siblings who all carry the variant. Two of the altogether four siblings additionally carry a heterozygous maternally inherited likely pathogenic variant in *MYH9* (NM_002473.4; c.1960C>G, p.(Leu654Val)). To our knowledge, the mother is healthy (Figure 2B). ATS-F787-II-1 was submitted as having AS confirmed by kidney biopsy (original biopsy report not available). No clinical data or medical records of the index and affected family members could be gathered (Figure 2C). Clinical data of ATS-F788-II-1 were also not available and a kidney biopsy confirming the clinical diagnosis of AS was not performed. This individual was part of the EARLY PRO-TECT

Alport trial and medicated with placebo. Under this treatment no disease progress was seen during the trial (30) (Figure 2D).

None of the 60 individuals with disease-causing variants in one of the *COL4A3*-5 genes had additional variants in one of the two alternate *COL4A3-5* genes (Figure 1).

Reclassification of individuals concerning their genetic result

Out of 56 individuals with a distinct genotype, the three individuals (5%) with FSGS were treated with cyclosporin A and mycophenolate mofetil. These individuals had to be re-classified as having a type-IV-collagen-related nephropathy questioning further extensive treatment with immunosuppressive drugs.

Discussion

Type-IV-collagen-related nephropathy is a phenotypically and genetically multifaceted disorder. It comprises various phenotypes like classic AS, a slower-progressive phenotype originally described as TBMN and unspecific biopsy phenotypes like FSGS. To add to this complexity, it features both autosomal and X-linked inheritance.

We sought to illustrate this complex phenotypic and genotypic picture: In 91% of the individuals with AS phenotypically and in 100% of the individuals with TBMN phenotypically, disease-causing variants could be identified explaining the clinical tentative diagnosis. This high detection rate might be caused by intensive clinical evaluation of the affected individuals and detailed family medical history. In 5% of the individuals with disease-causing variants in *COL4A3-5*, a clinical phenotype of FSGS could be observed. This finding was already described by Malone et al. in 2014 and others (31). In their study, 10% of individuals with single or compound heterozygous disease-causing variants in *COL4A3* or *COL4A4* had the clinical tentative diagnosis of hereditary FSGS. The main cause of this clinical and genetic difference may be the fact that FSGS is an unspecific histologic phenotype seen in the process of different kidney diseases.

Digenic inheritance is also proposed as a possible cause for type-IV-collagen-related nephropathy including AS and TBMN, although little evidence is available on this topic so far (12, 16, 18). In contrast to studies describing this inheritance pattern, we did not observe findings of this pattern of inheritance within this study. This might be due to the facts that the present study had a small cohort size, the affected individuals were of different origin compared to individuals described in the literature and was performed with different sequencing techniques. Of note, it cannot be excluded that a variant was not detected if it was located in one of the limited covered exons which mostly affects the exons 5, 11, 14, 23, 38, 41, and 48 of *COL4A5*.

Importantly, in 12% of individuals with AS as clinical tentative diagnosis and monoallelic disease-causing variants in *COL4A3*/*COL4A4*, a clear statement on autosomal dominant AS could not be made taking into account the genetic result and clinical data. There is an unresolved conflict concerning autosomal dominant AS and the frontlines seem to run across two standpoints: A "clinician-centered" view stating that clear diagnoses are important for surveillance and early treatment (12, 32); and a "geneticist-centered" view that AS is a monogenic disease with complete penetrance and progressive kidney failure (90% on dialysis by age 40 years in X-linked AS; comparable for autosomal recessive AS) (11, 14). For us and others on the genetics-side (13), there are many questions concerning the simple usage of autosomal dominant AS in any case with a monoallelic disease-causing variant in *COL4A3* and *COL4A4*: In cases with a clear AS phenotype (for example on kidney biopsy, ATS-F787-II-1 above) but only a monoallelic variant in *COL4A3*/*COL4A4*, could there be another variant on the other allele missed by routine genetic testing (e.g., intronic variant leading to a splicing defect, complex rearrangement missed by short-read-based NGS)? Should we use kidney biopsy specimen (or urine-derived renal cells) in these cases to run transcriptomics on to determine if there is a splicing defect on the other allele? Should we engage further in elucidation of rearrangements with chromosomal microarray/multiplex ligation-dependent probe amplification (MLPA) or even genome sequencing? Does the same variant cause the identical disorder, AS, in a homozygous and heterozygous state? In heterozygous carriers with monoallelic missense variants in *COL4A3*/*COL4A4*, might a dominant-negative effect come into play and explain a complete AS phenotype with high penetrance? Have phenocopies been taken into account? Has the pedigree been thoroughly investigated and relatives been tested? What if healthy parents and relatives carry the variant (as in cases ATS-F521-II-2, ATS-F663-II-1), how do we counsel these parents if they get another child? Do they have a 50% recurrence risk of AS, as in other clearly autosomal dominant diseases? And does the affected individual have a 50% risk of offspring with AS if he or she gets children? To what extent is there incomplete penetrance? The estimated prevalence of heterozygous disease-causing variants in *COL4A3*/*COL4A4* is 1 in 106, as has recently been shown (8). Are we to diagnose all of these people with autosomal dominant AS? All these questions are not satisfactorily addressed so far in the literature and need to be solved to optimize the medical care and genetic counseling of these individuals.

Undoubtedly, heterozygous carriers of disease-causing variants in *COL4A3*, *COL4A4*, and *COL4A5* have a higher risk of end-stage kidney failure than the general population (12, 24). They need surveillance and treatment with ACE inhibition once proteinuria/albuminuria develops (33, 34). But if we classify every of these cases as having autosomal

dominant AS, we are risking not making the considerations mentioned above impeding correct diagnoses and risk calculations. Hence, a unifying gene-centered nomenclature like type-IV-collagen-related nephropathy could steer free of this conflict (35), especially as TBMN is also an outdated term which is based on histologic findings not always present or preceding pathognomonic AS changes (12) (as seen by the fact that there are two cases with a hemizygous disease-causing variant in *COL4A5*, i.e., XLAS, but rated as TBMN by the referring clinician). We still used the term "TBMN" in lack of proper alternatives and as it was used by referring clinicians. In our opinion, genetic reports should state the genotype in the diagnosis and the designation AS should only be added if there is genotypic and phenotypic evidence for this diagnosis (e.g., "Diagnosis of type-IV-collagen-related nephropathy—X-linked Alport syndrome—hemizygous pathogenic frameshift variant in *COL4A5*").

There is lacking phenotype information (see Section Results) on a number of individuals in this study, which can be viewed as a limitation. However, the aim of this study was to illustrate the phenotypic and genotypic spectrum of type-IV-collagen related nephropathy and not a detailed genotype-phenotype correlation on AS, which have been published extensively (9–12, 36).

Finally, in this study, 57% of the identified variants were novel indicating that there are still many disease-causing variants in type-IV-collagen-releated-nephropathy unknown so far. Therefore, it is extremely important to submit identified variants to open genetic databases like ClinVar or LOVD to extend the knowledge of disease-causing variants and to optimize the clinical care of individuals with a type-IV-collagen-related nephropathy.

To conclude, this study illustrates the complex clinical and genetic spectrum of type-IV-collagen-related nephropathy including AS and TBMN in a small single tertiary-care center cohort. A refined nomenclature not impeding swift diagnosis, surveillance and treatment but owing to the diverse genetic considerations of this multifaceted disorder is direly needed and, by using the term "type-IV-collagen-related nephropathy", we propose a more gene-centered approach. Additionally, close cooperation of clinicians and geneticists is key to collect the necessary phenotypic and pedigree data needed to adequately assess individuals with suspected type-IV-collagen-related nephropathy.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee, Klinikum rechts der Isar, Technical University of Munich, Munich, Germany. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

Author contributions

Research and study design: KMR and JH. Data analysis/interpretation: JĆ, KMR, TM, and JH. Statistical analysis: JĆ. Patient acquisition: RG, PR, HS, DK, VT, NA-E, VN-S, JP, NS, AL, LR, UH, MB, CS, TM, and JH. Drafting and revising the article: JĆ, KMR, CS, TM, and JH. Supervision or mentorship and final approval of the version to be published: JH. All author contributed important intellectual content during manuscript drafting or revision, agrees to be personally accountable for the individual's own contributions, to ensure that questions pertaining to the accuracy or integrity of any portion of the work, even one in which the author was not directly involved, are appropriately investigated and resolved, and including with documentation in the literature if appropriate.

Funding

This work was supported with a research Grant by the European Society for Pediatric Nephrology (ESPN e.V.; ESPN #2.2020) and by the German Research Foundation (DFG) and the Technical University of Munich (TUM) in the framework of the Open Access Publishing Program.

Acknowledgments

We would like to thank the patients and their families for participation in this study.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.957733/full#supplementary-material

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