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ORIGINAL ARTICLE

COL4A3 mutation is an independent risk factor for poor prognosis in children with Alport syndrome

Gulsah Ozdemir[1] · Bora Gulhan[1] · Emine Atayar[2] · Seha Saygılı[3] · Oguz Soylemezoglu[4] · Zeynep Birsin Ozcakar[5] · Fehime Kara Eroglu[6] · Cengiz Candan[7] · Belde Kasap Demir[8] · Alper Soylu[9] · Selçuk Yüksel[10] · Harika Alpay[11] · Ayse Agbas[12] · Ali Duzova[1] · Mutlu Hayran[13] · Fatih Ozaltin[1,2] · Rezan Topaloglu[1]

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Abstract

Background Alport syndrome (AS) is an inherited glomerular disease caused by mutations in *COL4A3*, *COL4A4*, or *COL4A5*. Associations between clinical manifestations and genotype are not yet well defined. Our study aimed to define clinical and genetic characteristics, establish genotype–phenotype correlations, and determine prognosis of AS in children.

Methods A total of 87 children with AS from 10 pediatric nephrology centers, whom had genetic analyses performed at the Hacettepe University Nephrogenetics Laboratory between February 2017 and February 2019, were included. Data regarding demographics, family history, clinical and laboratory characteristics, histopathological and genetic test results, treatments, and yearly follow-up results were retrospectively analyzed.

Results Of 87 patients, 16% presented with nephrotic syndrome. In patients with nephrotic syndrome, kidney biopsy findings showed focal segmental glomerulosclerosis (FSGS) in 79%, and *COL4A3* mutations were the leading genetic abnormality (50%). Twenty-four percent of all patients progressed to chronic kidney disease (CKD). The rate of progression to CKD and the decline in the glomerular filtration rate of the patients with *COL4A3* mutation were higher than other mutation groups ($p < 0.001$ and $p = 0.04$, respectively). In kidney survival analysis, nephrotic syndrome presentation, histopathology of FSGS, *COL4A3* mutations, and autosomal recessive inheritance were found as independent risk factors for earlier progression to CKD. Cyclosporin A treatment did not improve kidney survival.

Conclusions We emphasize that genetic testing is important for patients suspected as having AS. Furthermore, *COL4A* mutations should be considered in patients with FSGS and steroid-resistant nephrotic syndrome. This approach will shed light on the prognosis of patients and help with definitive diagnosis, preventing unnecessary and potentially harmful medications.

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✉ Rezan Topaloglu
rezantopaloglu@hacettepe.edu.tr

[1] Division of Pediatric Nephrology, Faculty of Medicine, Hacettepe University, 06230 Ankara, Turkey

[2] Division of Pediatric Nephrology, Nephrogenetics Laboratory, Faculty of Medicine, Hacettepe University, Ankara, Turkey

[3] Division of Pediatric Nephrology, Faculty of Medicine, Istanbul University-Cerrahpaşa, Istanbul, Turkey

[4] Division of Pediatric Nephrology, Faculty of Medicine, Gazi University, Ankara, Turkey

[5] Division of Pediatric Nephrology, Faculty of Medicine, Ankara University, Ankara, Turkey

[6] Division of Pediatric Nephrology, Dr. Sami Ulus Maternity and Children's Health Hospital, Ankara, Turkey

[7] Division of Pediatric Nephrology, Göztepe Training and Research Hospital, Istanbul Medeniyet University, Istanbul, Turkey

[8] Division of Pediatric Nephrology, Tepecik Training and Research Hospital, Izmir, Turkey

[9] Division of Pediatric Nephrology, Faculty of Medicine, Dokuz Eylül University, Izmir, Turkey

[10] Division of Pediatric Nephrology, Faculty of Medicine, Pamukkale University, Denizli, Turkey

[11] Division of Pediatric Nephrology, Faculty of Medicine, Marmara University, Istanbul, Turkey

[12] Division of Pediatric Nephrology, Haseki Training and Research Hospital, Istanbul, Turkey

[13] Department of Preventive Oncology, Faculty of Medicine, Hacettepe University, Ankara, Turkey

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Keywords Alport syndrome · *COL4A* mutations · Focal segmental glomerulosclerosis · Nephrotic syndrome · Cyclosporin A

Introduction

Alport syndrome (AS) is an inherited glomerular basement membrane (GBM) disease characterized by progressive kidney failure and often occurring with sensorineural hearing loss and ocular abnormalities. It is caused by mutations in the *COL4A3*, *COL4A4*, or *COL4A5* genes encoding α3, α4, and α5 chains of the type IV collagen, respectively [1, 2]. According to the inheritance pattern, AS is classified as X-linked AS (XLAS), autosomal recessive AS (ARAS), or autosomal dominant AS (ADAS) [3]. XLAS comprises 80% of cases and is caused by mutations in the *COL4A5* gene. Rarely, ARAS (caused by homozygous or compound heterozygous mutations in the *COL4A3*/*COL4A4* genes) and ADAS (caused by heterozygous mutations in the *COL4A3*/*COL4A4* genes) affect about 15% and 5% of cases, respectively [4]. AS develops in approximately 1 in 5000 individuals and comprises 12.9% of cases of end-stage renal disease (ESRD) in children [5]. Recently, several studies have reported that *COL4A* mutations can also be detected in patients presenting with nephrotic syndrome (NS) and focal segmental glomerulosclerosis (FSGS). Malone et al. [6] reported that genetic analysis uncovered *COL4A3* or *COL4A4* mutations in approximately 10% of patients with familial FSGS. Gast et al. [7] reported that *COL4A* mutations were identified in the patients with familial and sporadic FSGS. However, the association between clinical manifestations and genotype has not as yet been well described.

In this study, we aimed to define the clinical characteristics of children with *COL4A3*, *COL4A4*, and *COL4A5* mutations in order to establish the genotype–phenotype correlation and determine the long-term prognosis.

Methods

Patients and data collection

A total of 87 children with genetically confirmed AS from 10 pediatric nephrology centers in Turkey, for whom genetic analyses were performed in the Hacettepe University Nephrogenetics Laboratory between February 2017 and February 2019, were included in the study. Patients without genetically confirmed AS diagnosis were not included (Fig. 1). We retrospectively collected data from medical records and requested other medical centers to fill out a questionnaire, which included data regarding patient demographic features, family history, clinical and laboratory characteristics at the first presentation, histopathological (if available) and genetic test results, treatments, and yearly follow-up results. The study protocol was approved by the Non-Interventional Clinical Researches Ethics Board of Hacettepe University (KA 19073), and written informed consent was provided by the patients' parents and by patients aged > 10 years. The study was conducted in accordance with the Helsinki Declaration.

Definitions

AS diagnosis was suspected in the presence of persistent hematuria and/or kidney failure and/or hearing loss and/or family history of AS. Genetically confirmed AS was diagnosed on the basis of the detection of *COL4A* mutations through genetic analysis. In addition to family history, ARAS and ADAS were diagnosed if the presence of homozygous and heterozygous *COL4A3*/*COL4A4* mutations was confirmed, respectively. Patients were classified as hypertensive if the office blood pressure measurement was above or equal to the 95th percentile for the age, gender, and height standards. Nephrotic-range proteinuria was defined as spot urine protein/creatinine ratio of > 2 mg/mg or 24-h urine protein > 40 mg/m^2/h. NS was defined by nephrotic-range proteinuria, hypoalbuminemia (< 2.5 g/dl), edema, and hyperlipidemia. Chronic kidney disease (CKD) stages were defined by the KDIGO clinical practice guidelines according to the glomerular filtration rate (GFR) and evidence of kidney damage [8]. GFR was calculated using the original Schwartz formula [9]. Delta GFR (ΔGFR) was defined as the difference between GFR at the last visit and GFR at the first visit. While evaluating kidney prognosis, a GFR value of equal to or less than 60 ml/min/m^2 was taken into consideration.

Genetic analysis

Genetic analyses were performed at the Hacettepe University Nephrogenetics Laboratory. DNA was extracted from peripheral blood using a commercial kit according to the manufacturer's recommendations (Invitrogen PureLink Genomic DNA Mini Kit). All patient samples were run on a gene panel containing 47 genes (*ACTN4*, *ADCK4*, *CFB*, *CFH*, *CFHR1*, *CFHR2*, *CFHR3*, *CFHR4*, *CFHR5*, *CFI*, *C3*, *COL4A3*, *COL4A4*, *COL4A5*, *COQ2*, *COQ6*, *DGKE*, *EMP2*, *FAN1*, *GLA*, *INF2*, *LAGE3*, *LAMB2*, *LMX1B*, *MCP*, *MYOE1*, *NPHS1*, *NPHS2*, *NUP93*, *NUP205*, *NXF5*, *OSGEP*, *PDSS2*, *PLCE1*, *PLG*,

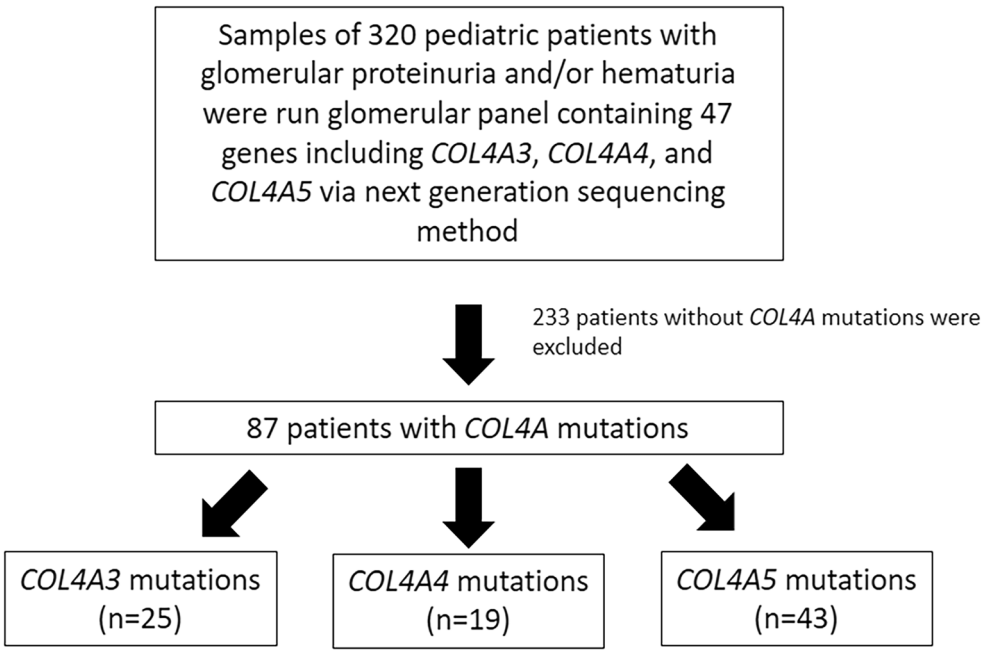

Fig. 1 Patient flowchart

PTPRO, *SCARB2*, *SGPL1*, *SMARCAL1*, *THBD*, *TMEM*, *TRPC6*, *TPRKB*, *TP53RK*, *TTC21B*, *WT1*, *XPO5*) via a next-generation sequencing method using the Ion S5 System® (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's recommendations. Data were analyzed using Ingenuity® Variant Analysis™ software (Qiagen, Redwood City, CA, USA). The following reference sequences of the National Center for Biotechnology Information (NCBI) (corresponding Ensembl) were used: *COL4A3* NM_000091 (ENST00000396578.7), *COL4A4* NM_000092.5 (ENST00000396625.4), and *COL4A5* NM_033380.3 (ENST00000328300.10). All mutations were confirmed by direct sequencing using BigDye v3.1 chemistry and an ABI3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Mutations detected were categorized into two groups as missense and non-missense (i.e., deletions, splice site, and non-sense).

Statistical analysis

Descriptive statistical analysis methods were used to evaluate demographic and clinical data. Mean, median, SD, and interquartile range (IQR) were calculated for numeric variables. Frequency tables were used to describe categorical data. The Mann–Whitney *U* test or independent samples *t* test was used to compare two independent samples. Survival analysis was performed using the Kaplan–Meier analysis with overall log-rank testing. All data were analyzed using IBM SPSS Statistics for Windows v.21 (IBM Corp., Armonk, NY, USA). p values < 0.05 in two-tailed tests were considered statistically significant in all analyses.

Results

Patient characteristics

The study included 87 children (46 males and 41 females). The mean age at first presentation was 7.6 years ± 4.1 years, and the median follow-up duration was 4.3 years (IQR 1.9–7.3). Genetic analyses uncovered mutations in *COL4A5* (n = 43, 49%), *COL4A3* (n = 25, 29%), and *COL4A4* (n = 19, 22%). Other clinical characteristics are shown in Table 1.

FSGS was detected in 16 patients (18%; 8 females and 8 males). The mean age at first presentation of the patients with FSGS was 9.2 years ± 2.7 years. Eleven (69%) of them presented with NS. While 6 (38%) patients presented with proteinuria alone, 10 (62%) had both hematuria and proteinuria at the first presentation. Genetic analyses revealed *COL4A3* mutations in 8 (50%), *COL4A4* mutations in 3 (19%), and *COL4A5* mutations in 5 (31%) patients. Other clinical characteristics are shown in Table 1.

Fourteen of 87 patients (16%; 10 females and 4 males) presented with NS. The mean age at first presentation of the patients with NS was 8.6 years ± 2.7 years. Eight (57%) of 14 patients presented with both hematuria and proteinuria. Histopathological evaluation showed FSGS in 11 of these cases (79%). Various immunosuppressive therapies were given to the patients with NS prior to the genetic diagnosis. Of all patients with NS, 3 received only steroids, 2 received only cyclosporin A (CsA), 5 received both steroids and CsA, and 4 received steroids, CsA, and another immunosuppressive drug (such as tacrolimus, cyclophosphamide, mycophenolate mofetil, or rituximab). Genetic analyses revealed *COL4A3* mutations in 7 patients (50%), *COL4A4* mutations in 4

Table 1 Demographic and clinical characteristics of all patients and patients with FSGS

	All patient cohort	Patients with FSGS
Total number of patients, *n* (%)	87 (100)	16 (18)
Female/male, *n* (%)	41 (47)/46 (53)	8 (50)/8 (50)
Age at first presentation (mean ± SD) (years)	7.6 ± 4.1	9.2 ± 2.7
Follow-up duration (median) (IQR) (years)	4.3 (1.9–7.3)	4.4 (2.2–9.1)
Urinalysis at first presentation, *n* (%)		
Hematuria and proteinuria	61 (70)	10 (63)
Hematuria alone	18 (21)	0 (0)
Proteinuria alone	8 (9)	6 (37)
Proteinuria at first presentation, *n* (%)		
None	18 (21)	0 (0)
Non-nephrotic range	40 (46)	3 (19)
Nephrotic range	29 (33)	13 (81)
Nephrotic syndrome at first presentation, *n* (%)	14 (16)	11 (69)
Hypertension at first presentation, *n* (%)	6 (8)	1 (6)
Hearing loss, *n* (%)	34 (39)	5 (31)
Histopathologic diagnosis, *n* (%)	58 (67)	
Alport syndrome	27/58 (47)	
FSGS	16/58 (28)	16 (100)
Mesangial cell proliferation	9/58 (15)	
Non-specific	6/58 (10)	
Genetic mutation, *n* (%)		
COL4A5	43 (49)	5 (31)
COL4A4	19 (22)*	3 (19)
COL4A3	25 (29)*	8 (50)
Mutation type, *n* (%)		
Missense	41 (47)	6 (38)
Deletion	26 (30)	5 (31)
Splice site	13 (15)	3 (19)
Nonsense	6 (7)	2 (12)
Inheritance pattern, *n* (%)		
XLAS	43 (49)	5 (31)
ARAS	33 (38)**	10 (63)
ADAS	11 (13)**	1 (6)
Renin–angiotensin–aldosterone system (RAAS) inhibitors, *n* (%)		
None	24 (28)	4 (25)
ACEi or ARB	44 (50)	9 (56)
ACEi and ARB	19 (22)	3 (19)
Cyclosporine treatment, *n* (%)		
Yes	16 (18)	10 (63)
No	71 (82)	6 (37)
Progression to CKD, *n* (%)		
eGFR < 60 ml/dk/1.73 m^2	21 (24)	9 (56)
eGFR < 15 ml/dk/1.73 m^2	11 (13)	3 (19)

FSGS focal segmental glomerulosclerosis, *ACEi* angiotensin-converting enzyme inhibitor, *ARB* angiotensin receptor blocker, *XLAS* X-linked Alport syndrome, *ARAS* autosomal recessive Alport syndrome, *ADAS* autosomal dominant Alport syndrome, *CKD* chronic kidney disease, *eGFR* estimated glomerular filtration rate

*The inheritance pattern of 19 patients with *COL4A4* mutation was ARAS in 13 (68%) patients and ADAS in 6 (32%) patients, while 20 (80%) of 25 patients with *COL4A3* mutation were ARAS and 5 (20%) were ADAS

**The mutation of 33 patients with the inheritance pattern of ARAS was *COL4A3* in 20 (60%) patients and *COL4A4* in 13 (40%) patients, while 5 (45%) of 11 patients with ADAS had *COL4A3* mutation and 6 (55%) had *COL4A4* mutation

patients (29%), and *COL4A5* mutations in 3 patients (21%). Their inheritance pattern was consistent with ARAS in 10 patients (71%), XLAS in 3 patients (21%), and ADAS in 1 patient (7%). During the follow-up, 9 of 14 patients (64%) progressed to CKD (CKD stage 3 ($n = 4$), CKD stage 4 ($n = 2$), and CKD stage 5 ($n = 3$)). Genetic results of NS patients who progressed to CKD were *COL4A3* in 6 (67%) patients, *COL4A4* in 2 (22%) patients, and *COL4A5* in 1 (11%) patient.

Of the 58 patients who underwent kidney biopsy, EM was performed in 27 patients and typical basal membrane changes consistent with AS were determined in 21 patients. The remaining 6 patients were genetically diagnosed with Alport syndrome.

Genetic analysis and clinical correlations

Among 87 patients, 43 (49%) had *COL4A5* mutations, 19 (22%) had *COL4A4* mutations, and 25 (29%) had *COL4A3* mutations (Table 1). In total, 93% of the patients with XLAS, 85% of the patients with ARAS, and all patients with ADAS had hematuria at first presentation. Furthermore, 79% of patients had proteinuria (with or without hematuria) at first presentation. Nephrotic-range proteinuria was present in 17 of 33 patients with ARAS (51%). However, it was only detected in 18% and 23% of the patients with ADAS and XLAS, respectively ($p = 0.01$). At first presentation, hypertension was detected in 6 patients. Nine patients were hypertensive at the last visit, and two of these patients were hypertensive at the first visit. There were no significant differences regarding genetic mutation and inheritance pattern in the patients with hypertension. Hearing loss, NS presentation, and FSGS histopathologic diagnosis were more common in patients with ARAS compared with the other groups ($p = 0.02$ for hearing loss, $p = 0.01$ for NS presentation, and $p = 0.18$ for FSGS). NS presentation and FSGS histopathologic diagnosis were more common but statistically insignificant in patients with *COL4A3* mutations compared with the other groups ($p = 0.06$ for NS presentation and $p = 0.14$ for FSGS). CsA was used more commonly in patients with *COL4A3* mutations than in the other groups ($p = 0.02$). During the follow-up, GFR decreased below 60 ml/min/1.73 m^2 in 21 of 87 patients (24%). *COL4A3* mutations were the leading genetic abnormality in patients who progressed to CKD. Of the 21 patients who progressed to CKD, 14, 4, and 3 patients had *COL4A3* (56%), *COL4A4* (21%), and *COL4A5* (7%) mutations, respectively ($p < 0.001$). The inheritance pattern of these patients was ARAS, XLAS, and ADAS in 17 (51%) patients, 3 (7%) patients, and 1 (1%) patient, respectively ($p < 0.001$). Other clinical features according to genetic analysis and inheritance pattern are shown in Table 2.

Predictors of progression to chronic kidney disease

After a median follow-up period of 4.3 years (IQR 1.9–7.3), 21 of 87 patients (24%) progressed to CKD (i.e., GFR ≤ 60 ml/min/1.73 m^2). The prevalence of CKD stages 3, 4, and 5 was 33%, 19%, and 48%, respectively. There was no gender difference between the CKD and non-CKD groups in the patients with *COL4A3* and *COL4A4* mutations. In the patients with *COL4A5* mutations, only male patients progressed to CKD in the follow-up.

Univariate analysis revealed that nephrotic-range proteinuria, NS presentation at first visit, hearing loss, FSGS in kidney biopsy, mutations in the *COL4A3* gene, ARAS inheritance pattern, and CsA treatment were significantly associated with CKD during the follow-up (Table 4). The follow-up duration of the patients with *COL4A3*, *COL4A4*, and *COL4A5* mutations was comparable; however, GFR loss was significantly higher in patients with *COL4A3* mutations when compared to patients with *COL4A4* and *COL4A5* mutations ($p = 0.04$). Also, patients with NS presentation, FSGS histopathology, and CsA treatment had a higher GFR decline at the last visit, all of which were statistically significant ($p < 0.05$). The CKD risk associated with *COL4A3* mutations persisted after adjustment for the presence of NS, presence of FSGS, and CsA use (OR 4.3, 95% CI 1.4–13.5).

During the follow-up, 9 of 16 patients (56%) with FSGS progressed to CKD (CKD stage 3 ($n = 4$), CKD stage 4 ($n = 2$), and CKD stage 5 ($n = 3$)). Genetic results of these FSGS patients were *COL4A3* in 7 (88%) patients, *COL4A4* in 1 (11%) patient, and *COL4A5* in 1 (11%) patient ($p = 0.03$). The mean age at the onset of CKD was 13.4 ± 4.1, 10.6 ± 0.0, and 10.8 ± 0.0 in *COL4A3*, *COL4A4*, and *COL4A5* patients, respectively. Other clinical characteristics regarding mutation and inheritance pattern of FSGS patients are shown in Table 3.

In terms of the rate of progression to CKD, there was no significant difference between the patients with missense mutations and those with non-missense mutations ($p = 0.06$). Due to the low number of the patients with hypertension, the risk of progression to CKD associated with hypertension could not be assessed. The proportion of patients who progressed to CKD without renin–angiotensin–aldosterone system (RAAS) inhibitors, with angiotensin-converting enzyme inhibitor (ACEi) or angiotensin receptor blocker (ARB) treatment, or with both ACEi and ARB treatments was 25%, 25%, and 21%, respectively ($p = 0.93$). Other results regarding progression to CKD are shown in Table 4.

Survival analysis

The overall median kidney survival rate without CKD was 12.1 years (95% CI 4.9–19.3). After the first presentation, the 3-year cumulative risk of CKD was 31%, 17%, and 13%

Table 2 Clinical characteristics of patients according to the *COL4A* genes and inheritance pattern

	Genetic analysis				Inheritance pattern			
	COL4A3 ($n = 25$)	*COL4A4* ($n = 19$)	*COL4A5* ($n = 43$)	*p* value	XLAS ($n = 43$)	ARAS ($n = 33$)	ADAS ($n = 11$)	*p* value
Female/male	12/13	13/6	16/27	0.07	16/27	16/17	9/2	0.03*
Age at first presentation, (mean ± SD) (years)	8.9 ± 3.9	7.6 ± 4.0	7.1 ± 4.7	0.26	7.1 ± 4.7	8.6 ± 3.8	7.6 ± 4.5	0.33
Follow-up duration, median (IQR) (years)	4.1 (1.4–6.9)	4.2 (2–11.1)	3.5 (1.9–7.4)	0.06	3.5 (1.9–7.4)	5.5 (2.2–7.6)	1.9 (1.1–4.2)	0.12
Proteinuria at first presentation, *n* (%)								
None	3 (12)	4 (21)	11 (26)	0.29	11 (26)	1 (3)	6 (55)	0.01**
Non-nephrotic range	10 (40)	8 (42)	22 (51)		22 (51)	15 (45)	3 (27)	
Nephrotic range	12 (48)	7 (37)	10 (23)		10 (23)	17 (52)	2 (18)	
NS at first presentation, *n* (%)	7 (28)	4 (21)	3 (7)	0.06	3 (7)	10 (30)	1 (9)	0.01**
Hypertension at first presentation, *n* (%)								
Yes	4 (16)	1 (5)	1 (2)	0.28	1 (2)	3 (9)	2 (18)	0.32
No	19 (76)	17 (90)	38 (89)		38 (89)	27 (81)	9 (82)	
Unknown	2 (8)	1 (5)	4 (9)		4 (9)	3 (9)	0 (0)	
Hearing loss, *n* (%)	11 (44)	11 (58)	12 (28)	0.07	12 (28)	19 (58)	3 (27)	0.02**
FSGS in kidney pathology, *n*[a] (%)	8/18 (44)	3/13 (23)	5/27 (19)	0.14	5/27 (19)	10/25 (40)	1/6 (17)	0.18
RAAS inhibitors, *n* (%)								
None	8 (32)	7 (37)	9 (21)	0.44	9 (21)	10 (30)	5 (45)	0.42
ACEi or ARB	11 (44)	11 (58)	22 (51)		22 (51)	17 (52)	5 (45)	
ACEi and ARB	6 (24)	1 (5)	12 (28)		12 (28)	6 (18)	1 (9)	
CsA treatment, *n* (%)	9 (36)	2 (11)	5 (12)	0.03***	5 (12)	10 (30)	1 (9)	0.07
Number of patients who developed CKD (GFR < 60 ml/min/1.73 m^2), *n* (%)	14 (56)	4 (21)	3 (7)	< 0.001***	3 (7)	17 (52)	1 (9)	< 0.001**
Age at onset of CKD (GFR < 60 ml/min/1.73 m^2) (mean ± SD)	13.9 ± 3.3	14.7 ± 7	15.5 ± 5.6	0.79	15.5 ± 5.6	13.9 ± 4.1	15.6 ± 0	0.78
Number of patients who developed CKD stage 5 (GFR < 15 ml/min/1.73 m^2), *n* (%)	6 (24)	3 (16)	2 (5)	0.06	2 (5)	8 (24)	1 (9)	0.03**
Age at onset of CKD stage 5 (GFR < 15 ml/min/1.73 m^2) (mean ± SD)	14.3 ± 2.9	18.1 ± 5.7	12.7 ± 1.9	0.28	12.7 ± 1.9	15.6 ± 4.4	15.6 ± 0	0.69
Kidney transplantation, *n* (%)	6 (24)	2 (11)	1 (2)	0.02***	1 (2)	7 (21)	1 (9)	0.02**
Mean ΔGFR (ml/min/1.73 m^2) (ΔGFR = GFR at the last visit − GFR at the first visit)	− 38.6 ± 59.9	4.2 ± 65.4	− 11.4 ± 50.8	0.04***	− 11.4 ± 50.8	− 31.5 ± 68.9	14 ± 37.3	0.06

IQR interquartile range, *NS* nephrotic syndrome, *FSGS* focal segmental glomerulosclerosis, *RAAS* renin–angiotensin–aldosterone system, *ACEi* angiotensin-converting enzyme inhibitor, *ARB* angiotensin receptor blockers, *CsA* cyclosporin A, *CKD* chronic kidney disease, *GFR* glomerular filtration rate, *XLAS* X-linked Alport syndrome, *ARAS* autosomal recessive Alport syndrome, *ADAS* autosomal dominant Alport syndrome

[a] Number of patients performed kidney biopsy

*Comparison of *ADAS* vs. *XLAS* and *ARAS*

**Comparison of *ARAS* vs. *XLAS* and *ADAS*

***Comparison of *COL4A3* vs. *COL4A4* and *COL4A5*

in *COL4A3* (*ARAS*), *COL4A4* (*ARAS*), and male *COL4A5* patients, respectively ($p < 0.001$). After the first presentation, the 5-year cumulative risk of CKD was 51%, 28%, and 14% in *COL4A3* (*ARAS*), *COL4A4* (*ARAS*), and male *COL4A5* patients, respectively ($p < 0.001$). Kidney survival rates of females and males with *COL4A3* mutations were also worse than those of the corresponding genders in the other mutation groups (females: $p = 0.005$; males: $p = 0.02$). The females with *COL4A5* mutation among all females and the males with *COL4A4* mutation among all male subjects had better kidney survival rates than those of other males and females of the other mutation groups (females: $p = 0.005$; males: $p = 0.02$).

Table 3 Clinical characteristics of patients with focal segmental glomerulosclerosis (FSGS) according to the *COL4A* genes and inheritance pattern

	Genetic analysis				Inheritance pattern			
	COL4A3 ($n=8$)	*COL4A4* ($n=3$)	*COL4A5* ($n=5$)	p value	XLAS ($n=5$)	ARAS ($n=10$)	ADAS ($n=1$)	p value
Female/male	4/4	2/1	2/3		2/3	6/4	1/0	
Age at first presentation, (mean ± SD) (years)	9.5 ± 3.0	6.7 ± 2.7	10.1 ± 1.6	0.22	10.1 ± 1.6	9.1 ± 2.9	5.0 ± 0.0	0.23
Follow-up duration, median (IQR) (years)	4.0 (1.7–6.8)	9.8 (2.2–12)*	4.6 (2.3–9.4)	0.47	4.6 (2.3–9.4)	5.3 (2.3–10.1)	1.5	0.57
Proteinuria at first presentation, *n* (%)								
Non-nephrotic range	2 (25)	0 (0)	1 (20)	0.63	1 (20)	2 (20)	0 (0)	0.88
Nephrotic range	6 (75)	3 (100)	4 (80)		4 (80)	8 (80)	1 (100)	
NS at first presentation, *n* (%)	5 (63)	3 (100)	3 (60)	0.43	3 (60)	7 (70)	1 (100)	0.72
Hypertension at first presentation, *n* (%)								
Yes	1 (12)	0 (0)	0 (0)	0.68	0 (0)	1 (10)	0 (0)	0.84
No	6 (75)	3 (100)	5 (100)		5 (100)	8 (80)	1 (100)	
Unknown	1 (12)	0 (0)	0 (0)		0 (0)	1 (10)	0 (0)	
Hearing loss, *n* (%)	3 (38)	2 (67)	0 (0)	0.23	0 (0)	5 (50)	0 (0)	0.21
RAAS inhibitors, *n* (%)								
None	2 (25)	2 (67)	0 (0)	0.23	0 (0)	4 (40)	0 (0)	0.32
ACEi or ARB	5 (63)	1 (33)	3 (60)		3 (60)	5 (50)	1 (100)	
ACEi and ARB	1 (12)	0 (0)	2 (40)		2 (40)	1 (10)	0 (0)	
CsA treatment, *n* (%)	6 (75)	2 (67)	2 (40)	0.44	2 (40)	7 (70)	1 (100)	0.38
Number of patients who developed CKD stage 3 (GFR < 60 ml/min/1.73 m^2), *n* (%)	7 (88)	1 (33)	1 (20)	0.03**	1 (20)	8 (80)	0 (0)	0.04***
Age at onset of CKD stage 3 (GFR < 60 ml/min/1.73 m^2) (mean ± SD)	13.4 ± 4.1	10.6 ± 0.0	10.8 ± 0.0	0.74	10.8 ± 0.0	13.0 ± 3.9	–	0.61
Number of patients who developed CKD stage 5 (GFR < 15 ml/min/1.73 m^2), *n* (%)	2 (25)	0 (0)	1 (20)	0.63	1 (20)	2 (20)	0 (0)	0.88
Age at onset of CKD stage 5 (GFR < 15 ml/min/1.73 m^2) (mean ± SD)	14.8 ± 5.8	–	11.3 ± 0.0	0,71	11.3 ± 0.0	14.8 ± 5.8	–	0.71
Kidney transplantation, *n* (%)	2 (25)	0 (0)	0 (0)	0.31	0 (0)	2 (20)	0 (0)	0.31
Mean ΔGFR (ml/min/1.73 m^2) (ΔGFR = GFR at the last visit − GFR at the first visit)	− 72.4 ± 72.9	− 46.5 ± 20.6	− 52.1 ± 35.6	0.73	− 52.1 ± 35.6	− 71.4 ± 62.8	− 5.0 ± 0.0	0.49

IQR interquartile range, *NS* nephrotic syndrome, *RAAS* renin–angiotensin–aldosterone system, *ACEi* angiotensin-converting enzyme inhibitor, *ARB* angiotensin receptor blockers, *CsA* cyclosporin A, *CKD* chronic kidney disease, *GFR* glomerular filtration rate, *XLAS* X-linked Alport syndrome, *ARAS* autosomal recessive Alport syndrome, *ADAS* autosomal dominant Alport syndrome

*IQR is not available; range is given

**Comparison of *COL4A3* vs. *COL4A4* and *COL4A5*

***Comparison of *ARAS* vs. *XLAS* and *ADAS*

In terms of time to progression to CKD, there was no significant difference between patients not receiving RAAS inhibitors and receiving ACEi and/or ARB treatment ($p=0.69$). Patients with *COL4A3* mutations, ARAS inheritance pattern, histopathology of FSGS, or NS presentation progressed to CKD earlier ($p<0.001$ for *COL4A3*, $p=0.01$ for ARAS, $p\leq0.001$ for FSGS, $p=0.01$ for NS presentation) when compared to those without. When we separately evaluated the effect of mutation and inheritance pattern, we observed the patients with *COL4A3* and ARAS inheritance pattern had the worse prognosis (Fig. 2a). The effects of CsA treatment on kidney survival were also evaluated in this cohort. CsA treatment did not improve kidney survival of patients with or without FSGS (Fig. 3).

Discussion

This is the first study identifying the clinical features of children with genetically confirmed AS in the largest Turkish

Table 4 Predictors of progression to chronic kidney disease

	Median follow-up duration (years) (IQR)	*p* value	Mean ΔGFR[a] (ml/min/1.73 m^2)	*p* value	Number of patients with mean GFR at the last visit, *n* (%)		*p* value
					< 60 ml/min/1.73 m^2	> 60 ml/min/1.73 m^2	
Gender							
Females (*n* = 41)	4.6 (1.9–10.2)	0.23	− 22.3 ± 63.4	0.36	10 (24)	31 (76)	0.95
Males (*n* = 46)	5.2 (2.5–7.8)		− 10.4 ± 54.5		11 (24)	35 (76)	
Proteinuria at first presentation							
None (*n* = 18)	3.1 (1.7–7.1)	0.61	− 7.7 ± 46.4	0.16	0 (0)	18 (100)	< 0.001
Non-nephrotic range (*n* = 40)	4.8 (2.5–7.2)		− 6.6 ± 58.6		7 (18)	33 (82)	
Nephrotic range (*n* = 29)	3.9 (1.4–8.4)		− 32.6 ± 63.6		14 (48)	15 (52)	
NS at first presentation							
Yes (*n* = 14)	5.5 (2.3–11.6)	0.18	− 75.3 ± 56.5	< 0.001	9 (64)	5 (36)	< 0.001
No (*n* = 73)	4.9 (1.9–8.4)		− 3.9 ± 51.7		12 (16)	61 (84)	
FSGS in kidney pathology							
Yes (*n* = 16)	4.4 (2.2–9.1)	0.69	− 61.2 ± 54.9	0.002	9 (56)	7 (44)	0.003
No (*n* = 42)	5.3 (1.9–8.7)		− 7.7 ± 57.6		12 (28)	30 (72)	
Hearing loss							
Yes (*n* = 34)	5.2 (2.8–10.5)	0.14	− 7.8 ± 49.1	0.28	14 (41)	20 (59)	0.003
No (*n* = 53)	4.3 (1.9–8.4)		− 21.9 ± 64.7		7 (13)	46 (86)	
Genetic mutation							
COL4A3 (*n* = 25)	4.1 (1.4–6.9)	0.06	− 38.6 ± 59.9	0.04	14 (56)	11 (44)	< 0.001
COL4A4 (*n* = 19)	4.2 (2–11.1)		4.2 ± 65.4		4 (21)	15 (79)	
COL4A5 (*n* = 43)	3.5 (1.9–7.4)		− 11.4 ± 50.8		3 (7)	40 (93)	
Mutation type							
Missense (*n* = 41)	4.1 (1.9–7.2)	0.38	− 28.3 + 47.1	0.07	12 (29)	29 (71)	0.06
Non-missense (*n* = 46)	4 (1.9–7.5)		− 4.9 + 66.3		9 (20)	37 (80)	
Inheritance pattern							
XLAS (*n* = 43)	3.5 (1.9–7.4)	0.12	− 11.4 ± 50.8	0.06	3 (7)	40 (93)	< 0.001
ARAS (*n* = 33)	5.5 (2.2–7.6)		− 31.5 ± 68.9		17 (51)	16 (49)	
ADAS (*n* = 11)	1.9 (1.1–4.2)		14.0 ± 37.3		1 (9)	10 (91)	
RAAS inhibitors							
None (*n* = 24)	6.9 (3–11.3)	0.08	0.3 ± 52.9	0.30	6 (25)	18 (75)	0.93
ACEi or ARB (*n* = 44)	2.6 (1.7–6.2)		− 17.7 ± 64.3		11 (25)	33 (75)	
ACEi and ARB (*n* = 19)	8.4 (5.2–10.4)		− 28.7 ± 49.1		4 (21)	15 (79)	
CsA treatment							
Yes (*n* = 16)	4 (2.3–9.1)	0.52	− 62.7 ± 57.6	< 0.001	10 (63)	6 (37)	< 0.001
No (*n* = 71)	5.4 (1.9–8.6)		− 4.8 ± 53.7		11 (15)	60 (85)	

IQR interquartile range, *NS* nephrotic syndrome, *FSGS* focal segmental glomerulosclerosis, *XLAS* X-linked Alport syndrome, *ARAS* autosomal recessive Alport syndrome, *ADAS* autosomal dominant Alport syndrome, *RAAS* renin–angiotensin–aldosterone system, *ACEi* angiotensin-converting enzyme inhibitor, *ARB* angiotensin receptor blockers, *CsA* cyclosporin A, *GFR* glomerular filtration rate

[a] ΔGFR = (GFR at the last visit − GFR at the first visit)

cohort. Most of the clinical features, including rate of hematuria and proteinuria at first presentation, were similar to those reported in the literature [3, 10]. However, this study clearly described prognostic factors for CKD in patients with AS, which include having *COL4A3* mutations, ARAS inheritance pattern, NS presentation, and FSGS confirmed by kidney biopsy.

Previously, it has been shown that FSGS can be seen in kidney biopsies of patients with AS and that *COL4A* mutations can be detected in patients with familial or sporadic FSGS [6, 7, 11]. It is still unclear whether FSGS is secondary to AS progression or a primary process related to kidney disease caused by *COL4A* mutations. Malone et al. [6] suggested that *COL4A* mutations may have a direct role in the pathogenesis of FSGS, or phenotypes of GBM caused by *COL4A* mutations could be phenocopies of primary FSGS. Gast et al. [7] stated that the probability of showing FSGS phenocopy in patients with AS may be more likely than the development

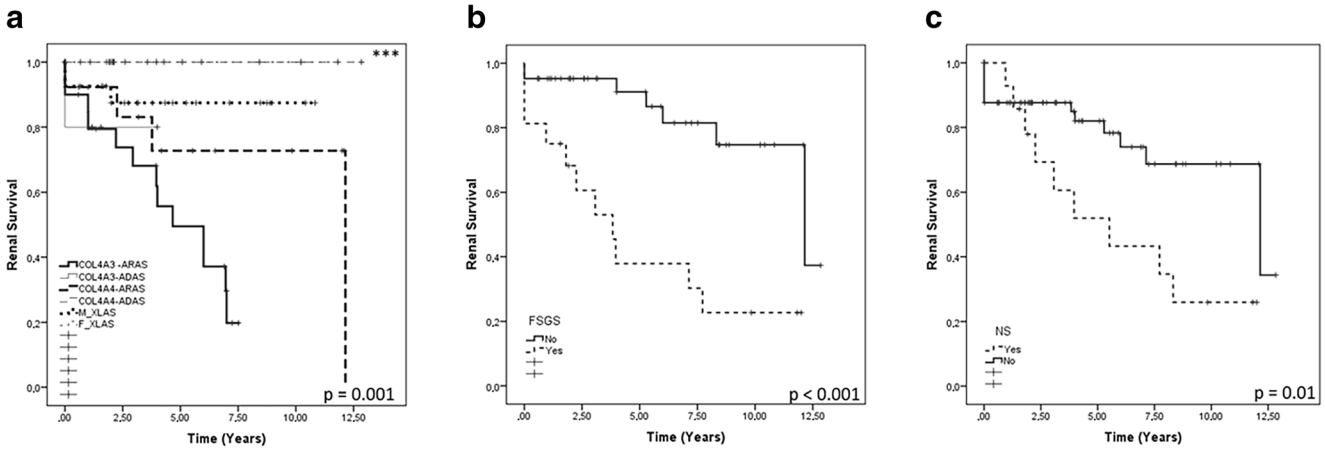

Fig. 2 Time to chronic kidney disease (years) in patients. Patients with autosomal recessive Alport syndrome (ARAS) and *COL4A3* mutation (**a**), histopathology of focal segmental glomerulosclerosis (FSGS) (**b**), and nephrotic syndrome (NS) presentation (**c**) progressed to chronic kidney disease significantly earlier compared to other groups. Triple asterisks indicate survival curves of the patients with *COL4A4*–ADAS and female XLAS completely overlapped. (It is seen in the upper curves)

of secondary FSGS. Warejko et al. [12] classified *COL4A3* and *COL4A5* genes as "phenocopy genes" and found mutations in genes, including *COL4A3* and *COL4A5*, caused phenocopy in 3.7% of patients with steroid-resistant nephrotic syndrome (SRNS). In our cohort, *COL4A3* mutations were detected in half of the FSGS patients. It is thought there might be a relationship between FSGS and *COL4A3* mutations. The relationship between cytotoxicity and apoptosis is presumed to be due to the accumulation of misfolded proteins in the podocytes caused by mutant *COL4A3* chains, as shown in recent studies [11, 13–15]. Taken together, it should be emphasized that *COL4A* mutations should be considered in the genetic diagnosis of patients with FSGS and SRNS, as this could prevent unnecessary use of immunosuppressive drugs in these patients.

In the literature, time to progression to CKD varies in different studies. It has been reported that 90% of male patients and 12% of female patients with XLAS develop CKD stage 5 by the age of 40 years [16, 17]. Regardless of gender, patients with ARAS have similar clinical features as male patients with XLAS [18], and the median age of CKD stage 5 development is 21 years [3]. In patients with ADAS, Kamiyoshi et al. [19] observed CKD stage 5 in 3 of 25 patients (12%) and defined kidney survival time as 70 years. In a cohort study by Marcocci et al. [20], 32.4% of patients with ADAS progressed to CKD and 24.3% reached stage 5 at a mean age of

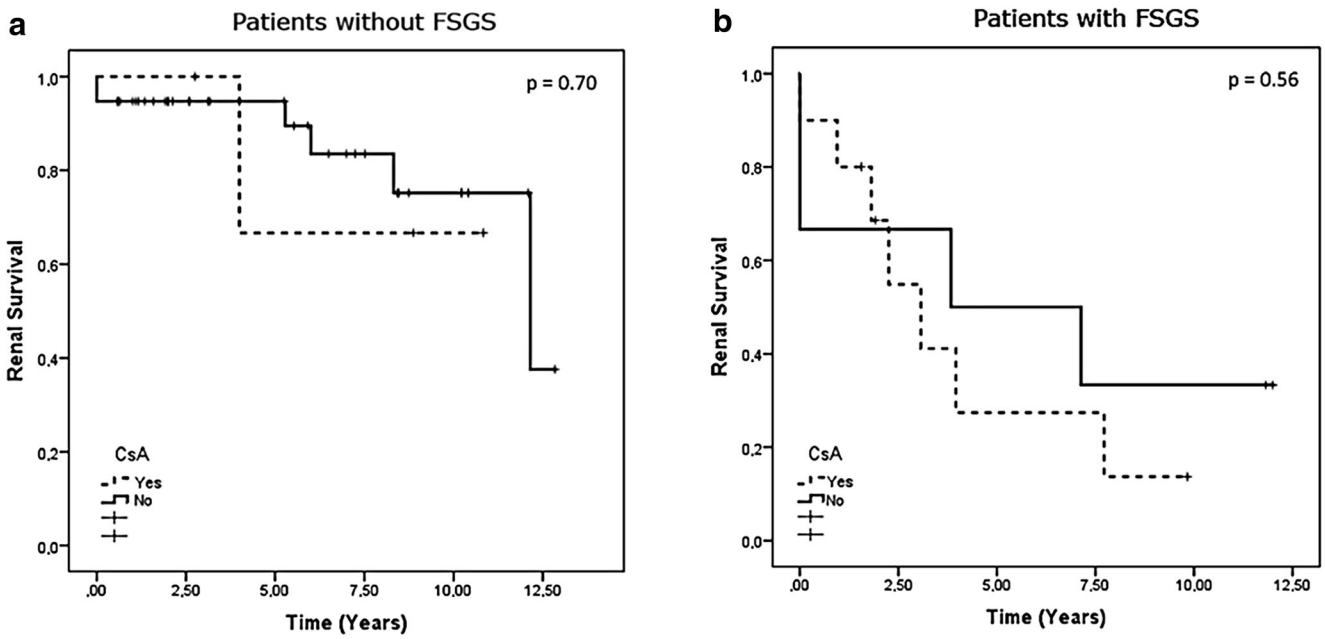

Fig. 3 **a**, **b** Effects of cyclosporin A on kidney survival

51.2 years. Our results were consistent with the literature in terms of progression time to CKD. There is still debate in the literature regarding how individuals who have a single autosomal mutation or females who carry an X-linked mutation should be referred. Some prefer to refer to them as "carriers" until they become significantly symptomatic. However, it has been well established that the long-term prognosis of these patients is not favorable [3, 20]. Similarly, in our cohort, those heterozygous patients were not completely asymptomatic but had greater or fewer urinary abnormalities (i.e., hematuria and/or proteinuria). Eleven patients were classified as ADAS, and hearing loss was detected in 3 of them. Five patients presented with proteinuria, 2 of whom had nephrotic-range proteinuria and one patient progressed to CKD at age 15.6 years. This observation would argue against the fact that dominant forms of Alport syndrome progress to CKD after the 5th decade of life. As we applied a gene panel in which coding regions and intron–exon boundaries were included, we might have missed another mutation in the intronic region of the same gene that would be pathogenic if outside of the sequenced regions. Another possibility would be that this individual might have another relevant mutation in another gene that was not included in the gene panel. Nevertheless, taken together, we believe that ADAS patients should not be considered as *carriers*, but they should be classified as Alport syndrome to be given timely and appropriate care. While agreeing with the fact that early CKD risk is low in the corresponding patient group compared to the patients with ARAS or males with XLAS, we believe that if our patients are followed up for longer, we would see an impairment in kidney function in accordance with established literature data.

We observed that progression to CKD of the patients with *COL4A3* mutation (also in ARAS and ADAS patients compared to corresponding patients with *COL4A4* mutation) was earlier and their GFR decline in the follow-up period was higher. Data regarding the clinical course of patients with *COL4A3* mutations and subsequent effects on CKD progression have not yet been well established. Zhang et al. [21] showed that the coexistence of heterozygous pathogenic *COL4A3* or *COL4A4* mutations caused more severe proteinuria in patients with *COL4A5* mutations. In addition, Bullich et al. [22] reported that patients with mutations in SRNS genes concomitant with *COL4A3* mutations had more severe phenotypes and earlier development of CKD stage 5 compared to patients without *COL4A3* mutations. In contrast to the literature data, we did not detect any mutations in a panel screening of common SRNS genes in our patients with *COL4A3* mutations. Therefore, unfavorable outcomes in this population may be related to *COL4A3* mutation itself.

The effect of mutation type on the prognosis of AS and genotype–phenotype correlation has been investigated previously. The rate of progression to CKD stage 5 was lower in patients with missense mutations than in those with non-missense mutations [16, 23]. In our study, we categorized mutations into two groups as missense and non-missense mutations. There was no significant difference regarding progression to CKD between patients with missense or non-missense mutations in all patient cohorts and FSGS patients, separately.

There is no specific treatment for AS. Studies have shown that RAAS inhibitors delay progression to CKD stage 5 due to their kidney-protective effects [24]. Two recent randomized controlled trials have shown that ACEi and ARB treatments have favorable effects on proteinuria in patients with AS [25, 26]. Recently, expert guidelines for the management of AS recommended initiation of ACEi at the time of diagnosis in male patients with XLAS and patients with ARAS due to the high risk of early CKD stage 5 development. In female patients with XLAS and patients with ADAS, ACEi treatment has been recommended at the time of overt proteinuria [27]. The Alport Syndrome Research Collaborative recommends to consider ACEi treatment when microalbuminuria occurs in patients with XLAS and a family history of early CKD stage 5 or severe (deletion, nonsense, or splicing) mutations [28]. In our study, RAAS inhibitors were prescribed to 63 patients (72%). The GFR decline was higher in patients treated with both ACEi and ARB compared to patients treated with either ACEi or ARB or those who were not treated. However, our study is not sufficient to make a final conclusion due to its retrospective nature and relatively short follow-up duration.

Although initial studies have suggested that CsA treatment may have a beneficial effect on proteinuria in patients with AS, subsequent studies have revealed that its nephrotoxic effects preclude its widespread use in the long term [29–33]. In our study, by the end of the follow-up period, the ratio of progression to CKD and GFR decline were higher in patients treated with CsA compared to those who did not receive CsA. However, no significant difference was found between CsA-treated and non-treated patients in kidney survival analysis. Our results indicate that CsA does not affect the duration of progression to CKD in patients with AS.

Our study has limitations, such as its retrospective character and relatively short follow-up duration (median 4.3 years).

In conclusion, detailed analyses of data from genetically confirmed Turkish patients with AS provide important clues regarding the presentation, course, and outcomes of the disease. *COL4A3* mutations, ARAS inheritance pattern, NS presentation, and FSGS findings on kidney biopsy are major risk factors for progression to CKD. CsA does not impair kidney functions nor does it improve kidney survival in patients with AS. We emphasize the importance of genetic testing in patients whose presentation and family history suggest AS. This could be performed as part of initial patient evaluation and could be considered prior to a kidney biopsy in order to make a definitive diagnosis, employ appropriate management, avoid unnecessary and potentially harmful medications, and provide genetic counseling.

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Authors' contributions RT, BG, FO, and AD: Research formulation and study design

RT, GO, SS, OS, ZBO, FKE, CC, BKD, AS, SY, HA, and AA: Data acquisition

RT, BG, FO, AD, GO, and MH: Data analysis/interpretation

FO and EA: Genetic analysis

MH, BG, and GO: Statistical analysis

RT: Supervision/mentorship

Each of the authors contributed important intellectual content during manuscript drafting and/or revision and approved the final version. Furthermore, they all accept responsibility for the overall work, including the accuracy and integrity of all portions of the work.

Availability of data and material The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

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

Ethics approval The study protocol was approved by the Non-Interventional Clinical Researches Ethics Board of Hacettepe University (KA 19073).

Consent to participate Written informed consent was provided by the patients' parents, as well as by patients aged > 10 years.

Consent for publication Patients signed informed consent regarding publishing their data.

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