

**ORIGINAL ARTICLE**

Precise variant interpretation, phenotype ascertainment, and genotype–phenotype correlation of children in the EARLY PRO-TECT Alport trial

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Abstract

Early initiation of therapy in patients with Alport syndrome (AS) slows down renal failure by many years. Genotype–phenotype correlations propose that the location and character of the individual's variant correlate with the renal outcome and any extra renal manifestations. In-depth clinical and genetic data of 60/62 children who participated in the EARLY PRO-TECT Alport trial were analyzed. Genetic variants were interpreted according to current guidelines and criteria. Genetically solved patients with X-linked inheritance were then classified according to the severity of their *COL4A5* variant into less-severe, intermediate, and severe groups and disease progress was compared. Almost 90% of patients were found to carry (likely) pathogenic variants and classified as genetically solved cases. Patients in the less-severe group demonstrated a borderline significant difference in disease progress compared to those in the severe group ($p = 0.05$). While having only limited power according to its sample size, an obvious strength is the precise clinical and genetic data of this well ascertained cohort. As in published data differences in clinical progress were shown between patients with *COL4A5* less-severe and severe variants. Therefore, clinical and segregational data are important for variant (re)classification. Genetic testing should be mandatory allowing early diagnosis and therapy of AS.

KEYWORDS

Alport syndrome, *COL4A3*, *COL4A4*, *COL4A5*, hereditary kidney disease

1 | INTRODUCTION

Alport syndrome (AS), a type IV collagen disease, is the second most common monogenic cause for end-stage renal failure (ESRF).^{1–3} AS is caused by pathogenic variants in the genes *COL4A3*, *COL4A4*, and *COL4A5*, each encoding an alpha-chain of type IV collagen, an important component of the basal membrane in kidney, cochlear, and eyes.^{1,2} The α (IV)-chain consists of a long collagen tail with an arrangement of repeating Gly-X-Y amino acid molecules, which is important for the structure of the triple helix, its flexibility and its unique mechanical properties.⁴ AS can be inherited in an X-linked (XLAS) or autosomal recessive (ARAS) form. Pathogenic variants in the *COL4A5* (Xq22.3) gene cause XLAS, which accounts for 80–85% of all patients with AS.^{5,6} Therefore, mostly males are affected and a distinct genotype–phenotype correlation could be detected.⁷ In female patients with a heterozygous variant in *COL4A5*, a variable intrafamilial and interfamilial penetrance exists resulting in a broad spectrum of clinical symptoms ranging from mild microscopic hematuria to severe AS.^{8–10} Autosomal recessive AS results from biallelic pathogenic variants in *COL4A3* (2q36.3) and *COL4A4* (2q36.3) in about 15%.^{3,7} In some cases a digenic inheritance was proposed.¹¹ The existence of an autosomal dominant inheritance (ADAS) (monoallelic pathogenic variants in *COL4A3* and *COL4A4*), which is also described in the literature, is contested.¹² Past literature mostly described it as a very rare form of AS, only one recent study postulated a frequency of 31%.¹³ The current recommendation is to describe these patients as carrier of AS or as patients with a thin basement membrane nephropathy (TBMN).¹² Heterozygous carriers of

pathogenic variants in *COL4A3* or *COL4A4* also have an increased risk for glomerular pathological changes presenting as focal segmental glomerular sclerosis (FSGS) and ESRF.^{8,14}

The disruption of type IV collagen structure is the pathophysiological hallmark in AS and leads to a dysfunction of the glomerular filter with hematuria, proteinuria, and progressive renal failure. Patients with AS can also develop (high-frequency-) hearing impairment and ocular findings, which include maculopathy and anterior lenticonus, which is pathognomonic for AS.^{15–18} Diagnostic criteria for patients with AS also include a positive family history of microscopic hematuria or renal failure, renal biopsy, and genetic testing.¹⁹ Genetic testing of *COL4A3*, *COL4A4*, and *COL4A5* is broadly available in most countries and has emerged as gold standard for the accurate diagnosis of AS.²⁰ To evaluate renal biopsy, electron microscopy (EM) should be performed. EM shows pathognomonic changes including thinning, areas of thinning and thickening and/or a complex splitting of the glomerular basement membrane (GBM).¹⁹ The age of ESRF differs among patients with different types of variants. Better genotype–phenotype correlations exist for patients with XLAS than for patients with ARAS, but as of the current literature there seems to be no significant difference in clinical course of male patients with XLAS and patients (both sexes) with ARAS.²⁰ The probability of ESRF for an untreated 30-year-old male patient with XLAS varies between 50% for missense variants, 70% for splice-site variants, and up to 90% for large rearrangements, nonsense variants, and frame shift variants.⁷

Registry data have shown that the progress of the renal manifestation in A scan be delayed by an early start of angiotensin-converting

enzyme inhibitor (ACEi) treatment in proteinuric patients with chronic kidney disease (CKD) stage 2.²¹ To clarify whether an even earlier start (CKD stage 0 or 1) is safe and effective, the EARLY PRO-TECT Alport trial (NCT01485978) was initiated in 2012.²² In the present study, we address the question, which arose during and after primary data analysis of the EARLY PRO-TECT Alport trial: can the classifications according to Gross et al.⁴ and Bekheirnia et al.⁶ be helpful in judging the clinical course and the response to therapy in genetically solved patients of the EARLY PRO-TECT Alport trial and a (likely) pathogenic variant in *COL4A5*?

Our data shall provide evidence for genetic counseling of families with AS in everyday clinical practice and shall underline the important duty of the clinician to provide complete assessment of renal and extra renal symptoms and, if available, biopsy results to allow the geneticist a precise and accurate assessment of identified variants.

2 | MATERIAL AND METHODS

2.1 | Patients

Patients' demographic and genetic data and data on family history of all affected family members were collected as part of the EARLY PRO-TECT Alport trial, whose design and primary endpoints have been published recently.²³ Briefly, starting in 2012, EARLY PRO-TECT Alport was the first randomized and placebo-controlled trial to evaluate safety and efficacy of ACEi in children with AS. At 14 trial sites in Germany, children were screened. According to the German Medicines Act, the trial was approved by all ethics committees and the Federal Institute for Drugs and Medical Devices (BfArM). Written informed consent was obtained from all legal representatives and assent from all patients ≥ 6 years of age.

2.2 | Phenotype ascertainment

During the trial, clinical information about the patient and the medical history of the family was obtained by using electronic case report forms (eCRF) and standardized questionnaires. Its accuracy and data quality were supervised online and controlled onsite twice a year by trial-monitors. Family history was considered positive when a family member presented with (microscopic) hematuria, proteinuria, renal failure, renal replacement therapy including dialysis or kidney transplantation or a kidney biopsy finding typical for AS.

2.3 | Inclusion criteria and procedure of the trial

The structure and proceedings of the trial have already been reported elsewhere.²³ In brief, children with definite diagnosis of AS by clinical assessment, kidney biopsy and/or genetic testing, aged between 24 months and 18 years and normal glomerular filtration rate were included in the trial. These children were either included in the

randomized, placebo-controlled, double-blind trial, or became open-arm control. The treatment phase was three up to 6 years. Disease progress during the trial was defined as either doubling or tripling of albuminuria depending on the stage of disease at beginning of the trial.

Data on previous therapies were collected. Filled-out questionnaires were obtained by study personnel. The results of genetic testing were collected by the trial's eCRF. All collected data were pseudonymized. In case of unclear findings or documentation, the trial site was contacted and asked for transfer of the results of genetic testing to the coordinating principal investigator in Goettingen.

2.4 | Genetic testing

Genetic testing was either performed by panel diagnostics or by exome sequencing (ES). For both, DNA was extracted from peripheral blood using the Gentra Puregene Blood Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

2.5 | Panel diagnostics

In 58 children, customized next generation sequencing gene panels of variable size were initiated by the local investigators using different accredited external laboratories (Table 1). These panels included copy number variant analysis of the *COL4A3-5* genes. Further information cannot be given as the results of these examinations were submitted from the local investigators in pseudonymized form.

2.6 | Exome sequencing

In two cases (93_01_02 and 93_03_05; Table 1) ES was initiated because genetic diagnostics were not performed prior to the participation in the trial. ES was done at the Institute of Human Genetics, Helmholtz Zentrum Munich, Neuherberg, Germany, using a Sure Select Human All Exon 60 Mb V6 Kit (Agilent) and a NovaSeq6000 (Illumina), as previously described.²⁴ The exome of patient 93_01_02 had an average coverage of 132 reads, 98% of the exome was covered at least 20x. The exome of patient 93_03_05 had an average coverage of 137 reads, 98% of the exome was covered at least 20x. Mitochondrial DNA was derived from off-target exome reads as previously described.²⁵ Reads were aligned to the human reference genome (UCSC Genome Browser build hg19) using Burrows-Wheeler Aligner (v.0.7.5a). Detection of single-nucleotide variants (SNVs) and small insertions and deletions (indels) was performed with SAM tools (version 0.1.19). Exome Depth was used for the detection of copy number variations (CNVs). A noise threshold of 2.5 was accepted for diagnostic analysis.²⁶ Called CNVs were visualized by the Integrative Genomics Viewer (IGV, <https://software.broadinstitute.org/software/igv/>) to check for sufficient coverage of the inspected region and plausibility of the CNV. CNVs were compared with publicly available control

TABLE 1 Overview about the patients included in the EARLY PRO-TECT Alport trial and their clinical and genetic results. ES, exome sequencing; n.a., not applicable; PD, panel diagnostics. Solved cases: patients with a “likely pathogenic” or “pathogenic” variant and a fitting genotype (i.e., biallelic variants in COL4A3 and COL4A4 in male and female patients or hemizygous variants in COL4A5 in male patients). Disease progress: doubling or tripling of albuminuria during the trial

Patient ID	Gender	Age at baseline (years)	Family history (years)	Kidney biopsy	Result	Kidney biopsy	Disease progress	Gene	Transcript	Nucleotide change	Amino acid change	Zygosity	Reference (PMID if available)	Variant classification due to reference	Variant classification to guidelines	ACMG	ACMG criteria	ClinVar	gnomAD
93_01_01	male	14	yes	no	-	no	yes	COL4A5	NM_000495.4	c.1871G > A	p.(Gly624Asp)	hemizygous	9 848 783	less-severe	less-severe	likely pathogenic	PS4 (moderate) PM1 (strong) PM2 PP3	pathogenic	16x heterozygous, 4x hemizygous (AF 0.00008743)
93_01_02	male	15	yes	yes	Alport syndrome	yes	yes	COL4A3	NM_000091.4	c.443G > T	p.(Gly148Val)	heterozygous	25 229 338	intermediate	intermediate	likely pathogenic	PS4 (moderate) PM1 (strong) PM2 PP3	VUS	4x heterozygous (AF 0.00001424)
93_01_03	male	13	yes	no	-	no	no	COL4A3	NM_000091.4	c.3724G > A; c.4421T > C	p.(Gly124Ser); p.(Leu1474Pro)	heterozygous	-; 24 130 771	intermediate	intermediate; less-severe	likely pathogenic; likely pathogenic	PS4 (moderate) PM2 PM5 PP3; PM1 PM2 PM3 PP3	not listed; VUS	not listed; 748x heterozygous (AF 0.0002664)
93_02_01	male	6	yes	no	-	no	no	COL4A5	NM_000495.4	c.3391G > A	p.(Gly1131Arg)	hemizygous	-	-	intermediate	likely pathogenic	PM1 (strong) PM2 PP3	not listed	not listed
93_03_01	male	5	yes	no	-	drop out	drop out	COL4A5	NM_000495.4	c.1130G > T	p.(Gly377Val)	hemizygous	-	-	intermediate	likely pathogenic	PM1 (strong) PM2 PP3	not listed	not listed
93_03_02	male	7	yes	no	-	yes	yes	COL4A5	NM_000495.4	c.2042-2A > G	p.(?)	hemizygous	-	-	severe	pathogenic	PV51 (strong) PM1 (strong) PM2 PM3	not listed	not listed
93_03_03	male	17	yes	no	-	yes	yes	COL4A5	NM_000495.4	c.3275G > A	p.(Gly1092Glu)	hemizygous	-	-	intermediate	likely pathogenic	PM1 (strong) PM2 PP3	not listed	not listed
93_03_04	male	15	yes	no	-	yes	yes	COL4A5	NM_000495.4	c.1871G > A	p.(Gly624Asp)	hemizygous	9 848 783	less-severe	less-severe	likely pathogenic	PS4 (moderate) PM1 (strong) PM2 PP3	pathogenic	16x heterozygous, 4x hemizygous (AF 0.00008743)
93_03_05	male	11	yes	no	-	no	no	COL4A3; COL4A4	NM_000091.4; NM_000092.4	c.2083G > A; c.2996G > A	p.(Gly695Arg); p.(Gly999Glu)	heterozygous; heterozygous	Biocientia Institute; -	n.d.; -	intermediate; intermediate	likely pathogenic; benign	PS4 (moderate) PM1 (strong) PM2 PP3; PM1 (strong) PM3	pathogenic; benign, 1x likely benign	31x heterozygous (AF 0.000114); 332x heterozygous, 33x homozygous (AF 0.01182)
93_03_06	male	4	yes	no	-	no	no	COL4A4	NM_000092.4	c.2270G > A; c.2906C > A	p.(Gly757Glu); p.(Ser969)	heterozygous	-; -	-; -	intermediate; severe	likely pathogenic; pathogenic	PM1 (strong) PM2 PP3; PV51 PSA (moderate) PM2 PP3	not listed; pathogenic	not listed; 18x heterozygous (AF 0.00006407)
93_05_01	male	11	yes	no	-	drop out	drop out	COL4A5	NM_000495.4	c.4324G > A	p.(Gly1442Ser)	hemizygous	-	-	intermediate	pathogenic	PM1 (strong) PM2 PM5 PP3	not listed, but other variants pathogen at the same position	not listed
93_05_02	male	3	yes	no	-	drop out	drop out	COL4A5	NM_000495.4	c.4324G > A	p.(Gly1442Ser)	hemizygous	-	-	intermediate	pathogenic	PM1 (strong) PM2 PM5 PP3	not listed, but other variants pathogen at the same position	not listed
93_05_03	male	14	yes	no	-	drop out	drop out	COL4A4	NM_000092.4	c.586G > A; c.3389G > A	p.(Gly196Ser); p.(Gly1130Glu)	heterozygous	-; -	-; -	intermediate; intermediate	likely pathogenic; likely pathogenic	PM1 (strong) PM2 PP3; PM1 (strong) PM2 PP3	not listed; not listed	not listed; not listed

TABLE 1 (Continued)

Patient ID	Gender	Age at baseline (years)	Family history	Kidney biopsy	Result kidney biopsy	Disease progress	Gene	Transcript	Nucleotide change	Amino acid change	Zygoty	Reference (PMID if available)	Variant classification due to reference	Variant classification due to guidelines	ACMG	ACMG criteria	ClinVar	gnomAD
93_05_04	male	5	yes	no	-	no	COL4A5	NM_000495.4	c.4298-1G > C	p.(?)	hemizygous	11 223 851	severe	severe	pathogenic	PVS1 (strong) PS4 (supporting) PM1 (strong) PM2	not listed	not listed
93_05_05	male	5	yes	no	-	no	COL4A5	NM_000495.4	c.3053G > A	p.(Gly1018Asp)	hemizygous	24 304 881	n.d	intermediate	likely pathogenic	PS4 (moderate) PM1 (strong) PM2 PP3	pathogenic	not listed
93_05_06	male	11	no	no	-	no	COL4A5	NM_000495.4	c.2395G > A	p.(Gly799Ser)	hemizygous	-	-	intermediate	likely pathogenic	PM1 (strong) PM2 PP3	not listed	not listed
93_05_07	male	6	no	no	-	no	COL4A5	NM_000495.4	c.2615G > A	p.(Gly872Asp)	hemizygous	-	-	intermediate	likely pathogenic	PM1 (strong) PM2 PM5 PP3	not listed, but other variants pathogen at the same position	not listed
93_06_01	male	14	yes	no	-	no	COL4A5	NM_000495.4	c.1871G > A	P.(Gly624Asp)	hemizygous	9 848 783	less-severe	less-severe	likely pathogenic	PS4 (moderate) PM1 (strong) PM2 PP3	pathogenic	16x heterozygous, 4x hemizygous (AF 0.00008743)
93_06_02	male	13	yes	no	-	yes	COL4A5	NM_000495.4	c.957_974dup	p.(Tyr320_Gly325dup)	hemizygous	-	-	intermediate	likely pathogenic	PM1 (strong) PM2 PM4	not listed	not listed
93_06_03	male	12	yes	no	-	yes	COL4A5	NM_000495.4	c.957_974dup	p.(Tyr320_Gly325dup)	hemizygous	-	-	intermediate	likely pathogenic	PM1 (strong) PM2 PM4	not listed	not listed
93_06_04	male	11	yes	no	-	yes	COL4A5	NM_000495.4	c.957_974dup	p.(Tyr320_Gly325dup)	hemizygous	-	-	intermediate	likely pathogenic	PM1 (strong) PM2 PM4	not listed	not listed
93_06_05	male	6	yes	no	-	no	COL4A3	NM_000091.4	c.2 T > C	p.(Met1Thr)	homozygous	23 297 803	severe	intermediate	likely pathogenic	PS4 (moderate) PM2 PM5 PP3	likely pathogenic	not listed
93_06_07	male	3	yes	no	-	no	COL4A5	NM_000495.4	duplication exon 44-49	p.(?)	hemizygous	-	-	severe	pathogenic	PVS1 (strong), PM1 (strong)	not listed	not listed (one partial duplication exon starting from exon 2 listed)
93_06_08	female	16	yes	no	-	yes	COL4A5	NM_000495.4	c.2255G > A	p.(Gly752Glu)	heterozygous	-	-	intermediate	likely pathogenic	PM1 (strong) PM2 PP3	not listed	not listed but other variant at the same position 2x heterozygous, 2x hemizygous
93_06_09	male	8	yes	no	-	no	COL4A5	NM_000495.4	c.1871G > A	p.(Gly624Asp)	hemizygous	9 848 783	less-severe	less-severe	likely pathogenic	PS4 (moderate) PM1 (strong) PM2 PP3	pathogenic	16x heterozygous, 4x hemizygous (AF 0.00008743)
93_07_01	male	13	yes	no	-	no	COL4A5	NM_000495.4	c.1781G > A	p.(Gly594Asp)	hemizygous	24 304 881	n.d	intermediate	pathogenic	PS4 (moderate) PM1 (strong) PM2 PM5 PP3	pathogenic	not listed
93_07_02	male	7	no	yes	-	yes	COL4A5	NM_000495.4	c.3154C > T	p.Gln1052*	hemizygous	24 854 265	severe	severe	pathogenic	PVS1 PM2 PP3	pathogenic	not listed
93_07_03	male	12	yes	no	-	no	COL4A5	NM_000495.4	c.3088G > A	p.(Gly1030Ser)	hemizygous	9 848 783	intermediate	intermediate	likely pathogenic	PS4 (moderate) PM1 (strong) PM2 PP3	pathogenic	not listed
93_07_04	male	5	yes	no	-	yes	COL4A5	NM_000495.4	c.3997 + 2 T > C	p.(?)	hemizygous	18 616 531	n.d	intermediate	likely pathogenic	PVS1 PM2	not listed	not listed
93_07_05	male	3	yes	no	-	yes	COL4A5	NM_000495.4	c.1912G > C	p.(Gly638Arg)	hemizygous	-	-	intermediate	pathogenic		not listed	not listed

(Continues)

TABLE 1 (Continued)

Patient ID	Gender	Age at baseline (years)	Family history	Kidney biopsy	Result kidney biopsy	Disease progress	Gene	Transcript	Nucleotide change	Amino acid change	Zygosity	Reference (PMID if available)	Variant classification due to reference	Variant classification due to guidelines	ACMG	ACMG criteria	ClinVar	gnomAD
93_07_06	male	13	yes	no	-	no	COL4A5	NM_000495.4	c.4672C > T	p.(Gln1558*)	hemizygous	-	-	severe	pathogenic	PVS1, PM2, PP3	not listed, but other variants pathogen at the same position	not listed
93_07_07	male	8	yes	no	-	no	COL4A5	NM_000495.4	c.1871G > A	p.(Gly624Asp)	hemizygous	9 848 783	less-severe	less-severe	likely pathogenic	PS4 (moderate), PM1 (strong), PM2, PP3	pathogenic	16x heterozygous, 4x hemizygous (AF 0.00008743)
93_07_08	male	2	yes	yes	-	no	COL4A5	NM_000495.4	c.1634G > A	p.(Gly545Asp)	hemizygous	-	-	intermediate	likely pathogenic	PM1 (strong), PM2, PM5, PP3	not listed, but other variants pathogen at the same position	not listed
93_09_01	male	5	yes	no	-	yes	COL4A5	NM_000495.4	c.2023G > A	p.(Gly675Ser)	hemizygous	15 954 103	n.d	intermediate	likely pathogenic	PS4 (moderate), PM1 (strong), PM2, PP3	pathogenic	not listed
93_09_02	male	7	yes	no	-	no	COL4A5	NM_000495.4	c.1904G > T	p.(Gly635Val)	hemizygous	-	-	intermediate	likely pathogenic	PM1 (strong), PM2, PM5, PP3	not listed, but other variants pathogen at the same position	not listed
93_10_01	male	7	no	no	-	yes	COL4A5	NM_000495.4	deletion exon 22-28	p(?)	hemizygous	-	severe	severe	likely pathogenic	PVS1, PM2	not listed	not listed
93_10_02	male	5	yes	no	-	no	COL4A5	NM_000495.4	deletion exon 2	p(?)	hemizygous	20 378 821	-	severe	pathogenic	PVS1 (strong), PM1 (strong)	pathogenic	not listed
93_10_03	male	12	yes	yes	TBMN	yes	COL4A5	NM_000495.4	c.761_762del	p.(Glu254Valfs*11)	hemizygous	-	-	severe	pathogenic	PVS1, PM2, PP3	not listed	not listed
93_11_01	male	6	yes	no	-	no	COL4A5	NM_000495.4	c.1871G > A	p.(Gly624Asp)	hemizygous	9 848 783	less-severe	less-severe	likely pathogenic	PS4 (moderate), PM1 (strong), PM2, PP3	pathogenic	16x heterozygous, 4x hemizygous (AF 0.00008743)
93_11_02	male	11	yes	no	-	no	COL4A5	NM_000495.4	c.1871G > A	p.(Gly624Asp)	hemizygous	9 848 783	less-severe	less-severe	likely pathogenic	PS4 (moderate), PM1 (strong), PM2, PP3	pathogenic	16x heterozygous, 4x hemizygous (AF 0.00008743)
93_11_03	male	9	yes	no	-	no	COL4A5	NM_000495.4	c.389G > A	p.(Lys128=)	hemizygous	22 921 432	n.d	unclear	VUS	PS4 (moderate), PM2, PP3	not listed	not listed
93_11_04	male	3	yes	no	-	no	COL4A5	NM_000495.4	c.81 + 4A > C	p(?)	hemizygous	26 809 805	intermediate	intermediate	VUS	PS4 (moderate), PM2	likely pathogenic	not listed
93_11_05	male	8	yes	yes	TBMN	no	COL4A5	NM_000495.4	c.81 + 4A > C	p(?)	hemizygous	26 809 805	intermediate	intermediate	VUS	PS4 (moderate), PM2	likely pathogenic	not listed
93_12_01	male	6	yes	no	-	no	COL4A5	NM_000495.4	c.982G > A	p.(Gly328Ser)	hemizygous	-	-	intermediate	likely pathogenic	PM1 (strong), PM2, PP3	not listed	not listed
93_12_02	male	7	yes	no	-	yes	COL4A5	NM_000495.4	c.1001G > T	p.(Gly334Val)	hemizygous	18 616 531	n.d	intermediate	pathogenic	PS4 (moderate), PM1 (strong), PM2, PM5, PP3	pathogenic	not listed
93_12_03	male	3	yes	no	-	no	COL4A5	NM_000495.4	c.1001G > T	p.(Gly334Val)	hemizygous	18 616 531	n.d	intermediate	pathogenic	pathogenic	pathogenic	not listed

TABLE 1 (Continued)

Patient ID	Gender	Age at baseline (years)	Family history	Kidney biopsy	Result	Disease progress	Gene	Transcript	Nucleotide change	Amino acid change	Zygoty	Reference (PMID if available)	Variant classification due to reference	Variant classification due to guidelines	ACMG	ACMG criteria	ClinVar	gnomAD
93_12_04	male	3	yes	no	-	no	COL4A5	NM_000495.4	c.2570G > A	p.(Gly857Glu)	hemizygous	-	-	intermediate	likely pathogenic	PM1 (strong) PM2 PM5 PP3	not listed	not listed
93_12_05	female	14	no	no	-	no	COL4A3	NM_000091.4	c.1408 + 1G > C; c.4803delT	p.(?)p. (Gly1602Ala)*13	heterozygous	-; 28 117 080	-; n.d	intermediate; severe	pathogenic; pathogenic	PVS1 (strong, de lbeI exon skipping in-frame) PM1 (strong) PM2 PM3; PVS1 PS4 (moderate) PM2	not listed; likely pathogenic/ pathogenic	not listed; not listed
93_13_01	male	8	yes	no	-	no	COL4A5	NM_000495.4	c.453_455del	p.(Pro152del)	hemizygous	-	-	intermediate	VUS	PM1 PM2 PM4 (supporting)	not listed	not listed
93_13_02	male	13	yes	no	-	yes	COL4A5	NM_000495.4	c.453_455del	p.(Pro152del)	hemizygous	-	-	intermediate	VUS	PM1 PM2 PM4 (supporting)	not listed	not listed
93_13_03	male	13	yes	no	-	drop out	COL4A5	NM_000495.4	c.1871G > A	p.(Gly624Asp)	hemizygous	9 848 783	less-severe	less-severe	likely pathogenic	PS4 (moderate) PM1 (strong) PM2 PP3	pathogenic	16x heterozygous, 4x hemizygous (AF 0.00008743)
93_13_04	male	11	yes	no	-	no	COL4A5	NM_000495.4	c.1948G > C	p.(Gly650Arg)	hemizygous	-	-	intermediate	likely pathogenic	PM1 (strong) PM2 PM5 PP3	not listed, but other variants pathogen at the same position	not listed
93_14_02	male	6	yes	no	-	yes	COL4A5	NM_000495.4	c.2042G > A	p.(Gly681Asp)	hemizygous	8 940 267	severe	intermediate	likely pathogenic	PS4 (moderate) PM1 (strong) PM2 PP3	pathogenic	not listed
93_14_03	male	12	yes	no	-	no	COL4A5	NM_000495.4	c.3808G > A	p.(Gly1270Ser)	hemizygous	11 462 238	intermediate	intermediate	likely pathogenic	PS4 (moderate) PM1 (strong) PM2 PP3	pathogenic	not listed
93_14_04	male	5	yes	no	-	no	COL4A5	NM_000495.4	c.4045dupG	p.(Glu1349Glyfs*24)	hemizygous	-	-	severe	pathogenic	PVS1 PM2 PP3	not listed	not listed
93_15_01	male	17	yes	yes	Alport syndrome	no	COL4A4	NM_000092.4	c.2662G > A; c.4922G > A	p.(Gly888Arg); p. (Cys1641Trp)	heterozygous	Bioscientia Institute; dbSNP	intermediate; -	intermediate; less-severe	likely pathogenic; likely pathogenic	PS4 (moderate) PM1 (strong) PM2 PP3; PM1 PM2 PM3 PP3	likely pathogenic; not listed	not listed; not listed, but other variant at the same position 3x heterozygous (AF 0.00004009)
93_15_03	male	14	yes	no	-	yes	COL4A4	NM_000092.4	c.4810-1G > A	p.(?)	homozygous	-	-	intermediate	VUS	PM1 PM2 PP3	not listed	not listed
93_16_01	male	4	no	yes	-	yes	COL4A5	NM_000495.4	c.4492_4505del	p.(Ala1498Leufs*12) (hemi)	hemizygous	-	-	severe	pathogenic	PVS1 PM2 PP3	not listed	not listed
93_16_02	male	4	yes	no	-	no	COL4A5	NM_000495.4	c.1871G > A	p.(Gly624Asp)	hemizygous	9 848 783	less-severe	less-severe	likely pathogenic	PS4 (moderate) PM1 (strong) PM2 PP3	pathogenic	16x heterozygous, 4x hemizygous (AF 0.00008743)
93_16_03	male	9	yes	yes	Alport syndrome	yes	COL4A3	NM_000091.4	c.1398del; c.4348C > T	p.(Asp466Gluufs*32); p. (Arg1450*)	heterozygous	-; dbSNP	-; -	severe; severe	pathogenic; pathogenic	PVS1 PM2 PM3; PVS1 PM2 PP3	not listed; not listed	not listed; not listed

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>) and 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, only variants with a minor allele frequency (MAF) of less than 0.1% in the in-house database of the Helmholtz Zentrum Munich containing over 20 000 exomes were considered. For the analysis of autosomal recessive and X-linked variants (homozygous, hemizygous or putative compound heterozygous) variants with a MAF of less than 1.0% were considered. As there are pathogenic alleles in hereditary nephropathies with a MAF of more than 1.0% like the *NPHS2*p. Arg229Gln allele (also known as p.R229Q) and as the two ES-cases were unsolved, an additional search for recessive and X-linked variants with a MAF up to 3% was used.

2.7 | Variant interpretation

All variants described in this study were compared with publicly available databases for (likely) pathogenic variants like ClinVar, the Human Gene Mutation Database (HGMD, <http://www.hgmd.cf.ac.uk>) and the Leiden Open Variation Database (LOVD, <https://www.lovd.nl>), and were rated in accordance to ACMG guidelines and current amendments such as recommendations of the ACGS guidelines.²⁷⁻³⁰ Patients with a “likely pathogenic” or “pathogenic” variant and a fitting genotype (i.e., biallelic variants in *COL4A3* and *COL4A4* in male and female patients, hemizygous variants in *COL4A5* in male patients or a heterozygous variants in *COL4A5* in female patients) were classified as genetically solved cases. ADAS was not considered as a definite genetic diagnosis and therefore these patients were not classified as genetically solved cases according to the literature recommendations.¹² Based on the available genotype–phenotype correlation, patients with variants in *COL4A5* were classified into the three categories less-severe, intermediate and severe according to Gross et al. and Bekheirnia et al.^{4,6} Patients with compound heterozygous or homozygous variants in *COL4A3* and *COL4A4* were not classified into these three categories as there were only few ARAS cases and because there is only limited evidence from genotype–phenotype correlations to divide patients into different clinical course categories. Therefore, this study only used those patients with causative variants in *COL4A5* for genotype–phenotype correlation.

3 | STATISTICAL ANALYSIS

Group comparisons were made by a two-tailed Fisher's exact test with a significance level (α) of 0.05. For the analysis, patients were divided in those who did receive treatment and those who received placebo. After that each subset was classified according to Gross et al.⁴ and Bekheirnia et al.⁶ and each group was tested for a possible association between disease progress and risk classification by Fisher's exact test. Descriptive statistics are displayed as median and inter-quartile range (IQR). Statistics were calculated by STATA version 14.2 (STATA, College Station, TX).

4 | RESULTS

Patients were evaluated and analyzed according to a flow chart (Figure 1). Out of 66 children who entered the treatment phase of the EARLY PRO-TECT Alport trial, five had to be excluded because of protocol violation or withdrawn consent. Twenty children qualified for randomization (all male) and 42 children (two girls and 40 boys) were openly treated. The patients' median age was 8 years (IQR 8). In 98% (60/61), molecular genetic testing was performed. One boy was not tested, because of his typical AS symptoms and two relatives with genetic testing, in which the variant causing AS could not be found. Therefore, the following results are based on these 60 patients (Table 1). See Table 1 for a complete account of the phenotypic and genotypic data including applied ACMG criteria for each variant.

4.1 | Genetic testing

Fifty-three different variants (67 in total) were reported in this study cohort of 60 children (Table 1). 31/53 (58%) variants were not described before in the literature, whereas the other 22/53 (42%) variants were already reported in the literature as being causative for AS.

9/53 (17%) of the variants (5x likely pathogenic, 4x pathogenic) were located in *COL4A3*, 8/53 [15%; 1x benign, 1x VUS (variant of uncertain significance), 5x likely pathogenic, 1x pathogenic] in *COL4A4*, and 36/53 (68%; 3x VUS, 21x likely pathogenic, 12x pathogenic) in *COL4A5* (X-linked AS). 1/53 variant (2%) is already known as SNP (single nucleotide polymorphism) and listed in dbSNP (<https://www.ncbi.nlm.nih.gov/snp/rs121912862>). 38/60 (63%) patients presented with missense variants leading to a substitution of a glycine residue in the triple helix domain. The missense variant p. (Gly624Asp) in *COL4A5*, which is known as being a mild (“hypomorphic”) variant, was observed in 9/38 (24%) patients in a hemizygous state, respectively.

According to the known inheritance patterns and the variant interpretation as per ACMG criteria (see methods), 52 out of 60 patients (87%) could be classified as “solved cases.” Eight children had to be classified as “unsolved” or not clearly solved cases because of class 3 variants or lacking evidence for biallelic variants in autosomal AS (Table 1).

4.2 | Variant interpretation using applied ACMG criteria

4.2.1 | Solved cases

According to ACMG, likely pathogenic variants could be found in two patients with variants in *COL4A3*, in two patients with variants in *COL4A4* and in 31 patients with variants in *COL4A5*. Furthermore, two patients had pathogenic variants in *COL4A3* and 14 patients pathogenic variants in *COL4A5*. In one patient a likely pathogenic and a pathogenic variant in *COL4A4* could be detected.

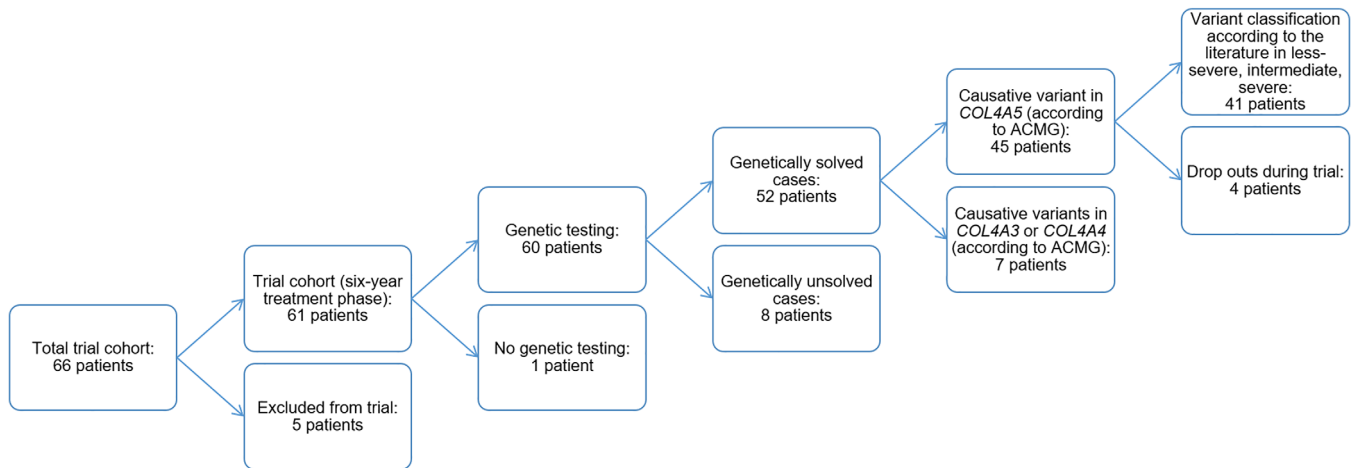


FIGURE 1 Flow chart and cohort overview [Colour figure can be viewed at wileyonlinelibrary.com]

4.2.2 | Unsolved or not unambiguously solved cases

In one patient a single heterozygous likely pathogenic variant in *COL4A3* (patient ID 93_01_02), in one patient a single heterozygous VUS in *COL4A4* (patient ID 93_15_03) and in one patient (patient ID 93_03_05) two single heterozygous variants (likely pathogenic and benign as per ACMG criteria) in two different genes (*COL4A3* and *COL4A4*) could be identified. These genetic results were all not compatible with a classical autosomal recessive inheritance. In six patients, a hemizygous VUS in *COL4A5* (patient IDs 93_11_03, 93_13_01, 93_13_02, 93_11_04, 93_11_05) was detected not sufficient for the diagnosis of XLAS.

4.3 | Variant classification

According to the recommendations of variant classification for *COL4A5* variants, the variants identified in the 45 solved cases were classified as follows (Tables 2 and 3): According to the classification of Gross et al.,⁴ 9/45 (20%) patients had a less-severe, 26/45 (58%) an

intermediate and 10/45 (22%) a severe variant. 1/4 (25%) patients without follow-up (drop outs) were classified as less-severe, 3/4 (75%) patients as intermediate. In comparison, the classification of Bekheirnia et al.⁶ classified 31/45 (69%) patients as having a less-severe, 3/45 (7%) as having an intermediate and 11/45 (24%) as having a severe variant. All four (100%) patients with loss to follow-up were grouped as having a less-severe variant.

4.4 | Clinical data

Most of the following data were already published by Gross et al.²³ Therefore, only a short overview of the clinical data of the patients, relevant for the present study, is shown.

4.5 | Kidney biopsy

Eight of 60 children (13%) received a kidney biopsy at a median age of 8.5 years (IQR 8) (Table 1). Two biopsy results were described as thin basement membrane (one of the two patients was an unsolved case)

TABLE 2 Classification of variants in *COL4A5* according to the literature (adapted to References 4 and 6)

	Less-severe variant	Intermediate variant	Severe variant
Gross et al. ⁴			
• Variant type	Glycine variant (exon 1–20)	Non-glycine variant, glycine variant (exon 21–47), acceptor splice-site variant	Large rearrangement, premature stop, frameshift, donor splice-site variant, variant involving the NC1-domain
• Mean age at onset of ESRF	30	26	20
Bekheirnia et al. ⁶			
• Variant type	Missense variant	Splice-site variant	Truncating variant, deletion, duplication
• Median age at onset of ESRF	37	28	25

Abbreviation: ESRF, end-stage renal failure.

TABLE 3 Genotype–phenotype correlation of the 41 genetically solved patients with variants in *COL4A5* according to the literature^{4,6}

	Less-severe variant (ramipril/placebo)	Intermediate variant (ramipril/placebo)	Severe variant (ramipril/placebo)
Gross et al. ⁴			
• Disease progress	2/0	7/3	5/0
• No disease progress	5/1	11/2	5/0
• Drop outs	1/0	3/0	0/0
Bekheirnia et al. ⁶			
• Disease progress	5/3	2/0	7/0
• No disease progress	16/3	1/0	4/0
• Drop outs	4/0	0	0

and 3/8 as AS (one of this case was a genetically unsolved case). In 3/8 patients the exact results of the kidney biopsy were not documented in the eCRF, but reported as consistent with AS.

4.6 | Co-morbidity

A relevant number of children (17/60, 28%) had at least one additional diagnosis besides AS, resulting in a total of 24 co-morbidities. The most common co-morbidities were allergies (4/17), followed by asthma (3/17) and skin diseases (3/17) (acne, atopic dermatitis, psoriasis). Malformations were documented in three children (persistent patent processus vaginalis, undescended testes, anorectal malformation). The remaining co-morbidities were attention deficit hyperactivity disorder ADHD (2/17), iron deficiency (2/17), anemia, sinus cyst, coloboma, enuresis, and short stature. In two children, further abnormalities of the kidneys were reported (pelvic kidney, hypertrophy of one kidney).

4.7 | Therapy

Although not significant because of the low number of randomized patients, the results of the EARLY PRO-TECT Alport trial cautiously indicated that ramipril therapy was effective: in the randomized arm, ramipril decreased the risk of disease progress by almost half (hazard ratio 0.51 (0.12–2.20), diminished the slope of albuminuria progression and the decline in glomerular filtration.²³

4.8 | Family history

A positive family history (microscopic hematuria, proteinuria or renal failure) was documented in 45/50 (90%) children with X-linked AS and 9/10 (90%) children with autosomal AS. The pedigrees in these families were in accordance with the pattern of inheritance (X-linked or autosomal AS). Of note, in all genetically unsolved/not unambiguously solved cases a positive family history was reported. Furthermore, 27/50 (54%) children with X-linked AS and 2/10 (20%) children

with autosomal AS had family members with ESRF (at a median age of 30 years, IQR 27). In total, 54 of the 60 children (90%) reported relatives with a positive family history for AS and half of the children (29/60) had relatives with AS, who developed ESRF.

4.9 | Genotype–phenotype correlation

Out of 60 patients who had participated in the trial for 3 years, 21/60 (35%) had a progress of disease. 3/21 (14%) patients were unsolved cases. 5/60 patients (8%) dropped out before being included in the trial for 3 years, all of them were solved cases.

The following descriptions will focus on the solved cases with (likely) pathogenic variants in *COL4A5* according to the ACMG criteria and without drop outs during the trial, altogether 41 patients. Concerning patients with variants in *COL4A3* or *COL4A4*, these groups will not be further evaluated.

The median age of the aforementioned 41 children who completed the trial was 7 years (IQR 7). 17/41 (41%) children had a progress of their disease during the trial. 35/41 (85%) children were treated with ramipril.

4.10 | Correlation using variant classification according to Gross et al.⁴

4.10.1 | Less-severe variant

The eight children with a less-severe variant had a median age of 9.5 years (IQR 7) and were the oldest patients in the trial. 7/8 (88%) children with a less-severe variant were on ramipril therapy. 2/8 (25%) children had a progress of their disease.

4.10.2 | Intermediate variant

The median age of 23 children with an intermediate variant was 7 years of age (IQR 7). 21/27 (78%) children were on ramipril and 8/21 (38%) showed disease progress.

TABLE 4 Fisher's exact test performed in the 35 genetically solved patients with ramipril therapy and a variant in *COL4A5* according to Bekheirnia et al.⁶ The highest percentage of patients without disease progress were found in the less-severe variant group compared to a higher percentage of patients with progress in the severe variant group ($p = 0.05$)

	Less-severe variant n %	Intermediate variant n %	Severe variant n %	Total n %
Disease progress	5 23.81	2 66.67	7 63.63	14 40.00
No disease progress	16 76.19	1 33.33	4 36.36	21 60.00
Total	21 100.00	3 100.00	11 100.00	35 100.00

4.10.3 | Severe variant

The 10 children with a severe variant were the youngest patients in the EARLY PRO-TTECT Alport trial using this classification system with a median age of 6 years (IQR 2). 5/10 (50%) were on ramipril therapy, and all of these children developed disease progress.

4.11 | Correlation using variant classification of Bekheirnia et al.⁶

4.11.1 | Less-severe variant

The 27 children with a less-severe variant had a median age of 7 years (IQR 7). 21/27 (78%) children with a less-severe variant were on ramipril therapy. 8/27 (30%) children had a progress of their disease, 5/8 (63%) children were treated with ramipril.

4.11.2 | Intermediate variant

The median age of three children with an intermediate variant was 5 years of age (IQR 2). They were the youngest children in this trial using this classification system. All (100%) children were on ramipril and 2/3 (67%) showed disease progress.

4.11.3 | Severe variant

The 11 children with a severe variant had a median age of 7 years (IQR 7). All (100%) children were on ramipril therapy, and 7/11 (64%) children developed disease progress.

4.12 | Comparison of disease course

The classification systems of Gross et al.⁴ and Bekheirnia et al.⁶ were compared with regard to the percentage of patients with and without disease progress. After using the classification of Bekheirnia et al.⁶ the highest percentage of patients without disease progress were found in the less-severe variant group compared to a higher percentage of patients with progress in the severe variant group (Table 4). This

discriminating classification was borderline significant ($p = 0.05$). In contrast, the classification according to Gross et al.⁴ did not significantly discriminate patients according to severity groups and disease progress (data not shown). This result could only be found in patients under treatment as numbers in the placebo group were very small.

5 | DISCUSSION

Early genetic diagnosis in children with AS is crucial for early therapy to delay renal failure and improve life-expectancy. The present study is a genotype-phenotype correlation of patients of the EARLY PRO-TTECT Alport trial. Although the study size is small, the patients are phenotypically very well ascertained and variant interpretation was transparent and reproducible using ACMG criteria with latest amendments.^{29,31}

In the present study, we address the question whether the classifications according to Gross et al.⁴ and Bekheirnia et al.⁶ are helpful in discriminating the clinical course and the response to therapy in genetically solved patients with AS and a causative variant in *COL4A5*.

First of all, in 8/60 (13%) patients of this study causative variants (or a fitting genotype) could not be identified leading to a genetically unsolved case. This could be due to several factors: (i) these patients carry their causative variant(s) within non-analyzed regions of the genes *COL4A3*, *COL4A4* or *COL4A5* (e.g., deep-intronic variants, which are usually not covered by sequencing methods capturing protein-coding and adjacent regions), (ii) they have a different monogenic disease mimicking AS which they were not tested for (e.g., hereditary FSGS) or they do not have a monogenic but a multifactorial disease resembling AS ("phenocopy"). To approach these facts, comprehensive genetic testing including genes associated with phenotypically related hereditary kidney disorders, for example, by comprehensive panel or ES testing, should be performed in patients with suspicion of AS and negative *COL4A3-5* genetic testing. Unfortunately, this was not included as part of the original trial and only two cases were analyzed by ES.

Of the remaining 52 patients, a genetic diagnosis could be made. Forty-five of these patients carried a causative variant in *COL4A5*, and 41/45 (91%) patients passed through the complete trial. Using the classification system of Gross et al.⁴ a statistically significant discrimination concerning the allocation to severity groups and disease progress of the 41 patients who passed through the complete trial was

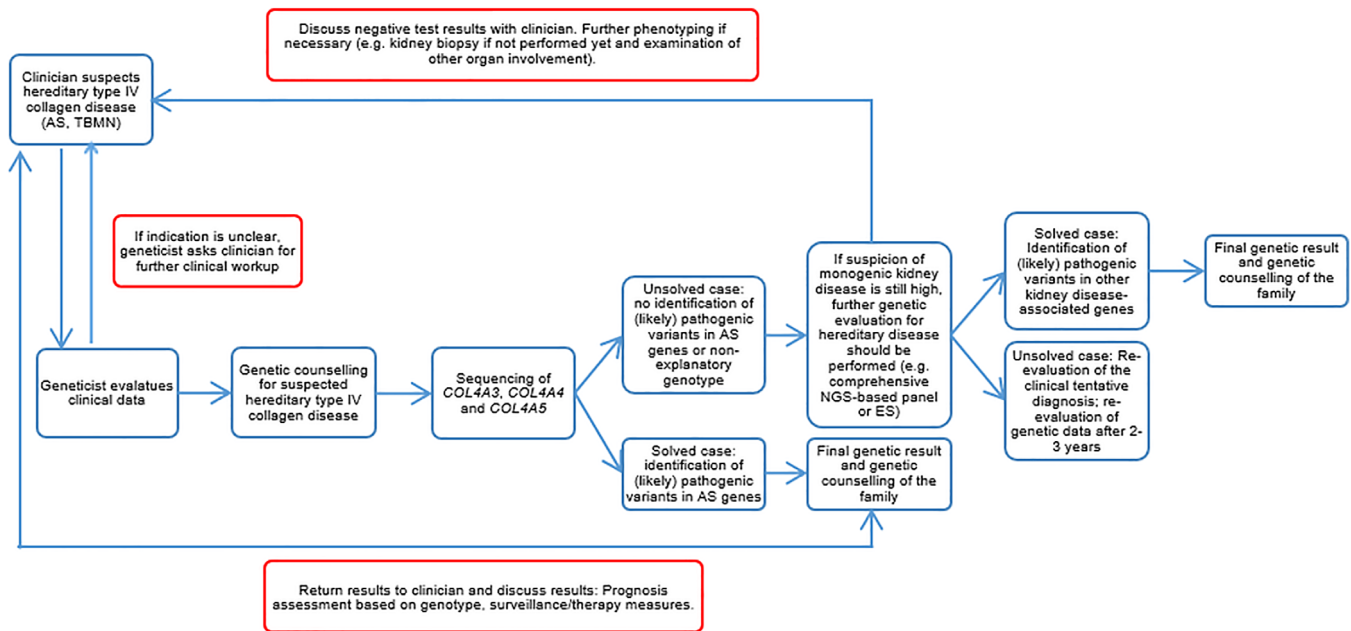


FIGURE 2 Evaluation of children with AS. Recommended interaction between clinician and geneticist. AS, Alport syndrome; ES, exome sequencing [Colour figure can be viewed at wileyonlinelibrary.com]

not possible. On the other hand, using the classification system of Bekheirnia et al.⁶ at least a borderline significance ($p = 0.05$) could be found when associating the severity groups with disease progress. The non-significant discrimination of clinical course by variant using Gross et al.⁴ in contrast to Bekheirnia et al.⁶ might be due to small sample size and/or the fact that Bekheirnia et al.⁶ classifies all missense variants in the less-severe group compared to Gross et al.⁴ Generally speaking, in both classification systems there was a trend in the group of treated patients that those with a less-severe or intermediate variant had less likely a progress compared to patients with a severe variant. Whether this trend concerning reduced disease progress in the less-severe or intermediate group is attributable to genotype combined with treatment or the genotype alone cannot be discerned by these data as the placebo group had too few patients to compare the outcome stratified according to the variant classifications. Unfortunately, the number of patients in this study was limited and maybe therefore significant results concerning the classification according to Gross et al.⁴ cannot be seen. Concerning other limitations, for most of the patients, no genetic report with specific information on testing method, quality parameters, and genes studied were available as the genetic analysis was performed before the patients were part of the trial.

For the VUS in our cohort, one can acknowledge that a further investigation of splicing effect on RNA level (synonymous variant NM_000495.4:c.384G > A, p.(Lys128=), near splice variant NM_000495.4:c.81 + 4A > C, p.(?) in COL4A5 and canonical splice variant NM_000092.4 c.[4810-1G > A];[4810-1G > A] affecting the acceptor splice site of the last exon of COL4A4) and segregation analysis (in-frame indel NM_000495.4 c.453_455del p.[Pro152del] in COL4A5) could lead to an upgrade of variant rating to “likely pathogenic.” This was not possible in this cohort, as index patients

and families were not accessible due to the study design of the EARLY PRO-TECT Alport trial.

The results of this study indicate, as other genotype–phenotype correlations showed before, that genetic testing is not only prime to diagnose AS but also for knowing the exact genotype to assess clinical course. Our data indicate that there is a possible better prognosis for missense (less-severe) variants, which is comparable with the literature data that patients with a severe variant develop ESRF earlier than patients with a less-severe variant.⁷ Of note, in our study population, the most common co-morbidities were allergies, asthma, and skin diseases, all of which can lead to recurrent infections, which also possibly could contribute to disease progression in children with AS.³² One can speculate that this individual effect can be explained by the distinct impact of missense variants on type IV collagen assembly, mechanical stability, and collagen degradation. For example, the less vulnerable the GBM is to hyperfiltration and mechanical stress, which is the case in glycine-missense variants compared to splice-site or truncating variants, the later AS progresses to (irreversible) loss of glomeruli and renal fibrosis and the better ACEis' effect on lowering filtration pressure will delay progression.⁴ Nevertheless, currently a precise prediction of the therapeutic response to nephro protective therapy in patients with specific variants is difficult just on the basis of the present study results.

Up to now, there are hundreds of different and individual variants in the huge COL4A3-5 genes with a size between 150 and 250 kb. Furthermore, the complexity of the type IV collagen triple helix extracellular suprastructure formation, function, and degradation is too little understood. Both facts make it difficult to judge the clinical course of an AS patient from a genetic test result alone. Therefore, this study should highlight the importance of interdisciplinary collaboration between clinicians and human geneticists in order to comprehensively

evaluate AS patients. The workup of these patients should include repeated comparison between genetic findings and clinical symptoms including family history to also identify phenocopies (Figure 2).

In addition, it should be pointed out that the most common variant in the trial wasp. (Gly624Asp) in COL4A5, identified in a hemizygous state in eight male XLAS patients. 2/8 (25%) children with this variant showed a progress of the disease during the trial. This variant is known in the literature to lead to a rather mild clinical phenotype ("hypomorphic" variant) with late onset of renal failure (median age of 50 years at ESRF).³³ In contrast, a recent case report described a more severe phenotype in patients carrying this variant.³⁴ The data of this clinical trial further support the doubt that this variant is "hypomorphic" in all carriers. Despite good response to ramipril therapy, according to our data, 2/8 patients with this variant also showed a disease progress. This is in contrast to the literature as there are only adult patients described.

6 | CONCLUSION

The complexity of genetic testing in AS make it imperative for clinicians and geneticists to work closely together. In the future, a patient's individual genotype might be helpful to predict response to therapy with a possible advantage for missense (less-severe) variants to be more responsive, but more data is currently still needed to confirm this hypothesis. We recommend to use a stringent workflow between geneticist and referring clinician. The early diagnosis by an experienced geneticist allows prompt surveillance and swift initiation of therapy once treatable symptoms of AS become apparent (proteinuria, hypertension), which has the potential to delay renal failure by decades and improve life-expectancy in our young patients. Furthermore, larger studies should be initiated on an international collaborative basis to further improve genotype-phenotype correlations in AS, especially concerning response to treatment.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

PEER REVIEW

The peer review history for this article is available at <https://publons.com/publon/10.1111/cge.13861>.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

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