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EDITED BY

Christopher E. Pedigo,
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REVIEWED BY

Jisook Yim,
The Catholic University of Korea, Korea
Dorin-Bogdan Borza,
Meharry Medical College, United States

*CORRESPONDENCE

Pavlina Plevova
✉ pavlina.plevova@fno.cz

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A founder *COL4A4* pathogenic variant resulting in autosomal recessive Alport syndrome accounts for most genetic kidney failure in Romani people

Pavlina Plevova^{1,2*}, Jana Indrakova¹, Judy Savige³, Petra Kuhnova¹, Petra Tvrda¹, Dita Cerna¹, Sarka Hilscherova¹, Monika Kudrejova¹, Daniela Polendova⁴, Radka Jaklova⁴, Martina Langova⁵, Helena Jahnova⁶, Jana Lastuvkova⁷, Jiri Dusek⁸, Josef Gut⁹, Marketa Vlckova¹⁰, Pavla Solarova¹¹, Gabriela Kreckova¹², Eva Kantorova¹³, Jana Soukalova¹⁴, Rastislav Slavkovsky¹⁵, Jana Zapletalova¹⁶, Tomas Tichy¹⁷ and Dana Thomasova¹⁰

¹Department of Clinical and Molecular Pathology and Medical Genetics, University Hospital Ostrava, Ostrava, Czechia, ²Department of Biomedical Sciences, Faculty of Medicine, University of Ostrava, Ostrava, Czechia, ³Department of Medicine (Melbourne Health and Northern Health), The University of Melbourne, Royal Melbourne Hospital, Melbourne, Australia, ⁴Department of Medical Genetics, Faculty of Medicine in Plzeň, Charles University and University Hospital Plzeň, Plzeň, Czechia, ⁵Department of Medical Genetics, Thomayer University Hospital, Prague, Czechia, ⁶Department of Pediatrics, Third Faculty of Medicine, Charles University and University Hospital Královské Vinohrady, Prague, Czechia, ⁷Department of Medical Genetics, Krajská zdravotní, a.s., Masaryk Hospital in Ústí nad Labem, Ústí nad Labem, Czechia, ⁸Department of Pediatrics, 2nd Faculty of Medicine, Charles University and Motol University Hospital, Prague, Czechia, ⁹Department of Pediatrics, Hospital Česká Lípa, Česká Lípa, Czechia, ¹⁰Department of Biology and Medical Genetics, 2nd Faculty of Medicine, Charles University and Motol University Hospital, Prague, Czechia, ¹¹Department of Medical Genetics, University Hospital Hradec Králové, Hradec Králové, Czechia, ¹²Department of Medical Genetics, Gennet, s.r.o., Liberec, Czechia, ¹³Department of Medical Genetics, Hospital České Budějovice a.s., České Budějovice, Czechia, ¹⁴Department of Medical Genetics and Genomics, University Hospital Brno, Brno, Czechia, ¹⁵Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry, Palacký University Olomouc, Olomouc, Czechia, ¹⁶Department of Medical Biophysics, Faculty of Medicine and Dentistry, Palacký University Olomouc, Olomouc, Czechia, ¹⁷Institute of Clinical and Molecular Pathology, Faculty of Medicine and Dentistry, Palacký University Olomouc, Olomouc, Czechia

Introduction: Romani people have a high prevalence of kidney failure. This study examined a Romani cohort for pathogenic variants in the *COL4A3*, *COL4A4*, and *COL4A5* genes that are affected in Alport syndrome (AS), a common cause of genetic kidney disease, characterized by hematuria, proteinuria, end-stage kidney failure, hearing loss, and eye anomalies.

Materials and methods: The study included 57 Romani from different families with clinical features that suggested AS who underwent next-generation sequencing (NGS) of the *COL4A3*, *COL4A4*, and *COL4A5* genes, and 83 family members.

Results: In total, 27 Romani (19%) had autosomal recessive AS caused by a homozygous pathogenic c.1598G>A, p.Gly533Asp variant in *COL4A4* ($n = 20$) or a homozygous c.415G>C, p.Gly139Arg variant in *COL4A3* ($n = 7$). For p.Gly533Asp, 12 (80%) had macroscopic hematuria, 12 (63%) developed end-stage kidney failure at a median age of 22 years, and 13 (67%) had hearing loss. For p.Gly139Arg, none had macroscopic hematuria ($p = 0.023$), three (50%) had end-stage kidney failure by a median age of 42 years ($p = 0.653$), and five (83%) had hearing loss ($p = 0.367$). The p.Gly533Asp variant was associated with a more severe phenotype than p.Gly139Arg,

with an earlier age at end-stage kidney failure and more macroscopic hematuria. Microscopic hematuria was very common in heterozygotes with both p.Gly533Asp (91%) and p.Gly139Arg (92%).

Conclusion: These two founder variants contribute to the high prevalence of kidney failure in Czech Romani. The estimated population frequency of autosomal recessive AS from these variants and consanguinity by descent is at least 1:11,000 in Czech Romani. This corresponds to a population frequency of autosomal dominant AS from these two variants alone of 1%. Romani with persistent hematuria should be offered genetic testing.

KEYWORDS

Alport syndrome, Romani, hematuria, proteinuria, end-stage kidney failure, hearing loss, consanguinity

Introduction

Alport syndrome (AS) is a genetic disease characterized by progressive kidney failure, sensorineural hearing loss, and ocular abnormalities (1). It results from pathogenic variants in the collagen IV genes that encode chains of the $\alpha3-4-5$ heterotrimer (2). Pathogenic variants in the *COL4A5* gene result in X-linked AS, whereas biallelic *COL4A3* or *COL4A4* variants are found in autosomal recessive AS (AR AS) (3). Individuals with heterozygous *COL4A3* or *COL4A4* mutations have thin basement membrane nephropathy or autosomal dominant AS (AD AS) with typically normal kidney function, but proteinuria may occur in later life. The *COL4A3* and *COL4A4* heterozygotes represent carriers of AR AS (3–5).

Romani children are often seen with AS or end-stage renal disease (ESRD) in Slovakia (6, 7), a European country neighboring the Czech Republic. The origins of the Romani are unclear, but the current understanding is that they have come to Europe from the Indian subcontinent centuries ago (8, 9), and several studies describe a high incidence of ESRD in India and Indian immigrants in Europe (10–12). Founder variants in the Alport genes have been reported in other isolated populations such as Ashkenazi (13–15), and the p.Gly624Asp variant in *COL4A5* accounts for nearly half the variants found in eastern Europe (16, 17).

This study examined whether pathogenic variants in the Alport genes (*COL4A3*, *COL4A4*, *COL4A5*) were responsible for the high prevalence of ESRD in Romani people living in the Czech Republic.

Materials and methods

This study included 57 self-identified Romani people from 57 families with clinical features that suggested AS and 83 family members who were known to be affected or at risk. They were recruited from the departments of pediatric nephrology, nephrology, or genetics from all regions of the Czech Republic between 1 January 2014 and 31 July 2022. This study was approved by the Institutional Ethics Committee of the University Hospital Ostrava according to the principles of the Declaration of Helsinki, and all study participants provided written informed consent.

Participants provided peripheral venous blood samples that were referred to the Laboratory of DNA diagnostics of the

Department of Medical Genetics, University Hospital of Ostrava for genetic analysis. Genomic DNA was isolated using conventional techniques and underwent sequence capture-based, next-generation sequencing (NGS) of the *COL4A3*, *COL4A4*, and *COL4A5* genes. The sequencing included all the coding exons, at least 50 bp of flanking intronic sequence, and the untranslated regions of the *COL4A3* (NM_000091.5), *COL4A4* (NM_000092.5), and *COL4A5* (NM_033380.3) genes using NimbleGen SeqCap EZ Target Enrichment System (Roche, Switzerland) as described previously (18). Sequencing was performed on an Illumina platform (Illumina, CA, USA), and data were examined with the standard Illumina base-calling procedure. Mapping to the human genome sequence, variant-calling, and copy number variation (CNV) analyses were performed with FinalistDx (IAB, Czech Republic). Pathogenic variant validation was performed with direct Sanger sequencing using the ABI Big Dye Terminator Cycle Sequencing Detection Kit v.3.1 and an ABI 3130 genetic analyzer (Applied Biosystems, CA, USA) according to the manufacturer's instructions. Multiplex Ligation-Dependent Probe Amplification (MLPA) was performed for the *COL4A3*, *COL4A4*, and *COL4A5* genes with SALSA kits P191/P192 (*COL4A5*), P439 (*COL4A3*), and P444 (*COL4A4*) (MRC-Holland, The Netherlands), and the results were analyzed using Coffalyzer software (MRC-Holland, The Netherlands).

DNA from three individuals was further examined for pathogenic variants by an NGS panel of 462 genes associated with genetic kidney diseases using the same strategy (Supplementary material 1). Targeted genotyping of parents and family members of affected individuals was performed where possible.

The genomic DNA of the first individual with a homozygous p.Gly533Asp variant in *COL4A4* was examined more in detail. This included a whole-genome microarray SNP analysis with HumanCytoSNP-12v2.1 BeadChips (Illumina, CA, USA) and examination of the array data with Bluefuse Multi Software (Illumina, CA, USA) according to the manufacturer's instructions.

Clinical data for patients with confirmed AS were provided by the referring physicians from the medical records. Age at ESRD was defined as the age at the clinical diagnosis of kidney failure, and where this was not available, the age at commencing dialysis or at the first kidney transplant.

In addition, DNA from 300 non-Romani individuals with clinical features that suggested AS and their 225 family members were examined.

Evaluation of the pathogenicity of sequence variants

The variants were described according to the recommendations of the Human Genome Variation Society (HGVS). Sequence variants were assessed according to the criteria of the American College of Medical Genetics (ACMG) (19). Pathogenicity was ascertained based on the following criteria: the presence of a missense variant involving a position 1 Gly or an in-frame Gly deletion in the collagen Gly-X-Y triple-helical domain; a splice-site or truncating variant; or a large genomic duplication or deletion (20). Variants were also assessed based on molecular, epidemiological, segregation, and computational criteria (Polyphen-2, SIFT, Mutation Taster, PhyloP100). The following online databases were searched to determine if variants were previously reported: Ensembl, gnomAD browser, the Human Gene Mutation Database (HGMD), Leiden Open (source) Variation Database (LOVD), and ClinVar. The potential impact of each variant was reported as one of the five categories: pathogenic, likely pathogenic, uncertain significance, likely benign, or benign.

Statistical analysis

Qualitative parameters in groups with different genotypes were compared using Fisher's exact test. Ages were compared using the Mann-Whitney *U* test. Statistical analysis was performed using SPSS version 23 software (Armonk, NY: IBM Corp., USA), and a *p*-value of less than 0.05 was considered significant.

Results

The study cohort comprised 57 unrelated individuals, including 56 with both parents Romani and one with only one Romani parent. They included 23 (40%) men and 34 (60%) women, with a median age at the referral of 16 years (range, 4–57 years) for men and 15.5 years (range, 4–60 years) for women. In addition, 83 affected or at-risk family members were examined, including 81 patients where both parents were Romani. These included 42 (51%) men and 41 (49%) women with an overall median age of 29.0 years (range 2–63 years).

In the Romani patients, only two pathogenic variants were detected in the *Alport* genes. These were c.1598G > A in *COL4A4* corresponding to p.Gly533Asp and c.415G > C in *COL4A3* corresponding to p.Gly139Arg. No pathogenic or likely pathogenic variant was found in *COL4A5*. A girl with one Romani parent had the p.Gly533Asp variant in *COL4A4* together with another pathogenic variant in trans in *COL4A4* (Table 1).

Both the p.Gly533Asp and p.Gly139Arg variants were considered to be pathogenic. These variants affected the position 1 glycine residue in the collagen Gly-X-Y triple-helical domain that was not adjacent to an interruption. They were not found in gnomAD. The computational tools all suggested pathogenicity (Polyphen-2–0.999 and 1.000, respectively; SIFT–Damaging; Mutation Taster–Disease causing); the affected glycines were highly conserved with a PhyloP100 score of 4.763 and 5.3, respectively. The p.Gly533Asp was considered pathogenic in ClinVar, likely pathogenic or pathogenic in LOVD, and was reported as pathogenic for AR AS in two patients

from the UK (21). The p.Gly139Arg had not been reported in ClinVar but was considered pathogenic in LOVD (22). Both variants segregated with the disease in the families.

Autosomal recessive AS was confirmed genetically in 22 of the 57 individuals in the cohort with clinical features of AS (39%). Another six individuals with AR AS were detected among the family members (Table 1). The family tree of one family is shown in Figure 1. A further nine patients identified from the 300 individuals from the non-Romani Czech cohort also had AR AS. In total, the 28 Romani represented 76% (28/37) of all patients with AR AS diagnosed in our laboratory.

In addition, there were 83 individuals heterozygous for one of these variants, with 70 (84%) heterozygous for p.Gly533Asp in *COL4A4* and 13 (16%) for p.Gly139Arg in *COL4A3* (Table 1), consistent with the diagnosis of AD AS or thin basement membrane nephropathy. One 10-year-old girl with isolated microscopic hematuria diagnosed at the age of three was a double heterozygote for the two variants. Altogether, monoallelic or biallelic pathogenic *COL4A4* or *COL4A3* variants were detected in 51 of 57 Romani with clinical features of AS (89%) and 61 of their 83 relatives (73%). This corresponded to an estimated prevalence of AR AS due to homozygous copies of these *COL4A4* and *COL4A3* variants in the Romani population in the Czech Republic of at least one in 11,000. These results also corresponded to a population frequency of these founder variants in at least one in 3,800 of the Czech Romani and probably higher (Table 2). These results also mean that AD AS due to these variants affects at least one in 3,800 Czech Romani.

In a group of 300 non-Romani Czech individuals and their 225 relatives, there was one individual who was a compound heterozygote for p.Gly533Asp in *COL4A4* and another pathogenic *COL4A4* variant in trans, 12 patients heterozygous for p.Gly533Asp *COL4A4*, and none with p.Gly139Arg in *COL4A3* (Table 1).

DNA from three Romani patients with AD AS whose phenotype did not correspond typically to the genotype was further examined for pathogenic variants by an NGS panel of 462 genes associated with genetic kidney diseases. The first patient was heterozygous for p.Gly533Asp in *COL4A4* and had isolated proteinuria from the age of 2 years, hearing loss from the age of 14 years, and had developed ESRD at the age of 18 years. His sister also had isolated proteinuria from adolescence and developed ESRD at the age of 61 years. The second patient also heterozygous for p.Gly533Asp in *COL4A4* developed ESRD at the age of 26 years. The third one was a 6-year-old boy heterozygous for p.Gly139Arg in *COL4A3* with microscopic hematuria, persistent proteinuria, and transient macroscopic hematuria. Other concurrent genetic etiologies for kidney disease were suspected in these individuals. However, testing with NGS for a kidney panel of 462 genes did not reveal any other pathogenic variants.

Clinical features associated with AR AS are summarized in Table 3. For p.Gly533Asp, 12 homozygotes (63%) developed ESRD at a median age of 22 years (range 12 to 55), and 13 (67%) had hearing loss, while all 20 (100%) had microscopic hematuria, 12 (80%) had macroscopic hematuria in childhood, and 17 (94%) had proteinuria with a median age of onset 5 years. For p.Gly139Arg, three homozygotes (50%) developed kidney failure by a median age of 42 years (range 17 to 56), five (83%) had hearing loss, six (100%) had microscopic hematuria, but none had macroscopic hematuria (*p* = 0.023), and five (83%) had proteinuria with a median age of

TABLE 1 Summary of pathogenic sequence variants detected in Romani, one parent Romani, and non-Romani patients.

Genotype	Probands	Family genetic testing positive	All pts. with the variant (probands + family testing positive)	Family genetic testing negative
	No. of pts.	No. of pts.	No. of pts. (% from the group)	No. of pts.
Romani				
COL4A4 c.1598G>A, p.Gly533Asp				
Homozygous (=AR AS)	19	1	20 (18)	23
Heterozygous (=AD AS)	24	44	68 (62)	
Double heterozygous: + COL4A3 p.Gly139Arg (=digenic AS)	1	0	1 (1)	
COL4A3 c.415G>C, p.Gly533Asp				
Homozygous (=AR AS)	2	5	7 (7)	1
Heterozygous (=AD AS)	4	9	13 (12)	
All Romani	50	59	109 (100)	24
One parent Romani				
COL4A4 c.1598G>A, p.Gly533Asp				
Compound heterozygous: + COL4A4 c.3707G>A, p.Gly1236Glu (=AR AS)	1	0	1 (33)	0
Heterozygous (=AD AS)	0	2	2 (66)	0
All one parent Romani	1	2	3 (100)	0
Non-Romani				
COL4A4 c.1598G>A, p.Gly533Asp				
Compound heterozygous: + COL4A4 c.3514_3515delinsTGAAA, p.Gly1172Ter (=AR AS)	1	0	1 (8)	0
Heterozygous (=AD AS)	4	8	12 (92)	4
COL4A3 c.415G>C, p.Gly533Asp				
Homozygous, heterozygous (=AR AS, AD AS)	0	0	0	0
All non-Romani	5	8	13 (100)	4
All pts from all subgroups	56	69	125	28

The above table shows the distribution of patients with homozygous and heterozygous p.Gly533Asp COL4A4 and p.Gly139Arg COL4A3 variants in the Romani ($n = 109$), one parent Romani ($n = 3$), and non-Romani ($n = 13$) patients. Homozygous (i.e., two identical) or compound heterozygous (i.e., two different) pathogenic variants in the same (COL4A4 or COL4A3) gene cause AR AS; heterozygous (i.e., one) pathogenic variants in COL4A4 or COL4A3 gene cause AD AS; the occurrence of one (heterozygous) pathogenic variant in COL4A4 and simultaneously one (heterozygous) pathogenic variant in COL4A3 (i.e., double heterozygous status) causes digenic Alport syndrome; numbers of all patients with the particular genotype are in bold. pts., patients; AR AS, autosomal recessive Alport syndrome; AD AS, autosomal dominant Alport syndrome; AS, Alport syndrome.

onset 20.5 years. There was only one person with a recognized ocular abnormality (lenticonus) and no data on the others.

Thus the p.Gly533Asp variant in COL4A4 was associated with a more severe phenotype than p.Gly139Arg in COL4A3, with an earlier onset of proteinuria ($p = 0.044$), earlier onset of ESRD ($p = 0.347$), and a greater risk of macroscopic hematuria ($p = 0.023$). However, there was also a large variability in the severity of the clinical features even within a family.

A total of six (9%) Romani patients heterozygous for c.1598G>A in COL4A4 did not have microscopic hematuria. Four (6%) did not have either microscopic hematuria or proteinuria or any other manifestation of AS. Two Romani siblings heterozygous for p.Gly533Asp COL4A4 variant had isolated proteinuria and progressed to renal failure at 18 and 61 years, respectively. No other pathogenic variant was detected in

one of the siblings by testing a panel of 462 genes for inherited kidney diseases, as mentioned above. Two other members of this family heterozygous for p.Gly533Asp had microscopic hematuria and concomitant proteinuria. They had normal renal functions at ages 36 and 40 years. Thus four of five patients with heterozygous p.Gly533Asp and proteinuria belonged to the same family.

Renal biopsy reports were available from eight patients with AR AS. The histological and electron microscopy findings included thin basement membrane nephropathy with the fusion of podocytes, thickening and splitting of the glomerular basement membrane, focal segmental glomerulosclerosis, or mesangioproliferative glomerulonephritis. Immunofluorescence $\alpha 3$ and $\alpha 5$ chain staining was negative in three patients with homozygous COL4A4 p.Gly533Asp variant (Table 4).

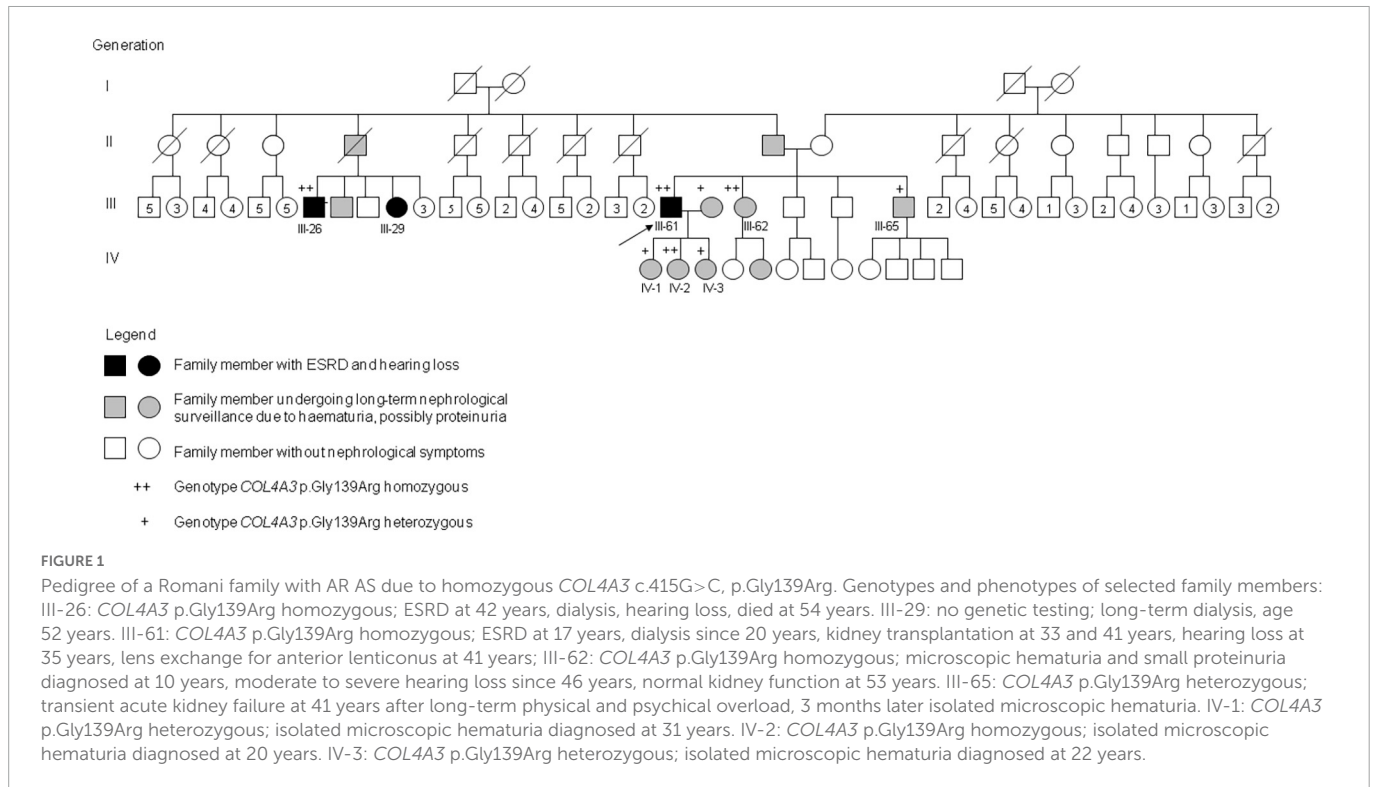


TABLE 2 Estimated prevalence of COL4A4 and COL4A3 variants in the Romani population in the Czech Republic.

Genotype or type of AS	Romani		Non-Romani	
	No. of pts.	Prevalence per 300,000 ^a	No. of pts.	Prevalence ^b
COL4A4 p.Gly533Asp homozygous	20	1:15,000	0	0
COL4A4 p.Gly533Asp heterozygous	68	1:4,400	11	1:950,000
COL4A4 p.Gly533Asp + COL4A3 p.Gly139Arg, double heterozygous	1	1:300,000	0	0
COL4A3 p.Gly139Arg homozygous	7	1:42,800	0	0
COL4A3 p.Gly139Arg heterozygous	13	1:23,000	0	0
Autosomal recessive AS ^c	27	1:11,000	n.a.	n.a.
Autosomal dominant AS (TBMN) ^d	81	1:3,800	n.a.	n.a.
Digenic COL4A4 + COL4A3	1	1:300 000	0	0

^aThe population of 300,000 Romani people in the Czech Republic was used for calculation.

^bThe population of 10,500,000 non-Romani people in the Czech Republic was used for calculation.

^cCOL4A4 p.Gly533Asp homozygous or COL4A3 p.Gly139Arg homozygous.

^dCOL4A4 p.Gly533Asp heterozygous or COL4A3 p.Gly139Arg heterozygous.

AS, Alport syndrome; n.a., non-applicable; TBMN, thin basement membrane nephropathy.

In patients with homozygous p.Gly533Asp in COL4A4, 45 homozygous benign variants (Supplementary material 2) were also detected, with 16 in COL4A3 and 29 in COL4A4. Both are neighboring genes located in a head-to-head orientation. In the first patient, in whom a homozygous c.1598G > A COL4A4 variant was detected, an SNP microarray analysis was performed. Loss of heterozygosity of 250.57 Mb (equivalent to 9% of the genome) was demonstrated in 17 regions, including a 47,837,907-bp loss of heterozygosity at the 2q32.1–2q37.1 region (chr2: 183,690,254–231,528,160), where the COL4A3 and COL4A4 genes are located. This suggested consanguinity of the third degree. Despite this fact, the parents of the patient claimed to be fourth-degree relatives because the grandfather of the patient’s mother was

the brother of her father’s father. Fourth-degree relatives usually display a loss of heterozygosity of 80–90 Mb (23), while here we observed 250.57 Mb. This corresponds approximately to the first-cousin relationship.

Discussion

In many European countries, Romani people constitute a major ethnic minority (7). According to the European Romani Rights Center, the estimated number of Romani in Czechia is between 250,000 and 300,000 (24), which represents 2.8% of the 10.5 million inhabitants. However,

TABLE 3 Summary of clinical features in Alport syndrome patients with respect to the genotype.

	Clinical features	Microscopic hematuria	Macroscopic hematuria	Proteinuria	ESRD	Hearing loss
	Subgroup of patients according to the genotype and ethnic group	No. of pts. (% in the subgroup)	No. of pts. (% in the subgroup)	No. of pts. (% in the subgroup)	No. of pts. (% in the subgroup)	No. of pts. (% in the subgroup)
AR AS						
1.	COL4A4 p.Gly533Asp homozygous, Romani	<i>n</i> = 20				
1.1	Feature: Yes	20 (100)	12 (80)	17 (94)	12 (63)	13 (67)
1.2	Feature: No	0	3 (20)	1 (6)	7 (37)	6 (33)
1.3	Feature: Information not available	0	5	2	1	1
1.4	Median age at feature diagnosis (years) (range) ^a	4.0 (8 m-6)	5.0 (2-12)	5.0 (1-13)	22.0 (15-55)	14.5 (10-20)
2.	COL4A4 p.Gly533Asp + other COL4A4 variant (compound heterozygous) one parent Romani, non-Romani	<i>n</i> = 2				
2.1	Feature: Yes	2 (100)	2 (100) ^b	1 (50) ^c	0	1 (50)
2.2	Feature: No	0	0	1 (50) ^d	2	1 (50)
2.3	Feature: Information not available	0	0	0	0	0
2.4	Median age at feature diagnosis (years) (range) ^a	2.75 (2.5-3)	2.75 (2.5-3)	3 (3)	n.a.	n.a.
3.	COL4A3 p.Gly139Arg homozygous, Romani	<i>n</i> = 7				
3.1	Feature: Yes	6 (100)	0 (0)	5 (83)	3 (50)	5 (83)
3.2	Feature: No	0	6 (100)	1 (17)	3 (50) ^e	1 (17)
3.3	Feature: Information not available	1	1	1	1	1
3.4	Median age at feature diagnosis (years) (range) ^a	8.0 (5-20)	-	20.5 (10-31)	42.0 (17-56)	35.0 (25-46)
	Statistics 3.1 vs. 1.1 (Fisher's exact test)	n.a.	<i>p</i> = 0.023	<i>p</i> = 0.446	<i>p</i> = 0.653	<i>p</i> = 0.367
	Statistics 3.4 vs. 1.4 (Mann-Whitney <i>U</i> test)	<i>p</i> = 0.010	n.a.	<i>p</i> = 0.044	<i>p</i> = 0.347	<i>p</i> = 0.001
Digenic AS						
4.	COL4A4 p.Gly533Asp, COL4A3 p.Gly139Arg double heterozygous Romani	<i>n</i> = 1				
4.1	Feature: Yes	1	0	1	0	0
4.2	Feature: No	0	1	0	1	1
4.3	Feature: Information not available	0	0	0	0	0
4.4	Median age at feature diagnosis (years) (range) ^a	3	n.a.	3	n.a.	n.a.
AD AS						
5.	COL4A4 p.Gly533Asp heterozygous Romani, one parent Romani	<i>n</i> = 70 (68 Romani, 2 one parent Romani)				
5.1	Feature: Yes	61 (91)	1 (1) ^f	5 (7) ^g	3 (4) ^h	6 (9) ⁱ
5.2	Feature: No	6 (9) ^j	66 (99)	62 (93)	66 (96)	61 (91)
5.3	Feature: Information not available	3	3	3	1	3
5.4	Median age at feature diagnosis (years) (range) ^a	7.0 (1-58)	n.a.	n.a.	26.0 (18-61)	12.0 (1-35)
	Statistics 5.1 vs. 1.1 (Fisher's exact test)	<i>p</i> = 0.329	<i>p</i> < 0.0001	<i>p</i> < 0.0001	<i>p</i> < 0.0001	<i>p</i> < 0.0001
	Statistics 5.4 vs. 1.4 (Mann-Whitney <i>U</i> test)	<i>p</i> < 0.0001	n.a.	n.a.	<i>p</i> = 0.515	<i>p</i> = 0.420

(Continued)

TABLE 3 (Continued)

	Clinical features	Microscopic hematuria	Macroscopic hematuria	Proteinuria	ESRD	Hearing loss
	Subgroup of patients according to the genotype and ethnic group	No. of pts. (% in the subgroup)	No. of pts. (% in the subgroup)	No. of pts. (% in the subgroup)	No. of pts. (% in the subgroup)	No. of pts. (% in the subgroup)
6.	COL4A4 p.Gly533Asp heterozygous Non-Romani	<i>n</i> = 12				
6.1	Feature: Yes	11 (92)	0	2 (17)	0	2 (17)
6.2	Feature: No	1 (8) ^k	12 (100)	10 (83) ^l	12 (100)	10 (83)
6.3	Feature: Information not available	0	0	0	0	0
6.4	Median age at feature diagnosis (years) (range) ^a	9.0 (4–30)	n.a.	26.5 (23–30)	n.a.	3 (3)
	Statistics 6.1 vs. 5.1 (Fisher's exact test)	<i>p</i> = 1.000	<i>p</i> = 1.000	<i>p</i> = 0.287	<i>p</i> = 1.000	<i>p</i> = 0.600
	Statistics 6.4 vs. 5.4 (Mann-Whitney <i>U</i> test)	<i>p</i> = 0.528	n.a.	n.a.	n.a.	n.a.
7.	COL4A3 p.Gly139Arg heterozygous Romani	<i>n</i> = 13				
7.1	Feature: Yes	11 (92)	1 (8) ^m	2 (17) ⁿ	0 (1 acute transient renal failure) ^o	1 (8) ^p
7.2	Feature: No	1 (8) ^q	11 (92)	10 (83)	12 (100)	11 (92)
7.3	Feature: Information not available	1	1	1	1	1
7.4	Median age at feature diagnosis (years) (range) ^a	14.0 (5–41)	6 (6)	31.0 (5–57) ^r	n.a.	n.a.
	Statistics 7.1 vs. 5.1 (Fisher's exact test)	<i>p</i> = 1.000	<i>p</i> = 0.282	<i>p</i> = 0.287	<i>p</i> = 1.000	<i>p</i> = 1.000
	Statistics 7.4 vs. 5.4 (Mann-Whitney <i>U</i> test)	<i>p</i> < 0.0001	n.a.	n.a.	n.a.	n.a.
	Statistics 7.1 vs. 6.1 (Fisher's exact test)	<i>p</i> = 1.000	<i>p</i> = 1.000	<i>p</i> = 1.000	<i>p</i> = 1.000	<i>p</i> = 1.000
	Statistics 7.4 vs. 6.4 (Mann-Whitney <i>U</i> test)	<i>p</i> < 0.0001	n.a.	n.a.	n.a.	n.a.

The above table shows the occurrence of clinical features of Alport syndrome in the patients with respect to the different types of AS, including AR AS, digenic AS and AD AS, to the particular genetic variant and ethnic type of population—Romani, one parent Romani and non-Romani. The recorded features include microscopic and macroscopic hematuria, proteinuria, end-stage renal disease, and hearing loss. The age of onset of each of the features was documented whenever possible. AR AS patients were divided into three groups according to the genotype. The occurrence and age of onset of the features was statistically analyzed between the patients with homozygous p.Gly533Asp *COL4A4* variant (subgroup 1, *n* = 20) and the patients with homozygous p.Gly139Arg *COL4A3* variant (subgroup 3, *n* = 7) using Fisher's exact test, and Mann-Whitney *U* test, respectively. AR AS due to compound heterozygous *COL4A4* variants (subgroup 2, *n* = 2) could not be included in the statistical analysis because of the low number of patients. Digenic AS (subgroup 4) was found in only one patient, thus statistical analysis was also not possible. Patients with AD AS were divided into three groups. The data from Romani patients with heterozygous p.Gly139Arg *COL4A3* variant (subgroup 7, *n* = 13) were compared with those of Romani plus one parent Romani patients with heterozygous *COL4A4* p.Gly533Asp variant (subgroup 5, *n* = 70) and then to those of non-Romani patients with heterozygous *COL4A4* p.Gly533Asp (subgroup 6, *n* = 12). Again, the occurrence of the features was statistically analyzed using Fisher's exact test, and the Mann-Whitney *U* test was used to compare the ages of onset of the features between the groups. As regards results of statistical analyses, a *p*-value less than 0.05 was considered significant (bold).

^aInformation not available in all pts.

^bTransient, isolated attack accompanying a respiratory infection.

^cGross proteinuria at 11 years, renal biopsy disclosed focal glomerulosclerosis.

^dAge 14 years.

^e3 pts without ESRD at 25, 33, and 54 years.

^fSince 9 years, no proteinuria.

^g4 of them were members of the same family, 2 of them isolated proteinuria and ESRD at 18 and 61 years; other concurrent genetic etiology is possible but was not disclosed; NGS kidney gene panel of 462 genes testing did not reveal any other pathogenic variant in the pts with ESRD at 18 and 26 years.

^hThe pts. with ESRD at 18 and 61 years are relatives (see g).

ⁱThe pt with isolated proteinuria and ESRD at 18 years (see g, h)—hearing loss since 14 years; other etiology of hearing loss is probable in two siblings with moderate hearing loss since 1 year and in the other two patients.

^j4 pts without features of AS at 3, 7, 29 and 40 years, 2 pts isolated proteinuria with ESRD (see g).

^kWithout features of AS at 20 years.

^lIn 1 pt, transient PU in pregnancy at 29 years.

^mTransient, isolated attack.

ⁿOne of the pts also had transient macroscopic hematuria (see m); NGS kidney gene panel of 462 genes testing did not reveal any other pathogenic variant in the pt.

^oThe patient developed transient acute kidney failure at 41 years after long-term physical and psychological overload; 3 months later, isolated microscopic hematuria was present in this patient; he has persistent isolated microscopic hematuria and normal kidney function 1 year after the acute kidney failure episode.

^pOther etiology of hearing loss is probable

^qWithout features of AS at 6 years.

^rThe 57-year-old pt was treated for diabetes mellitus for 30 years.

ESRD, end-stage renal disease; pts, patients; AR AS, autosomal recessive Alport syndrome; AD AS, autosomal dominant Alport syndrome; years, years; m, months; n.a., non-applicable.

we have found that Romani represent 76% of all individuals with AR AS diagnosed in our laboratory and that this is due to two founder pathogenic variants and a high rate of consanguinity by descent.

Our data are similar to those observed in Slovakia, where Kolvek et al. frequently treated Romani children with ESRD (7). Although the estimated Romani population in Slovakia is at most 7% (8), 11 of their 14 patients with AS (79%) were Romani (7). They, too, hypothesized

TABLE 4 Summary of renal biopsy histology and $\alpha 3$, $\alpha 5$ chain immunofluorescence staining in patients with autosomal recessive Alport syndrome with respect to the genotype.

Renal biopsy details							
Genotype	Renal biopsy report available no. of pts. (%)	TBMN, fusion of podocytes no. of pts. (% of pts. with renal biopsy report)	Thickening and splitting of the GBM no. of pts. (% of pts. with renal biopsy report)	Focal segmental glomerular sclerosis no. of pts. (% of pts. with renal biopsy report)	Mesangio-proliferative glomerulonephritis no. of pts. (% of pts. with renal biopsy report)	$\alpha 3$ chain immunofluorescence staining no. of pts. (% of pts. with renal biopsy report)	$\alpha 5$ chain immunofluorescence staining no. of pts. (% of pts. with renal biopsy report)
<i>COL4A4</i> c.1598G>A, p.Gly533Asp homozygous	7/20 (35)	2/7 (28)	2/7 (28)	2/7 (28)	1/7 (14)	3/7 ^{1a,b,c} (43)	2/7 ^{1a,b} (29)
<i>COL4A4</i> c.1598G>A, p.Gly533Asp + c.3707G>A, p.Gly1236Glu	1/1 (100)	0/1	0/1	0/1	1 ² /1 (100)	0/1	0/1
<i>COL4A4</i> c.1598G>A, p.Gly533Asp + c.3514_3515delinsTGAAA, p.Gly1172Ter	0/1	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
<i>COL4A3</i> c.415G>C, p.Gly139Arg homozygous	0/7	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

^{1a}1st sample: $\alpha 3$: apparent defects of positivity in glomeruli, some glomeruli completely negative, Bowman's capsule and tubular basement membranes (BM) negative, $\alpha 5$: glomerular BM negative, Bowman's capsule BM positive, tubular BM only focally positive. ^{1b}2nd sample: $\alpha 3$: low expression intensity or complete defects of positivity in capillary glomerular loops, Bowman's capsule BM positive, $\alpha 5$: staining not performed. ^{1c}3rd sample: $\alpha 3$ negative, $\alpha 5$ negative. ²segmental glomerular sclerosis also detected. Pts, patients; TBMN, thin basement membrane nephropathy; GBM, glomerular basement membrane; BM, basement membrane.

that this resulted from a founder effect (25). In an earlier report, AS has been reported in 14 families from Eastern Slovakia where about one in four were Romani (6). It is likely that p.Gly533Asp was responsible for many of their patients, as well as those reported in the UK (21) and the Netherlands (22) where Romani is also found. This variant has already been reported ten times in LOVD (22), which suggests that it is relatively common in Europe.

Based on our data, the estimated prevalence of AR AS in the Czech Romani population is at least 1:11,000 due to these founder variants. Thus, the corresponding heterozygous variants and AD AS occur in about 1% of the Romani population according to the Hardy–Weinberg equation. This is equivalent to the population frequency of AD AS due to multiple different variants in other populations. The true prevalence is likely to be even greater since there may be other less common variants also. Thus, individuals with heterozygous variants may be undetected. However, they too have a risk of hypertension, proteinuria, and renal impairment, and, even if small, the risk of ESRD should be assessed and monitored (26). The demonstration of p.Gly533Asp in *COL4A4* in the non-Romani population suggests that it will be widespread in Romani and non-Romani throughout Europe and maybe also in North America and Australia, wherever there has been Romani immigration.

Only two pathogenic variants were detected in the Romani in our study. The c.1598G>A variant in *COL4A4* corresponding to p.Gly533Asp was nearly four times more common than the c.415G>C variant in *COL4A3* corresponding to p.Gly139Arg.

Two individuals with AR AS due to homozygous variants have been found in the UK (21), and this variant was also found in Czech people not known to be Romani. Based on our data, the *COL4A4* variant described here is the commonest cause of AR AS at least in the Czech Republic. Another variant c.3114C>G, p.Ser969Ter in *COL4A4* has been reported frequently in British people causing not only AR AS but also AD AS in the heterozygous form (27).

The p.Gly139Arg in *COL4A3* has not been reported previously. There was only one individual with both founder variants, which suggests that these occurred in different subpopulations of the Romani people. Another explanation is that the p.Gly139Arg is uncommon. This variant was only found in Romani. In Romani, AR AS was caused only by homozygous *COL4A4* c.1598G>A, whereas in non-Romani, c.1598G>A was found solely in combination with another pathogenic variant. This suggests the mixing of the Romani and Czech populations.

The degree of consanguinity in the Romani population may be higher than suspected because their population has been a relatively small community in the Czech Republic (7). This is important for clinicians to remember when undertaking genetic counseling for these families, and genetic testing and preconception analysis should be offered to the spouse or partner of a Romani who has the p.Gly533Asp or p.Gly139Arg variant.

The published data suggest that missense variants in *COL4A3* and *COL4A4* genes have a less severe phenotype than loss-of-function,

splice site variants, or large deletions. Individuals with two truncating variants have an earlier onset of kidney failure or hearing loss than those with only one truncating variant, who are in turn more likely to develop ESRD than those with no truncating variant (5, 21, 28, 29). However, this genotype-phenotype correlation is not always seen (30).

The Romani cohort represents the opportunity to study modifying factors that worsen disease severity in AD AS. Overall the median age for the *COL4A4* variant was similar to that reported previously. However, both intra- and interfamilial variability in age at kidney failure has already been described (5, 31, 32), and was also seen here, with the age at ESRD varying from 17 to 42 and with normal renal function in another affected individual.

There are several large studies and a meta-analysis of genotype-phenotype analyses in individuals with AD AS (30–33). The likelihood of isolated microscopic hematuria, macroscopic hematuria, ESRD, and hearing loss in this cohort is similar to previous reports, but proteinuria occurred less often (5, 31). Early onset ESRD has been reported in AD AS, but it is rare and may result from coincidental diseases. For instance, in a meta-analysis of 777 individuals with AD AS, there were four patients with ESRD before the age of 25 years (31).

Overall, the variant features that are associated with a higher penetrance of hematuria are known. Both the Gly substitutions reported here are highly destabilizing variants, namely, Arg and Asp, and are consistent with an increased risk of hematuria (33).

Other AR diseases also occur in Romani including neuropathy, myopathy, and hearing loss (9, 34–36). It has been suggested that newborn screening for these diseases should be performed in Romani because of the sometimes 5% carrier rates (9).

In conclusion, this study identified two founder pathogenic variants, p.Gly533Asp in *COL4A4* and p.Gly139Arg in *COL4A3* in the Romani population. These variants explain the high proportion of Romani people among the Czech cohort with features suggesting AS. The estimated population frequency of AR AS from these variants is at least 1:11,000 in the Czech Romani. This corresponds to a population frequency of AD AS from these two variants alone of 1%. Our data suggest that consanguinity by descent is common in the Romani. Romani with persistent hematuria should be offered genetic testing, and preconception genetic testing should be offered to the partners of Romani who have one of these founder variants.

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 Institutional Ethics Committee of the University Hospital Ostrava. 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) for the publication of any potentially identifiable images or data included in this article.

Author contributions

PP, JI, PK, PT, DC, SH, MK, DP, RJ, ML, HJ, JL, JD, JG, MV, PS, GK, EK, JS, RS, JZ, TT, and DT performed the material preparation, data collection, and analysis. JS helped to analysis and writing. PP wrote the first draft of the manuscript and was the principal investigator of IP/RVO-FNOs/2015. DT was the principal investigator of NV19-06-00443. All authors contributed to the study conception and design, commented on previous versions of the manuscript, read, and approved the final manuscript.

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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.

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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.2023.1096869/full#supplementary-material>

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