https://doi.org/10.15388/vu.thesis.506
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Genotype – Phenotype Correlation and Prognostic Impact of Collagen IV α345 Nephropathies

DOCTORAL DISSERTATION

Medicine and Health Sciences,
Medicine (M 001)

VILNIUS 2023

The dissertation was prepared between 2018 and 2022 at Vilnius University Faculty of Medicine.

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The text of this dissertation can be accessed at the library of Vilnius University, as well as on the website of Vilnius University:
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VILNIAUS UNIVERSITETAS

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Kolageno IV alfa 345 nefropatijų genotipo – fenotipo sąsajos ir prognostiniai veiksniai

DAKTARO DISERTACIJA

Medicinos ir sveikatos mokslai,
Medicina (M 001)

VILNIUS 2023

Disertacija rengta 2018–2022 metais Vilniaus universiteto Medicinos Fakultete.

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Disertacija giname viešame Gynimo tarybos posėdyje 2023 m. rugsėjo mėn. 13 d. 14:00 val. VšĮ Vilniaus universiteto ligoninės Santaros Klinikos Raudonojoje auditorijoje (E122)
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Disertaciją galima peržiūrėti VU bibliotekoje ir VU interneto svetainėje adresu: *https://www.vu.lt/naujienos/ivykiu-kalendorius*

ABREVIATIONS

ACMG – The American College of Medical Genetics and Genomics
ADAS – autosomal dominant Alport syndrome
ADPKD – autosomal dominant polycystic kidney disease
AH – arterial hypertension
AMP – Association for molecular pathology
ARAS – autosomal recessive Alport syndrome
ARB – angiotensin receptor blocker
Arg – arginine
AS – Alport syndrome
Asn – asparagine
Asp – aspartic acid
BFH – benign familial haematuria
BM – basement membrane
Bn – benign
BP – blood pressure
CKD – chronic kidney disease
CKD-EPI – Chronic Kidney Disease Epidemiology Collaboration
COL4 – type IV collagen
COL4A1 – type IV collagen α1 chain gene, and other genes accordingly
COL4A3-A5 – *COL4A3, COL4A4* and *COL4A5* genes
Cys – cysteine
DNA – Deoxyribonucleic acid
EBM – epidermal basement membrane
ECM – extracellular matrix
eGFR – estimated glomerular filtration rate
EM – electron microscopy
F – female
FH – family history
Fig – figure
FPE – foot process effacement
FSGS – focal segmental glomerulosclerosis
GBM – glomerular basement membrane
GFB – glomerular filtration barrier
Glu – glutamic acid
Gln – glutamine
Gly – glycine
His – histidine

HL – hearing loss
IF – immunofluorescence
Ile – isoleucine
KB – kidney biopsy
KF – kidney failure
KRT – kidney replacement therapy
LBn – likely benign
Leu – leucine
LM – light microscopy
LP – likely pathogenic
Lys – lysine
M – male
Met – methionine
NGS – next generation sequencing
OCT – optical coherence tomography
ORL – otorhinolaryngologist
P – pathogenic
Phe – phenylalanine
Pro – proteinuria
RAAS – renin angiotensin aldosterone system
RBC – red blood cells
R – recessive
SGLT2 inhibitors - sodium-glucose cotransporter-2 inhibitors
Ser – Serine
TBMD – thin basement membrane disease
TBM – thin basement membrane
Thr – threonine
Trp – tryptophan
Tyr – tyrosine
Val – valine
VUH SK – Vilnius University Hospital Santaros Klinikos
VUS – variant of uncertain significance
XLAS – X-linked Alport syndrome

CONTENTS

1. INTRODUCTION 11

1.1. Brief introduction 11
1.2. Aim and objectives 12
1.3. Defended statements 12
1.4. Significance and scientific novelty of the study 13

2. LITERATURE REVIEW 14

2.1. Type IV collagen 14
2.2. Structure of type IV collagen 14
2.3. Basement membranes 16
2.4. Distribution of the BM in the human body 17
2.5. Glomerular basement membrane 17
2.6. Type IV collagen genes 18
2.7. Role of type IV collagen in diseases 18
2.8. Alport syndrome 19
2.9. Epidemiology and prevalence of Alport syndrome 19
2.10. Pathogenesis of Alport syndrome 20
2.11. Genetics of Alport syndrome 21
2.12. Clinical features of Alport syndrome 24
2.13. Diagnostics of Alport syndrome 25
2.14. Genotype – phenotype correlations in Alport syndrome 26
2.15. Treatment of Alport syndrome 27
2.16. Differential diagnosis of Alport syndrome 32

3. METHODS 34

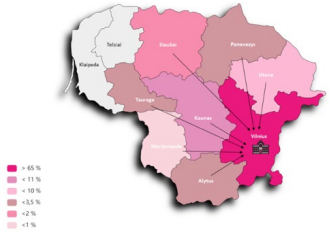
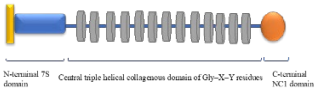
3.1. Study organization 34
3.2 Ethical statement 36
3.3. Study design and setting 36
3.4. Molecular analysis and genetic data 41
3.5. Statistical analysis 42

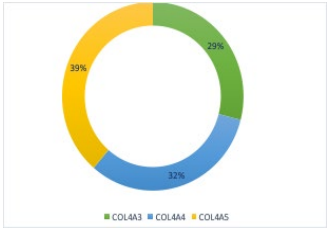
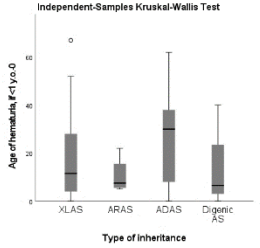
4. RESULTS 45

4.1. Distribution and diagnostic screening of AS 45
4.2. General and clinical characteristics of individuals with AS 46
4.3. Molecular characteristics of changes in *COL4A3-A5* genes 52
4.3.1. General characteristics 52
4.3.2. Novel genetic changes 56
4.3.3. Shared *COL4A3-A5* variants with general population 67

4.4. Genotype-phenotype correlations and clinical characteristics of individuals with AS 70
4.4.1. Comparison between different types of AS 70
4.4.2 Genotype-phenotype characteristics of individuals with XLAS 72
4.4.3. Genotype-phenotype characteristics of individuals with ARAS 80
4.4.4. Genotype-phenotype characteristics of individuals with ADAS 82
4.4.5. Genotype-phenotype characteristics of individuals with digenic AS 90
5. DISCUSSION 93
6. CONCLUSIONS 106
7. PRACTICAL RECOMMENDATIONS 107
8. LIMITATIONS 110
SANTRAUKA LIETUVIŲ KALBA 111
REFERENCES 150
SUPPLEMENTS 163
ABOUT THE AUTHOR 185
PUBLICATION LIST 186
PRESENTATION LIST 187
AKNOWLEDGEMENTS 189

DISERTATION AT – A – GLANCE

The aim of this thesis is to evaluate the diagnostic yield, molecular characteristics, genotype – phenotype associations and risk factors for progression of collagen IV α345 nephropathies.				
Graphics	Aims	Methods	Conclusions	Practical insight
 *(n=114 AS from 221 suspected)*	To assess the diagnostic yield of Alport syndrome in the Lithuanian cohort.	AS screening algorithm: pathognomonic features in kidney biopsy, family pedigree and clinical features (ex. persistent hematuria).	Molecular analysis of the *COL4A3-A5* gene is the first-line diagnostic method for suspected AS. The diagnosis was confirmed in more than half of the individuals with persistent microscopic hematuria. Renal biopsy was performed in 43% of AS subjects with advanced CKD. Renal biopsy was significantly less common in children than in adults.	Indication for following epidemiological studies of AS in LT. AS diagnostic algorithm was proposed.
 *Published: please see Ref. No. [88]. International cohort.*	To evaluate molecular characteristics of pathogenic variants in *COL4A3-A5* genes and to determine its impact on disease progression.	DBS cards, blood sample collection, DNA extraction, NGS. Prediction tool of pathogenicity: MutationTaster, PolyPhen-2 and SIFT.	Molecular features including being adjacent to a non-collagenous region, degree of instability caused by the replacement residue of Gly substitutions of *COL4A3-A5* contribute to the clinical severity and disease progression in AS.	The evaluation of variant pathogenicity should include clinical features, localization, and the type of genetic variant to enable early initiation of RAAS inhibitors.
	To perform the clinical characteristics of novel *COL4A3-A5* variants found in Lithuanian cohort.	Evaluation of ACMG/AMP criteria and comparison with ClinVar, Varsome, LVOD, Franklin, InterVar, Centogene databases.	After analysing the *COL4A3-A5* genes of 221 individuals using NGS, we identified 44 different genetic changes. Among these changes, 28 variants (63.6%) were considered new and had not been previously	Identifying novel variants increases the data in global genetic databases and helps researchers better understand the disease

The aim of this thesis is to evaluate the diagnostic yield, molecular characteristics, genotype – phenotype associations and risk factors for progression of collagen IV α345 nephropathies.				
Graphics	Aims	Methods	Conclusions	Practical insight
 *Published: please see Ref. No. [118].*		Insertion of novel variants into LVOD and ClinVar databases.	described. Genotype-phenotype correlation analysis revealed that some variants exhibited phenotypic variation within the affected families. Furthermore, over a third of the genetic changes found were shared with the global population and had been documented in international genetic databases.	manifestations and develop new clinical trials and targeting therapies.
 *Digenic AS was evaluated within international cohort. Published: please see Ref. No. [85].*	To stratify the risk of CKD progression among different types of Alport syndrome.	Description of genotype-phenotype correlation.	Molecular analysis is the gold standard for assessing the risk of AS progression. Males with XLAS and individuals with ARAS have the highest risk of CKD progression, while individuals with ADAS, females with XLAS, and individuals with digenic AS have a varying risk of KF ranging from moderate to mild, unless proteinuria is present.	Therapy with RAAS inhibitors is recommended for XLAS males, individuals with ARAS, or digenic AS at the time of diagnosis and for XLAS females and ADAS at the presence of proteinuria. Kidney donation of individuals with AS is not recommended.

AS – Alport syndrome; CKD – chronic kidney disease; KF – kidney failure; XLAS – X-linked AS; ARAS – autosomal recessive AS; ADAS – autosomal dominant AS; DBS – dry blood spot, KB – kidney biopsy; NGS – next generation sequencing; LT – Lithuania; RAAS – renin angiotensin aldosterone system

1. INTRODUCTION

1.1. Brief introduction

Collagen IV α345 nephropathy, commonly known as Alport syndrome (AS), is the second most common cause of inherited kidney failure (KF), following autosomal dominant polycystic kidney disease (ADPKD) (1). AS is associated with approximately 1–2% of all cases of kidney failure (KF) (2). It is characterized by glomerular microhematuria, proteinuria, and progressive chronic kidney disease (CKD) that eventually leads to KF. Additionally, extrarenal manifestations may also be observed (3).

The *COL4A3, COL4A4* and *COL4A5 (COL4A3-A5)* genes encode the α3, α4, and α5 chains of collagen IV (COL4), respectively, which are crucial components of the basement membrane (BM) in the glomerulus, cochlea, lens, and retina. Consequently, pathogenic variants in *COL4A5* typically lead to X-linked Alport syndrome (XLAS), while alterations in *COL4A3* and *COL4A4* genes are associated with autosomal dominant (ADAS) or autosomal recessive AS (ARAS) (4–6). There is also evidence of digenic inheritance in AS due to the transmission of variants in two of the three *COL4A3-A5* genes (7). The prevalence of AS ranges from 1 in 5,000 to 1 in 53,000 in the general population (8,9). However, recent studies have revealed that the prevalence of AS is at least one in 2,000 for the X-linked form and one in 100 for ADAS (10). The implications of these findings indicate that numerous variants may remain unnoticed, and even individuals who show no obvious symptoms could possess pathogenic variants in the *COL4A3-A5* genes (11). The presentation of AS can be influenced not only by the affected gene but also by various genetic alterations, including missense, nonsense, splice site, or frameshift variants within the *COL4A3-A5* genes. Various genetic alterations contribute to a broad spectrum of kidney disease courses, influencing the age of onset of KF and the occurrence of extrarenal manifestations (12–17). Early detection of AS in individuals with persistent hematuria and proteinuria has a substantial effect on clinical outcomes. Several studies have demonstrated significant improvement in kidney function among AS individuals through early initiation and long-term use of renin-angiotensin-aldosterone system (RAAS) blockade agents (18–20). Accurate diagnosis, prognosis, genetic counselling, and treatment options can be facilitated by molecular analysis of the *COL4A3-A5* genes. A recent consensus statement emphasized that a combination of clinical features, family history of KF or persistent hematuria,

and identification of specific genetic variants provide the strongest evidence for diagnosing AS (21).

Insufficient data on AS in various countries has resulted in a lack of knowledge regarding numerous potential variants of the genes linked to AS. Genetic diagnosis plays a crucial role in understanding the disease burden, determining the epidemiological prevalence of AS, and guiding treatment and prognosis. Therefore, it is important to increase the identification of disease-causing variants and understand their clinical phenotypes. This study aims to evaluate diagnostic yield, genotype-phenotype correlations, prognostic impact, and genetic distribution in a large Lithuanian cohort with AS, marking the first such study in Lithuania so far. The longitudinal and standardized approach of this study is expected to provide robust evidence regarding the variability of genetic variants in the *COL4A3-A5* genes and their prognostic implications compared to other countries. Through a detailed analysis of genetic variations and molecular pathology, this study will contribute to more comprehensive molecular research and tailored diagnostic approaches for individuals in Lithuania with a clinically suspected AS.

1.2. Aim and objectives

The aim of this thesis is to evaluate the diagnostic yield, molecular characteristics, genotype – phenotype associations and risk factors for progression of collagen IV α345 nephropathies.

Objectives:

1. To assess the diagnostic yield of Alport syndrome in the Lithuanian cohort.
2. To evaluate molecular characteristics of pathogenic variants in *COL4A3-A5* genes and to determine its impact on disease progression.
3. To perform the clinical characterization of novel *COL4A3-A5* variants found in Lithuanian cohort.
4. To stratify the risk of CKD progression among different types of Alport syndrome.

1.3. Defended statements

1. Molecular analysis is essential for diagnosing AS and indicating the risks of progression, associated with genetic background.
2. Molecular features of *COL4A3-A5* contribute to the clinical severity and disease progression in AS.

3. Novel and previously unreported variants detected in the *COL4A3-A5* genes in the Lithuanian families distinct from those observed in other populations.
4. Risk of CKD progression in individuals with disease causing variants in *COL4A3-A5* genes differs among different types of AS and gender.

1.4. Significance and scientific novelty of the study

The growing number of population-based scientific publications and clinical trials focusing on therapeutic options for individuals with genetically confirmed AS has emphasized the importance of identifying disease-causing variants in the *COL4A3-A5* genes, responsible for collagen IV α345 nephropathies, in the Lithuanian population. While many treatment studies target inflammation and fibrosis, common lesions in other chronic kidney diseases, AS holds promise as a valuable model for exploring potential drugs in other CKD-associated conditions. This is particularly advantageous considering that individuals with AS are typically young and have fewer comorbidities, which can greatly facilitate clinical trials (22). Molecular *COL4A3-A5* analysis can provide crucial information not only for therapeutic options but also for diagnosis, prognosis, and genetic counselling. Although this diagnostic method is no longer considered innovative, performing genetic testing for *COL4A3-A5* genes in a previously unscreened Lithuanian population was expected to uncover new insights into the molecular pathology of collagen IV α345 nephropathies due to their allelic heterogeneity and the variability observed across different populations. The identification of both known and novel *COL4A3-A5* variants in this study has significant implications. It extends our understanding of genotype-phenotype correlations, enriches global genetic databases for new drug development of industrial drug companies, elucidates the molecular mechanisms underlying collagen IV α345 nephropathies, and enables practical applications in clinical settings. These applications include accurate clinical diagnosis, minimizing the need for invasive procedures, selecting appropriate treatments, including the disease course modifying agents to manage the risk of CKD progression, monitoring individuals with AS, and providing genetic counselling for identifying at-risk family members. Moreover, a clinical diagnosis of AS helps prevent asymptomatic individuals with AS from donating their kidneys, thereby avoiding an increased risk of developing CKD later in life. Additionally, a confirmed genetic diagnosis of AS plays a crucial role in family planning, allowing discussions on pregnancy, reproductive issues, and the assessment of the risk of having children affected by AS.

2. LITERATURE REVIEW

2.1. Type IV collagen

Type IV collagen is a protein molecule that belongs to the collagen superfamily, which comprises at least 28 different types of proteins. These proteins play a vital role in providing structural support to the human body by forming extracellular fibbers and network structures (23). Due to its stiffness and resistance to stretching, collagen IV serves as an ideal matrix and backbone for BM. It forms a cross-linked network that is connected to laminin, nidogen, and perlecan (24,25). Collagen IV is a crucial carrier of signalling molecules, playing a vital role in various physiological and pathological functions of the BM (26,27).

2.2. Structure of type IV collagen

Collagen IV is a tertiary structured triple helical molecule containing of three α chains. Six different α chains, α1 to α6, encoded by six genes *COL4A1* to *COL4A6,* respectively (28), have been identified (29,30). Each α chain of collagen IV consists of three distinct domains: a N-terminal 7S-domain rich in cysteine (Cys) and lysine (Lys), a central collagenous domain with repeated Gly–X–Y residues (where X and Y represent any amino acids) interrupted by short non-collagenous (NC) domains, and a C-terminal non-collagenous (NC1) domain composed of approximately 230 amino acids (30) (Fig. 1).

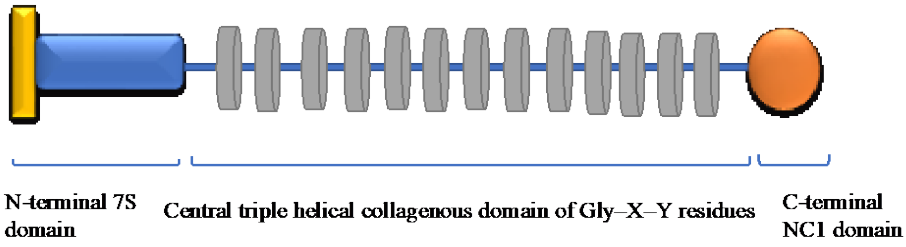


Figure 1. Schematic structure of α1-6 chains in type IV collagen.

The presence of glycine as every third amino acid in the central collagenous domain is crucial because this small residue can fit into the collagen IV triple helix. Additionally, the small interruptions in the NC1 region between the Gly-X-Y repeats can act as specific sites for cell binding and crosslinking between the α chains. This process leads to a less rigid

structure of collagen IV, providing flexibility and enabling the formation of a network (31).

Three out of the six α chains come together to form a heterotrimeric helix through the assembly of their NC1 domains, while the 7S domains are responsible for the covalent construction of heterotrimers, creating a complex mesh-like network that serves as the foundation for the basement membrane (32). To date, three different combinations of α chains, also known as protomer isoforms, have been characterized: α1α1α2, α3α4α5, and α5α5α6 (Fig. 2).

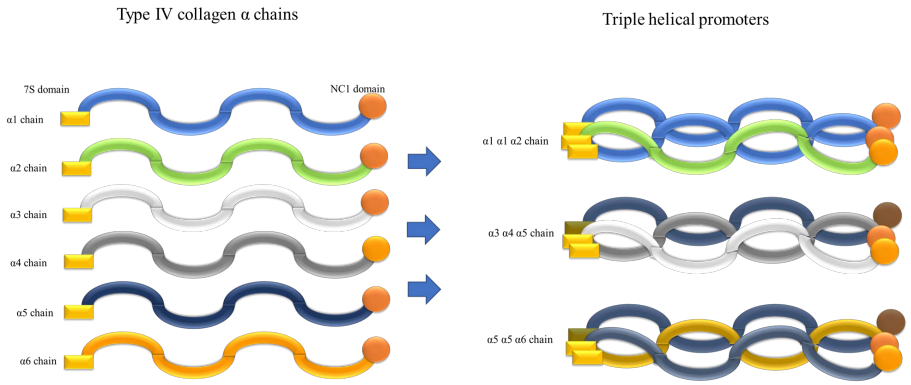


Figure 2. Schematic structure of type IV collagen and its isomers α1α1α2, α3α4α5, and α5α5α6. Adapted by J.M.B. Sand 2019 (27)

The 7S domains of four protomers, which are heavily glycosylated for better resistance to collagenase activity, connect to each other by forming a 7S tetramer. Meanwhile, the NC1 domains of two protomers assemble in an end-to-end fashion, forming an NC1 dimer (33–35). Finally, the presence of disulfide bonds and lysine-hydroxylysine crosslinks between cysteine and lysine residues in the 7S tetramers results in the typical lattice-shaped network structure of type IV collagen. This structure contributes to the formation of stable and tightly bound basement membranes (36) (Fig. 3).

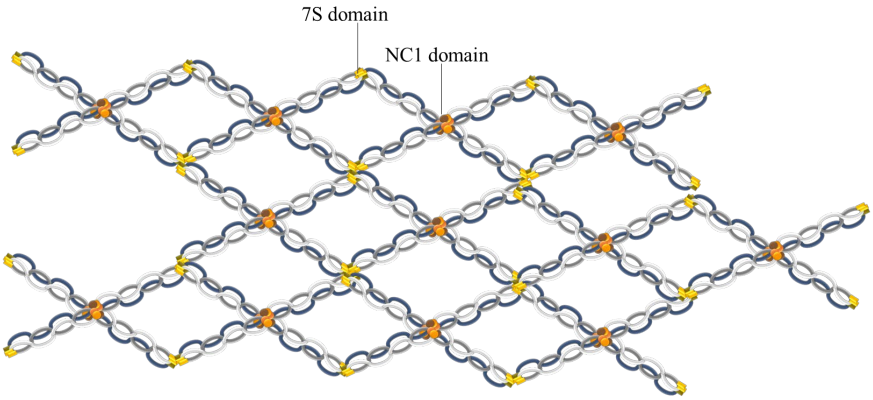


Figure 3. Schematic illustration of structure of network in type IV collagen. Adapted by J.M.B. Sand 2019 (27)

2.3. Basement membranes

Type IV collagen is a major and highly significant structural component of basement membranes. BMs, also known as basal laminae, are thin structures in the extracellular matrix (ECM) with a thickness ranging from 50-350 nm (typically 60-80 nm). They surround different cells and tissues, serving as an anatomical barrier for cells of various origins and providing physical support to the tissues (37,38). In addition to type IV collagen, the basement membrane is composed of various other components, such as laminin, nidogen, proteoglycans, and other minor elements (39). Laminin is a major non-collagenous heterotrimeric molecule found BM. It possesses a cross-shaped structure formed through the assembly of α, β and γ chains. The unique construction of laminin isoforms supports many biological functions, including attachment, proliferation, and differentiation of the cells (40). Nidogen is a glycoprotein with a dumbbell-shaped structure that plays a crucial role in connecting the networks of type IV collagen and laminin. It also supports other components, including perlecan and fibulins, within the basement membrane (24,39,41). Other structural components of the BM include proteoglycans, such as heparan sulphate (agrin), perlecan, and chondroitin sulphate, as well as integrins and other molecules (39). The BM participates in various cellular processes, including development, attachment, migration, survival, apoptosis, signalling, and polarization of cells. It also plays an important role in tissue repair and regeneration, and serves as a storage site for enzymes, signalling molecules, and plasma proteins (39). The BM in glomeruli serves an additional essential role by acting as a molecular mesh that selectively eliminates small molecules from the blood (42).

2.4. Distribution of the BM in the human body

The six α chains of collagen IV, including their isomers α1α1α2(IV), α3α4α5(IV), and α5α5α6(IV), exhibit significant differences in tissue distribution throughout the human body (27). The α1α1α2(IV) isomer of collagen IV is predominantly present in all tissues, while other isomers show tissue-specific and limited distribution. The α3α4α5(IV) isomer is mainly localized in the BM of glomeruli, alveoli, testis, eye, and inner ear. In contrast, α5α5α6(IV) is found in the BM of the skin, bronchial epithelium cells, smooth muscle, and Bowman's capsule, along with the tubular membranes in the kidneys (27,30,43). During the embryonic period, the premature and ubiquitous α1α1α2(IV) isomer is replaced by α3α4α5(IV) and α5α5α6(IV) in the specific tissues mentioned earlier for further development. The differentiation of the BM from the α1α1α2(IV) network to the mature forms, including α3α4α5(IV), leads to better adaptation to increased stress, likely due to a higher degree of disulfide crosslinking, providing stronger resistance to proteolysis (44).

2.5. Glomerular basement membrane

The molecular structure of the glomerular basement membrane (GBM), which is one of the most studied BMs, is very similar to that of other BMs, with collagen IV being its major component. The GBM is located between podocytes, which are specialized cells in the kidneys, and endothelial cells. Together, they form the glomerular filtration barrier (GFB), which selectively eliminates water and small molecules into the urinary space while retaining macromolecules and cells in the circulation (42). The presence of proteinuria and hematuria indicates dysfunction of the GBM. Within and surrounding glomeruli, additional matrix components are identified, including the BM of the Bowman’s capsule and the mesangial matrix. The mesangial matrix, produced by mesangial cells that connect the capillary loops in the glomeruli, differs from the typical sheet-like structure of the BMs but contains the same major elements of the BM (42,45). The structure and major components of the GBM were initially described using ultrastructural and biochemical analyses (45,46), however recent studies of mass spectrometry-based proteomics, have demonstrated that GBM differs from BMs in other tissues (47). Most importantly, the replacement of the α1α1α2 (IV) isomers, which are found in most tissues, with the mature α3α4α5 network of type IV collagen is crucial for the specific functioning of the glomerular barrier. In addition, minor elements such as nephronectin and fibulin 1 contribute to the composition of

the GBM. These and other potentially undiscovered differences between the GBM and other basement membranes may provide valuable knowledge for future therapeutic approaches (47).

2.6. Type IV collagen genes

Collagen IV α1-6 chains are encoded by *COL4A1-6* genes, respectively. Human collagen IV genes are organized in three pairs with head-to-head orientation: *COL4A1* and *COL4A2* on chromosome 13, *COL4A3* and *COL4A4* on chromosome 2, and *COL4A5* and C*OL4A6* on chromosome X (Table 1) (29). The α1-4 chains are encoded by autosomal chromosomes, while α5-6 by the sex chromosome X.

Table 1. Characteristics of the six type IV collagen genes. *According to ncbi.nlm.nih.gov/gene, last review January 2023*

Gene	Chromosome	Transcript	Genetic locus	Size of the gene (kb)	Number of exons	Protein (α chain)
COL4A1	13	NM_001845.6	13q34	<100	53	α1
COL4A2	13	NM_001846.4	13q34	<100	48	α2
COL4A3	2	NM_000091.3	2q36	~250	54	α3
COL4A4	2	NM_000092.4	2q36	>113	57	α4
COL4A5	X	NM_033380.1	Xq22	250	57	α5
COL4A6	X	NM_001847.4	Xq22	~425	50	α6

Several studies of sequence analyses from mammalian species have demonstrated the presence of a common ancestral gene that, through three successive gene duplications, has evolved into six related genes (29). Based on sequence similarity, the genes can be classified into two groups: the α1-like group, which includes *COL4A1, COL4A3,* and *COL4A5* genes, and the α2-like group, which comprises *COL4A2, COL4A4,* and *COL4A6* genes. Both groups exhibit specific exon-intron organization, indicating their evolutionary relationships (30). The paired genes of collagen IV possess a unique feature that sets them apart from other collagen families. This feature is characterized by bidirectional promoters, which enable the regulation of the expression of two different collagen IV chains in a cell-specific manner (48,49).

2.7. Role of type IV collagen in diseases

Increased knowledge of molecular structure and function of the BM has expanded the understanding of genes and gene defects associated with the

pathogenetic mechanisms in many inherited diseases. Since type IV collagen is the major structural component of the BM, many linked disorders, including collagen IV α345 nephropathies also known as Alport syndrome, have been recognized.

2.8. Alport syndrome

Alport syndrome is a progressive hereditary kidney disease frequently associated with sensorineural hearing loss and ocular abnormalities (3). AS was discovered by Arthur C. Alport in 1927 and characterized by hematuria, kidney disease and bilateral hearing loss (50). Ocular manifestations were reported as part of the syndrome in 1954 (51). Based on the type of inheritance Alport syndrome is classified into X-linked Alport syndrome (XLAS), autosomal recessive AS (ARAS), autosomal dominant AS (ADAS) and digenic AS (16).

2.9. Epidemiology and prevalence of Alport syndrome

AS is one of the most frequent hereditary disorders causing end stage kidney disease. It has been described as rare disease due to affecting less than one in 2000 individuals (52). The most widely accepted estimate of the prevalence of AS is 1 in 5,000 individuals, based on data from the United States. This estimate is derived from the observation of approximately 300 cases in Utah and southern Idaho, within a population of 1,500,000 individuals (53). However, due to lack of generally accepted diagnostic criteria, availability of genetic tests and diverse manifestation of a disease, exact prevalence of AS sill remain unclear (54).

AS has been identified in various ethnic groups and does not seem to be limited to specific geographic regions (53). Population-based studies have proposed incidence of AS of one in 53,000 in Finland (55) and 17,000 in southern Sweden (56). Other studies on Rhode Island and Portugal described 77 and 124 individuals (including most types of AS) within the populations of approximately 1,000,000 and 10,400,000 habitants, respectively (57–60). Individuals with AS were reported to contain 2.3% and 1.3% of whose having kidney transplantation, according to the Mayo Clinic (61) and study from Sweden (62). Approximately 0.56 % of the European dialysis population has AS (63), including 18% in French (64), and 0 to 1.21% in Denmark (Report on Dialysis and Transplantation in Denmark from Danish National Registry in 2001). Meanwhile permanent hematuria and thin GBM leading mostly to ADAS, appear in 1%–2.5% of children and adults based on population

screening (65,66), and in 4%–7% of normal donor kidney transplant histological specimens (67).

Recent large study by Gibson J and his colleagues, reported the prevalence of predicted pathogenic *COL4A3–COL4A5* variants using population Sequencing Database to be at least one in 2320 for *COL4A5* pathogenic variants and one in 106 of individuals with *COL4A3* and *COL4A4* variants. They also reached the conclusion that the true prevalence of potentially pathogenic *COL4A3–COL4A5* variants is even greater and are often unrecognized (10).

2.10. Pathogenesis of Alport syndrome

AS is caused by disease causing variants (previously termed as mutations) in the three type IV collagen genes *COL4A3, COL4A4* and *COL4A5*, encoding the α3, α4 and α5 chains, respectively. These α chains form the collagen IV network with three isomers: α1α1α2, α3α4α5, and α5α5α6, which are localized in the kidneys (68). During normal embryogenesis and kidney development, most of the premature α1α1α2 (IV) network is replaced by α3α4α5 in the GBM and by a5-a6-a5 in Bowman's capsule (69,70). Experimental models in mice have shown that the mature α3α4α5 (IV) network in glomerular cells are produced by glomerular cells podocytes instead of endothelial cells, that was previously thought (69).

The α3α4α5 (IV) network in mature GBM is essential for providing the availability to support the highest glomerular capillary pressure, therefore if any of *COL4A3-A5* genes are genetically changed, the normal assembly of the heterotrimer α3α4α5 (IV) network in the GBM is damaged (71). In AS unnormal α3α4α5 (IV) network is partially switched to primary and vulnerable to filtration pressure α1α1α2 network, which together with ectopic laminin isoforms alter the expression of glomerular matrix receptors and change the distribution of cytoskeletal proteins, leading to the foot process effacement (FPE) and increased secretion of matrix metalloproteinases in glomerular podocytes (70).

Arterial hypertension (AH) induces endothelin-1 secretion by endothelial cells, that activates mesangial cells in AS (72). All these changes lead to progressive degradation of glomerular filtration barrier, thin basement membranes, focal segmental glomerulosclerosis (FSGS) and other damaging changes resulting the hematuria, proteinuria, and progressive kidney disease (70). Current data suggest that the type of pathogenic variant and the different zygosity, including heterozygotes, hemizygotes, homozygotes, or compound heterozygotes, variably modify the structure of GBM and, consequently, the

severity of the disease (13,73). The progression of AS is related to the proportion of damage in the GBM (22). The impairment of glomerular function and the podocyte stress response result in the secretion and circulation of profibrotic chemokines and cytokines, which are the main triggers for interstitial fibrosis and tubular atrophy, considered as key features in the progression of the disease to KF. Additionally, recent data suggest that the quantity and quality of pro-inflammatory and profibrotic proteins in the urine determine the course of AS (74). Defects in collagen IV also lead to hearing and eyes abnormalities, where predominant α3α4α5 (IV) network is damaged (3).

2.11. Genetics of Alport syndrome

AS is caused by genetic changes in *COL4A3, COL4A4* and *COL4A5*. Pathogenic changes in *COL4A5* are associated with XLAS, while alterations in *COL4A3* or *COL4A4* genes cause autosomal AS. Compound heterozygous or homozygous variants in *COL4A3* or *COL4A4* genes result ARAS, while one single variant in either of these genes are associated with so called ADAS, although this term is still debatable (16,75). Recently, new form of AS has been identified and named as digenic AS caused by transmission of variants in two of the three genes (Table 2) (7).

Table 2. Classification of Alport syndrome due to genetic changes in *COL4A3-A5* genes and type of inheritance, based on Kashtan CE, 2021 (16)

Type of inheritance	Gene affected	Genetic state	Naming
X-linked	*COL4A5*	Hemizygous (males) Heterozygous (females)	X-linked AS (XLAS)
Autosomal	*COL4A3* or *COL4A4*	Recessive (homozygous or compound heterozygous)	Autosomal recessive AS (ARAS)
		Dominant (heterozygous)	Autosomal dominant AS (ADAS) or thin basement membrane disease (TBMD) or benign familial hematuria (BFH) or autosomal recessive "carrier"
Digenic	*COL4A3* and *COL4A4* or *COL4A3* and	*COL4A3* and *COL4A4* pathogenic variants in trans (recessive)	Digenic AS

Type of inheritance	Gene affected	Genetic state	Naming
	COL4A5 or *COL4A4* and *COL4A5*	*COL4A3* and *COL4A4* pathogenic variants in cis (dominant) Pathogenic variants in *COL4A5* and in *COL4A3* or *COL4A4*	

XLAS arises from pathogenic variants in the *COL4A5* gene, localized on the X chromosome and coding collagen IV alpha-5 chain. Affected father transmits mutated gene with his X chromosome to all his daughters while all sons remain healthy due to transmission of unaffected Y chromosome. Males with XLAS have only one X chromosome which is mutated and leads to more severe phenotype comparing to affected females who are described as heterozygous for disease-causing *COL4A5* variants. Most of heterozygotes manifest only microhematuria, whereas others may develop KF (76). The variability of phenotypes in females with AS is associated to random X chromosome inactivation by which only one X chromosome stays active per cell, resulting expression the mutated X chromosome in one-half of cells while other cells remain normal (77). ARAS accounts for about 10-15% of all individuals with AS and is caused by genetic defects in either *COL4A3* or *COL4A4* genes, coding α3 and α4 chains (IV), respectively. *COL4A3* and *COL4A4* genes are localized in both copies (alleles) of chromosome 2, inherited by both parents. In the case of ARAS, usually two different genetic changes, each inherited from both parents (compound heterozygous variants), are found on different chromosomes (in trans position). Two genetic variants found in cis position are inherited together from one parent and usually cause less severe phenotype comparing with ARAS in trans position (78). Homozygous pathogenic variants causing ARAS is associated with consanguinity. Females and males with ARAS are affected equally (79–81) and severity of the disease in both genders are virtually alike to those of classic XLAS in males (76,80). Heterozygous pathogenic variant in *COL4A3* or *COL4A4* shows wide phenotypic spectrum of symptoms, ranging from asymptomatic isolated hematuria to progressive kidney disease and extrarenal manifestations (16). The historical term “thin basement membrane disease (TBMD),” “familial benign hematuria,” and “carriers of ARAS” have been used for isolated persistent hematuria (11,82). However, many individuals with single variant in *COL4A3* or *COL4A4*, previously defined as having disease with „benign course“, eventually progress to severe proteinuria and

finally to KF (82). Therefore, aim of redefining nomenclature from TBMD to ADAS was proposed by Kashtan CE, to simplify the diagnostic naming, identification, monitoring, and early initiation of the treatment for AS (16,82–84). Digenic AS is a rare form of AS which is caused by pathogenic variant in *COL4A5* plus one in *COL4A3* or *COL4A4* or pathogenic variants in *COL4A3* and *COL4A4* genes. Combinations of digenic AS are different from each other by frequencies in population, mode of inheritance and molecular and clinical outcomes. Some variants, which include *COL4A5* and one of the *COL4A3* or *COL4A3* variant show different severity of disease course depending on the gender (85).

To date, over 5000 different pathogenic variants in the *COL4A3, COL4A4*, and *COL4A5* genes have been observed (LVOD database, last review 01/2023, Table 3). Most of genetic variants are reported in the official genetic databases including LOVD, HGMD, ARUP, ClinVar and others.

Table 3. Number of *COL4A3-A5* pathogenic variants reported in Genetic databases *(last review 01/2023)*

Gene	LOVD	HGMD	ARUP
COL4A5	2593	1286	807
COL4A4	1075	321	-
COL4A3	1050	335	-

ARUP database: https://arup.utah.edu/database/ALPORT/ALPORT_welcome.php
HGMD database: https://www.hgmd.cf.ac.uk/ac/gene.php?gene=COL4A3
LOVD database: https://databases.lovd.nl/shared/genes/COL4A5

Pathogenic variants that may cause AS are classified into missense (including Gly substitution and non-Gly substitution), nonsense, frameshift, splice site, and other pathogenic changes. Missense variants are the most frequent changes found in *COL4A3-A5* genes. To date, approximately 84-88 % of these changes, many of which are glycine substitution (95%) are found in intermediated collagenous domain of α5 (IV) chain. Missense changes also can be found in carboxy non-collagenous (NC1 terminus) domain and non-collagenous interruptions and usually contain other than glycine change (non-Gly substitution). The position 1 Gly substitutions in collagenous domain change the normal structure of Gly-Xaa-Yaa triplets which is crucial for collagen IV stability and functioning. Cysteine substitutions in the carboxy NC domain also play a critical role for collagen IV stability as these types of pathogenic variants disturb the normal crosslinking in the carboxy NC domain (86–88). These types of changes are referred to as pathogenic hotspots and are associated with more severe phenotypes of AS, in addition to other genetic

changes including nonsense, splice site, and frameshift variants. There is also evidence of deletions involving *COL4A5* and *COL4A6* genes, which are associated with a very rare and severe form of AS related to leiomyomatosis (78). Genetic variants are usually valued for their pathogenicity using the American College of Medical Genetics and Genomics/Association for Molecular Pathology (ACMG/AMP) criteria and is classified as pathogenic (P), likely pathogenic (LP), variant of uncertain significance (VUS), likely benign (LBn) and benign (Bn) (89). Also, the term hypomorphic variants have been recently proposed for genetic changes as they are not fully destructive and therefore obtain a residual protein function. One of commonly known hypomorphic variant is p.(Gly624Asp) in *COL4A5* (1,11,87).

2.12. Clinical features of Alport syndrome

Individuals with AS may present permanent microhematuria, different amount of proteinuria (often nephrotic range), oedema, hypertension, progressive decline of kidney function eventually leading to KF. Permanent microhematuria appear in 95% of cases, usually in the first years of life. Gradually, the symptoms exacerbate, therefore individuals develop worsening proteinuria, hypertension leading to deterioration in eGFR and progressing to KF. Kidney survival of AS patients is usually around 16 to 35 years of age (90). Less often, individuals with AS may have macrohematuria which may appear after upper respiratory tract infections (9). Later in childhood or during time of adolescence, individuals with AS commonly develop hearing loss associated with abnormalities of collagen IV in the inner ear (Izzedine et al., 2004). Bilateral sensorineural hearing abnormalities appear before the onset of KF and starts with high-tone loss (90). Specific ocular findings such as anterior lenticonus, subcapsular cataracts, abnormal pigmentary changes in the retina with yellow or white flecks (dot-and-fleck retinopathy), posterior polymorphous dystrophy, corneal erosions, temporal retinal thinning, dull macular reflex, lozenge, foveopathy, macular hole may be present in individuals with AS. However, some studies demonstrated that presence of ocular findings may be associated with worsen progression of kidney disease in individuals with AS (91). Clinical features of AS strongly depend on type of inheritance, gender (in case of XLAS), affected collagen IV gene, type of genetic variants and its pathogenicity, therefore clinical manifestation, and progression of different types of AS, including XLAS, ARAS, ADAS and digenic AS, are described based on genotype - phenotype correlation.

2.13. Diagnostics of Alport syndrome

The golden standard for diagnosis of AS is performing a genetic analysis of the entire coding sequences of all three *COL4A3-A5* genes. When AS is suspected (Table 4), next generation sequencing (NGS) should be performed over even a kidney biopsy (KB). Finding a *COL4A3-A5* genetic variant, causing structure and function abnormalities of collagen IV confirms the diagnosis of AS and indicates the importance of family screening. Genetic analysis allows to identify not only affected collagen IV gene, type of change but also mode of inheritance leading to further examination of family members who are at risk of being affected and let to avoid unnecessary KB (11,87). Genetic analysis is also recommended in the first-degree relatives of individual with known pathogenic variants in *COL4A3-A5* genes (87).

Table 4. Criteria for clinical suspicion of AS *(adapted by Savige J, <...>, Cerkauskaite A et al., 2022)* (87)

Clinical criteria	Comment
Permanent microhematuria	Persistent dysmorphic hematuria for longer than 6 months
Persistent proteinuria	Persistent proteinuria >0.5 g/g; steroid-resistant nephrotic syndrome
Hearing impairment	Hearing abnormalities with hematuria
Ocular lesions	Retinal fleck, lenticonus, or temporal retinal thinning
Family history	Family history of hematuria or kidney impairment
Kidney biopsy	Biopsy proven FSGS
	GBM lamellation or thickening or thinning in EM; or abnormal collagen IV α-chain if immunostaining is available
Kidney failure	KF with unknown aetiology, especially with hematuria and family history of hematuria or kidney impairment
Familial IgA	IgA nephritis in index individual with family history of hematuria

FSGS – focal segmental glomerulosclerosis; GBM – glomerular basement membrane; EM – electron microscopy, KF – kidney failure, IgA – immunoglobulin A

Examination for clinical symptoms may be performed before molecular analysis primarily to confirm the suspicion of AS, and additionally afterward, particularly to identify extrarenal features. Presence of typical to AS manifestations including ocular and hearing abnormalities may encourage genetic testing. External examination should include audiometry, retinal photography, and retinal optical coherence tomography (OCT) (91).

Kidney examination is assessed through serum creatinine levels, estimation of glomerular filtration rate (eGFR), urine analysis for red blood cell count, and measurement of protein/creatinine or albumin/creatinine ratio, as well as monitoring of blood pressure. While kidney biopsy is not essential for AS diagnosis compared to genetic analysis, it can be helpful in cases where genetic analysis results are inconclusive or there is a high suspicion of AS despite negative molecular analysis, providing additional clinical data including the identification of VUS (1,18). Evaluation of KB should include light microscopy (LM), routine immunofluorescence (IF) and electron microscopy (EM). The typical kidney histological abnormalities in individuals with AS is reported on EM as irregular thinning and thickening GBM with lamellation which is characterized as "basket-weave" lesions (92). Immunofluorescence usually is normal or shows nonspecific immunoreactant deposition, while collagen IV immunostaining, if available, has diagnostic and prognostic issues showing missing collagen IV α chains in the tissue. Individuals with AS may have FSGS, tubular atrophy, mesangial expansion, and other non-specific lesions in LM, however, evidence of AS specific foam cells is also described (18,83,90).

2.14. Genotype – phenotype correlations in Alport syndrome

The probability of presence of extrarenal manifestations and progression of kidney disease in individuals with AS is highly variable and basically determined by the type of pathogenic variant in *COL4A3-A5* genes (93). Nonsense or small frameshift variants that result in the absence or decreased functionality of the protein indicate a high risk of progressing to kidney failure by the age of 30. The risk is lower with missense, particularly non-Gly substitutions, or splice site variants (13).

Males with XLAS and all individuals with ARAS have similar or "classic" manifestation of AS and develop typical AS symptoms including progressive kidney impairment with hematuria and proteinuria, bilateral sensorineural hearing loss and specific ocular abnormalities such as lenticonus or retinal maculopathy. On the other hand, females with XLAS and individuals with ADAS usually manifest less severe phenotype of AS and have a lower risk at development of KF (94).

Gender plays an important role with respect to CKD progression in individuals with XLAS. Fifty percent of males with XLAS reach KF at the age of 25 while almost 90 % percent are under kidney replacement therapy by their 40, whereas females with AS show variable phenotype due to random X chromosome inactivation. Approximately 12% of women with XLAS reach

KF by the age of 40, while by the age of 80, the proportion increases significantly and reaches 40% (13). Large rearrangement, premature stops, frameshift, and truncation variants are associated with development of KF at the median age of 19.8 years in XLAS males, according to Gross O and his team (73). Individuals with ARAS often progress to KF by the age of 40 independently on gender (12).

Individuals with ADAS have variable phenotypes which range from asymptomatic to isolated microhematuria or proteinuria and in some cases progression to KF. The manifestation related to heterozygous pathogenic variants in *COL4A3* or *COL4A4* differ significantly even in the same family, probably due to presence of modifier genes, ameliorating or worsening the effect of identified pathogenic variant, in addition to AH, high sodium intake, smoking and obesity which can be independent contributors to kidney disease (16,82,93). The estimated risk for KF in individuals with ADAS is less than 1% unless certain risk factors for progression are present. These risk factors include FSGS, GBM lamellation or thickening, proteinuria, sensorineural hearing loss, positive family history for KF, or presence of genetic modifiers. In the presence of these risk factors, the risk of KF increases to 20% (16).

The risk for KF in individuals with digenic AS is highly different and significantly relies on affected genes. Pathogenic variants found in *COL4A3* and *COL4A4* can be found in trans or cis positions with an imitation of ARAS and ADAS with the 100% and up to 20% risk for KF, respectively. The pathogenic variants in *COL4A5* and *COL4A3* or *COL4A4* in males are associated with worsen course of AS and usually counts 100 % of risk for KF (16,75,93).

2.15. Treatment of Alport syndrome

At the present time, there is no curing treatment for AS, therefore all males with XLAS, all individuals with ARAS, as well as a particular percentage of females with XLAS, digenic AS and ADAS, will eventually progress to KF (22).

Symptomatic treatment for Alport syndrome

Symptomatic therapy includes hearing aid for hearing loss, ophthalmological interventions for ocular lesions (i.e., cataract removal), kidney replacement therapy (KRT) including dialysis and kidney transplantation for KF. Individuals with AS who progress to KF should undergo pre-emptive kidney transplantation if it is possible and recommended. However, the graft survival of individuals with AS is similar or even better comparing with general population

which requires transplantation (93). Several studies reported that family members even with only persistent hematuria or "carrier status" in all kinds of AS including *COL4A3* and *COL4A4* heterozygotes should not act as kidney donors due to possible impairment of kidney function over the time (87,95,96). Cases of posttransplant anti-GBM nephritis also were reported (97).

Disease course modifying agents

Currently the only recommendations for slowing progression of AS are based on early investigation of RAAS blockade and recently proposed sodium-glucose cotransporter-2 inhibitors (SGLT2 inhibitors) (93,98).

RAAS blockade is widely used for all proteinuric nephropathies due to its anti-proteinuric effect additionally to its main function for reducing the blood pressure. Decrease of proteinuria was shown to slow the progression to KF by several studies from 1997 (22,99,100). Many trials with animals and human models studied the effect of angiotensin receptor blockers (ARBs) and angiotensin converting enzyme inhibitors (ACEi) on reducing the proteinuria and progression in AS, however the largest study for evidence on the use RAAS blockers came from European Alport Registry (22). The results indicated a significantly slower progression of kidney disease and a later onset of KRT in the group treated with RAAS blockade (101). Additionally, Gross O and colleagues demonstrated similar results in a cohort of 174 individuals with all types of AS compared to the placebo group, with a follow-up period of approximately two decades. This study particularly highlighted the importance of early ACE inhibitor use in the early stages of CKD (93). Food and Drug Administration (FDA) approved most ACEi for therapy of hypertension in both adults and children older than 6 years old (93). However, the EARLY PROTECT trial indicated the use of ramipril in children with AS who have isolated microscopic hematuria or microalbuminuria and are aged ≥2 years (102). Recent clinical recommendations indicate early initiation of ACE inhibitors in individuals with AS at the time of diagnosis in males with XLAS and in all individuals with ARAS if they are aged ≥24 months. In females with XLAS and in all individuals with ADAS, therapy can be started at the detection of microalbuminuria (1,18).

Bomback AS with his colleagues proposed the term "aldosterone breakthrough phenomenon" for increased levels of aldosterone in some individuals using RAAS inhibitors. This phenomenon showed association with left ventricular hypertrophy and higher amounts of albuminuria (103), therefore another study by Rubel et al. searched the efficacy in use of dual therapy of using mineral corticoid receptor blockers (MCRB) spironolactone

and ramipril in *COL4*α3 −/− mice. This study showed slower progression of CKD in favour of reducing proteinuria and fibrosis, however probably due to premature death caused by hyperkalaemia, survival rate did not change (104). Another MCRB finerenone shows promising results in slowing progression of CKD in Diabetes Mellitus (DM), therefore further studies are needed for evaluation of this effect in non-diabetic individuals (105,106).

SGLT2 inhibitors are drugs that block glucose absorption in the proximal convoluted tubule of the kidneys, thereby inducing glucose osmotic diuresis along with natriuresis. These mechanisms mediate the vasoconstriction of the afferent glomerular arteriole, resulting in a reduction of intraglomerular pressure and albuminuria (107–109). Several recent studies have shown promising effects of SGLT2 inhibitors in lowering proteinuria and slowing the progression of CKD in individuals with AS. However, due to the small number of participants in these studies, further trials are needed to confirm these findings (98).

Clinical trials on therapy of Alport syndrome

AS has become very attractive to industrial drug companies for several reasons. Firstly, AS represents a typical form of CKD characterized by proteinuria and fibrosis. Additionally, individuals with AS are usually young and have fewer co-morbidities, making them ideal candidates for clinical trials. Furthermore, since there is currently no specific treatment for AS, any new drug developed for this condition would likely receive orphan drug status with associated benefits. Lastly, there is a relatively large cohort of individuals affected by AS, providing a significant patient population for research and development (22,93). The main clinical trials and its drugs for AS are summarized in Table 5.

Table 5. Clinical trials related to the therapies of AS (clinicaltrials.gov)

Drug	Mechanism of action	Phase, status, number of participants	Name of clinical trial, (number of ClinicalTrials.gov)	Results
Bardoxolone	Nrf2 activator NF-κB inhibitor	Phase III, completed, n=157	CARDINAL (NCT03019185)	FDA not approved. No evidence that bardoxolone is effective at slowing kidney disease progression in patients with AS.
		Phase III, recruiting, n=480	EAGLE (NCT03749447)	No results yet
Lademirsen (RG-012, SAR339375)	Anti-microRNA21 drug. Reduces P42/P44 MAPK pathway activation	Phase II, active, not recruiting, n=43	HERA (NCT02855268)	No results yet
Sparsentan	Dual endothelin type A receptor and angiotensin II type 1 receptor antagonist	Phase II, recruiting, n=57	EPPIK (NCT05003986)	Sparsentan extended lifespan and reduced proteinuria in AS mice + auditory benefit. EPPIK trial showed greater reduction in proteinuria in children
Atrasentan	Endothelin type A receptor antagonist	Phase II, recruiting, n=80	AFFINITY (NCT04573920)	Evaluating reduction of proteinuria
Hydroxychloroquine	Immunomodulatory drug	Phase II, recruiting, n=50	CHXLAS (NCT04937907)	Evaluating reduction of proteinuria
Benazepril, Valsartan and Fluvastatin	Drug combination inhibits ACE, ARB and HMG CoA reductase	Phase II, completed, n=9	Effects of an Intensified treatment with ACE-I, ATA II, and statins in	Evaluating reduction of proteinuria

Drug	Mechanism of action	Phase, status, number of participants	Name of clinical trial, (number of ClinicalTrials.gov)	Results
			AS (NCT00309257)	
Ramipril	Angiotensin converting enzyme inhibitor	Phase III, completed, n=66	EARLY PROTECT (Early prospective therapy trial to delay kidney failure in Children with AS) (NCT01485978)	Reduction of proteinuria in children
R3R01 & River 3 Renal	Lipid-modifying drug	Phase II, not yet recruiting, n=50	Study to evaluate R3R01 in patients with AS and patients with FSGS (NCT05267262)	Reduction of lipotoxity in the kidneys

Experimental therapies for AS include targeting several aspects such as reducing fibrosis, proteinuria, and lipotoxicity in the kidneys. Other approaches involve altering the pathological pathways, stabilizing the abnormal proteins themselves, and generating tissues and organs that could be immunocompatible through correction of the abnormal genes (1,22).

2.16. Differential diagnosis of Alport syndrome

The differential diagnoses of AS include hematuria related urologic diseases and cancer, IgA nephropathy (although it does not exclude co-existence of AS) and other nephropathies (Table 6) (78).

Table 6. Differential diagnosis of Alport syndrome. Similarities and differences to AS (78)

Differential diagnosis	Gene	Similarities	Differences
Complement related disease: deposit dense disease, *CFHR5* nephropathy	*CFH, CFHR5*	Hematuria, proteinuria, kidney failure	No hearing loss
Fechtner syndrome	*MYH9*	Hematuria, proteinuria, kidney failure, hearing loss, lamellated GBM	Neutrophil inclusions, thrombocytopenia, large platelets
Fibronectin glomerulopathy	*FN1*	Proteinuria, nephrotic syndrome, kidney failure	Hematuria rare, disease recurrence post-transplantation
Hereditary angiopathy, nephropathy and cramps (HANAC)	*COL4A1, COL4A2*	Hematuria, kidney cysts, irregularly thickened tubular, and Bowman's capsular membranes, retinal angiopathy	Neurovascular abnormalities and cramps
IgA nephropathy	Not identified	Hematuria, proteinuria, progressive CKD, positive family history	No ocular and hearing abnormalities

Differential diagnosis	Gene	Similarities	Differences
SLE	-	Hematuria, proteinuria, KF	Positive serological findings
Bartter syndrome	*SLC12A1; CLCNKB, BSND, CASR*	Kidney failure, hearing abnormalities	Polyuria-polydipsia, hypokalaemia, hypercalciuria, nephrocalcinosis
Nail-patella syndrome	*LMX1B*	Ocular, kidney involvement	Extra skeletal abnormalities, nail abnormalities
Mitochondrial syndromes (MELAS)	Mitochondrial DNA	Nephrotic syndrome, ocular, hearing abnormalities	Cardiac and muscular weakness, stroke like symptoms
Fabry disease	*GLA*	Proteinuria, KF, positive family history, tinnitus, ocular abnormalities	No hematuria, CNS, cardiac involvement, hypohidrosis
Branchio-oto-renal syndrome (BOR) syndrome	*EYA1*	Ocular, hearing abnormalities, CKD	Urinary tract malformation, characteristic craniofacial features

CKD – chronic kidney disease; SLE – systemic lupus erythematosus; KF – kidney failure

3. METHODS

This research was performed as a part of an international, multicentre, observational, longitudinal Alport syndrome biomarker study BioAlport (ClinicalTrials.gov Identifier: NCT02718027). The analyses conducted for this thesis included only Lithuanian participants and their genetic data. However, in order to overcome the limitation of a limited number of patients in specific types of AS and achieve more significant results, we collaborated with international working groups. These collaborations involved the Australian AS working group, supervised by Prof. Dr. J. Savige, the British AS working group (Alport syndrome Workshops), and the European Rare Kidney Disease Reference Network (ERKNet). The purpose of these collaborations was to analyse the genotype-phenotype correlations of glycine substitutions and digenic AS.

3.1. Study organization

Study cohort analysis focused on pathogenic changes in *COL4A3-A5* genes, clinical characteristics, and genotype-phenotype correlations in Lithuanian individuals with clinical and genetic diagnoses of AS. This study included Paediatric and Adult Nephrology centres at Vilnius University Hospital Santaros Klinikos (VUH SK). All study participants were screened, evaluated, and examined followed by the same standardized protocol for adult and children (described below). All investigators were trained at the beginning of the study to provide the equivalent approach of the research procedures (described below). The study organization included clinical and molecular data collection, evaluation techniques of the sample and data analysis. Study organization is shown in Figure 4.

The responsibilities of the doctoral student included: overall organization of the study idea, process; collection of the samples for DNA analysis; nephrological examination of the study adult participants; all required medical data collection, storing, and analysis; working into international AS working groups.

The study was conducted in collaboration with national and international scientists from genetic laboratories, clinical geneticists, bioinformatics experts, adult and paediatric nephrologists, ophthalmologists, and audiology specialists (surdologists).

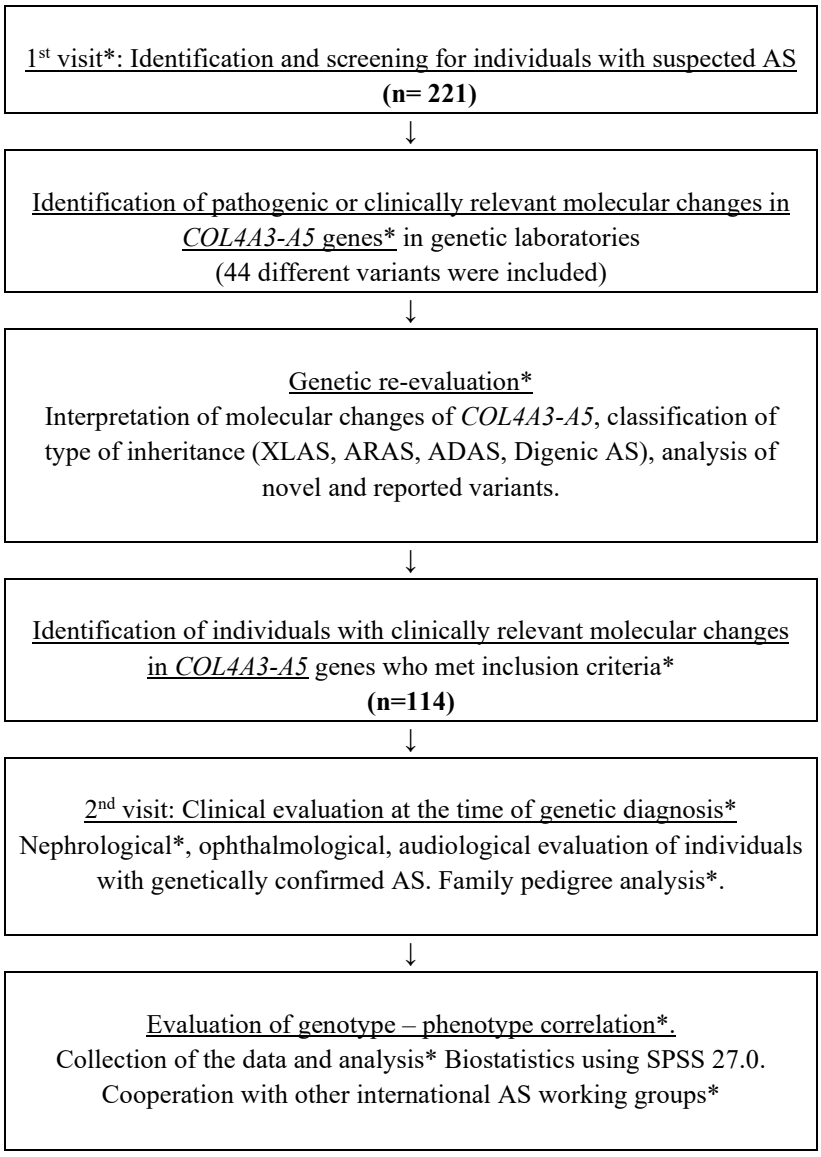


Figure 4. Study organization

Out of the 221 individuals who met the eligibility criteria for AS, Alport syndrome was diagnosed in 114 individuals, showing 44 different genetic changes in the *COL4A3-A5* genes. One hundred seven individuals were excluded either due to the absence of clinically relevant molecular changes (benign, likely benign, and polymorphism) in the *COL4A3-A5* genes, or due to the presence of AS-unrelated pathogenic changes in the *NPHP1*, *CLCN5*, or *CFHR5* genes. These changes were associated with nephronophthisis (n=1), Dent disease (n=2 unrelated patients), or *CFHR5* nephropathy (n=1), respectively. * Performed by PhD student (except the nephrological evaluation during the 1st and 2nd visit for children)

3.2 Ethical statement

The study protocol was approved by the Vilnius Regional Biomedical Research Ethics Committee of Lithuania (BioAlport, No 158200-16-857-367). Written informed consent to participate in this research was provided by all participants. Informed consent from minors (individuals under 18 years old) was obtained from their parents or legal guardians.

3.3. Study design and setting

Eligibility criteria

The study cohort included the individuals from all over Lithuania treated by adult or paediatric nephrologists at VUH SK in Vilnius, Lithuania from 2016 to 2022. The age of participants ranged from 2 months to 82 years old. The research design for this study included observational (descriptive) and analytic studies. Adults or children who met the eligibility criteria (Table 7) for AS were evaluated during the first visit by either an adult nephrologist (PhD student) or a paediatric nephrologist.

Table 7. Eligibility criteria for suspicion of AS

Eligibility criteria	Description
Clinical features	– Persistent isolated microscopic hematuria >6 months; – Persistent hematuria with proteinuria of unknown etiology; – Nephrotic range proteinuria or steroid-resistant NS; – Hearing abnormalities with persistent hematuria or CKD with unknown etiology; – AS specific ocular lesions (retinal flecks, lenticonus, and others); – CKD or KF with unknown etiology, particularly if there is a positive family history of CKD, hearing abnormalities, or persistent hematuria.
Kidney biopsy	Findings of kidney biopsy, specific to AS: foam cells; unexplained FSGS; thin, thickened, lamellated GBM; FPE
AS in family	Individuals in the family with genetically diagnosed AS

CKD – chronic kidney disease; KF – kidney failure (eGFR <15 ml); FSGS – focal segmental glomerulosclerosis; GBM – glomerular basement membrane; NS – nephrotic syndrome; FPE – foot process effacement

All individuals with a high level of suspicion for AS, who met one or several eligibility criteria, provided a blood sample for deoxyribonucleic acid

(DNA) extraction. During the first visit, clinical and demographic data, and family history from individuals with suspected AS were collected retrospectively (Table 8). Moreover, according to Levy and Feingold, retrospective studies are considered the most efficient approach for rare diseases or diseases with low prevalence (110).

Table 8. Retrospective demographic, clinical and family history data collected during the first visit for individuals with suspected of AS

Demographic data	1. Date of birth; age years; gender; ethnicity; living place (city)
Clinical data`	1. Nephrological data: a. Presence and age at detection of haematuria b. Presence, amount, and age of detection of proteinuria c. Age at kidney failure (V stage CKD) d. Presence and detection of AH e. Use of RAAS inhibitors (name, dosage) f. Performance and findings of kidney biopsy (Table 9). Amount of proteinuria (g/L), eGFR, age at the time of kidney biopsy 2. Ophthalmological data: presence of ocular lesions and time at their detection 3. Audiometric data: presence of hearing abnormalities, time at their detection, previous audiograms
Family history	Kidney diseases, haematuria, AS in the family

Table 9. Kidney biopsy data considered as specific or related to AS.

Method of kidney histological evaluation	Pathological findings in kidney biopsy
Light microscopy (LM)	Foam cells, FSGS, expansion of the mesangial matrix
Immunofluorescence (IF)	Unspecific immunoglobulin M, G and C3 deposits or/and IgA deposits
Electron microscopy (EM)	Thinning, thickening, lamellation of GMB

FSGS – focal segmental glomerulosclerosis; GBM – glomerular basement membrane; IgA – immunoglobulin A

The DNA samples (n=178) were sent to Centogene laboratory in Rostock, Germany. If unable to send the samples to Centogene laboratory, the patients (n=43) were referred to clinical geneticists at VUH SK in Vilnius, Lithuania, for genetic evaluation and DNA collection.

Sample selection and evaluation for the present analysis

Molecular results obtained from genetic laboratories using Genetic Databases (described in the Genetic section) were reevaluated by the PhD student. Only individuals with pathogenic, likely pathogenic, and potentially clinically relevant variants of uncertain significance (VUS) in *COL4A3-A5* genes were included in further analysis. Additionally, individuals with incidental genetic findings in *COL4A3-A5* genes during screening for other genetic disorders were also included in the study cohort. No individuals who met the exclusion criteria were involved in the present study (Table 10).

Table 10. Inclusion and exclusion criteria for the study

Inclusion criteria
1. Individuals with pathogenic, likely pathogenic or and potentially clinically relevant VUS in *COL4A3-A5* genes involving: a. Individuals screened for AS who met eligibility criteria b. Individuals with accidental molecular findings in *COL4A3-A5* genes during screening for other genetic disorders 2. Individuals of both genders over the age of 2 months (criteria by "BioAlport") 3. Signed informed consent form to participate in the study
Exclusion criteria
1. Individuals with benign, likely benign variants or polymorphisms in *COL4A3-A5* genes 2. Individuals suspected to have AS but genetically confirmed to have other genetic disorders 3. Individuals with pathogenic variants in *COL4A3-A5* genes and coexisting genetic disorders

Clinical evaluation of the study participants

Only individuals who met inclusion criteria were referred to a second visit with the adult (PhD student) or paediatric nephrologist. During the second visit, nephrological evaluation was performed, and all individuals were referred to specially trained specialists for hearing and ocular evaluation. Nephrological evaluation for adults and children was performed by adult and paediatric nephrologists, respectively.

Nephrological evaluation:

1. Haematuria using spot urine samples
2. Spot urinary protein and creatinine ratio* (uPr/uCr; PCR) expressed in mg/mmol; and albumin and creatinine ratio (uA/Cr; ACR) in mg/mmol.

*Reference values were used according to the to the Kidney Disease Improving Global Outcome (KDIGO) definitions, 2021.

3. Amount of proteinuria (spot urinary PCR, according to KDIGO, 2020 and 2021):
    a. A1: PCR <15 mg/mmol (normal to mildly increased)
    b. A2: PCR 15 – 49 mg/mmol (moderate proteinuria)
    c. A3: PCR 50 – 299 mg/mmol (severe proteinuria)
    d. Nephrotic range: PCR ≥300 mg/mmol in adults and ≥ 200 mg/mmol in children)
4. Stage of CKD based on eGFR (using Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula for adults and updated Schwartz equation formula (2009) for children) (Table 11)

Table 11. Stage of CKD based on eGFR (KDIGO, 2012) (111)

CKD stage	eGFR (ml/min/1.73 m2)
1	≥90
2	60-89
3a*	45-59
3b*	30-44
4	15-29
5	<15

*CKD stage 3 was not divided into 3a or 3b and was counted together in our study, as it did not have a significant impact on our results or aims to the study

5. Presence of arterial hypertension (AH) (*in adults:* when repeated blood pressure measurements (BPM) were >130/90 mmHg; *in children:* BP was standardized to calculating age-, sex- and height standard deviation score. AH by office BMP was defined as office systolic and/or diastolic BP values over 95th percentile). Individuals with AH who had normal BP while using RAAS inhibitors were also included

<u>Nephrological definitions used in this study:</u>

- Kidney failure (KF) – referred to as the 5 stage of CKD according to the updated nomenclature proposed by the KDIGO consensus in 2020 (KDIGO, 2020). It replaces the previously used term "end-stage kidney disease (ESKD)" and is defined by an eGFR less than 15 ml/min/1.73 m² (112).
- Microalbuminuria – defined as an abnormal increase in albumin excretion rate within the specific range of 30–299 mg of albumin/g of creatinine (mg/g), using the calculation of urine albumin-creatinine ratio (mg/mmol) (113).

Individuals with genetically confirmed diagnosis of AS were referred to ophthalmological evaluation based on prof. Savige J (91) (Table 12). Individuals were considered to have AS related ophthalmopathy if one or more criteria were present. The onset of ocular abnormalities is often poorly recognized and may occur earlier than detected. Therefore, in order to describe genotype-phenotype correlations, we used the age at diagnosis of ocular abnormalities, which coincided with the time of molecular diagnosis of AS.

Table 12. Ocular examination for individuals with AS.

Ocular examination	Possible phenotype
Slit lamp examination	Cornea: recurrent corneal ulcers and erosions; corneal clouding; posterior polymorphous corneal dystrophy
Ophthalmoscopy, retinoscopy	Lens: anterior lenticonus; cataracts; perimacular fleck retinopathy
Retinal photography or redfree images	Central fleck retinopathy: asymmetric patches of confluent flecks; peripheral coalescing retinopathy
Optical coherence tomography	Temporal retinal thinning, dull macular reflex, lozenge, foveopathy, macular hole

Hearing evaluation: Audiograms for screening bilateral hearing abnormalities were performed in most of the individuals by surdologists at the time of molecular diagnosis. Only individuals with bilateral hearing abnormalities loss in the range of 2.000-8.000 Hz were considered as having AS specific hearing lesion. The onset of hearing abnormalities is often poorly recognized and may occur earlier than detected. Therefore, in order to describe genotype-phenotype correlations, we used the age at detection of hearing loss, which coincided with the time of molecular diagnosis of AS.

Familial evaluation: Pedigree analysis was conducted for all participants. Family members with a high degree of suspicion for AS were referred for molecular analysis and additional evaluation.

3.4. Molecular analysis and genetic data

Molecular analysis

The genetic analysis was performed in two genetic laboratories – Centogene laboratory (85 % of samples, Rostock, Germany) and the laboratory of VUH SK Medical Genetics centre (15 % of samples, Vilnius, Lithuania). The main diagnostic method for molecular analysis in both laboratories was NGS using similar techniques. NGS was used for all individuals in the Centogene laboratory, even when familial genetic variants were known. On the other hand, the VUH SK Medical Genetics Centre performed the Sanger method to detect pathogenic variants in familial cases for AS. Using NGS, genomic DNA was enzymatically fragmented, and regions of interest were enriched using DNA capture probes targeted against the coding regions and known pathogenic/likely pathogenic changes of panel genes. The libraries were then sequenced on an Illumina platform.

The entire coding region of the *COL4A5, COL4A3*, and *COL4A4*, genes, including 10 bp of flanking intronic sequences, were amplified and sequenced. Missing fragments were completed using classical Sanger sequencing to achieve 100 % coverage of all genes of this panel. Data analysis, including alignment to the hg19 human reference genome (Genome Reference Consortium GRCh37), variant calling, and annotation was performed using validated software from Centogene or VUH SK genetic laboratories.

The original findings presented in the study have been made publicly accessible and located in the ClinVar Database, specifically under the accession SUB11112191 (SCV002098105 - SCV002098129). However, it should be noted that the authors acknowledge a partial unavailability of raw data within this study. This is primarily due to the Centogene laboratory's-imposed time restriction, wherein variants and their corresponding raw data older than 5 years are not stored. The limited accessibility of data represents a potential significant constraint within the study.

Evaluation of pathogenicity of genetic variant

Data bioinformatics analysis and screening were performed depending on the targeted gene capture and high-throughput sequencing from DNA samples. All variants with minor allele frequency of less than 1% in gnomAD database, and pathogenic variants reported in HGMD, in ClinVar or in CentoMD were assessed. The in-silico prediction of the damaging effects of each identified genetic change was assessed using MutationTaster, PolyPhen-2 and SIFT.

Splicing variants were analysed by Human Splicing Finder (http://www.umd.be/HSF3/index.html) additional to MutationTaster. Pathogenic changes which could not be classified with either of the in-silico tools were declared as not applicable (N/a).

Genetic changes in *COL4A3-A5* were checked and compared in five databases – Centogene, Franklin, ClinVar, InterVar and Varsome (last review in December, 2022), and further classified into five categories due to their pathogenicity using ACMG/AMP criteria – pathogenic (P, class 1), likely pathogenic (LP, class 2), variant of uncertain significance (VUS, class 3), likely benign variant (LBn, class 4), and benign (Bn) variants/polymorphism (Plm, class 5) by PhD candidate. It should be noted that although the identification of VUS does not confirm the diagnosis of AS, we decided to include these variants in our analysis due to their potential implications for clinically relevant manifestations of AS. This decision was based on clinical evaluations and data from other genetic databases. Therefore, we thoroughly evaluated these variants and reclassified them according to our clinical practice.

Naming of Alport syndrome

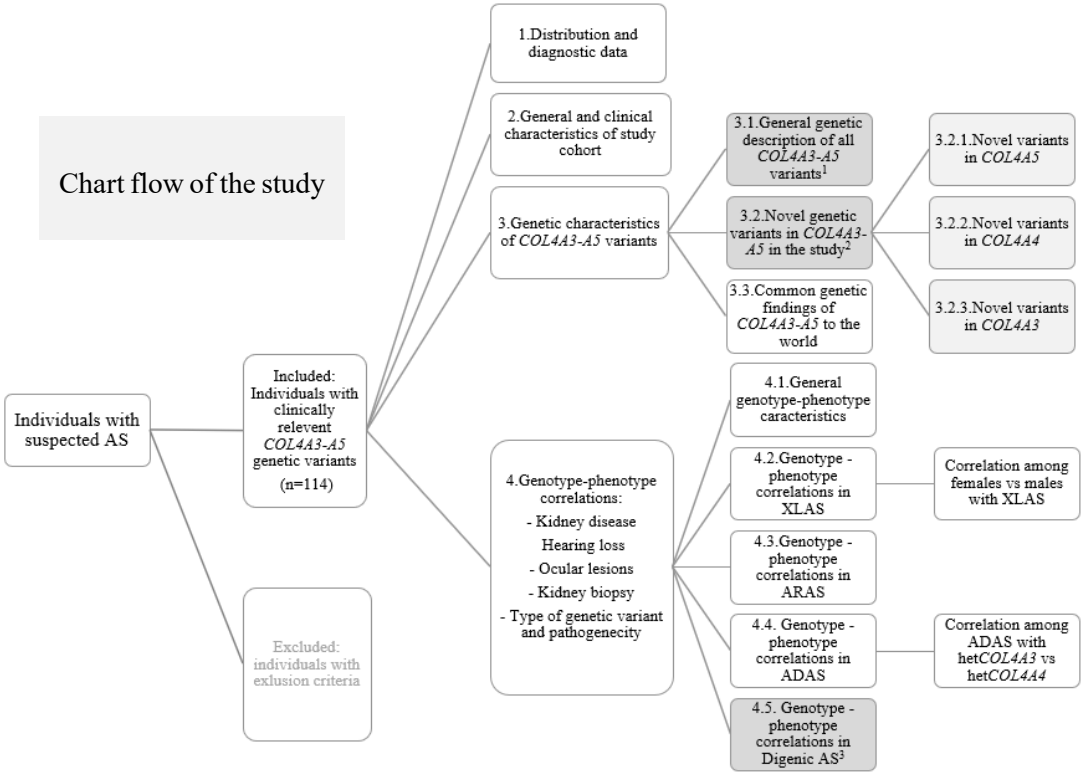
Individuals with hetero/hemizygous pathogenic changes in *COL4A5 (1)* compound heterozygous or homozygous variants in *COL4A3* or *COL4A4 (2)*, singular heterozygous variants in *COL4A3* or *COL4A4 (3)*, or digenic variants in two of the following genes *COL4A3/A4/A5 (4)* were named as having XLAS, ARAS, ADAS, or digenic AS, respectively. Although there is still some debate about naming the individuals with heterozygous *COL4A3/A4* changes with ADAS, thin basement membrane disease or carriers of autosomal recessive AS, in this study we decided to use the term ADAS following the Kashtan et al. proposed unified classification of AS in 2018 (16).

3.5. Statistical analysis

The collected data was securely stored in a database for further analysis. To assess the quantitative data, descriptive statistical analysis was conducted using the SPSS 27.0 (Statistical Package for the Social Sciences) software package. Continuous variables were presented as either mean (V) ± standard deviation (SD) or median (interquartile range [IQR]), depending on their distribution. Categorical data were presented as frequencies and percentages. Parametric criteria were applied to compare normally distributed quantitative values, while non-parametric tests were used for non-normally distributed

quantitative values and ordinal variables. The chosen level of confidence was 95%, with a significance level set at p=0.05 (two-sided). Statistically significant results were identified if $p \leq 0.05$. Independent sample comparisons were performed using parametric analysis of variance (ANOVA) or the non-parametric Dunn's Kruskal-Wallis test. The Chi-square (x2) criterion was employed to assess the independence between nominal scale and tabulated frequency characteristics. The Fisher's exact test was employed to compare categorical variables, while the Mann-Whitney test was used to compare continuous variables between different sexes and the presence of *COL4A3-A5* disease-causing variant.

No kidney survival could be evaluate using Kaplan-Meier tests having only small number of individuals with KF.



1. Joel T Gibson, <...>, Agne Cerkauskaite et al. Genotype-phenotype correlations for *COL4A3-COL4A5* variants resulting in Gly substitutions in Alport syndrome. Sci Rep. 2022 Feb 17;12(1):2722. doi: 10.1038/s41598-022-06525-9.
2. Cerkauskaite A et al. Identification of 27 novel variants in genes *COL4A3, COL4A4* and *COL4A5* in Lithuanian families with Alport syndrome. Front Med (Lausanne). 2022 Mar 28; 9:859521. doi: 10.3389/fmed.2022.859521. ECollection 2022.
3. Savige J, <...>, Cerkauskaite A et al. Digenic Alport syndrome – additional pathogenic heterozygous variants worsen X-linked disease in females and autosomal dominant disease. Clin J Am Soc Nephrol. 2022 Jun 8; CJN.03120322. doi: 10.2215/CJN.03120322.

4. RESULTS

4.1. Distribution and diagnostic screening of AS

A distributional analysis of the residential locations of all study participants revealed 16 Lithuanian cities. The majority of individuals resided in the Vilnius region, while no patients were identified from the North-West Lithuania region (Fig. 5).

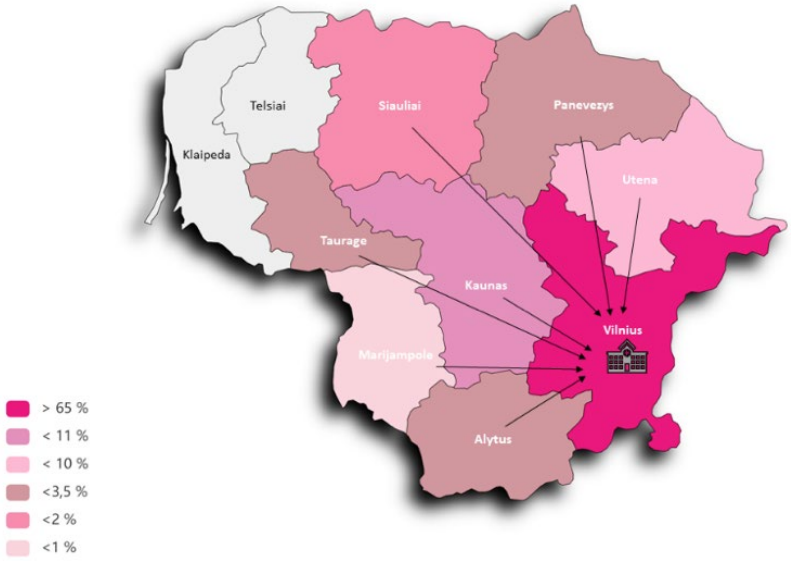


Figure 5. Distribution of study population according to Lithuanian cities (n=114)

Molecular analysis was carried out in all (100%) study participants. Fifty-three percent of them were identified as probands. Table 13 presents the diagnostic pathways leading to genetic testing for individuals with clinical suspicion of AS.

Among the probands, 70% were referred for genetic testing following the detection of pathological lesions in kidney biopsies. Pedigree analysis led to the identification of 54 affected relatives of the probands. Interestingly, 3% of individuals were enrolled in the study were unexpectedly confirmed of AS during the screening for ADPKD.

Table 13. Diagnostic ways leading to genetic diagnosis of AS in our study (n=114)

Type of individuals	No	Steps of diagnostic ways to genetic testing
Probands (n=60)	15	(1) Clinical features* → (2) genetic analysis
	42	(1) Clinical features* → (2) kidney biopsy → (3) genetic analysis
	3	(1) Suspicion of other disease (ADPKD) → (2) genetic testing
Affected relatives identified due to family screening (n=54)	54	(1) Positive family history for AS → (2) genetic analysis

No – number of individuals; ADPKD – autosomal dominant polycystic kidney disease, AS – Alport syndrome. * Clinical features included persistent hematuria, proteinuria, unknown aetiology of CKD

Fifteen individuals were referred for molecular analysis based solely on their clinical features. Among this group five individuals had only isolated microhematuria. Nine individuals had persistent microhematuria with proteinuria and normal kidney function. Out of these nine individuals, two had both hearing and ocular abnormalities, while two had either hearing or ocular lesions. Finally, one individual with stage 3 CKD and hearing abnormalities was directly referred for genetic analysis.

During the study, two participants with XLAS, a female and a male, passed away at the ages of 66 and 19, respectively. The female patient's death was attributed to complications following kidney transplantation, while the male patient's death was due to heart insufficiency.

4.2. General and clinical characteristics of individuals with AS

Genetic and clinical data were obtained from 114 participants from 63 unrelated families and included 53 (46.5%) males. It is important to note that three probands belonged to two different families due to the identification of distinct genetic variants in both parents of each proband (e.g., in ARAS). Consequently, the number of probands does not correspond to the number of different families. Clinical and demographic data are shown in Table 14.

Table 14. General and clinical characteristics of 114 individuals with AS depending on gender

Demographic and clinical features	Total individuals with AS	Males	Females	P-value
Total No of individuals, %	114 (100.0)	53 (46.5)	61 (53.5)	0.454
Probands, %	60 (52.6)	32 (60.4)	28 (45.9)	0,123
Suspected AS by FS, %	54 (47.4)	21 (39.6)	33 (54.1)	0,123
Mean age at GD*	32.5±18.4	28.8±17.6	35.6±18.6	**0.048**
Hematuria, %	114 (100.0)	53 (100.0)	61 (100.0)	-
Age at detection of hematuria	20.99±17.7	19.37±17.4	19.37±18.0	0.187
Proteinuria, %	81 (71.1)	36 (67.9)	45 (73.8)	0.492
Age at detection of Pro	26±17.7	24.0±19.5	27.5±16.2	0.381
Nephrotic range Pro, %	11 (9.6)	5 (9.4)	6 (9.8)	0.942
CKD I, %	69 (60.5)	34 (64.2)	35 (57.4)	$\times^2$=7.321, df=4, p=0.12
CKD II, %	17 (14.9)	4 (7.5)	13 (21.3)	
CKD III, %	15 (13.2)	7 (13.2)	8 (13.1)	
CKD IV, %	3 (2.6)	3 (5.7)	0 (0.0)	
KF (CKD V), %	10 (8.8)	5 (9.4)	5 (8.2)	
Mean age at KF, %	36.1±14.1	27.6±10.3	44.6±12.7	**0.048**
AH, %	53 (46.5)	29 (54.7)	24 (39.3)	0.101
RAAS inhibition, %	66 (58.4)	33 (63.5)	33 (54.1)	0.314
KB, %	49 (43.0)	23 (43.4)	26 (42.6)	0.934
Age at KB	34.1±16.3	30.5±14.8	37.3±17.1	0.73
Hearing abnormalities, %	31 (30.1)	19 (38.8)	12 (22.2)	0.067
Ocular abnormalities, %	18 (18.2)	11 (22.9)	7 (13.7)	0.236

GD – genetic diagnosis, No – number, Pro – proteinuria, AH- arterial hypertension, KF – kidney failure, KB kidney biopsy, FS – family screening, CKD – chronic kidney disease, No – number.
* Individuals with clinically significant molecular changes in *COL4A3-A5* genes were enrolled immediately after receiving a positive genetic diagnosis. No individuals with a prior molecular diagnosis were included in the study

Individuals under 18 years old accounted for 20% of all study participants (Table 15).

Table 15. General and clinical characteristics of adults and children with AS

Demographic and clinical features	Total individuals with AS	Adult (age>18 y.o.)	Children (age <18 y.o.)	P-value
Total No of individuals, %	114 (100.0)	91 (79.8)	23 (20.2)	<0.01
Probands, %	60 (52.6)	48 (52.7)	12 52.2)	0.961
Suspected AS by FS, %	54 (47.4)	43 (47.3)	11 (47.8)	0.961
Mean age at GD*	32.5±18.4	38.5±15.4	8.5±4.6	<0.001
Hematuria, %	114 (100.0)	91 (100.0)	23 (100.0)	
Age at detection of hematuria	21±17.7	25.0±17.6	5.4±4.2	<0.001

Demographic and clinical features	Total individuals with AS	Adult (age>18 y.o.)	Children (age <18 y.o.)	P-value
Proteinuria, %	81 (71.1)	68 (74.7)	13 (56.5)	0.085
Age at detection of Pro	26.0±17.7	30.0±16.6	6.2±4.9	<0.001
Nephrotic range Pro, %	11 (9.6)	10 (11.0)	1 (4.3)	0.456
CKD I, %	69 (60.5)	49 (53.8)	20 (87.0)	$\times^2$=9.058, df=4, p=0.06
CKD II, %	17 (14.9)	15 (16.5)	2 (8.7)	
CKD III, %	15 (13.2)	14 (15.4)	1 (4.3)	
CKD IV, %	3 (2.6)	3 (3.3)	0 (0.0)	
KF (CKD V), %	10 (8.8)	10 (11.0)	0 (0.0)	
Mean age at KF, %	36.1±14.1	36.1±14.1	-	
AH, %	53 (46.5)	51 (56.0)	2 (8.7)	<0.001
RAAS inhibition, %	66 (58.4)	56 (62.2)	10 (43.5)	0.104
KB, %	49 (43.0)	45 (49.5)	4 (17.4)	**0.009**
Age at KB	34.1±16.3	36.2±15.3	11.0±4.2	<0.001
Hearing abnormalities, %	31 (30.1)	28 (33.7)	3 (15.0)	0.173
Ocular abnormalities, %	18 (18.2)	13 (16.3)	5 (26.3)	0.306

GD – genetic diagnosis, No – number, Pro – proteinuria, AH- arterial hypertension, KF – kidney failure, KB kidney biopsy, FS – family screening, CKD – chronic kidney disease, No – number
* Individuals with clinically significant molecular changes in *COL4A3-A5* genes were enrolled immediately after receiving a positive genetic diagnosis. No individuals with a prior molecular diagnosis were included in the study

Age at genetic diagnosis ranged from 1 to 70 years (the mean age was 32.5±18.4 years) and considerably differed among gender. Mean age at genetic diagnosis of all AS women was 35.64 ± 18.65 years, while in AS males it was established significantly earlier at 28,82 ± 17.6 (p=0.048, Fig. 6).

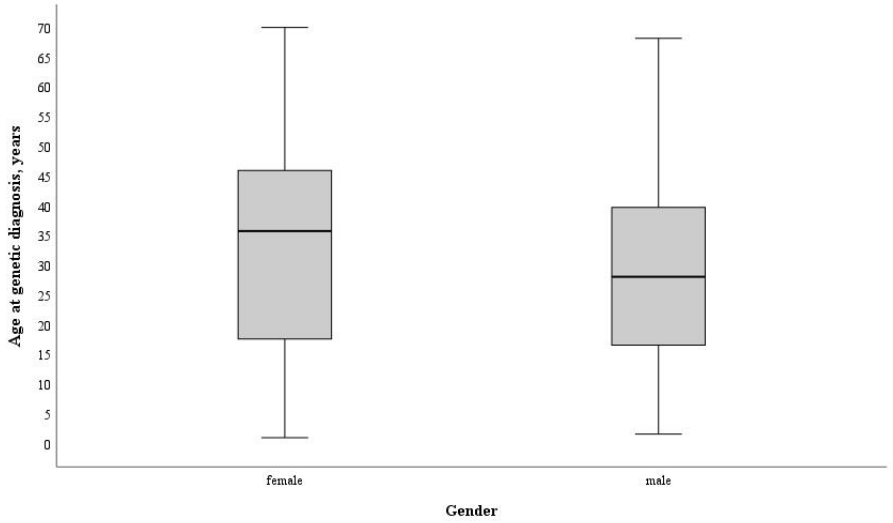


Figure 6. Distribution of age at genetic diagnosis of individuals with AS by gender (p=0.048)

General characteristics of kidney disease in AS

Microscopic persistent haematuria was detected in all individuals independently on age or gender. Proteinuria was detected in 71.1% of patients and was associated with lower eGFR levels comparing with individuals without it (Fig. 7 and Fig. 8).

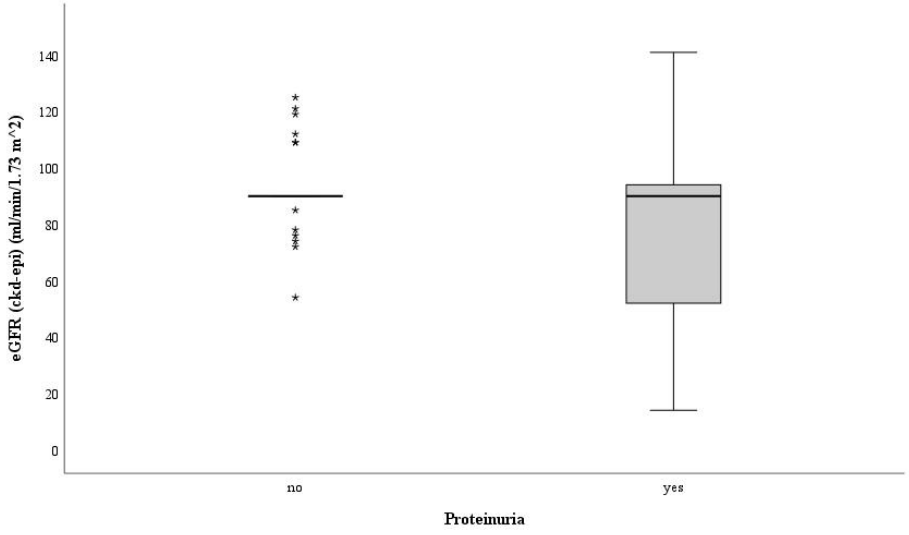


Figure 7. The comparison of eGFR in different individuals with AS in proteinuric vs non-proteinuric group at one time measurement (p=0.048)

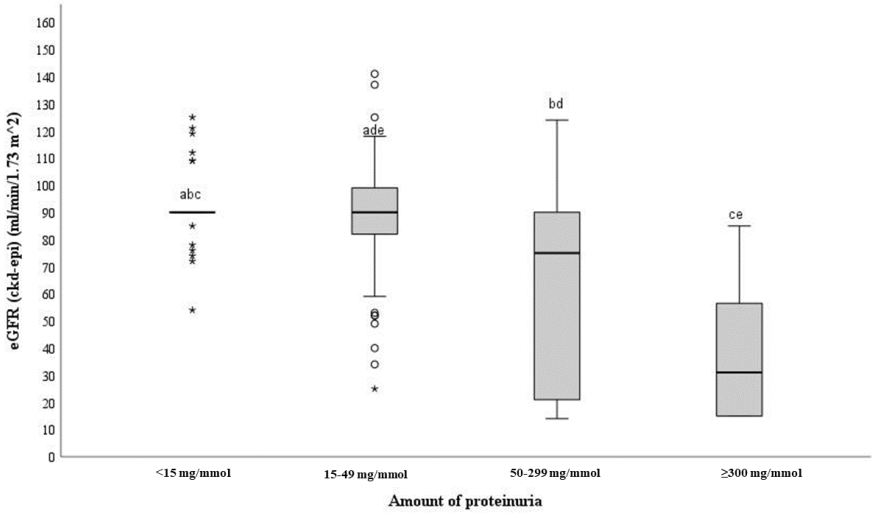


Figure 8. The correlation between of eGFR and proteinuria at one time measurement levels in individuals with AS. Independent-Samples Kruskal-Wallis Test Summary (×2= 26.732, df=3, p<0.001, ^{abcdep}<0.05)). Proteinuria was measured using spot protein-creatine ration (PCR) in mg/mmol

The detection of hypertension was typically observed at a later age (42 y.o. r=-0.434, p<0.001) and did not vary by gender; however, individuals with hypertension exhibited lower eGFR levels compared to those without it (r=-0.294, p=0.021). Among the 53 individuals with arterial hypertension, 48 were receiving treatment with RAAS blockade. Additionally, 13 individuals without hypertension received ACE inhibitors or ARBs due to the detection of proteinuria.

No KF was observed in paediatric group, whereas eGFR <15 ml/min/1.73 presented in 9.3 % and 8.2% of adult males and females, respectively. KF appeared significantly later in females compared to adult males (p=0.048). Kidney biopsy was performed in 43% of individuals with AS, with a significantly lower frequency observed in children compared to adults (p=0.009).

General characteristics of ocular and hearing abnormalities in AS

Ophthalmological evaluation at the time of genetic diagnosis was available in 86.8% (99 out of 114) individuals, among which 18 had AS specific ocular lesions detected at the mean age at 24.6±18.3 years (Fig. 9). Ocular abnormalities were more detected in individuals older than 32.5 years (p=0.02, 95% CI [1.23-11.62], and did not differ between the genders (p=0.12).

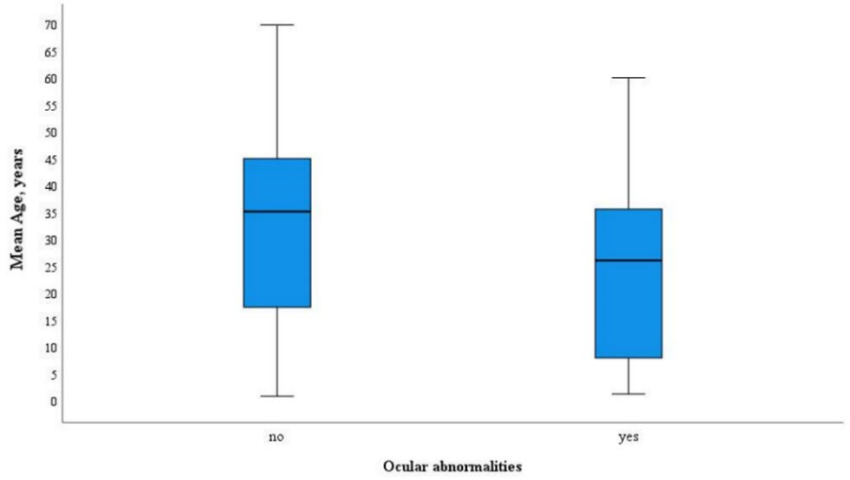


Figure 9. The age distribution among individuals with ocular lesions compared to those without it. Ophthalmological evaluation was performed at the time of genetic diagnosis

Hearing evaluation using audiograms was available for 90.4% (103 out of 114) individuals, among whom 31 had bilateral sensorineural hearing loss, which was diagnosed at a mean age of 38.6±17.1 (Fig. 10).

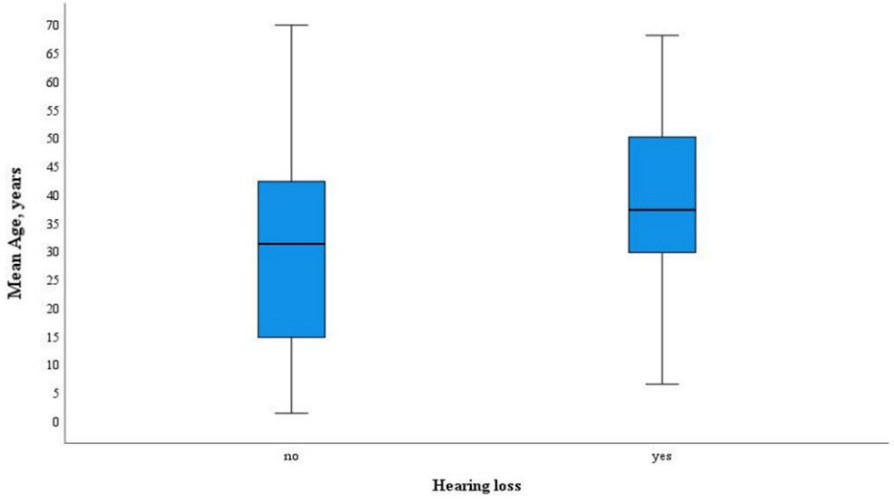


Figure 10. The age distribution among individuals with hearing abnormalities compared to those without it. Hearing evaluation was performed at the time of genetic diagnosis

Hearing abnormalities were more detected in individuals older than 20.4 y.o (p=0.012, 95% CI [1.4-13.2]) and did not differ between genders (p=0.114).

4.3. Molecular characteristics of changes in *COL4A3-A5* genes

4.3.1. General characteristics

NGS of all exons in the *COL4A3*, *COL4A4*, and *COL4A5* genes of 221 individuals from 120 different families led to the identification of 44 different genetic variants in 63 families (Table 16, Supp. Table 1). Twenty-eight (63.6%) variant was declared as novel or previously undescribed (Section 4.3.2) whereas 36.7% of genetic changes were reported and listed in genetic databases. Eight of the reported genetic variants were found to be shared with the world population (Section 4.3.3).

Table 16. General characteristics of genetic variants of *COL4A3, COL4A4* and *COL4A5* genes in 114 individuals

General features of novel variants	Total No	*COL4A3*	*COL4A4*	*COL4A5*
No of different variants, %	44 (100.0)	12 (27.2)	16 (36.4)	16 (36.4)
No of all Ind. with variant in particular gene, %	114 (100.0)	21* (18.4)	39* (34.2)	61*(53.5)
No of families with total GV*, %	63**(100.0)	14**(22.2)	35**(55.6)	30*(47.6)
No of Ind. with single GV, %	106 (100.0)	16 (15.1)	32 (30.2)	58 (54.7)
No of ind. with >1 gene (or allele) affected, %	8 (100.0)	3 (37.5)	6 (75.0)	3 (37.5)
No of total compound GV in the same gene, %	4 (100.0)	2 (50.0)	2 (50.0)	None
No of different families with compound GV, %	2 (100.0)	1 (50.0)	1 (50.0)	None
No of homozygous GV, %	1 (100.0)	None	1 (100.0)	None
No of different digenic GV with affected gene, %	8 (100.0)	1 (12.5)	4 (50.0)	3 (37.5)
No of Ind. with digenic variants with affected gene*, %	4* (100.0)	1* (25.0)	4* (100.0)	3* (75.0)
Novel GV, %	28 (100.0)	9 (32.1)	9 (32.1)	10 (35.7)
Reported GV, %	16 (100.0)	3 (18.8)	7 (43.8)	6 (37.5)
Described and shared with other populations GV, %	7 (100.0)	2 (28.6)	3 (42.9)	2 (28.6)

* Including the same individuals with digenic AS or ARAS (cross-groups); ** It is important to note that three probands belonged to two different families due to the identification of distinct genetic variants in both parents of each proband (e.g., in ARAS). Consequently, the number of probands does not correspond to the number of different families. GV – genetic variant; Ind. – individuals; No – number

Most of individuals (53.5%) had molecular changes in *COL4A5* gene, whereas distribution of genetic changes in individuals with *COL4A3* (18.4 %,) and *COL4A4* (34.2%) were significantly lower (p=0.042). To be noted, some individuals had more than one changes in the same or other *COL4A3-A5* genes. The frequency of different variants in each group of three *COL4A3*, *COL4A4*, and *COL4A5* genes was 27.2%, 36.4%, and 36.4%, respectively.

In this study, four different types of genetic variants including missense (75.0%), frameshift (9.1%), splice site (13.6%) and unknown (2.3%) variants in *COL4A3-A5* genes were identified (Table 17).

Table 17. Distribution of 44 different genetic variants and its types in *COL4A3-A5* genes

Type of GV	Total No of diff. GV	*COL4A3*	*COL4A4*	*COL4A5*
Total missense (%)*:	33 (75.0)	12 (100.0)	12 (75.0)	9 (56.3)
1. Gly subs	25 (56.8)	9 (75.0)	7 (43.8)	9 (56.3)
2. Non-Gly subs	8 (18.2)	3 (25.0)	5 (31.3)	0 (0.0)
Frameshift (%)	4 (9.1)	0 (0.0)	1 (6.3)	3 (18.8)
Splice site (%)	6 (13.6)	0 (0.0)	2 (12.5)	4 (25.0)
Unknown (%)	1 (2.3)	0 (0.0)	1 (6.3)	0 (0.0)
Total No of diff. GV (%)	44 (100.0)	12 (27.3)	16 (36.4)	16 (36.4)

* The total number of individuals with missense variants includes both Gly substitution and non-Gly substitution variants. No – number; diff. – different; GV – genetic variant; Gly – glycine; subs. – substitution

Different missense variants including Gly substitutions (n=25) and non-Gly substitutions (other amino acid change, n=8) were the most frequent types of genetic changes in all *COLA4A3-A5* genes and accounted for 76% of all individuals with AS (Table 18).

Table 18. Distribution of 114 individuals with different types of genetic changes in *COL4A3-A5*

Type of GV	Total No of Ind.	*COL4A3*	*COL4A4*	*COL4A5*
Total missense (%):	92 (76.0)	21 (100)	26 (66.7)	45 (73.8)
1. Gly subs	77 (63.6)	16 (76.2)	16 (41.0)	45 (73.8)
2. Non-Gly subs	15 (12.4)	5 (23.8)	10 (25.6)	0 (0.0)
Frameshift (%)	13 (11.4)	0 (0.0)	4 (10.3)	9 (14.8)
Splice site (%)	15 (12.4)	0 (0.0)	8 (20.5)	7 (11.5)
Unknown (%)	1 (0.8)	0 (0.0)	1 (2.6)	0 (0.0)
Total No of Ind. (%)	121* (106.1)	21* (18.4)	39* (39.2)	61* (53.5)

*To be noted: The number of individuals is counted based on the presence of a specific gene alteration. Therefore, individuals with ARAS or digenic AS may be counted twice. The total number of individuals with missense variants includes both Gly subs. and non-Gly subs. GV. No – number; GV – genetic variant; Gly – glycine; subs. – substitution; Ind. – individuals

The amino acid changes in different Gly substitutions distributed as follows 44% arginine (Arg, R), 12% valine (Val, V), 12% glutamic acid (Glu, E), 16% serine (Ser, S), 12% aspartic acid (Asp, D), 4% cysteine (Cys, C). The frequency of amino acid change in individuals with AS are indicated in Table 19.

Table 19. The frequency of amino acid changes in 77 individuals with Gly substitutions in *COL4A3-A5* genes

Amino acid which changed glycine	Total No of Ind.	*COL4A3*	*COL4A4*	*COL4A5*
Arginine (%)	27 (35.1)	11 (68.8)	6 (37.5)	10 (22.2)
Valine (%)	5 (6.5)	2 (12.5)	0 (0.0)	3 (6.7)
Glutamic acid (%)	5 (6.5)	1 (6.3)	4 (25.0)	0 (0.0)
Serine (%)	10 (13.0)	2 (12.5)	3 (18.8)	5 (11.1)
Aspartic acid (%)	27 (35.1))	0 (0.0)	0 (0.0)	27 (60.0)
Cysteine (%)	3 (3.9)	0 (0.0)	3 (18.8)	0 (0.0)
Total No of Ind. (%) *	77 (100.0)	16 (20.8)	16 (20.8)	45 (58.4)

No – number, Ind. – individuals. *To be noted: The number of individuals is counted based on the presence of a specific gene alteration. Therefore, individuals with ARAS or digenic AS may be counted twice

In collaboration with Australian colleagues, we discovered a significant correlation between instability in the *COL4A5* gene due to altered amino acid residues and the progression of AS. The location of the exon did not have a significant impact. Pathogenic variants in *COL4A5* with highly destabilizing glycine substitutions (Arg, Val, Glu, Asp, Trp) were associated with earlier onset of kidney failure and hearing loss. Substitutions near non-collagenous regions delayed kidney failure, while substitutions near non-collagenous regions in *COL4A3* and *COL4A4* reduced the risk of hematuria (88). The LVOD database, which included nine different molecular changes in *COL4A5* (45 individuals) from the Lithuanian cohort, was combined with the gnomAD database for the general evaluation, selection, and analysis. More details about the study can be found in the "Discussion" section.

The pathogenicity of the variants in *COL4A3-A5* genes, described in Genetic laboratories using ACMG classification, is shown in Table 20.

Table 20. Distribution of pathogenicity of *COL4A3-A5* genetic variants in 114 individuals

Pathogenicity	Total No of Ind.*	*COL4A3*	*COL4A4*	*COL4A5*
P (%)	35 (28.9)	2 (9.5)	3 (7.7)	30 (49.2)
LP (%)	67 (55.4)	14 (66.7)	26 (66.7)	27 (44.3)
VUS (%)	19 (15.7)	5 (23.8)	10 (25.6)	4 (6.6)
Total No of Ind. (%)	121* (106.1)	21* (18.4)	39* (39.2)	61* (53.5)

* The number of individuals is counted based on the presence of a specific gene alteration. Therefore, individuals with ARAS or digenic AS may be counted twice. P – pathogenic; LP – likely pathogenic; VUS – variant of uncertain significance; No – number, Ind. – individuals

Likely pathogenic variants were the most common variants found in the study among all individuals with *COL4A3 (n=14), COL4A4 (n=26)* and *COL4A5 (n=27)* changes. Distribution of pathogenicity of 44 different *COL4A3-A5* genetic variants found in the study is shown in Table 21 and Supp. Tables 1-2.

Table 21. The distribution of pathogenicity, based on genetic laboratory analysis, for 44 different novel genetic variants

Pathogenicity	Total No of diff. GV	*COL4A3*	*COL4A4*	*COL4A5*
P (%)	6 (13.6)	1 (8.3)	1 (6.3)	4 (25.0)
LP (%)	27 (61.4)	8 (66.7)	9 (56.3)	10 (62.5)
VUS (%)	11 (25.0)	3 (25.0)	6 (37.5)	2 (12.5)
Total No of diff. GV (%)	44 (100.0)	12 (27.3)	16 (36.4)	16 (36.4)

P – pathogenic; LP – likely pathogenic; VUS – variant of uncertain significance; No – number; diff – different; GV – genetic variant

A single heterozygous variant in *COL4A3* was found in 16 individuals, while in *COL4A4* it was found in 32 individuals, leading to the diagnosis of autosomal dominant Alport syndrome (ADAS, n=48), whereas single genetic change in heterozygous or hemizygous state of *COL4A5* gene was associated with X-linked AS (XLAS, n=58) and was identified in 31 females and 27 males, respectively. Autosomal recessive inheritance was detected in three individuals with compound heterozygous *COL4A3* (2 individuals) or *COL4A4* (1 individual) variants; and in one female with homozygous variants in *COL4A4* gene. Four individuals had digenic Alport syndrome following by identification of two variants in different *COL4A3-A5* genes (Table 22).

Table 22. Distribution of individuals with genetic variants in *COL4A3-A5* into different types of AS

Gene	ADAS (n=48)	ARAS (n=4)		XLAS (n=58)		Digenic AS* (n=4)
	Het	Compound Het	Homo	Het	Hemi	
COL4A3	16	2	None	-	-	1
COL4A4	32	1	1	-	-	3
COL4A5	-	-	-	31	27	3
Total	48	3	1	31	27	4*

* The same individual has more than one gene affected and constructs *COL4A3 +COL4A4*; *COL4A3+COL4A5* and *COL4A4+COL4A5* variation. Het – heterozygous; homo-homozygous; hemi – hemizygous; ADAS – autosomal dominant AS; ARAS – autosomal recessive AS; XLAS – X-linked AS; AS – Alport syndrome

Distribution of different AS types in this cohort is shown in Fig. 11 and will be discussed in more details in Chapter 4.4.

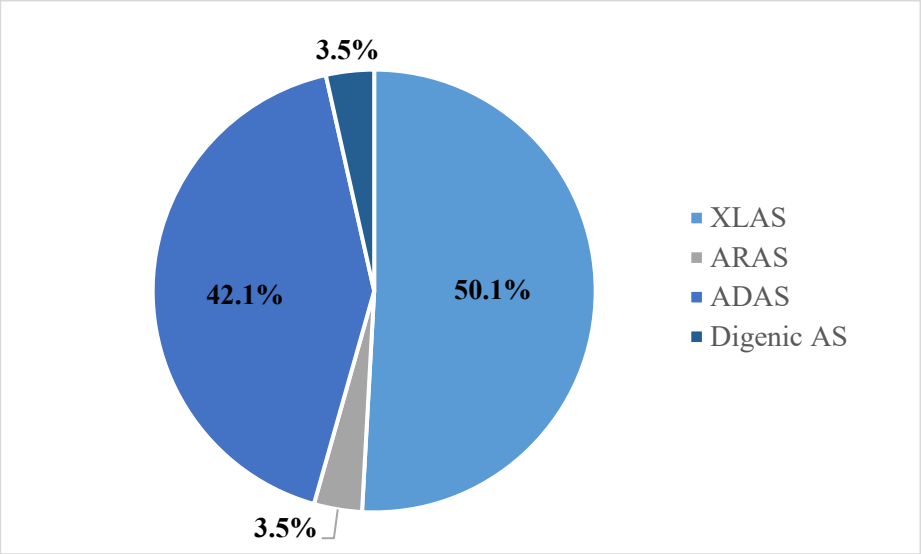


Figure 11. Distribution of different types of Alport syndrome in the study population

4.3.2. Novel genetic changes

Novel changes were found in 60 individuals from 28 different families. General characteristics of novel variants is shown in Table 23.

Table 23. General characteristics of 28 novel genetic variants of *COL4A3, COL4A4* and *COL4A5* genes

General characteristics of NV	Total No	*COL4A3*	*COL4A4*	*COL4A5*
NV, %	28 (63.6)	9 (75.0)	9 (56.3)	10 (62.5)
No of Ind. with NV, %	60 (52.6)	11 (52.4)	23 (59.0)	28 (45.9)
No of families with NV, %	28 (44.4)	8 (57.1)	11 (31.4)	11 (36.7)
No of Ind.* with ARAS and NV, %	3 (75.0)	2 (100.0)	1 (50.0)	0 (0.0)
No of Ind.* with digenic AS and NV, %	3 (75.0)	1 (100.0)	2 (50.0)	2 (66.7)

NV – novel variant; Ind – individual; No – number; ARAS – autosomal recessive Alport syndrome; AS – Alport syndrome. *The number of individuals is counted based on the presence of a specific gene alteration. Therefore, individuals with ARAS or digenic AS may be counted twice

The frequency of the novel variants among all variants found in the study (n=44) in *COL4A5, COL4A3* and *COL4A4* genes accounted for 62.5% (10/16), 75.0% (9/12) and 56.3% (9/16), respectively, and did not differ significantly between the genes (Fig. 12).

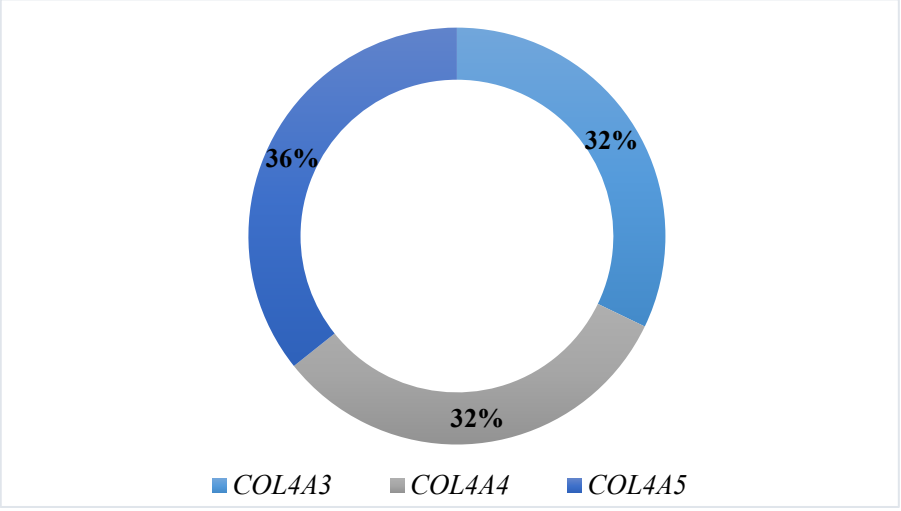


Figure 12. Distribution of novel variants in different *COL4A3-A5* genes

Type of novel changes in all *COL4A3-A5* genes

Twenty-eight (63.6 %) novel variants in *COL4A3-A5* genes were found in the study comprising 21 different missense variants (including Gly substitutions (76.2 %) and other amino acid changes (23.8%)), 3 frameshift, 3 splice site and 1 unknown type genetic changes (Table 24).

Table 24. Distribution of 28 different novel genetic changes in *COL4A3-A5*

Type of GV	Total no of diff. GV	*COL4A3*	*COL4A4*	*COL4A5*
Missense: Gly subs. (%)	16 (57.1)	7 (77.8)	4 (44.4)	5 (50.0)
Missense: non-Gly subs. (%)	5 (17.9)	2 (22.2)	3 (33.3)	0 (0.0)
Frameshift (%)	3 (10.7)	0 (0.0)	0 (0.0)	3 (30.0)
Splice site (%)	3 (10.7)	0 (0.0)	1 (11.1)	2 (20.0)
Unknown (%)	1 (3.6)	0 (0.0)	1 (11.1)	0 (0.0)
Total no of GV (%)	28 (100.0)	9 (32.1)	9 (32.1)	10 (35.7)

No – number; diff. – different; GV – genetic variant; Gly – glycine; subs. – substitution

The most frequent change in individuals with novel variants was Gly substitution and comprised 54.7 % of all individuals with novel changes in *COL4A5* gene. Distribution of 60 individuals with novel genetic changes in *COL4A3-A5* is shown in Table 25.

Table 25. Distribution of 60 different individuals with novel genetic changes in *COL4A3-A5*

Type of var.	Total No of Ind.	*COL4A3*	*COL4A4*	*COL4A5*
Missense: Gly subs. (%)	35 (54.7)	10 (76.9)	11 (47.8)	14 (50.0)
Missense: non-Gly subs. (%)	8 (12.5)	3 (23.1)	5 (21.7)	0 (0.0)
Frameshift (%)	8 (12.5)	0 (0.0)	0 (0.0)	9 (32.1)
Splice site (%)	11 (17.2)	0 (0.0)	6 (26.1)	5 (17.9)
Unknown (%)	1 (1.6)	0 (0.0)	1 (4.3)	0 (0.0)
Total No of Ind.* (%)	64* (106.7)	13* (20.3)	23* (35.9)	28* (43.8)

* The number of individuals is counted based on the presence of a specific gene alteration. Therefore, individuals with ARAS or digenic AS may be counted twice. No – number; Ind. – individual; subs. – substitution; Gly – glycine

Novel Glycine substitutions and amino acid changes

The most frequent amino acid change among the 35 individuals with Gly substitution was Arginine, accounting for more than half of the novel cases. Serine, valine, and glutamic acid accounted for 17.1%, 14.3%, and 11.4% of cases, respectively (Table 26). No Glycine change into aspartic acid or cysteine was observed among the novel missense variants. The distribution of amino acid changes in different Gly substitutions in *COL4A3-A5* genes is shown in Table 27.

Table 26. The frequency of amino acid changes in 35 individuals with novel Gly substitutions

Amino acid which changed glycine	Total No of Ind.	*COL4A3*	*COL4A4*	*COL4A5*
Arginine (%)	20 (57.1)	5 (50.0)	5 (45.5)	10 (71.4)
Valine (%)	5 (14.3)	2 (20.0)	0 (0.0)	3 (21.4)
Glutamic acid (%)	4 (11.4)	1 (10.0)	3 (27.3)	0 (0.0)
Serine (%)	6 (17.1)	2 (20.0)	3 (27.3)	1 (7.1)
Aspartic acid (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Cysteine (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Total No of Ind.* (%)	35 (100.0)	10 (28.6)	11 (31.4)	14 (40.0)

No – number; Ind. – individual. * The number of individuals is counted based on the presence of a specific gene alteration. Therefore, individuals with ARAS or digenic AS may be counted twice

Table 27. The frequency of amino acid changes in 16 novel Gly substitutions

Amino acid which changed glycine	Total No of diff. Gly subs.	*COL4A3*	*COL4A4*	*COL4A5*
Arginine (%)	8 (50.0)	3 (42.9)	2 (50.0)	3 (60.0)
Valine (%)	3 (18.8)	2 (28.6)	0 (0.0)	1 (20.0)
Glutamic acid (%)	2 (12.5)	1 (14.3)	1 (25.0)	0 (0.0)
Serine (%)	3 (18.8)	1 (14.3)	1 (25.0)	1 (20.0)
Aspartic acid (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Cysteine (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Total No of diff. Gly subs. (%)	16 (100.0)	7 (43.8)	4 (25.0)	5 (31.3)

No – number; diff. – different; subs. – substitution; Gly - glycine

General pathogenicity of novel variants

In our study, the novel molecular changes were classified into likely pathogenic (LP) variants and variants of uncertain significance (VUS), accounting for 71.4% (20 out of 28 variants) and 28.6% (8 out of 28 variants) of the novel cases, respectively. No pathogenic (P) variants were found. LP variants were the most frequent changes identified in all *COL4A3-A5* genes. The general distribution of variant pathogenicity among the 60 individuals is shown in Table 28 and Supp. Tables 1-2.

Table 28. The distribution of pathogenicity, based on genetic laboratory analysis, for all novel genetic variants in 60 individuals

Pathogenicity	Total No of Ind.	*COL4A3*	*COL4A4*	*COL4A5*
P (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
LP (%)	50 (78.1)	8 (61.5)	18 (78.3)	24 (85.7)
VUS (%)	16 (25.0)	5 (38.5)	5 (21.7)	4 (14.3)
Total No of Ind.* (%)	64* (106.7)	13* (20.3)	23* (35.9)	28* (43.8)

* The number of individuals is counted based on the presence of a specific gene alteration. Therefore, individuals with ARAS or digenic AS may be counted twice

Our clinical interpretation and reclassification of novel *COL4A3-A5* changes was based on ACMG criteria as defined by different genetic databases (ClinVar, InterVar, Franklin, Centogene), as well as the results of mutation predictive tools (PPT, SIFT, MT, Align-GVGD), and consideration of clinical course, family history, kidney biopsy results, sex, and age. Clinical features associated with reclassification from VUS to likely pathogenic variants included nephrotic range proteinuria, proteinuria with eGFR <60 ml/min/1.73 m^2, III-V stage CKD, kidney function alterations along with hearing and/or ocular abnormalities, presence of CKD or KF in family members, considering their age. It should be noted that individuals with comorbidities potentially associated with worsening kidney function were excluded from the evaluation. Our proposed reclassification of novel variants is described in sections 4.3.2.1, 4.3.2.2, and 4.3.2.3.

Distribution of novel variants into different AS types

Novel variants in *COL4A5* genes were found in 39.2% (11/28) of all families with XLAS, whereas one heterozygous *COL4A3* or *COL4A4* change led to the diagnosis of ADAS to 48.4 % (15/31) of families. Three individuals had one or both novel compound heterozygous *COL4A3* or *COL4A4* variants, thus ARAS. Three individuals were identified as having digenic AS due to more than one *COL4A3-A5* gene affected (Table 29).

Table 29. Distribution of individuals with novel genetic variants in *COL4A3-A5* and type of AS

Gene	ADAS (n=28)	ARAS (n=3)		XLAS (n=26)		Digenic AS* (n=3)
	Het	Compound Het	Homo	Het	Hemi	
COL4A3	8	2	0	-	-	1
COL4A4	20	1	0	-	-	2
COL4A5	-	-	-	17	9	2
Total	28	3	0	17	9	3*

* The same individual has more than one gene affected and constructs *COL4A3 +COL4A4*; *COL4A3+COL4A5* and *COL4A4+COL4A5* variation. Het – heterozygous; homo-homozygous; hemi – hemizygous; ADAS – autosomal dominant AS; ARAS – autosomal recessive AS; XLAS – X-linked AS; AS – Alport syndrome

4.3.2.1. Novel variants in *COL4A5* gene

In total, 28 individuals with 10 novel *COL4A5* variants were identified. Among them, there were 26 individuals with XLAS (9 males and 17 females) from 11 different families, and they presented with 10 different novel *COL4A5* variants. Additionally, two females from the same XLAS families exhibited digenic AS, which involved changes in both *COL4A5* and *COL4A4* genes (Table 30). Additionally, 31% of individuals with novel *COL4A5* variants were younger than 18 years old at the time of diagnosis.

Table 30. Distribution of AS types with novel *COL4A5* variants in 28 individuals

Type of AS	Subtype of AS	No of individuals (No of diff. families)	Note
XLAS males	-	9 (8)	-
XLAS females	-	17 (10)	-
Digenic	*COL4A5+COL4A3*	0	-
	COL4A5+ COL4A4	2 (2)	Also mentioned in Chapter 4.3.2.2.

Five novel glycine substitutions were discovered in *COL4A5* gene, while frameshift and splice site changes accounted for 3 and 2 novel variants in *COL4A5*, respectively. The summary of novel *COL4A5* changes found in the Lithuanian cohort are shown in Table 29 and with more detailed information provided in Supp. Tables 12-14.

Table 31. ACMG classification of novel *COL4A5* variants

GV in *COL4A5* gene	Type, (GV identification number)	M/F with GV	No of diff. fam.	ACMG by LAB	Our interpretation
c.3508G>C (p.Gly1170Arg)	Gly subs. (3)	1M/2F	1	LP	P
3106+1G>A	Splice site (4)	1M/2F	1	LP	LP
c.1417_1418del (p.Val473Glufs*3)	Frameshift (6)	4F*	1	LP	P
c.2623G>A (p.Gly875Arg)	Gly subs.(13)	2M/4F	2	LP	P
c.347delC (p.Pro116Glnfs*39)	Frameshift (14)	1M/1F	1	LP	LP
c.883G>A (p.Gly295Ser)	Gly subs.(18)	1M	1	VUS	LP
c.2777G>T (p.Gly926Val)	Gly subs.(21)	2M/1F	1	VUS	P
c.1374delinsTT (p.Pro459Serfs*6)	Frameshift(26)	1M/2F	1	LP	VUS
c.3554-2A>G	Splice site (33)	2F	1	LP	LP
c.466G>C (p.Gly156Arg)	Gly subs.(34)	1F	1	LP	P

LAB – laboratory; No – number; M – male; F – female; P – pathogenic; LP – likely pathogenic; VUS – variant of uncertain significance; GV – genetic variant; Ind. – individuals; fam. – family; diff. – different. * Including one female with digenic AS

The Centogene laboratory classified eight novel variants in *COL4A5* as likely pathogenic. Among these variants, four were described as disease-causing or probably damaging by PolyPhen-2 (PP2), while the remaining variants were not applicable. Franklin, ClinVar, and InterVar databases considered 7, 1, and 4 changes as likely pathogenic, respectively. Two novel changes in *COL4A*5 were identified as VUS by Centogene (PP2, MT, SIFT was not applicable). Both of these variants were classified as unknown significance using ACMG criteria in the InterVar database, while Franklin described one of these changes as LP.

Our clinical interpretation of novel *COL4A5* changes resulted in reclassifying more than half of the likely pathogenic variants as pathogenic. Three LP variants remained unchanged. Both VUS variants were classified as having a more pathogenic effect than benign, as shown in Table 32. In total, pathogenicity was changed in 7 (70%) of the genetic variants out of the 10 novel *COL4A5* changes.

Table 32. Laboratory-based pathogenicity changes in *COL4A5* from study results

Pathogenicity	Total No of GV by lab. (%)	Changed into Pst	Changed into LPst	Changed into VUSst	Total No of GV by our st. (%)
P (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	5 (50.0)
LP (%)	8 (80.0)	5 (62.5)	3 (37.5)	0 (0.0.)	4 (40.0)
VUS (%)	2 (20.0)	1 (50.0)	1 (50.0)	0 (0.0)	1 (10.0)
Total No of GV	10 (100.0)	6 (60.0)	1 (10.0)	0 (0.0)	10 (100.0)

^{lab} – ACMG and pathogenicity reported by genetic laboratory; st – changed ACMG and pathogenicity by our study interpretation; P – pathogenic; LP – likely pathogenic; VUS – variant of uncertain significance; GV – genetic variant. Blue colour indicates the altered pathogenicity from the laboratory to our study

4.3.2.2. Novel variants in *COL4A4* gene

In total, 23 individuals with 9 novel *COL4A4* variants were identified. Twenty individuals from 9 different families had a single heterozygous *COL4A4* variant, indicating ADAS with a 7 different novel *COL4A4* changes. In one ARAS male patient, two compound heterozygous *COL4A4* variants were identified, one of which was novel. Additionally, two females had digenic AS, with novel changes found in both *COL4A4* (2 novel variants) and *COL4A5* genes (Table 33). Approximately 28% of patients with novel *COL4A4* variants were younger than 18 years old at the time of diagnosis.

Table 33. AS types with novel *COL4A4* variants in 23 individuals

Type of AS	Subtype of AS	No of Ind. (No of diff. families). M/F	Note
ADAS	-	20 (9) 11M/9F	ADAS with heterozygous *COL4A4* variant
ARAS	Compound heterozygous	1M (from the same family as ADAS)	2 variants in *COL4A4*: 1 novel and 1 previously described.
Digenic AS	*COL4A4+COL4A5*	2F (2)	Also mentioned in Chapter 4.3.2.1.

Four novel glycine substitutions were discovered in *COL4A4* gene, while non-Gly amino acid changes, splice site and unknown variants accounted for 3, 1 and 1 novel variants in *COL4A4*, respectively. The summary of novel *COL4A4* changes found in the Lithuanian cohort are shown in Table 34 and with more detailed information provided in Supp. Tables.1-2.

Table 34. ACMG classification novel of *COL4A4* genetic variants

GV in *COL4A4*	Type, (GV identification number)	M/F with GV	No of diff. fam.	ACMG by LAB	Our clinical interpretation
c.657+2dup	Unknown (5)	1F	1	LP	LBn
c.4151C>T (p.Ala1384Val)	Non-Gly subs.(7)	2F*	2	VUS	LP*
c.594+1G>A	Splice site (9)	3M */3F	2	LP	LP*
c.1987G>C (p.Gly663Arg)	Gly subs.(16)	2M	1	LP	LBn
c.4910G>A (p.Arg1637Gln)	Non-Gly subs.(27)	1F*	1	VUS	LP*
c.2756A>G (p.Glu919Gly)	Non-Gly subs.(32)	1M/1F	1	VUS	LBn
c.4315G>A (p.Gly1439Ser)	Gly subs. (36)	2M/1F	1	LP	LBn
c.3044G>A (Gly1015Glu)	Gly subs. (40)	3M	1	LP	LBn
c.2347G>A (p.Gly783Arg)	Gly subs. (41)	2M/1F	1	LP	LP

* Including individuals with ARAS and digenic AS. LAB – laboratory; No – number; M – male; F – female; P – pathogenic; LP – likely pathogenic; VUS – variant of uncertain significance; LBn – likely benign; Ind. – individuals; diff. – different; fam. – family; GV – genetic variant

The genetic laboratory classified six novel variants in *COL4A4* as likely pathogenic. Three of these variants were described as pathogenic or probably damaging by PolyPhen-2 (PP2), while the remaining variants were not applicable. According to Franklin and InterVar databases, three of these

changes were considered variants of uncertain significance (VUS), while one was declared as pathogenic. ClinVar was not applicable for two likely pathogenic changes, and two variants were identified as either pathogenic or VUS. Three novel changes in *COL4A4* were identified as variants of uncertain significance (VUS). PolyPhen2 described these variants as either probably damaging or benign, although MutationTester mostly indicated polymorphism. All three variants were classified as VUS by genetic databases including ClinVar, Franklin, and InterVar.

Our clinical interpretation of novel *COL4A4* variants resulted in reclassifying more than half of the likely pathogenic variants as likely benign. One VUS variant was considered more likely benign, while another was classified as likely pathogenic, as shown in Table 35. In total, pathogenicity was changed in 6 (66.7%) of the genetic variants out of the 9 novel *COL4A4* changes.

Table 35. Laboratory-based pathogenicity changes in *COL4A4* from study results

Pathogenicity	Total No of GV by lab. (%)	Changed into P^{st}	Changed into LP^{st}	Changed into VUS^{st}	Changed into LBn^{st}	Total No of GV by our st. (%)
P (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
LP (%)	6 (66.7)	0 (0.0)	2 (33.3)	0 (0.0)	4 (66.6)	4 (44.4)
VUS (%)	3 (33.3)	0 (0.0)	1 (33.3)	0 (0.0)	1 (33.3)	0 (0.0)
LBn (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	5 (55.6)
No of GV	9 (100.0)	0 (0.0)	1 (11.1)	0 (0.0)	5 (55.6)	9 (100.0)

^{lab} – ACMG and pathogenicity reported by genetic laboratory; st – changed ACMG and pathogenicity by our study interpretation; P – pathogenic; LP – likely pathogenic; VUS – variant of uncertain significance; LBn – likely benign; GV – genetic variant. Blue colour indicates the altered pathogenicity from the laboratory to our study

4.3.2.3 Novel variants in *COL4A3* gene

In total, 11 individuals with 9 novel *COL4A3* variants were identified. Eight individuals from 6 different families had one heterozygous *COL4A3* variant, leading to ADAS. These individuals accounted for 6 different novel *COL4A3* changes. Additionally, twin sisters had two novel compound heterozygous *COL4A3* variants. Furthermore, one individual had digenic AS, which included one novel change in the *COL4A3* gene and one known variant in the *COL4A4* gene (Table 36). Eighteen percent of the individuals with novel *COL4A3* variants were younger than 18 years old at the time of diagnosis.

Table 36. AS types with novel *COL4A3* variants in 11 individuals

Type of AS	Subtype of AS	No of Ind. (No of diff. families). M/F	Note
ADAS	-	8 (6) 4M/4F	With het. *COL4A3* variant
ARAS	Compound heterozygous	2 (1) 2F	Twin sisters
Digenic	*COL4A3+COL4A4*	1M (1)	One novel in *COL4A3*

Seven novel glycine substitutions and two other amino acid changes were identified in the *COL4A3* genes. A summary of the novel *COL4A3* changes found in the Lithuanian cohort is shown in Table 37, and in Supp. Tables 1-2.

Table 37. ACMG classification of novel *COL4A3* genetic variants

GV in *COL4A3*	Type, (GV identification number)	M/F with GV	No of diff. fam.	ACMG by LAB	Our clinical interpretation
c.520G>A (p.Gly174Arg)	Gly subs.(19)	1M/1F	1	LP	LP
c.4702C>T (p.Pro1568Ser)	Non-Gly (23)	2F*	1	VUS	P*
c.3247G>C (p.Gly1083Arg)	Gly subs. (24)	2F *	1	VUS	P*
c.2711G>T (p.Gly904Val)	Gly subs.(31)	1M	1	LP	LBn
c.4717G>A (p.Gly1573Ser)	Gly subs.(35)	1M/1F	1	LP	LBn
c.416G>A (p.Gly139Glu)	Gly subs.(37)	1F	1	LP	LP
c.1021C>T (p.Arg341Cys)	Non-Gly (38)	1M *	1	VUS	LP*
c.593G>T (p.Gly198Val)	Gly subs.(39)	1M	1	LP	LP
c.2188G>C (p.Gly730Arg)	Gly subs.(43)	1F	1	LP	LBn

* Including individuals with ARAS and digenic inheritance. LAB- laboratory; No – number; M- male; F- female; P – pathogenic; LP- likely pathogenic; VUS – variant of uncertain significance; LBn – likely benign; Bn – benign; non-Gly – non glycine substitution

Centogene laboratory classified six novel variants in *COL4A3* as LP, with four of these variants described as disease-causing or probably damaging by PolyPhen-2 (PP2), while two were labelled as not applicable. InterVar declared all these LP variants as VUS, while the Franklin database considered 83.3% of these variants as VUS and one as pathogenic. Three novel changes in *COL4A3* were identified as VUS, despite all of them being described as probably damaging or disease-causing by both PP2 and MutationTester. InterVar, ClinVar, and Franklin databases classified these three variants as VUS.

Our clinical interpretation of novel *COL4A3* changes resulted in reclassifying half of the likely pathogenic variants as likely benign and the rest half remained unchanged, as indicated in Table 38. All VUS were also reclassified, with two being classified as pathogenic and one as likely benign.

In total, pathogenicity was changed was changed in 6 (66.7%) of genetic variants out of 9 novel *COL4A3* changes.

Table 38. Laboratory-based pathogenicity changes in *COL4A3* from study results

Pathogenicity	Total No of GV by lab. (%)	Changed into P^{st}	Changed into LP^{st}	Changed into VUS^{st}	Changed into LBn^{st}	Total No of GV in our st. (%)
P^{lab} (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (22.2)
LP^{lab} (%)	6 (66.7)	0 (0.0)	3 (50.0)	0 (0.0)	3 (50.0)	4 (44.4)
VUS^{lab} (%)	3 (33.3)	2 (66.7)	1 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)
LBn^{lab} (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (33.3)
Total No of GV	9 (100.0)	2 (22.2)	1 (11.1)	0 (0.0)	3 (33.3)	9 (100.0)

^{lab} – ACMG and pathogenicity reported by genetic laboratory; st – changed ACMG and pathogenicity by our study interpretation; P – pathogenic; LP – likely pathogenic; VUS – variant of uncertain significance; LBn – likely benign; GV – genetic variant. Blue colour indicates the altered pathogenicity from the laboratory to our study

4.3.3. Shared *COL4A3-A5* variants with general population

To enhance the validity of our statistical and descriptive analysis, we examined all variants identified in the study for novelty and frequency using genetic population-based databases such as ClinVar, Varsome, Franklin, and LVOD. Seven genetic variants identified in the Lithuanian cohort were found to be shared with the general population worldwide. The genetic variants and our clinical interpretation of their pathogenicity are presented in Table 39 and Supp. Tables 1-2.

Table 39. Distribution of genetic findings in the Lithuanian cohort shared with the global population

Gene	GV ID	GV	Type of AS	M/F (No of fam.)	ACMG by LAB	Study ACMG
COL4A3	(44)	p.Leu1474Pro (c.4421T>C)	ADAS	2M (2)	LP	P
COL4A3	(29)	p.Gly1167Arg (c.3499G>A)	ADAS	2F (2)	P	LP
COL4A4	(20)	c.-101-4A>G	ADAS/ digenic AS	1M*, 1F (2)	VUS	LBn
COL4A4	(11)	p.Gly999Glu (c.2996G>A)	ADAS/ digenic AS	1F* (1)	VUS	LBn

Gene	GV ID	GV	Type of AS	M/F (No of fam.)	ACMG by LAB	Study ACMG
COL4A4	(10)	p.Gly527Cys (c.1579G>T)	ADAS/ ARAS	3M*(2)	P	LP
COL4A5	(12)	p.Gly624Asp (c.1871G>A)	XLAS/ digenic AS	10F*/12M (14)	P	P
COL4A5	(17)	p.Gly1229Ser (c.3685G>A)	XLAS	2M/2F (1)	P	P

No – number; M – male; F – female; P – pathogenic; LP – likely pathogenic; VUS – variant of uncertain significance; LBn – likely benign; GV – genetic variant; ID – identification code in the study; fam. – family. * Individuals with ARAS or digenic AS

Heterozygous *COL4A3* p.Leu1474Pro (c.4421T>C) variant was identified in two males from different families. According to the genetic laboratory (VUH SK) this change was described as pathogenic by MutationTaster and SIFT. To compare, Franklin and InterVar identified this change as VUS, whereas ClinVar indicated conflicting pathogenicity (from benign to pathogenic) . Both of males had hematuria and proteinuria. One male at the age of 45 reached KF while other individual had eGFR >90 ml/min/1.73 m3 at the age of 34. Kidney biopsy of both males (30 and 33 y.o.) revealed TBM, and thickness and lamellation of GBM, while FSGS was only identified in male with KF at the age of 30. Bilateral hearing loss was found in one male while ocular lesions were not common for any of these individuals.

Heterozygous *COL4A3* p.Gly1167Arg (c.3499G>A) variant was found in 2 females with ADAS. According to the Centogene laboratory this change was described as pathogenic by PolyPhen-2 (probably damaging), MutationTaster (Disease causing) and SIFT (deleterious) tools. To compare, Franklin and ClinVar described this variant as pathogenic while InterVar indicated it as likely pathogenic. Both of the females had proteinuria, while no KF was observed. Hearing abnormalities was found in one female. None of women had ocular lesions.

Heterozygous *COL4A4* c.-101-4A>G variant was found in one female (32 y.o) with ADAS and in one male (A3/438376; 44 y.o.) with digenic AS sharing phenotype with other *COL4A3* variant (genotype-phenotype correlation of digenic AS is described in digenic AS section). According to the Centogene laboratory this change was described as variant with unknown significance (PolyPhen-2, MutationTaster and SIFT were not applicable). To compare, Franklin identified this change as likely benign, whereas ClinVar declared conflicting data. Female with this variant had hematuria and proteinuria with normal kidney function (eGFR >90 ml/min/1.73 m3). Ocular and hearing abnormalities was not observed.

Heterozygous *COL4A4* p.Gly999Glu (c.2996G>A) variant was found in individual A5/412818 with digenic AS and shared its phenotype with other variant in *COL4A5* gene. According to the Centogene laboratory this change was described as VUS and probably damaging by PolyPhen-2, MutationTaster and SIFT tools. To compare, Franklin and InterVar identified this change as benign, while ClinVar indicated it as VUS or LP. Individual with digenic change is described in digenic section and is not included in description above.

One heterozygous *COL4A4* p.Gly527Cys (c.1579G>T) variant was found in two males with ADAS. According to the Centogene laboratory this change was described as pathogenic by PolyPhen-2 (probably damaging), MutationTaster (Disease causing) and SIFT (deleterious) tools. To compare, Franklin and ClinVar identified this change as likely pathogenic, whereas InterVar indicated this variants as VUS. Both of males (46 and 20 y.o.) had hematuria, while none of them had proteinuria or impaired kidney function. One male had hearing loss, while ocular abnormalities were not observed in any of individuals. One male (13 y.o.) had this variant together with other *COL4A4* variant leading to more severe and complex manifestation of ARAS (genotype-phenotype correlation of ARAS is described in ARAS section).

The most prevalent variant in our cohort shared with the world population was *COL4A5* p. Gly624Asp change (61 %) in exon 25 which accounted for 22 individuals from 14 different families. Twenty-one patient having this variants was diagnosed with XLAS while one female had digenic AS and shared her phenotype with other *COL4A4* variant. According to the Centogene laboratory this change was described as pathogenic by PolyPhen-2 (probably damaging), MutationTaster (disease causing) and SIFT (deleterious) tools. To compare, Franklin identified this change as pathogenic, InterVar as likely pathogenic, whereas ClinVar described this variant as conflicting pathogenicity. Among 12 males and 9 females with single *COL4A5* Gly624Asp variant, all individuals had hematuria, while proteinuria appeared in 83.3% (10/12) and 66.6% (6/9) of individuals, respectively. None of these patients reached KF, however 8 (38%) out of 21 had eGFR less than 60 ml/min/1.73 m3. Bilateral sensorineural hearing loss and ocular abnormalities were found in 9 and 2 individuals, respectively. Individual with digenic change is described in digenic section and is not included in description above.

COL4A5 p.Gly1229Ser (c.3685G>A) variant was found in 4 individuals from the same family (1 female and 3 males). According to the Centogene laboratory this change was described as variant with unknown significance although PolyPhen-2, MutationTaster and SFIT identified this variant as probably damaging, disease causing and deleterious, respectively. To

compare, Franklin and ClinVar identified this variants as pathogenic, while InterVar described it as likely pathogenic. All individuals had hematuria, 3 of 4 had proteinuria. None of family members reached KF nor had hearing or ocular abnormalities.

Links to the shared genetic variants in COL4A3-A5 genes:

1.https://www.ncbi.nlm.nih.gov/clinvar/variation/449540/?oq=COL4A3+p.Leu1474Pro&m=NM_000091.5(COL4A3):c.4421T%3EC%20(p.Leu1474Pro)
2. https://www.ncbi.nlm.nih.gov/clinvar/variation/17492/
3.https://www.ncbi.nlm.nih.gov/clinvar/variation/683162/?oq=COL4A4+c.-101-4A%3EG&m=NM_000092.5(COL4A4):c.-101-4A%3EG
4.https://www.ncbi.nlm.nih.gov/clinvar/variation/255027/?oq=COL4A4+p.Gly999Glu&m=NM_000092.5(COL4A4):c.2996G%3EA%20(p.Gly999Glu)
5.https://www.ncbi.nlm.nih.gov/clinvar/variation/554661/?oq=COL4A4+p.Gly527Cys&m=NM_000092.5(COL4A4):c.1579G%3ET%20(p.Gly527Cys)
6.https://www.ncbi.nlm.nih.gov/clinvar/variation/24455/?oq=Gly624Asp+COL4A5&m=NM_033380.3(COL4A5):c.1871G%3EA%20(p.Gly624Asp)
7.https://www.ncbi.nlm.nih.gov/clinvar/variation/562403/?oq=COL4A5+p.Gly1229Ser&m=NM_033380.3(COL4A5):c.3685G%3EA%20(p.Gly1229Ser)

4.4. Genotype-phenotype correlations and clinical characteristics of individuals with AS

4.4.1. Comparison between different types of AS

In this study, the genetic analysis of *COL4A3-A5* genes identified 44 distinct genetic changes inherited in an X-linked, autosomal dominant, autosomal recessive, and digenic manner by 58, 48, 4, and 4 individuals, respectively. Consequently, four different groups of Alport syndrome, namely XLAS, ARAS, ADAS, and digenic AS were categorized. The distribution of gender in the different types of AS is illustrated in Figure 13.

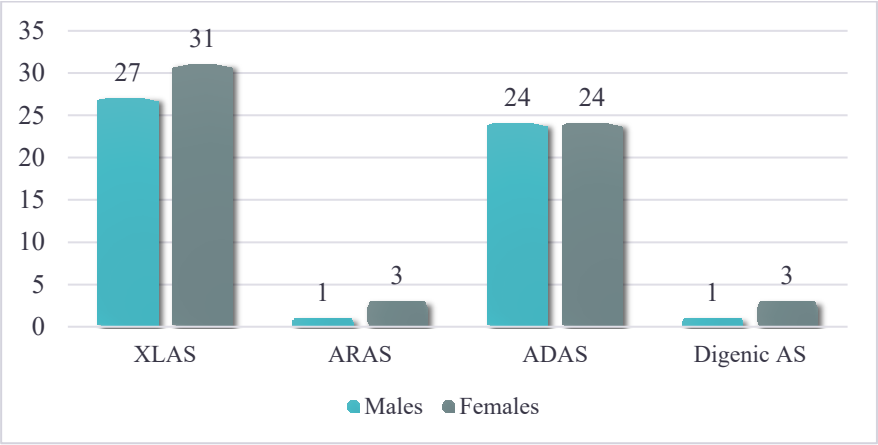


Figure 13. Distribution of gender in different types of AS

Probands comprised for 46.6%, 75%, 54.2% and 100% of individuals in XLAS, ARAS, ADAS and digenic AS group, respectively. The most common diagnostic tool of AS in XLAS (51.7%) and ADAS (45.8%) groups was family screening and did not differ among these groups.

Hematuria was detected significantly earlier than proteinuria in all AS groups, with the earliest diagnosis observed in the XLAS group (p=0.05), as depicted in Figure 14.

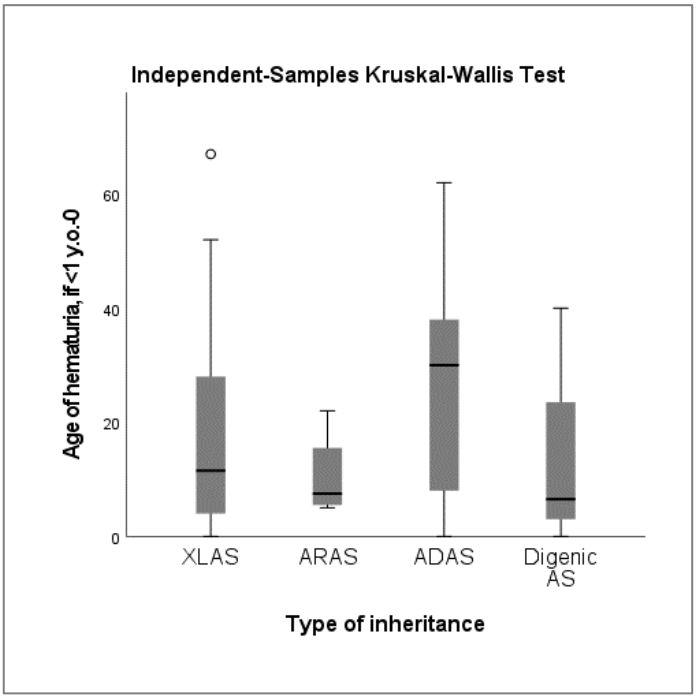


Figure 14. Age at detection of haematuria depending on type of inheritance of AS

The majority of individuals with XLAS, ADAS, and digenic AS exhibited normal kidney function, indicated by eGFR >90 ml/1.73/m^2. However, individuals with ARAS experienced more severe kidney disease, despite the relatively small number of individuals in this group. Kidney failure was observed in 6 (10%) individuals with XLAS, 2 (50%) individuals with ARAS, and 2 (4%) individuals with ADAS. None of the individuals with digenic AS reached kidney failure. It should be noted that a direct comparison of all four types of AS could not be performed due to the smaller cohort sizes of ARAS and digenic AS in comparison to XLAS and ADAS.

4.4.2 Genotype-phenotype characteristics of individuals with XLAS

In total, 58 individuals with XLAS were identified in the study, accounting for 50.9% of all study participants (Table 40 and Supp. Tables 12-13). Among them, there were 27 males with a mean age at diagnosis of 29.3 ± 17.0 years. The majority of XLAS females (71.0%) were diagnosed through family screening, while males were primarily referred to molecular analysis, often preceded by pathologic findings in KB (40.7%). There was a higher proportion of women diagnosed with AS through family screening compared to males (p = 0.04; 95% CI [1.6-14.9]), whereas males exhibited more significant clinical symptoms leading to the diagnosis of AS (p = 0.04; 95% CI [0.01-1.04]).

Table 40. Clinical characteristics of 58 individuals with XLAS

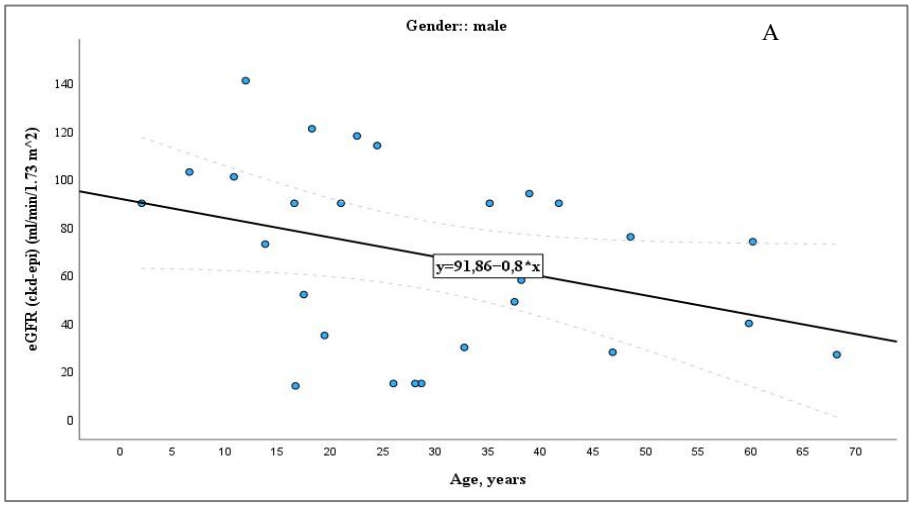
Demographic and clinical features of XLAS	Total	Hemizygotes (males)	Heterozygotes (females)	P-value
Number of subjects, %	58 (100.0)	27 (46.6)	31 (53.4)	>0.05
Age at genetic diagnosis, %	32.4±18.1	29.3±17.0	35.1±19.0	0.229
Probands, %	27 (46.6)	18 (66.7)	9 (29.0)	**0.04**
Dgn. of AS by CF& KB, %	19 (32.8)	11 (40.7)	8 (25.8)	0.227
Dgn. of AS by CF only, %	7 (12.1)	6 (22.2)	2 (3.2)	**0.04**
Dgn. of AS by FS, %	31 (53.4)	9 (33.3)	22 (71.0)	**0.004**
Hematuria, %	58 (100.0)	27 (100.0)	31 (100.0)	>0.05
Age at dgn. of hematuria, %	17.4±16.4	14.0±15.2	20.4±17.0	0.139
Proteinuria, %	48 (82.8)	23 (85.2)	25 (80.6)	0.648
Age at dgn. of proteinuria	23.2±18.1	20.1±19.8	25.2±16.6	0.207
Nephrotic range proteinuria, %	7 (12.0)	5 (18.5)	2 (6.5)	0.233
CKD I, %	30 (51.7)	12 (44.4)	18 (58.1)	
CKD II, %	9 (15.5)	3 (11.1)	6 (19.4)	x^2=4.7
CKD III, %	11 (19.0)	6 (22.2)	5 (16.1)	p=0.32
CKD IV, %	2 (3.4)	2 (7.4)	0 (0.0)	df.=4
CKD V (KF) , %	6 (10.3)	4 (14.8)	2 (6.5)	
Mean age at KF, %	32.8±16.0	23.5±5.4	51.5±12.0	0.06

Demographic and clinical features of XLAS	Total	Hemizygotes (males)	Heterozygotes (females)	P-value
Arterial hypertension, %	31 (53.4)	17 (63.0)	14 (45.2)	0.175
RAAS inhibition, %	39 (67.2)	20 (74.1)	19 (61.3)	0.301
KB, %	22 (37.9)	14 (51.9)	8 (25.8)	**0.041**
Age at kidney biopsy, %	26.8±15.1	26.4±14.2	27.6±17.5	0.785
Hearing abnormalities, %	18 (31.0)	15 (60.0)	3 (11.5)	**<0.001**
Ocular lesions, %	7 (12.1)	6 (25.0)	1 (3.7)	**0.042**

GD – genetic diagnosis, No – number, Pro – proteinuria, AH- arterial hypertension, KF – kidney failure, KB kidney biopsy, FS – family screening, CKD – chronic kidney disease, No – number; dgn. – diagnosis; CF – clinical features; FS – family screening

Kidney disease

Haematuria was detected in all individuals with XLAS, while proteinuria was observed in more than 80 % of both hemizygotes and heterozygotes. KF developed in four males with XLAS at a mean age of 23.5 ± 5.4 years (range: 19-31), while 6.5% of females reached KF later in years (mean age of 51.5 ± 12.0 years). However, a significant difference of KF between genders in our study was not found (p=0.064). In our study, no significant correlation between age and eGFR levels was observed in males with XLAS (Fig. 15A, n=27, p=0.063; r=-0.362) while in females a reasonable association between lower eGFR levels and older age was detected (Fig. 15 B, n=31, p=0.001; r=-0.553).



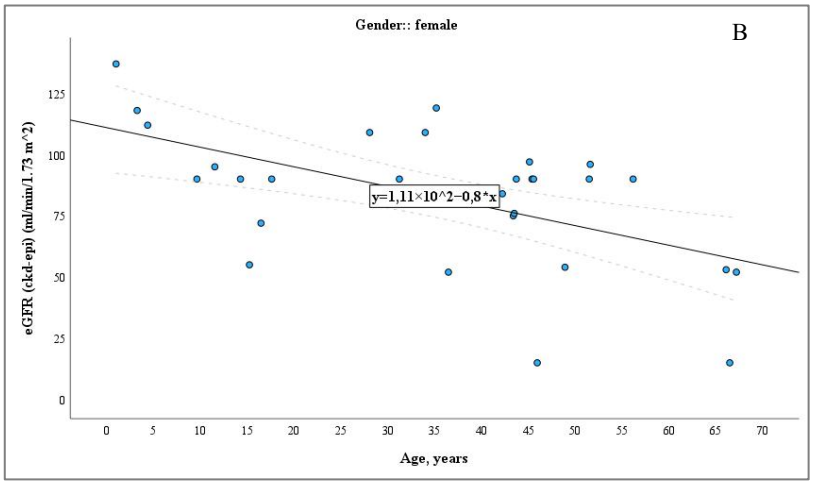


Figure 15. Correlation of age and eGFR levels in different male patients (A, n=27, p=0.063; r=-0.362) and female patients (B, n=31, p=0.001; r=-0.553) at one time measurement

Lower eGFR levels in individuals with XLAS strongly depended on the presence of proteinuria (p=0.043). A negative correlation was found between older age and lower eGFR levels in females with XLAS and proteinuria (n=25; r=-0.478; p=0.016, Fig.16), while no difference was observed in non-proteinuric group of females (n=6; r=-0.429; p=0.397). In the group of men, we did not find a relationship between men's age, eGFG and proteinuria.

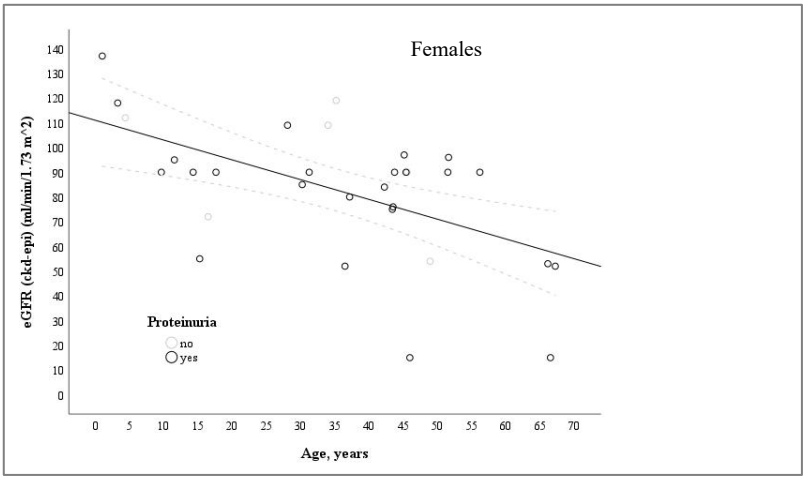


Figure 16. Correlation of age and eGFR levels in proteinuric (n=25; r=-0.478; p=0.016) and non-proteinuric (n=6; r=-0,429, p=0,397) groups of different females with XLAS at one time measurement

Nineteen individuals with proteinuria underwent a kidney biopsy, while two males and one female with XLAS had only hematuria without proteinuria. The kidney biopsy findings in individuals with XLAS were categorized based on histological changes observed in light and electron microscopy, as well as the presence or absence of unspecific immunoglobulins in immunofluorescence. General findings in individuals with XLAS are presented in Table 41.

Table 41. General findings of kidney biopsy in 22 individuals with XLAS

Findings in kidney biopsy	Total XLAS (n=58)	Females (n=31)	Males (n=27)	P-value
KB, % (n_o/n_t)	22 (37.9)	8 (13.8)	14 (24.1)	0.04
Mean age at KB, %	26.8±15.1	27.6±17.5	26.4±14.2	0.785
FSGS, %	9 (40.9)	4 (50.0)	5 (35.7)	0.662
Expansion of mesangial matrix, %	11 (50.0)	3 (37.5)	8 (57.1)	0.659
Foam cells in LM, %	5 (22.7)	0 (0.0)	5 (41.7)	0.068
Positive non-specific IF, %	1 (4.8)	1 (12.5)	0 (0.0)	0.381
Negative non-specific IF, %	20 (95.2)	7 (87.5)	13 (100.0)	
Positive IgA in IF, %	3 (13.6)	2 (25.0)	1 (7.1)	0.527
EM, %	17 (77.3)	7 (87.5)	10 (71.4)	0.613
TBM in EM, %	17 (77.3)	7 (87.5)	10 (71.4)	0.613
Lamellated GBM in EM, %	12 (54.5)	4 (50.0)	8 (57.1)	0.593
FPE in EM, %	17 (77.3)	7 (87.5)	10 (71.4)	0.613

KB – kidney biopsy, FSGS – focal segmental glomerulosclerosis, LM – light microscopy, IF – immunofluorescence, EM – electron microscopy, TBM- thin basement membrane, GBM-glomerular basement membrane, FPE – Foot process effacement

The mean age at the time of KB for males was 26.4 ± 14.2 years (median 21, range 6-49), while for females it was 27.6 ± 17.5 years (median 22, range 9-55). The mean eGFR was 68.3 ± 37.7 mL/min/1.73 m² for males and 82.9 ± 27.4 mL/min/1.73 m² for females. The quantity of proteinuria at the time of KB ranged from none to 3.0 g/L, with no significant difference between the genders.

EM results were available for 71.4% of males and 87.5% of females with XLAS. The most common features observed in EM were TBM and foot process effacement. Additionally, more than half of the individuals with XLAS exhibited lamellation or thickened glomerular basement membrane, which was associated with higher eGFR values for both genders compared to FSGS. Clinical characteristics of kidney biopsy of males and females are listed in Table 42 and Table 43, respectively.

Table 42. Clinical characteristics of kidney biopsy in 14 males with XLAS

Variables		FSGS	Expansion of mesangial matrix	TBM	Lamellated or thickened GBM
Age at KB	N	5	8	10	8
	Mean (SD)	29.2±12.7	19.3±10.0	24.1±13.2	19.6±9.8
	Median (P25,P75)	32.0 (16.5;40.5)	17.5 (12.0; 29.0)	20.0 (14.8; 35.0)	17.5 (12.3; 29.5)
	min, max	16; 46	6; 35	6; 49	6; 35
Pro at the time of KB	N	5	8	10	8
	Mean (SD)	2.2±0.84	1.75±0.9	1.7±0.82	1.0±0.0
	Median (P25,P75)	2.0 (1.5; 3.0)	2.0 (1.25; 2.0)	2.0 (1.0; 2.0)	1.0 (1.0; 1.0)
	min, max	1; 3	0; 3	0; 3	1; 1
Kidney function (eGFR) at the time of KB	N	5	8	10	8
	Mean (SD)	34.6±15.8	52.5±42.2	61.8±36.0	116.1±53.2
	Median (P25,P75)	30.0 (21.0; 50.5)	39.5 (15; 95.5)	62.5 (26.3; 93.3)	107.0 (76.5; 166.8)
	min, max	14; 52	14; 121	14; 118	46; 204

Table 43. Clinical characteristics of kidney biopsy in 8 females with XLAS

Variables		FSGS	Expansion of mesangial matrix	TBM	Lamellated or thickened GBM
Age at KB	N	4	3	7	4
	Mean (SD)	27.25±18.5	22.6±20.3	25.0±17.1	26.8±20.7
	Median (P25,P75)	27.0 (10.3;44.5)	13.0 (9.0; -)	15.0 (13.0; 40.0)	21.5 (10.3; 48.5)
	min, max	9; 46	9; 46	9; 55	9; 55
Pro at the time of KB	N	4	3	7	4
	Mean (SD)	1.5±1.3	0.67±0.6	1.71±1.1	0.75±0.5
	Median (P25,P75)	1.5 (0.25;2.75)	0.3 (0.0; -)	0.4 (1.0; 3,0)	1.0 (0.25; 1.0)
	min, max	0; 3	0; 1	0; 3	0; 1
Kidney function (eGFR) at the time of KB	N	4	3	7	4
	Mean (SD)	81.3±17.5	90.0±0.00	81.7±13.5	99.25±45.1
	Median (P25,P75)	90.0 (63.8; 90.0)	90.0 (90.0; 90.0)	90.0 (72.0; 90.0)	84.0 (67.5; 146.3)
	min, max	55; 90	90; 90	55; 90	64; 165

KB – kidney biopsy; FSGS – focal segmental glomerulosclerosis; Pro – proteinuria; eGFR – estimated glomerular filtration rate; TBM – thin basement membrane; GBM – glomerular basement membrane. Proteinuria was measured in urine spot samples and expressed in g/L

Hearing and ocular abnormalities

Among the 27 males with XLAS, 55.6% exhibited hearing loss which was detected at a mean age of 34.7 ± 18.4 years (median 32.7, range 7 to 61.8 years). In contrast, hearing abnormalities were relatively uncommon in females, with only 9.7% experiencing them at a mean age of 53.3 ± 15.6 years (median 56, range 36.5 to 67.2 years). The estimated mean age for the diagnosis of hearing loss was 40.1 years (95% CI [31.6-48.6]) in males and 61.7 years (95% CI [53.8-69.4]) in females, respectively ($p<0.001$). Three men with bilateral sensorineural hearing loss developed KF at the age of 27.7 [26-29] years, while none of the females with hearing loss reached KF, although two of them had stage 3 CKD. We did not find a correlation between eye damage and kidney function in both gender groups. Among the 27 males with XLAS, 25.0% exhibited ocular lesions at a mean age of 29.1 ± 16.6 years (median 27.0, range 10.8-60.2 years), while only one female had ocular lesions at the mean age of 56 years. The estimated mean age for the diagnosis of ocular lesions was 52.4 years (95% CI [42.8-62.1]) in males and 63.5 years (95% CI [57.4-69.4]) in females, respectively ($p=0.016$). The risk of having KF did not differ significantly between males with ocular lesions and those without them ($p=0.486$), and similarly for females ($p=0.349$).

Genotype-phenotype correlations

The most common type of genetic changes in *COL4A5* for males and females with XLAS was Gly substitutions and counted for 85.2% and 67.7% of all cases, respectively. No other amino acid substitutions were found in both groups. In 4 males with XLAS and KF Gly was substituted by Arg and Val residues, while in 2 females with KF by Arg.

Hearing abnormalities were detected more frequently in males with Gly substitutions compared to those with other types of genetic changes in *COL4A5* ($p<0.001$). Similarly, ocular lesions were also more common in males with Gly substitutions compared to those with other genetic changes ($p=0.006$). However, no significant difference was observed across different types of genetic changes in women with regards to hearing abnormalities and ocular lesions. Genotype-phenotype correlations in males and females are shown in Tables 44 and 45.

Table 44. Genotype-phenotype correlations in 27 males with *COL4A5* gene

Features	Total	Missense Gly	Frameshift	Splice-site
Hematuria, %	27 (100.0)	23 (100.0)	2 (100.0)	2 (100.0)
Proteinuria, %	23 (85.2)	19 (82.6)	2 (100.0)	2 (100.0)
Nephrotic Pro, %	5 (18.5)	5 (21.7)	0 (0.0)	0 (0.0)
CKD 1, %	12 (44.4)	9 (39.1)	2 (100.0)	1 (50.0)
CKD 2, %	3 (11.1)	2 (8.7)	0 (0.0)	1 (50.0)
CKD 3, %	6 (22.2)	6 (26.1)	0 (0.0)	0 (0.0)
CKD 4, %	2 (7.4)	2 (8.7)	0 (0.0)	0 (0.0)
CKD 5 (KF), %	4 (14.8)	**4 (17.4)**	0 (0.0)	0 (0.0)
KB, %	14 (51.9)	12 (52.2)	1 (50.0)	1 (50.0)
TBM, %	10 (37.0)	8 (34.8)	1 (50.0)	1 (50.0)
TcGBM, %	8 (29.6)	6 (26.1)	1 (50.0)	1 (50.0)
FSGS, %	5 (18.5)	5 (21.7)	0 (0.0)	0 (0.0)
Hearing ab., %	15 (55.6)	11 (47.8)	2 (100.0)	2 (100.0)
Ocular ab., %	6 (22.2)	4 (17.4)	1 (50.0)	1 (50.0)
Total, %	27 (100.0)	23 (85.2)	2 (7.4)	2 (7.4)

Table 45. Genotype-phenotype correlations in 31 females with *COL4A5* gene

Features	Total	Missense Gly	Frameshift	Splice-site
Hematuria, %	31 (100.0)	21 (100.0)	5 (100.0)	5 (100.0)
Proteinuria, %	25 (80.6)	18 (85.7)	3 (60.0)	4 (80.0)
Nephrotic Pro, %	2 (6.5)	1 (4.8)	0 (0.0)	1 (20.0)
CKD 1, %	18 (58.1)	11 (52.4)	4 (80.0)	3 (60.0)
CKD 2, %	6 (19.4)	4 (19.0)	0 (0.0)	2 (40.0)
CKD 3, %	5 (16.1)	4 (19.0)	1	0 (0.0)
CKD 4, %	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
CKD 5 (KF), %	2 (6.5)	**2 (9.5)**	0 (0.0)	0 (0.0)
KB, %	8 (25.8)	6 (28.6)	0 (0.0)	2 (40.0)
TBM, %	7 (22.6)	5 (23.8)	0 (0.0)	2 (40.0)
TcGBM, %	4 (12.9)	2 (9.5)	0 (0.0)	2 (40.0)
FSGS, %	4 (12.9)	3 (9.7)	0 (0.0)	1 (20.0)
Hearing ab., %	3 (9.7)	2 (9.5)	1 (20.0)	0 (0.0)
Ocular ab., %	1 (3.2)	1 (4.8)	0 (0.0)	0 (0.0)
Total, %	31 (100.0)	21 (67.7)	5 (16.1)	5 (16.1)

Pro – proteinuria; CKD – chronic kidney disease; KB – kidney biopsy; TBM – thin basement membrane; TcGBM – thickened and lamellated GBM; GBM – glomerular basement membrane; FSGS – focal segmental glomerulosclerosis; ab. – abnormalities

In females and males, no significant differences in eGFR levels depending on different type of genetic variant was observed (Fig. 17 and Fig. 18, respectively).

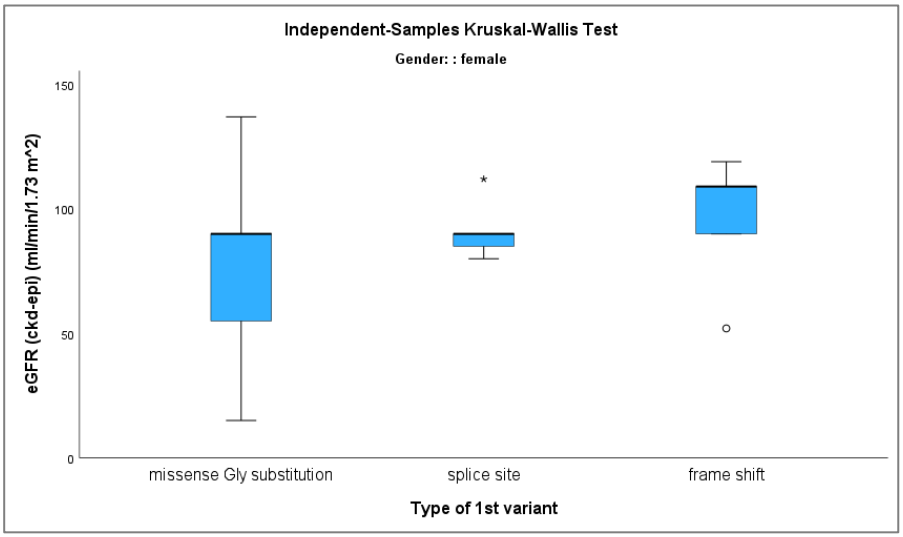


Figure 17. Distribution of eGFR levels at one time measurement depending on different types of *COL4A5* genes in females (p=0.32)

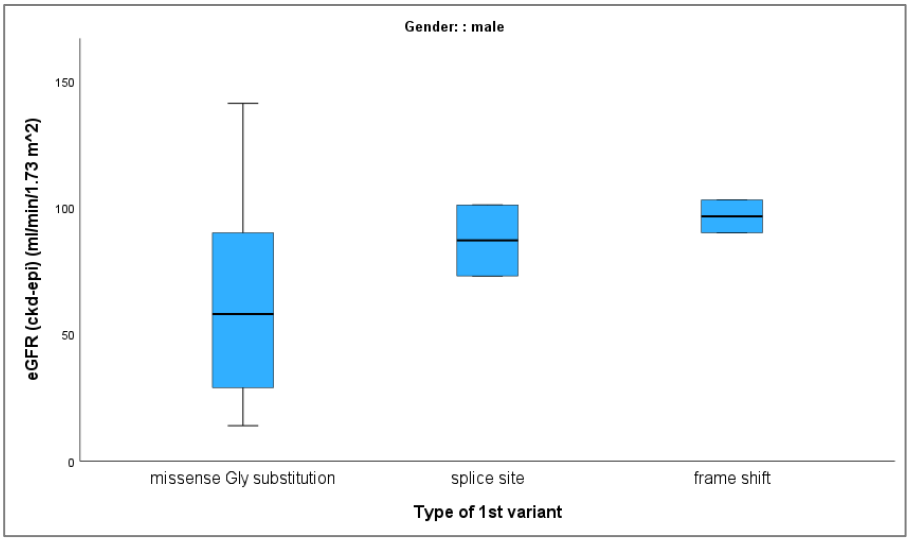


Figure 18. Distribution of eGFR levels at one time measurement depending on different types of *COL4A5* genes in males (p=0.364)

4.4.3. Genotype-phenotype characteristics of individuals with ARAS

In total, four (3.5%) individuals with ARAS were identified in the study, including one male and three females. Three individuals were diagnosed based on clinical features alone or with a kidney biopsy, while one female was referred for genetic analysis due to her twin sister's positive AS. One male had compound heterozygous variants, each inherited from his parents, who were also included in the study and described in the ADAS section (4.4.). The general characteristics of individuals with ARAS can be found in Table 46 and Supp. Tables 6-8.

Table 46. Clinical characteristics of 4 individuals with ARAS

Demographic and clinical features	Total No of individuals with ARAS
Number of subjects, %	4 (100.0)
Males, %	1 (25.0)
Females, %	3 (75.0)
Age at genetic diagnosis	26.7±6.7
Probands, %	3 (75.0)
Suspected AS by CF, %	1 (25.0)
Suspected AS by CF+KF, %	3 (75.0)
Suspected AS by FS, %	1 (25.0)
Hematuria, %	4 (100.0)
Age at detection of hematuria	10.5±7.9
Proteinuria, %	4 (100.0)
Age at detection of Pro	20.2±10.1
Nephrotic range Pro, %	3 (75.0)
CKD I, %	1 (25.0)
CKD II, %	0 (0.0)
CKD III, %	1 (25.0)
CKD IV, %	0 (0.0)
KF (CKD V), %	2 (50.0)
Mean age at KF	32.5±0.7
Arterial hypertension, %	3 (75.0)
RAAS inhibition, %	4 (100.0)
KB, %	3 (75.0)
Age at KB	30.0±3.2
Hearing ab., %	4 (100.0)
Ocular ab., %	2 (50.0)

Pro – proteinuria; KF – kidney failure; KB kidney biopsy; FS – family screening; CKD – chronic kidney disease; CF – clinical features; FS – family screening; ab. – abnormalities; No – number

Haematuria and proteinuria were detected in all four individuals with ARAS (A4/497163, A3/3232145, A3/3232146, A4/4/283867). However, nephrotic-range proteinuria was more common in adult individuals (75%, 3 out of 4) compared to a 7-year-old boy who had a proteinuria less than 50 mg/mmol (PCR) and normal kidney function (eGFR >90 ml/min/1.73 m^2, Supp. Table 7). Treatment for RAAS blockade was investigated for all individuals with ARAS following the detection of proteinuria, regardless of the genetic diagnosis. Among the four individuals with ARAS, A3/3232145 and A4/4/283867 reached KF at the ages of 33 and 32, respectively.

Kidney biopsy was performed in three females with ARAS at a mean age of 30.0±3.2. All three females exhibited proteinuria exceeding 1 g/L at the time of the KB, and two of them had abnormal kidney function (median eGFR was 50.0, with a minimum of 45 and a maximum of 55). The general findings of the KB are listed in Table 47 and Supp. Table 8.

Table 47. General findings of kidney biopsy in 3 individuals with ARAS

Findings in kidney biopsy	No of Ind.
Number of kidney biopsy, %	3 (100.0)
Males, %	0
Females, %	3 (100.0)
FSGS, %	2 (66.7)
Expansion of mesangial matrix, %	3 (100.0)
Foam cells in LM, %	1 (33.3)
Positive non-specific IF, %	1 (33.3)
Negative non-specific IF, %	2 (66.7)
Electron microscopy, %	3 (100.0)
TBM in EM, %	3 (100.0)
Lamellated GBM in EM, %	3 (100.0)
Foot process effacement in EM, %	3 (100.0)

LM – light microscopy; FSGS – focal segmental glomerulosclerosis; IF – immunofluorescence; TBM – thin basement membrane; EM – electron microscopy; GBM – glomerular basement membrane; No – number; Ind. – individuals

Hearing and ocular abnormalities

In the group of individuals with ARAS, hearing loss developed in all four individuals, with the youngest being 7 years old. Individuals who had KF also exhibited hearing abnormalities since childhood. Two out of the four individuals with ARAS had ocular lesions at the ages of 7 and 32. Both patients had pathogenic variants in the *COL4A3* genes. One of these patients

developed KF at the age of 32, while the other had an eGFR greater than 90 ml/min/1.73 m^2.

Genotype-phenotype correlations

ARAS was observed in two individuals with pathogenic *COL4A4* variants and two individuals with *COL4A3* genetic alterations (Table 48). Two individuals had compound heterozygous variants of *COL4A3*, and one patient had compound heterozygous variants of *COL4A4*. Additionally, one female had homozygous *COL4A4* variants. One variant was classified as pathogenic, three as likely pathogenic, and four as VUS. Seven individuals inherited missense GV, three of which involved Gly substitution, and one individual had a splice-site variant. The homozygous variant p.Ala607Val in *COL4A4* resulted in KF at the age of 32, similar to the case of a female with compound p.Pro1568Ser and p.Gly1083Arg variants in *COL4A3*, who reached KF at the age of 33 (Supp. Tables 6-7).

Table 48. Summarized genetic description of 4 individuals with ARAS

Type of AS	Individuals	1st gene/alleles° (GV ID)			2nd gene/alleles° (var. no)		
		Hetero geneity	Type	ACMG	Hetero geneity	Type	ACMG
ARAS	A3/3232145	*COL4A3* (23)			*COL4A3* (24)		
		Het	Missense	VUS	Het	Missense	VUS
	A3/3232146	*COL4A3* (23)			*COL4A3* (24)		
		Het	Missense	VUS	Het	Missense	VUS
	A4/49716	*COL4A4* (9)			*COL4A4* (10)		
		Het	Splice site	LP	Het	Missense	P
	A4/4/283867	*COL4A4* (28)			*COL4A4* (28)		
		Homo	Missense	LP	Homo	Missense	LP

° Naming of gene covers: different alleles of the same gene in ARAS. *Description according to the genetic laboratory analysis, ** Twin sisters sharing the same genetic variants

4.4.4. Genotype-phenotype characteristics of individuals with ADAS

In total, 48 (82.8%) individuals with one heterozygous *COL4A3* or *COL4A4* variant, thus ADAS, were identified and included 24 males. The mean age at diagnosis was 24±18.7 for males and 37.7±20.3 for females. The majority of individuals with ADAS (45.8%) were diagnosed through family screening. Two males were diagnosed with AS accidentally while genetically screening

for ADPKD. The general characteristics of males and females with ADAS are shown in Table 49 and Supp. Tables 3-5.

Table 49. General characteristics of 48 individuals with ADAS depending on gender

Demographic and clinical features	Total No of Ind.	Females	Males	P-value
Number of subjects, %,	48 (100.0)	24 (50.0)	24 (50.0)	>0.05
Age at genetic diagnosis	33.2±19.8	37.7±20.3	24±18.7	0.113
Probands, %	26 (54.2)	14 (58.3)	12 (50.0)	0.562
Dgn. of AS by CF &KB, %	18 (37.5)	11 (45.8)	7 (29.2)	0.233
Dgn. of AS by CF only, %	6 (12.5)	3 (12.5)	3 (12.5)	0.667
Dgn, of AS by FS, %	22 (45.8)	10 (41.7)	12 (50.0)	0.562
Hematuria, %	48 (100.0)	24 (100.0)	24 (100.0)	>0.05
Age at dgn. of hematuria	27.0±18.3	25.3±18.0	28.6±18.8	0.572
Proteinuria, %	25 (52.1)	14 (58.3)	11 (45.8)	0.386
Age at dgn. of proteinuria	32.8±17.3	34.4±16.9	30.7±18.5	0.476
Nephrotic range Pro, %	1 (2.1)	1 (4.2)	0 (0.0)	0.156
CKD I, %	35 (72.9)	15 (62.5)	20 (83.3)	x^2=5.62 p=0.229 df.=4
CKD II, %	7 (15.6)	6 (25.0)	1 (4.2)	
CKD III, %	3 (6.2)	2 (8.3)	1 (4.2)	
CKD IV, %	1 (2.1)	0 (0.0)	1 (4.2)	
CKD V (KF), %	2 (4.2)	1 (4.2)	1 (4.2)	
Mean age at KF	49.5±7.8	55.0±0	44.0±0	1.000
Arterial hypertension, %	18 (37.5)	6 (25.0)	12 (50.0)	0.074
RAAS inhibition, %	19 (39.6)	8 (33.3)	11 (47.8)	0.312
Kidney biopsy, %	21 (43.8)	13 (54.2)	8 (33.3)	0.146
Age at kidney biopsy	43.0±15.1	46.9±14.5	36.6±14.9	0.069
Hearing abnormalities, %	8 (16.7)	5 (22.7)	3 (13.6)	0.698
Ocular lesions, %	8 (16.7)	4 (22.2)	4 (18.2)	1.00

GD – genetic diagnosis; No – number; Pro – proteinuria; AH- arterial hypertension; KF – kidney failure; KB kidney biopsy; FS – family screening; CKD – chronic kidney disease; dgn. – diagnosis; CF – clinical features; Ind. – individuals

General comparison of individuals with one heterozygous *COL4A3* or *COL4A4* variants is shown in Table 50 and Supp. Tables 3-4.

Table 50. General characteristics of 48 individuals with ADAS depending on affected gene

Demographic and clinical features	Total No of Ind.	*COL4A3*het	*COL4A*4het	P-value
Number of subjects, %,	48 (100.0)	16 (33.3)	32 (66.7)	p>0.05
Males, %,	24 (50.0)	8 (50.0)	16 (50.0)	1.000
Females, %,	24 (50.0)	8 (50.0)	16 (50.0)	1.000
Age at genetic diagnosis	33.2±19.8	33.3±18.9	33.2±21.0	0.984

Probands, %	26 (54.2)	10 (62.5)	16 (50.0)	0.413
Dgn. of AS by CF &KB, %	18 (37.5)	7 (43.8)	11 (34.4)	0.527
Dgn. of AS by CF only, %	6 (12.5)	2 (12.5)	4 (12.5)	1.000
Dgn, of AS by FS, %	22 (45.8)	6 (37.5)	16 (50.0)	0.413
Hematuria, %	48 (100.0)	16 (100.0)	32 (100.0)	p>0.05
Age at dgn. of hematuria	27.0±18.3	23.4±16.3	28.8±19.2	0.344
Proteinuria, %	25 (52.1)	10 (62.5)	15 (46.9)	0.307
Age at dgn. of Pro, %	23.0±17.3	25.1±14.2	37.9±17.8	0.70
Nephrotic range Pro, %	1 (2.1)	0 (0.0)	1 (3.1)	0.237
CKD I, %	35 (72.9)	11 (68.8)	24 (75.0)	
CKD II, %	7 (14.6)	4 (25.0)	3 (9.4)	x^2=4.093
CKD III, %	3 (6.3)	0 (0.0)	3 (9.4)	p=0.444
CKD IV, %	1 (2.1)	0 (0.0)	1 (3.1)	df.=4
CKD V (KF), %	2 (4.2)	1 (6.3)	1 (3.1)	
Mean age at KF	49.5±7.8	44.0±0	55.0±0	1.000
Arterial hypertension, %	18 (37.5)	6 (37.5)	12 (37.5)	1.000
RAAS inhibition, %	19 (40.4)	8 (50.0)	11 (35.5)	0.337
Kidney biopsy, %	21 (43.8)	9 (56.3)	12 (37.5)	0.217
Age at kidney biopsy	43.0±15.1	38.0±11.6	46.7±16.9	0.117
Hearing abnormalities, %	8 (16.7)	4 (9.1)	4 (13.8)	0.414
Ocular lesions, %	8 (16.7)	1 (7.7)	7 (25.9)	0.236

No – number; Pro – proteinuria; KF – kidney failure; KB – kidney biopsy; FS – family screening; CF – clinical features; CKD – chronic kidney disease; dgn. – diagnosis; Ind. – individuals

In our study, no significant differences in kidney function or other features were observed based on gender (p= 0.13, Table 47) or different *COL4A3* or *COL4A4* genes (p=0.56, Table 48). Therefore, genotype-phenotype correlations of females and males with ADAS and different *COL4A3* and *COL4A4* genes were considered together in further analysis.

Kidney disease

Haematuria was detected in all 48 individuals with ADAS, while more than half of the heterozygotes developed proteinuria, including only one (3.8%) individual with nephrotic-range proteinuria and CKD stage 2. Among the 26 individuals with ADAS and proteinuria, 61.5% had an eGFR greater than 90 ml/min/1.73 m^2. Two individuals with a proteinuria of from 50 mg/mmol to 350 mg/mmol reached KF at the ages of 55 and 44, respectively. None of the individuals without proteinuria had KF, although 3 of them reached CKD stage 2.

In our study, a reasonable association between lower eGFR levels and older age in individuals with ADAS was observed (n=48, r=-0.542, p<0.001, Fig. 19).

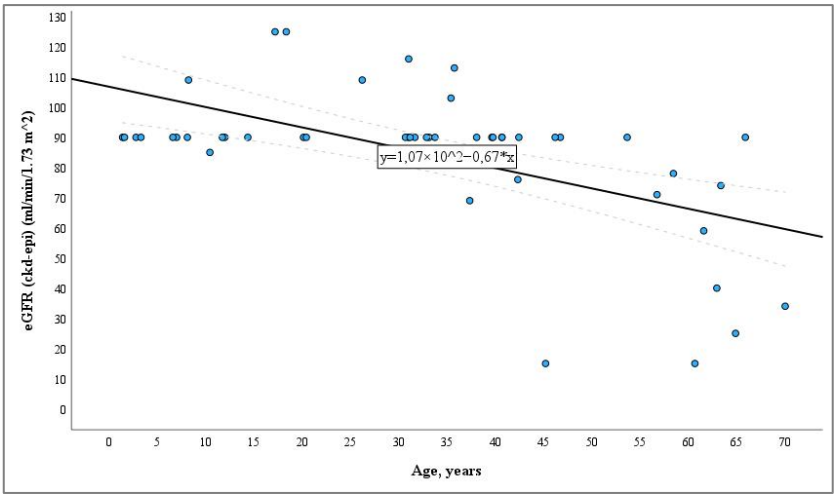


Figure 19. Correlation of age and eGFR levels in 48 individuals with ADAS at the one-time measurement (r=0.542, p<0.001)

Statistical analysis revealed a highly significant correlation between older age and lower eGFR levels in individuals with proteinuria (n=25; r=-0.737; p=0.013), as illustrated in Figure 20. While no significant correlation in eGFR levels and age was observed in individuals without proteinuria (n=23; r=-0,321, p=0,135).

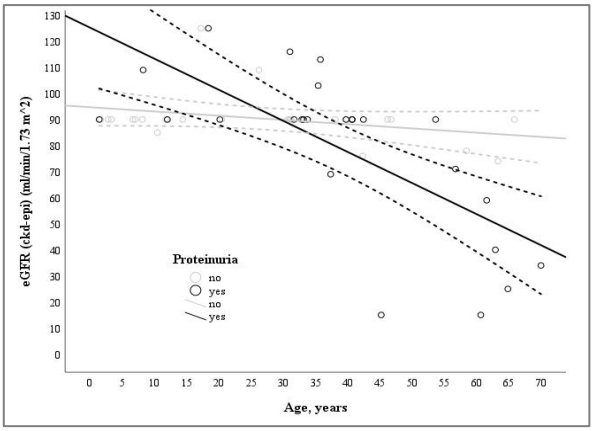


Figure 20. Correlation of age and eGFR levels in proteinuric (n=25; r=-0.737; p=0.013) and non-proteinuric (n=23; r=-0,321, p=0,135) group of individuals with ADAS at the one-time measurement

Twenty-one individuals (43.8%) underwent a kidney biopsy, among whom 76.2% had proteinuria. The kidney biopsy findings in individuals with ADAS were stratified by the affected gene, histological changes in light and electron microscopies (LM, EM), and the presence or absence of unspecific immunoglobulins in immunofluorescence. The general findings of kidney biopsy in individuals with ADAS are shown in Table 51 and Supp. Table 5.

Table 51. General findings of kidney biopsy in 21 individuals with ADAS

Clinical characteristics and KB findings	Total No of Ind.	Het *COL4A3*	Het *COL4A4*	P-value
No of KB	21 (100.0)	9 (42.9)	12 (57.1)	0.217
Age at KB	43.0±15.1	38.0±11.6	46.7 ±16.9	0.117
Females	13 (61.9)	4 (44.4)	9 (75.0)	0.203
Males	8 (38.1)	5 (55.6)	3 (25.0)	0.203
FSGS	8 (38.1)	3 (14.3)	5 (23.8)	>0.05
Mean age at FSGS	57±11.0	54.1±9.0	58.9±12.6	0.456
Expansion of MM	6 (30.0)	3 (33.3)	3 (27.3)	1.000
Foam cells in LM	0 (0.0)	0 (0.0)	0 (0.0)	-
Positive non-specific IF	3 (15.0)	1 (11.1)	2 (18.2)	1.000
Negative non-specific IF	17 (85.0)	8 (88.9)	9 (81.8)	1.000
IgA (+) immunostaining	3 (15.0)	2 (22.2)	1 (9.1)	0.566
EM	19 (90.5)	8 (88.9)	11 (91.7)	>0.05
TBM in EM	19 (90.5)	8 (88.9)	11 (91.7)	>0.05
Lamellated GBM in EM	6 (28.6)	2 (9.5)	4 (19.0)	>0.05
FPE in EM	19 (90.5)	8 (38.1)	11 (52.4)	>0.05

KB – kidney biopsy; FSGS – focal segmental glomerulosclerosis; MM – mesangial matrix; LM – light microscopy; IF – immunofluorescence; EM – electron microscopy; TBM – thin basement membrane; GBM – glomerular basement membrane; FPE – foot process effacement

The mean age at the time of kidney biopsy for individuals with ADAS was 43.0 ± 15.1 (median, 36 [IQR, 17–69]) years, and mean eGFR was 85.3 ± 30.9 mL/min/1.73 m2. Quantity of proteinuria at the time of KB varied from 0.0 to 3.0 (mean, 0.6 ± 0.9) g/L (Table 52).

Table 52. Clinical characteristics of 21 individual stratified by findings in LM and EM in kidney biopsy

Variables		FSGS	EMM	Thickened and lamellated GBM	TBM
Age at KB	N	8	6	6	19
	Mean (SD)	53.4±13.7	42.3±13.5	49±17.4	43±15.5
	Median (P25,P75)	57.0 (40.3; 63.3)	38.0 (30.8; 55.8)	48.5 (32.5; 65.3)	36 (31.0; 61,0)

Variables		FSGS	EMM	Thickened and lamellated GBM	TBM
	min; max	30; 69	30; 64	31; 69	17; 69
Pro at the time of KB	N	8	6	6	19
	Mean (SD)	0.93±1.2	0.6±1.2	1.0±1.2	0.5±0.9
	Median (P25,P75)	0.2 (0.0; 2.0)	0.2 (0.0; 1.0)	0.4 (0.0; 2.3)	0.2 (0.0; 0.5)
	min; max	0.0; 3.0	0.0; 3.0	0.0; 3.0	0.0; 3.0)
Kidney function (eGFR) at the time of KB	N	8	6	6	19
	Mean (SD)	58.6±31.4	85.8±33.2	85.5±38.8	90.3±28.0
	Median (P25,P75)	61.5 (25; 87.8)	94.0 (66.5; 108.8)	85.5 (23.0; 106.0)	93 (78.0; 110)
	min; max	23; 98	23; 117	23; 116	23; 125

KB – kidney biopsy; FSGS – focal segmental glomerulosclerosis; Pro – proteinuria; eGFR – estimated glomerular filtration rate (expressed in ml/min/1/73m^2, CKD-EPI); TBM – thin basement membrane; GBM – glomerular basement membrane. Proteinuria was measured in urine spot samples and expressed in g/L

Electron microscopy was available for 90.5% of individuals with ADAS. The most common features observed in EM were thin basement membrane and foot process effacement. Additionally, lamellation or thickened glomerular basement membrane appeared in 28.6% of individuals with ADAS at the mean age of 49±17.4, and it was associated with higher eGFR values compared to FSGS.

Hearing and ocular abnormalities

Eight (16.7%) individuals with ADAS exhibited bilateral sensorineural hearing abnormalities which were detected at a mean age of 46.5 ± 11.8 years. The diagnosis of hearing abnormalities was higher in individuals with ADAS who were older than 35.4 years (p=0.02, 95% CI [1.22-99.3], Fig. 21).

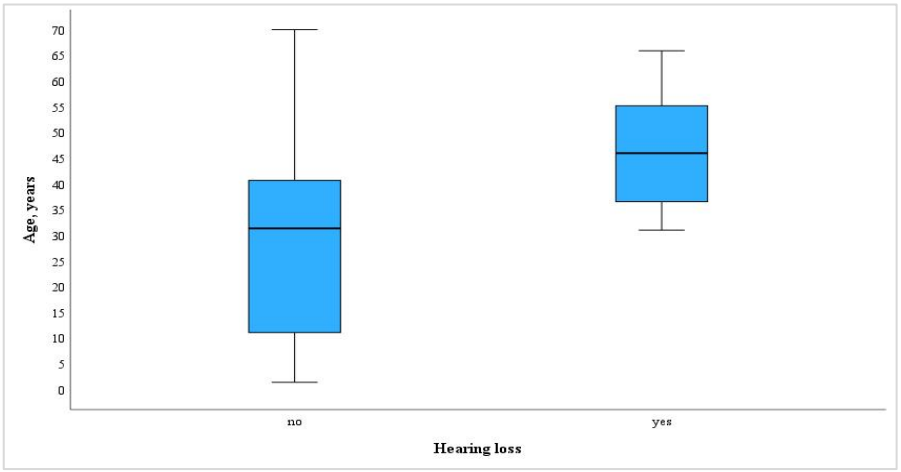


Figure 21. Distribution of hearing abnormalities in individuals with ADAS. Mann-Whitney test, p=0.02

Eight (16.7%) individuals with ADAS exhibited ocular lesions specific to AS which were detected at a mean age of 16.6 ± 17.3 years. The diagnosis of ocular abnormalities was higher in individuals with ADAS who were older than 6.6 years (p = 0.02, 95% CI [1.6 - 216.1], Fig. 22).

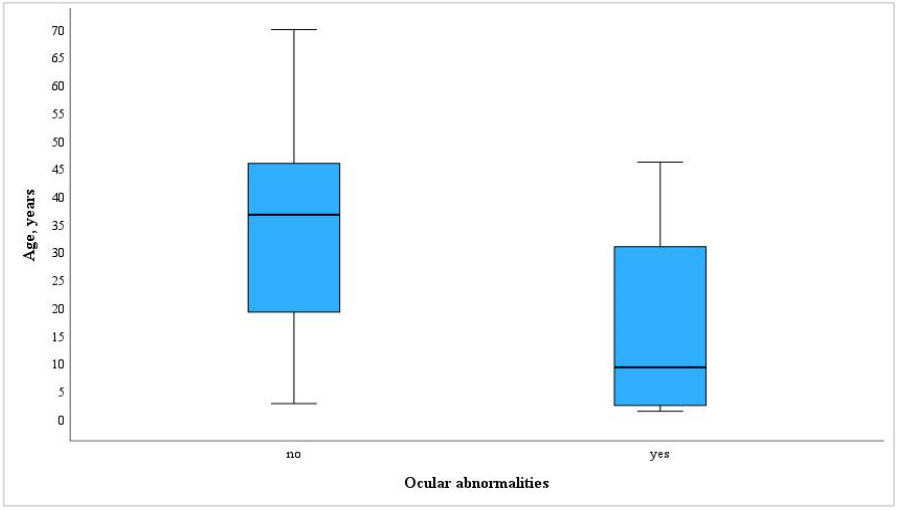


Figure 22. Distribution of ocular abnormalities and age. Mann-Whitney test, p=0.02

Distribution of different genetic variants depending on affected gene is listed in Table 53.

Table 53. Distribution of different types of GV depending on affected gene

Type of GV	*COL4A3*het	*COL4A4*het	Total No of Ind.
Missense: Gly subs., %	13 (81.3)	14 (43.8)	27 (56.3)
Missense: Non-Gly., %	3 (18.8)	7 (21.9)	10 (20.8)
Frameshift, %	0 (0.0)	4 (12.5)	4 (8.3)
Splice site, %	0 (0.0)	6 (18.8)	6 (12.5)
Unknown, %	0 (0.0)	1 (3.1)	1 (2.1)
Total No of Ind., %	16 (33.3)	32 (66.7)	48 (100.0)

GV – genetic variant; Gly – glycine; subs. – substitution; No – number; Ind. – individuals; het – heterozygous

No significant difference was observed across different types of genetic changes in ADAS with regards to hearing abnormalities and ocular lesions. KF was associated with 1 non-Gly substitution, where Leu was changed into Pro (*COL4A3* p.Leu1474Pro (c.4421T>C)); and 1 splice site variant c.594+1G>A in *COL4A4*. Genotype-phenotype correlations are shown in Table 54.

Table 54. Genotype-phenotype correlations in individuals with ADAS

Features	Total (%)	Missense Gly (%)	Non-Gly (%)	Frameshift (%)	Splice site (%)	Unknown (%)
Hematuria	48 (100)	27 (100.0)	10 (100.0)	4 (100.0)	6 (100)	1 (100)
Pro	25 (52.1)	11 (40.0)	7 (70.0)	2 (50.0)	5 (83.3)	0 (0.0)
Neph.pro	1 (2.1)	0 (0.0)	1 (10.0)	0 (0.0)	0 (0.0)	0 (0.0)
CKD 1	35 (72.9)	20 (74.1)	6 (60.0)	4 (100.0)	4 (66.7)	1 (100.0)
CKD 2	7 (14.6)	5 (18.5)	2 (20.0)	0 (0.0)	0 (0.0)	0 (0.0)
CKD 3	3 (6.3)	2 (7.4)	1 (10.0)	0 (0.0)	0 (0.0)	0 (0.0)
CKD 4	1 (2.1)	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)
CKD 5	2 (4.2)	0 (0.0)	**1 (10.0)**	0 (0.0)	**1 (16.7)**	0 (0.0)
KB	21 (43.8)	7 (25.9)	8 (80.0)	2 (50.0)	3 (50.0)	1 (100)
TBM	18 (37.5)	7 (25.9)	7 (70.0)	2 (50.0)	2 (33.3)	1 (100)
TcGBM	6 (12.5)	2 (7.4)	3 (30.0)	0 (0.0)	1 (16.7)	0 (0.0)
FSGS	8 (16.7)	3 (11.1)	3 (30.0)	0 (0.0)	2 (33.3)	0 (0.0)
Hearing a.	8 (16.7)	4 (14.8)	2 (20.0)	0 (0.0)	1 (16.7)	1 (100)
Ocular a.	8 (16.7)	5 (18.5)	0 (0.0)	0 (0.0)	3 (50.0)	0 (0.0)
Total	48 (100.0)	27 (56.3)	10 (20.8)	4 (8.3)	6 (12.5)	1 (2.1)

Pro – proteinuria; CKD – chronic kidney disease; KB – kidney biopsy; TBM – thin basement membrane; TcGBM – thickened and lamellated GBM; FSGS – focal segmental glomerulosclerosis; a. – abnormalities

Similar eGFR rate levels were observed among individuals with different types of genetic variants in *COL4A3-A4* genes, and there were no significant differences detected between the groups (Fig. 23).

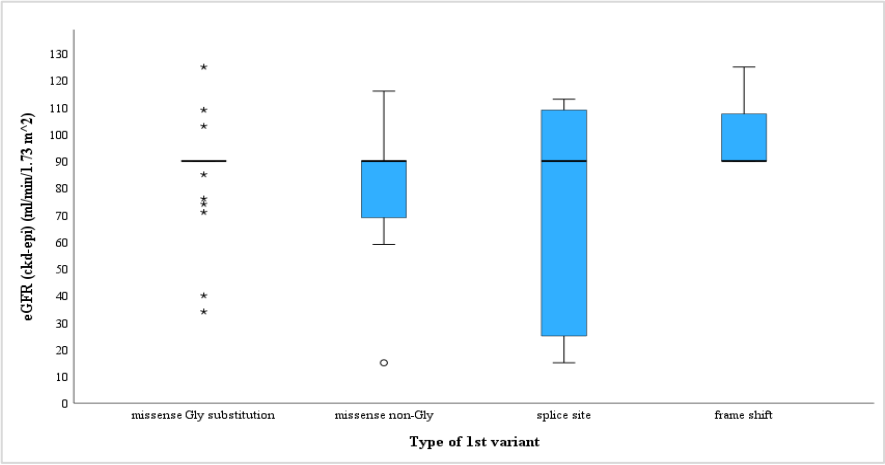


Figure 23. Distribution of different types of genetic changes in *COL4A3-A4* genes according to eGFR values (p=0.484)

4.4.5. Genotype-phenotype characteristics of individuals with digenic AS

In total, four (3.5%) individuals with digenic AS were identified, including one male and three females (Table 55; Supp. Tables 9-11). Three individuals were diagnosed based on kidney biopsies, while one female underwent genetic analysis solely due to clinical features. Among the three females, digenic variants in both *COL4A4* and *COL4A5* genes were detected. Additionally, two of them also belonged to the families with XLAS and therefore, were described in the XLAS section.

Table 55. Clinical characteristics of digenic AS

Demographic and clinical features	Total No of individuals with digenic AS
Number of subjects, %	4 (3.5)
Males, %	1 (25.0)
Females, %	3 (75.0)
Age at genetic diagnosis	30.3±12.6
Probands, %	4 (100.0)
Clinical diagnosis by kidney biopsy. %	3 (75.0)
Clinical diagnosis by clinical features, %	1 (25.0)
Clinical diagnosis by family screening, %	0 (0.0)
Hematuria, %	4 (100.0)
Mean age at diagnosis of hematuria	13.2±18

Demographic and clinical features	Total No of individuals with digenic AS
Proteinuria, %	4 (100.0)
Nephrotic range proteinuria	0 (0.0)
Mean age at diagnosis of proteinuria	23±11.8
CKD I, %	3 (75.0)
CKD II, %	1 (25.0)
CKD III, %	0 (0.0)
CKD IV, %	0 (0.0)
CKD V (KF), %	0 (0.0)
Mean age at KF	-
Arterial hypertension, %	1 (25.0)
RAAS inhibition, %	4 (100.0)
Kidney biopsy, %	3 (75.0)
Age at kidney biopsy	25.5±16.3
Hearing abnormalities, %	1 (25.0)
Ocular lesions, %	1 (25.0)

CKD – chronic kidney disease; KF – kidney failure

Kidney disease

Digenic AS variants induced haematuria and proteinuria in all four individuals. Among the three individuals (two females and one male), kidney function was normal, with an eGFR greater than 90 ml/min/1.73 m², and their PCR was less than 20 mg/mmol. However, only one female had proteinuria exceeding 50 mg/mmol (PCR) and was classified as having the 2nd stage of CKD. None of the individuals reached KF.

Kidney biopsy was performed in 3 of 4 individuals with digenic AS and included two females and one male at the mean age at 25.5±16.3 and 40, respectively. General findings of kidney biopsy are shown in Table 56.

Table 56. General findings of kidney biopsy in 3 individuals with digenic AS

Histological findings	Digenic AS
Number of kidney biopsy	3 (75.0)
Total number of kidney biopsies by gender: F/M	2F/1M
Abnormal kidney biopsy at light microscopy	3 (75.0)
Focal segmental glomerulosclerosis	1 (25.0)
Mesangioproliferative changes	1 (25.0)
Foam cells in LM	1 (25.0)
Positive non-specific immunofluorescence	0 (0.0)
Negative non-specific immunofluorescence	3 (75.0)
Positive IgA immunostaining	1 (25.0)
Electron microscopy	3 (75.0)
Thin basement membrane in electron microscopy	3 (75.0)

Histological findings	Digenic AS
Lamellated GBM in electron microscopy	2 (50.0)
Foot process effacement in electron microscopy	3 (75.0)

M – male; F – female

Hearing and ocular abnormalities

In the group of individuals with digenic AS, hearing loss only developed in one female at the age of 39, who also had CKD stage 2. Ocular changes were found in the same female who had hearing abnormalities. None of the other individuals with digenic AS had ocular lesions.

Genotype-phenotype correlations

Among individuals with digenic AS, the following gene combinations were identified: *COL4A3* and *COL4A4* in one case, and three individuals had *COL4A4* and *COL4A5* variants. One change was classified as pathogenic, two as likely pathogenic, and five as VUS. Missense alterations affected five individuals, two of whom had glycine substitutions. Additionally, one splice site variant and one frameshift variant were detected. The classification of genetic changes in the four individuals with digenic AS is shown in Table 57 and Supp. Table 10.

Table 57. Classification of genetic changes in 4 individuals with digenic AS

Type of AS	ID (gender)	1st gene/alleles° (GV ID)			2nd gene/alleles° (GV ID)		
		Hetero geneity	Type	ACMG	Hetero geneity	Type	ACMG
Digenic AS	A5/412818 F	*COL4A5* (12)			*COL4A4* (11)		
		Het	Missense	P	Het	Missense	VUS
	A5/46512 F	*COL4A5* (6)			*COL4A4* (7)		
		Het	Frameshift	LP	Het	Missense	VUS
	A5/4262450 F	*COL4A5* (26)			*COL4A4* (27)		
		Het	Frameshift	LP	Het	Missense	VUS
	A3/438376 M	*COL4A3* (38)			*COL4A4* (20)		
		Het	Missense	VUS	Het	Splice site	VUS

° Naming of gene covers: different alleles of the same gene in different genes in digenic AS
GV – genetic variant; ID – genetic variant identification number in the study; Het – heterozygous; P – pathogenic; LP – likely pathogenic; VUS – variant of uncertain significance; F – female; M – male

5. DISCUSSION

The identification of disease-causing variants in the *COL4A3*, *COL4A4*, and *COL4A5* genes is critical for the diagnosis and management of Alport syndrome. Although more than 5000 pathogenic variants have been recorded (114), it is likely that many more remain undiscovered (4). To improve our understanding of the distribution, diagnostics, genetic background, similarities to other populations and genotype-phenotype correlations in Alport syndrome, we conducted an extensive mutational analysis of 221 individuals from 120 different families and identified 44 different genetic variants in the type IV collagen genes in 63 Lithuanian families. Out of all the variants detected in this study, 64 % were classified as novel or previously unreported, while 37 % were observed in the global population and thought to have originated from Central/East Europe as a result of a founder effect that occurred 750-900 years ago (115). These findings expand the spectrum of known pathogenic variants in *COL4A3-A5* genes and provide important insights into the genetic basis of Alport syndrome in Lithuanian population.

In recent years, the identification of an increased number of disease-causing variants in *COL4A3, COL4A4,* and *COL4A5* has expanded our understanding of the phenotypic spectrum of AS and increased the estimated prevalence of Alport syndrome from being "very rare" to accounting for at least one in 2320 for *COL4A5* pathogenic variants and one in 106 of individuals with *COL4A3* and *COL4A4* variants. However, the true prevalence of potentially pathogenic *COL4A3–A5* variants is even greater and are often undiscovered (10). AS has been observed in individuals from different ethnic groups and does not seem to be limited to particular geographic regions (53). Due to limited population-based studies data, the exact prevalence of AS in different countries is unknown. Therefore, one of our aims was to evaluate its distribution in Lithuania. Genetic analysis for suspected genetic diseases was available in two main Lithuanian centres – in VUH SK (Vilnius city) and Lithuanian University of Health Science (LSMU, Kaunas city), thus individuals with highly suspicion of genetic disease were referred to these centres from all over Lithuania. Although we had no data about AS in LSMU, 63 families having disease-causing variants in *COL4A3-A5* genes during five-years period were discovered in VUH SK. Most individuals with AS were from the Vilnius region, while the distribution of AS in other Lithuanian cities was significantly lower, likely due to the difficulty in diagnosing AS, the rarity of the disease, and the lack of knowledge about it. Our study described 114 individuals with AS in a population of approximately 2,795,700 inhabitants (according to osp.stat.gov.lt, 2021), supporting the hypothesis that AS is a

prevalent disease in our population. However, further epidemiological studies need to be conducted to accurately estimate the prevalence of AS in Lithuania.

In our study, 60.5% of individuals diagnosed with AS demonstrated normal kidney function and 23.7% presented only isolated hematuria. Kidney failure was observed in 9% of the participants, with the age range ranging from 19 to 60 years. The remaining individuals had varying severity of CKD or proteinuria, and only 30.1 % and 18.2 % had hearing and ocular manifestations, respectively. Therefore, individuals with AS may experience a high degree of phenotypic diversity, which makes the diagnosis of the disease more challenging. Flinter EA et al. proposed AS diagnostic criteria including clinical features (hematuria, unknown etiology CKD, ocular and hearing abnormalities), pathological lesions in kidney biopsy and positive family history for AS (116). In our study, nearly half of the patients, comprising 48% of all the children in the study, were diagnosed with AS through family screening. This highlights the significance of analysing the family pedigree of individuals at risk, as it enables early diagnosis, potential initiation of renoprotective treatment, and the possibility of avoiding future diagnostic interventions (75,84). Comparing different AS types, most of XLAS females and individuals with ADAS were diagnosed with AS due to family screening (71.0%), while males with XLAS and individuals with ARAS and digenic AS were referred to molecular analysis mostly followed by pathologic findings in kidney biopsy (40.7%). These findings indicate that males with XLAS and individuals with ARAS or occasionally digenic AS exhibit similar and more severe manifestations of AS, which typically appear earlier in life. As a result, they often require kidney biopsy or other diagnostic approaches. In contrast, females with XLAS and individuals with ADAS are typically asymptomatic or present with a milder phenotype of AS that manifests later in life. Consequently, they are often diagnosed through family screening rather than experiencing noticeable symptoms (94). We also observed that males with AS are diagnosed significantly earlier than females (n=0.048), suggesting a similar pattern of more severe phenotype in males compared to females (75).

Before the advent of genomics, kidney biopsy was a significant diagnostic tool for AS as it revealed characteristic lesions, such as a TBM or other GBM abnormalities observed through electron microscopy. However, despite the presence of these distinct lesions in EM, the pathological diagnosis of AS patients sometimes led to misdiagnosis. This resulted in cases where AS patients, who had previously undergone renal biopsy, received treatment for other conditions due to the initial misdiagnosis (117). However, in our study, nearly half of the individuals were suspected to have AS based on the findings

from kidney biopsy, although the frequency of performing kidney biopsy in children was considerably lower (p=0.009), probably due to less severe phenotype comparing with adults. Hence, although genetic analysis is the primary method for diagnosing AS, kidney biopsy can be valuable tool in raising suspicion of AS and offering supplementary clinical information in situations where genetic analysis results are inconclusive, such as in cases involving variants of unknown significance or negative molecular analysis despite a high suspicion of AS (1,83). Therefore, although NGS is widely regarded as the preferred method for diagnosing AS, there are other valuable diagnostic approaches, including recognizing clinical features such as persistent microscopic hematuria, CKD, hearing, and ocular abnormalities, identifying pathogenic lesions through kidney biopsy, and conducting family screening, which can also contribute significantly to the diagnosis of AS.

This study performed NGS testing on all three *COL4A3-A5* genes in individuals who were suspected of having AS. The analysis identified 28 novel genetic changes, which accounted for more than half of all the variants detected in a cohort of 221 individuals from 120 unrelated families. These novel changes, with a frequency of 9 in *COL4A3* and *COL4A4,* equally, and 10 in *COL4A5*, have been included in the LVOD genetic database, thereby allowing researchers to expand their understanding of the genetic basis of diseases and traits (118). This contributes to improved accuracy in genetic testing and diagnosis (86). To be noted, the description of novel variants and their clinical presentation is highly influenced by several factors, including the mode of inheritance, the affected collagen IV gene, type of genetic variants and its pathogenicity (11). Previous guidelines have determined that identifying a pathogenic variant is the most reliable evidence for diagnosing AS (75,78). However, the definition of pathogenicity for genetic variants in *COL4A3-A5* has become less certain (21). Genetic laboratories, including Centogene laboratory, where we performed our genetic testing, primarily use SIFT, PolyPhen2, MutationTaster software and web-based tools (Alamut, Varsome, ClinVar, InterVar, Centogene database) to assess the pathogenicity of genetic variants and provide conclusions to clinicians. Despite the proposed ACMG/AMP criteria (89) for interpreting sequence variants, there are cases where insufficient data hinders a conclusive determination of pathogenicity (86). In our study, six novel VUS were reclassified as likely pathogenic or pathogenic due to their association with severe phenotypic manifestations, highlighting the importance of clinical evaluation in addition to laboratory results. Among these variants, the c.2777G>T p.Gly926Val variant in *COL4A5* was initially classified as a VUS. However, both males carrying this variant reached kidney failure at the ages of 24 and 31, suggesting a more

significant impact on the disease progression than previously anticipated. Therefore, it is essential to recognize that not all predictions regarding the pathogenicity of genetic variants can be considered reliable. This underscores the continued significance of clinical evaluation by healthcare professionals in assessing phenotypic characteristics and the importance of reporting novel findings in genetic databases (119). Our study identified a high rate of detecting pathogenic and likely pathogenic novel *COL4A3-A5* variants in individuals exhibiting KF or ocular abnormalities. These findings have facilitated the implementation of more precise and early targeted therapy for individuals affected by AS or their family members. However, the detection rate of P or LP variants was lower in individuals with persistent hematuria alone. Therefore, determination of the pathogenicity and description of novel variant has the crucial impact on the onset and advancement of a disease (11).

In addition to pathogenicity, the location and type of genetic variants are crucial factors that contribute to the risk of progressive kidney impairment (75,78,120). Therefore, we conducted an analysis of various types of genetic changes in the *COL4A3-A5* genes to search for genotype-phenotype correlations within our cohort. Among all *COL4A3-A5* genes, the most common types of genetic changes observed in our study with AS were missense variants, which included both Gly and non-Gly substitutions. These variants accounted for 76.0 % of all individuals with AS. These findings aligned with previous studies indicating that approximately more than half of AS patients inherit single amino acid substitutions in *COL4A3-A5* genes, with glycine substitutions representing 73% of these cases (121,122). Therefore, together with Australian working group we analysed the relationship between molecular characteristics of glycine substitutions in *COL4A3-A5* genes and the clinical severity and progression of XLAS and ADAS (88). The LVOD database, which included nine different molecular changes (45 individuals) from the Lithuanian cohort, was combined with the gnomAD database for the general analysis. The genetic variants of *COL4A3* and *COL4A4* were sourced from the Genomics England 100,000 Genomes project. We examined three molecular features for each Gly substitution. These features included the molecular location of the variant (exons 1 to 20 or exons 21 to carboxyl terminus), whether the variant was adjacent to a non-collagenous interruption or terminus (referred to as "non-collagenous boundary" variants), and the extent of instability caused by the residue replacing Gly. The residues Ala, Ser, and Cys were classified as mildly destabilizing, whereas Arg, Val, Glu, Asp, and Trp were classified as highly destabilizing (123). These findings aligned with the data from Lithuanian cohort with *COL4A5* molecular changes, where the most common residues substituting glycine were arginine

and valine, which were associated with a more severe disease progression (Supp. Tables 1-2; 12-14). Together with Australian colleagues we found significant correlation in *COL4A5* gene instability caused by the changed residue and AS progression, while the exon location did not have a significant impact. Pathogenic variants in *COL4A5* that resulted in Gly substitutions with highly destabilizing residues were associated with earlier onset of kidney failure and hearing loss (p=0.002 and p=0.004, respectively). Substitutions near non-collagenous regions delayed kidney failure for 19 years (p=0.014). In heterozygous pathogenic variants of *COL4A3* and *COL4A4*, Gly substitutions with highly destabilizing residues were linked to an increased risk of hematuria, while substitutions near non-collagenous regions reduced the risk. *COL4A5* variants adjacent to non-collagenous regions were more common in the normal population. The specific substitution and nearby residues determine the risk of hematuria, early onset of kidney failure, and hearing loss in AS. Overall, this study provided insights into the clinical impact of specific molecular features of Gly missense variants in AS, although other genetic and environmental factors were also considered to contribute to disease severity and progression (88).

Although it is critical to establish a strategy for accurately interpreting the clinical significance of rare or novel variants (118), it is equally important to avoid making incorrect clinical decisions based solely on common and well-known variants worldwide. We discovered seven genetic variants in the Lithuanian cohort that were prevalent in the general population worldwide. These variants were further examined and compared to findings from other studies in terms of their phenotypic implications. The two most prevalent variants identified in our study were *COL4A5* Gly624Asp (c.1871G>A) and *COL4A3* p.Leu1474Pro (c.4421T>C). These variants have also been reported as hypomorphic disease-causing variants and were commonly observed in other populations (1). *COL4A5* Gly624Asp (c.1871G>A) variant was found in 22 individuals from 14 different families. Although the Gly624Asp variant has been traditionally considered less severe, recent evidence has brought attention to cases where this variant was associated with noticeable KF indicating a potential for increased severity (1). In our study, a total of 21 patients with this variant were diagnosed with XLAS, while one female patient had digenic AS and shared her phenotype with another *COL4A4* variant. Among the 12 males and 9 females with a single *COL4A5* Gly624Asp variant, none of these patients reached KF although 38% of individuals had an eGFR less than 60 ml/min/1.73 m^2. These findings are consistent with previous studies, indicating that this variant is associated with a milder phenotype and a later onset of kidney failure (115). However, Macheroux et al. reported on a

severe phenotype of *COL4A5* Gly624Asp variant in an X-linked family, with affected individuals showing early onset of microscopic hematuria and proteinuria (124). In our study all individuals with this variant had hematuria, while proteinuria appeared in 83.3% males and 66.6% females. Boeckhaus et al. found that hemizygous patients with the variant have an increased risk of developing KF if left untreated, but early treatment with ACE inhibitors can reduce this risk (125). Therefore, all individuals in our *COL4A5* Gly624Asp group received ACE inhibitor treatment if albuminuria was detected. The EARLY PRO-TECT Alport trial observed disease progression in some children with the variant (126)These findings highlight the challenges in interpreting the clinical significance of the variant, which is considered a milder form of the disease but can still lead to KF in midlife (1). Additionally, this variant has a prevalence of one in 3403 among individuals of European ancestry and is predominantly found in Europeans, therefore indicating that Lithuanians have common ancestors to other European countries (10,88,115). We also found two individuals with one heterozygous *COL4A3* p.Leu1474Pro (c.4421T>C) variant. Although this variant is commonly associated with isolated microscopic hematuria, it typically leads to kidney failure when present alongside another disease-causing *COL4A3* variant in trans or due to digenic inheritance (85,121). However, in our study, we observed a case where a male individual (A34460114) with only one heterozygous pathogenic variant experienced KF at the age of 45 years old. This demonstrates phenotypic variability even within the same genotype (114). In addition to the previously reported variants, we identified several other variants including *COL4A5* p.Gly1229Ser (c.3685G>A), *COL4A3* p.Gly1167Arg (c.3499G>A), *COL4A4* c.-101-4A>G, *COL4A4* p.Gly999Glu (c.2996G>A), and *COL4A4* p.Gly527Cys (c.1579G>T). These variants have been observed in other European and Asian populations, suggesting a common founder effect. (Source: nih.gov, (88)).

The clinical presentation of AS is strongly influenced by the genotype, thus the clinical features and progression of AS subtypes, including XLAS, ARAS, ADAS, and digenic AS, were characterized based on genotype-phenotype correlation. Approximately half of the affected individuals in our study had XLAS, while the rest had ARAS, ADAS, or digenic disease. Hematuria was detected significantly earlier compared to the diagnosis of proteinuria in all types of AS, with the earliest presentation observed in the XLAS group (p=0,05), indicating that XLAS has more severe phenotype in comparison with other types of AS (127). In our study, KF was observed in only 9% of individuals with AS, including 10% of XLAS, 50% of ARAS, and 4% of ADAS cases. These findings contrast with other studies that indicate a higher

prevalence of KF in males with XLAS, as well as in individuals with ARAS and 10-30% of ADAS or females with XLAS (14,101,127–129). The discrepancy in our study results may be attributed to the limited number of individuals with kidney failure, which could be influenced by the relatively young age of the study cohort. However, we did identify male gender, presence of proteinuria, and arterial hypertension as the main risk factors for CKD progression in individuals with AS. Hypertension and proteinuria significantly worsen kidney survival in individuals with AS compared to those without these conditions. This finding aligns with what is observed in the general population with hypertension and proteinuria, as well as in CKD progression in other diseases (22). Therefore, individuals with AS, who are relatively young and have CKD without significant comorbidities, would be ideal candidates for clinical trials targeting CKD therapy.

Several extensive studies and a meta-analysis have been conducted to analyse the genotype-phenotype relationships in individuals with ADAS (22,88,130,131). Heterozygous disease-causing variants in *COL4A3* or *COL4A4* result in a broad range of phenotypic manifestations. These can vary from asymptomatic isolated hematuria to progressive kidney disease and extrarenal lesions (16). According to Kashtan, approximately 1% of individuals with ADAS reach KF, while the risk increases up to 20% in those with risk factors such as proteinuria, FSGS, GBM thickening and lamellation, and family history of progressive kidney disease (16). However, molecular studies in unbiased cohorts indicates that KF is much less common (132). In our study, only two individuals out of 48 developed KF at the age of 55 years (A49737, *COL4A4* c.594+1G>A; NM_000092.5) and 44 years (A34460114, *COL4A3* p. Leu1474Pro, c.4421T>C; NM_000091.5). In a similar study, individuals with ADAS reached KF at a median age of 47-70 years 39; (82,127). The burden of ADAS depends on the type of pathogenic change, as nonsense variants are more often associated with KF compared to missense variants (12). In our study, a female who developed KF had a splice site variant in *COL4A4*, and also presented other risk factors such as FSGS in the kidney biopsy, severe proteinuria, and a late diagnosis of AS, without early initiation of renoprotective treatment. However, this person's offspring had only persistent hematuria and minimal proteinuria, even in adulthood. Interestingly, the grandson of another study participant (A4/497163*bi) was diagnosed with ARAS due to compound heterozygous variants in the *COL4A4* gene, inherited from his mother and father. The same variant (*COL4A4* c.594+1G>A; NM_000092.5) was also found in a 64-year-old male (A494487) from a different, unrelated family, who was diagnosed with CKD stage IV, and his kidney histology revealed FSGS. Despite severe kidney

function impairment, the proteinuria level was low (PCR 20 mg/mmol). Therefore, the same variant may be associated with both interfamilial and extrafamilial variation, highlighting the importance of careful evaluation and monitoring of individuals with heterozygous *COL4A3* or *COL4A4* variants. Although no differences in the affected gene (*COL4A3* or *COL4A4*) and gender-related phenotype were observed among individuals with ADAS in our and other cohorts (82), it is interesting to note that a male with the missense *COL4A3* p.Leu1474Pro (c.4421T>C) variant reached KF 10 years earlier compared to a woman with a *COL4A4* splice site pathogenic variant, contradicting the findings of Mathaiou's meta-analysis, which revealed a median age at KF of 55 years for missense variants and 47 years for non-missense variants (131). Proteinuria was also detected significantly earlier in the male at the age of 30, compared to the female who developed proteinuria at the age of 53. These findings are based on individual cases and do not demonstrate statistical significance. However, a study by Furlano et al. indicated that proteinuria and its onset may serve as a surrogate marker for CKD progression leading to kidney failure (82).Therefore, in collaboration with the Alport syndrome workshop group, we have stratified proteinuria as a risk factor for CKD progression in individuals with ADAS, suggesting early initiation of ACE inhibitors (1).

Although extrarenal manifestations in ADAS are rare, one study reported that 18 % of individuals with ADAS developed sensorineural hearing loss (133). However, the findings in our study agree with those of previous research. In another study, abnormal ocular findings were reported in 23% of individuals, but only 2 of them (<1%) had anomalies that could be attributed to AS (82). On the other hand, in our study, ocular abnormalities were observed in 17 % of individuals with heterozygous *COL4A3* and *COL4A4* variants. These findings suggest that the phenotypic manifestations in individuals with ADAS vary greatly. However, extrarenal expression is usually less severe and less frequent than in males with XLAS and individuals with ARAS (82). Nevertheless, the development of risk factors can significantly increase the burden of the disease (3,22). Alternatively, it is possible that our individuals with hearing loss and ocular abnormalities had a second pathogenic variant that went undetected, leading to a diagnosis of ARAS or digenic AS.

Although the exact prevalence of ARAS is not known, some studies suggest that this type of AS comprises fifteen percent of patients with AS [12]. In our study, only four individuals with ARAS were detected, representing only 3.5 percent of the total cohort. Currently, there is only emerging information on genotype-phenotype correlations among individuals with

ARAS (134). One study found that individuals with truncating variants often develop KF earlier in life and have a higher incidence of nephrotic range proteinuria, as well as more ocular and sensorineural hearing changes compared to those with compound or biallelic missense variants (135). In our study, we observed KF in two women with ARAS. The first one (A3/3232145) with heterozygous missense variants c.4702C>T (p.Pro1568Ser) and c.3247G>C (p.Gly1083Arg) in *COL4A*3, developed KF at age 33. Her biopsy revealed FSGS and a thinned and lamellated glomerular basement membrane, which is consistent with histological findings described in other studies (16,136). Interestingly, her heterozygous twin sister (A3/3232146) with the same variants had a milder clinical phenotype but similar kidney biopsy features, which might be due to the dizygotic form of the twins and modifying environmental factors. Alport twin studies could provide more insight into this. Another study reported that all individuals with ARAS had hematuria and proteinuria, with 43% developing KF (134). Both twins in our study had nephrotic range proteinuria, which is a prognostic factor for KF. Another female (A4/4/283867) with ARAS, homozygous for the *COL4A4* p.Ala607Val (c.1820C>T; NM_000092.5) variant, developed KF at a similar age of 32 years old. During the pedigree analysis, we discovered a consanguinity relationship in this case, which is consistent with a higher frequency of autosomal recessive disorders in consanguineous families. Both females who developed KF, despite having different heterogeneity and involvement of autosomal genes (*COL4A3* and *COL4A*4), exhibited similar progression of chronic CKD, with comparable ages at diagnosis of proteinuria (22 and 23 years old). These findings are consistent with other studies that demonstrate no significant difference in the age of KF among individuals with ARAS who have different *COL4A3* or *COL4A4* gene variants, although the average age of kidney failure is noted to be earlier at 23.2±9.3 years (n=35) for *COL4A3* and 25.4±10.3 (n=26, p=0.39) years for *COL4A4* (12,80) compared to our study patients. The fourth individual (A4/49716) diagnosed with ARAS at age 6 had two compound heterozygous *COL4A4* variants. A novel pathogenic splice site variant c.594+1G>A (NM_000092.5) was found in *COL4A4* and shared its phenotype with the likely pathogenic variant in *COL4A4* c.1579G>T (p.Gly527Cys) that had already been described (127). This boy (A4/49716) had both hematuria and proteinuria detected from age 1, without any deterioration of kidney function at age 12. Patients with ARAS often go undetected due to the lack of a positive family history and different genotype-phenotype correlations among affected relatives. Both males and females with ARAS have a similarly high risk of kidney failure, and ocular and hearing abnormalities are also frequently observed (114). This is

consistent with our study where all individuals, regardless of age, exhibited bilateral hearing loss, while ocular lesions were present in half of the individuals. Therefore, individuals with ARAS display a severe phenotype, often comparable to males with XLAS.

The risk of developing KF is higher in individuals with large fragment deletions, nonsense variants or frameshift variants in the *COL4A5* gene, compared to those with splicing or missense variants (13). However, in this study, the most severe phenotype leading to KF was associated with a missense variants. All males with XLAS who developed KF (n=4) had glycine substitutions with highly destabilizing residues in *COL4A5*, including Asp (p.Gly766Asp), Arg (p.Gly875Arg), and Val (p.Gly926Val), and reached KF at a mean age of 23.5±5.4 years. These findings from our cohort are consistent with our larger study conducted with Australian colleagues, where we also observed that males with highly destabilizing residues reached KF at a mean age of 24.6±12.4 years (n=52) and had a worse outcome compared to those with non-glycine substitutions (p=0.03) (88). We also found that glycine substitutions located away from a non-collagenous sequence (interruption, amino, or carboxy terminus) were associated with a more severe phenotype, characterized by earlier onset of KF, compared to Gly substitutions situated near a non-collagenous sequence (88). Therefore, we also identified novel highly pathogenic *COL4A5* p.Gly926Val (c.2777G>T; NM_033380.3) variant [118] . This novel variant changes the amino acid from Gly to Val at position 926 and is classified as a variant of uncertain significance in Centogene and Franklin's databases, but the Varsome database describes it as likely pathogenic (PM2, MM1, PP2, PP3). Two brothers from the same family with this variant developed KF at the ages of 24 and 32, respectively. Interestingly, the younger brother developed KF eight years earlier than his older brother, suggesting that the same gene variant can lead to significantly different phenotypes. In general, the age of KF in individuals with AS depends on the type of variant (137). In our study, only 4 out of 27 males (15 %) with XLAS developed KF, which is consistent with a better prognosis than previously reported (15% compared with 90%) (14). The mean age of KF in individuals with missense variants, splicing site variants, and truncation variants has been reported as 37 years, 28 years, and 25 years, respectively (17). The males in our study were relatively young (median age of 29.3±17.0 years) at the time of diagnosis and phenotype evaluation, which might explain the lower rate of KF than in other studies. However, nineteen percent of men with *COL4A5* variants had significant nephrotic range proteinuria (PCR ≥300 mg/mmol), which can also be associated with a future decline in kidney function and is the main predictor of KF (138,139). Bilateral sensorineural

hearing loss was observed in 60% of males with XLAS and in 75% of all XLAS males KF, which is consistent with findings from other studies (9,14). However, further studies are needed to determine the exact relationship between kidney and extrarenal manifestations in males with XLAS [(14)]. Additionally, given the severe disease manifestations and high risk of CKD progression observed in males with XLAS and individuals with ARAS, our recommendations from the Alport Syndrome Workshops group suggest initiating ACE inhibitor treatment as soon as a genetic diagnosis of AS is made, regardless of the presence or absence of proteinuria (1).

It is generally known that women with XLAS tend to have a milder phenotype (13,140), but they still have a risk of developing KF and other extrarenal manifestations (141). In our study, most of the females with XLAS only had hematuria, but two women (mother and her daughter) from the same family with heterozygous variant p.Gly875Arg (c.2623G>A) in *COL4A5* reached KF at the age of 40 and 63. The relationship between the location, type, and phenotype of *COL4A5* variants has been less clear for females with XLAS. In fact, an early large study revealed no correlation between genotype and phenotype in women with X-linked disease (13). Furthermore, there was no correlation between earlier age at kidney failure in men and women from the same family, which is consistent with our study, as one male with XLAS, from the same family as the previously described females, reached KF significantly earlier, at the age of 20. However, predicting the disease course is complicated due to the high intrafamilial heterogeneity caused by random X-chromosomal inactivation (13). Additionally, in our cohort, only 12% of females had hearing, and 4% had ocular abnormalities. These findings indicate that women have a lower frequency of developing extrarenal manifestations in AS (114). We did not find any significant difference among the types of genetic variants and the occurrence of hearing or ocular abnormalities in women with XLAS. These findings support other studies that have also not demonstrated any genotype-phenotype correlations for hearing loss or ocular abnormalities in women with pathogenic *COL4A5* variants (114). Therefore, recent studies have shifted their focus towards examining the association between genotype and proteinuria, as proteinuria is commonly observed prior to the onset of KF (142). In our study, 81% of females had proteinuria at the mean age at 25.2±16.6 years. However, a recent extensive study involving 336 females revealed that 73% of women developed proteinuria at a mean age of 7 years old, and 15% of all females eventually progressed to KF (142). These findings align with our recommendations from the Alport Syndrome Workshops group, which suggest that proteinuria should be considered a risk factor for CKD progression in females with AS. Therefore, it is advised to

initiate ACE inhibitor treatment as soon as proteinuria is detected in these individuals (1).

The inheritance pattern of digenic variants remains complex and raises many questions that need to be addressed when considering prognosis and the risk to affected relatives (6). The consequences of digenic AS vary depending on the sex of the affected individual, the "severity" of the *COL4A5* variant, and the nature of the *COL4A3* or *COL4A4* change (143). In our study, we identified four individuals, aged 14 to 42, from four different families, who carried digenic variants in two of the three *COL4A3-A5* genes. Two out of the four individuals were from families with XLAS. Digenic AS is considered extremely rare, and there is a lack of knowledge about the natural course of this type of AS. Therefore, we collaborated with our Australian colleagues to study digenic AS and evaluate genotype-phenotype correlations (143). According to the current guidelines for Genetic Testing and Management of AS, digenic inheritance may result in more severe phenotypes, including kidney impairment (87). However, in our study, none of the individuals with digenic variants, regardless of the variant type (missense and splicing changes), developed KF. Three women with a combination of variants in *COL4A5* and *COL4A3* had microalbuminuria, although only one had mild kidney impairment in CKD stage 2, while the other two had eGFR above 90 ml/min/1.73 m^2. This is consistent with our study with Australian colleagues, where we found that in females (n=26) with a pathogenic *COL4A5* variant, on average 50% of heterotrimers are affected, which increases to 75% with an additional *COL4A3* or *COL4A4* variant, leading to a higher risk of proteinuria and, therefore, increasing the risk of kidney failure (143). In the Lithuanian cohort, two out of three women underwent kidney biopsy at the ages of 14 and 37, which both revealed FSGS, lamellation, and thickening of GBM. These findings indicate a higher risk of disease progression later in life. Therefore, we emphasize the importance of initiating ACE inhibitors for females with a *COL4A5* variant and an additional *COL4A4* or *COL4A3* variant at the time of diagnosis (143). Although we did not find males with a *COL4A5* variant and another autosomal variant in the Lithuanian cohort, in our larger digenic study, we found that in males with a pathogenic *COL4A5* variant, all collagen IV a345- heterotrimers are affected, and an additional *COL4A3* or *COL4A4* variant may not worsen the disease. On the other hand, in Lithuanian cohort we identified one male with genetic changes in *COL4A3* and *COL4A4,* who only had mild proteinuria and hematuria with normal kidney function at the age of 40. Kidney biopsy revealed only thin basement membrane and foot process effacement. According to our study with Australian colleagues, in digenic AS with pathogenic *COL4A3* and *COL4A4* variants, 75% of the

heterotrimers are affected (143). The *COL4A3* and *COL4A4* genes are located head-to-head on chromosome 2, and inheritance is autosomal dominant when both variants affect the same chromosome (in cis) or recessive when they affect different chromosomes (in trans). The autosomal recessive form is more similar to ARAS, resulting in a higher prevalence of KF than in the cis position. Therefore, our male patient, having normal kidney function at the age of 42, was associated with a more benign manifestation. However, digenic AS through autosomal inheritance may cause increased proteinuria and a median age of kidney failure that is intermediate between ADAS and ARAS. Therefore, molecular analysis of all *COL4A3-A5* genes is crucial for all individuals, including those with a known pathogenic familial variant in one particular gene, as the risk and therapy initiation for digenic AS considerably differ compared to other types of AS.

In conclusion, this is the first study on Alport syndrome conducted in Lithuania. Firstly, we identified 114 patients out of 221 individuals with suspected AS, demonstrating that AS is a prevalent kidney disease among other rare inherited kidney disorders in Lithuania. This research provides a solid foundation for further epidemiological studies on AS in Lithuania. Secondly, we showed that molecular analysis is a valuable tool for AS diagnosis, prognosis, and treatment initiation. Thirdly, the ability to predict the severity of AS based on genetic variants has significant implications for personalized treatment and interventions. Fourthly, identifying severe variants allows for early interventions that can delay the onset of kidney failure and guide therapeutic choices. Fifth, identifying novel variants increases the data in global genetic databases and helps researchers better understand the disease manifestations. Sixth, identifying common genetic variants in other populations may aid future researchers in exploring common ancestry in Europe and other countries. Seventh, recognizing genetic variants also plays a vital role in clinical trials by standardizing the risk of KF, facilitating the comparison of treatment outcomes, and informing the development of targeted therapies. Additionally, the identification of genetic variants in *COL4A3-A5* can dissuade affected relatives from acting as kidney donors and ensure appropriate counselling regarding family planning. Overall, predicting the severity of AS variants enhances patient management, improves clinical decision-making, and advances research in this field.

6. CONCLUSIONS

1. Molecular analysis of the *COL4A3-A5* gene is the first-line diagnostic method for suspected Alport syndrome. The diagnosis was confirmed in more than half of the individuals with persistent microscopic hematuria. Renal biopsy was performed in 43% of AS subjects with advanced CKD. Renal biopsy was significantly less common in children than in adults.
2. Molecular characteristics influence the clinical severity and disease progression of AS. Pathogenic *COL4A5* variants involving substitution of glycine with destabilizing amino acids (Arg, Val, Glu, Asp, Trp) were correlated with earlier development of end-stage CKD and hearing loss. A glycine substitution near the non-collagenous region in the *COL4A5* gene was associated with subsequent deterioration of renal function, while in the *COL4A3* and *COL4A4* genes reduced the risk of hematuria.
3. After analysing the *COL4A3-A5* genes of 221 individuals using NGS, we identified 44 different genetic changes. Among these changes, 28 variants (63.6%) were considered new and had not been previously described. Genotype-phenotype correlation analysis revealed that some variants exhibited phenotypic variation within the affected families. Furthermore, over a third of the genetic changes found were shared with the global population and had been documented in international genetic databases.
4. Molecular analysis is the gold standard for assessing the risk of AS progression. Males with XLAS and individuals with ARAS have the highest risk of CKD progression, while individuals with ADAS, females with XLAS, and individuals with digenic AS have a varying risk of KF ranging from moderate to mild, unless proteinuria is present.

7. PRACTICAL RECOMMENDATIONS

1. Persistent glomerular hematuria, when other glomerular diseases have been ruled out, can be a strong indicator of AS, particularly in the presence of proteinuria, positive family history of kidney failure, or extrarenal manifestations. Therefore, we recommend following the AS diagnostic algorithm outlined in Figure 24.
2. Performing a genetic kidney disease panel is recommended, even in patients with a strong suspicion of a known inherited kidney disease like ADPKD, as AS can be masked by other genetic kidney disease diagnoses. Additionally, screening for all *COL4A3-A5* variants is recommended, even if the exact familial variant has been identified, to avoid overlooking digenic AS and account for different types of AS that may occur within the same family.
3. While the identification of VUS does not confirm a diagnosis of AS, it is recommended to evaluate clinical manifestations, family history, and consider kidney biopsy after their identification. This is important because not all VUS variants result in better outcomes in CKD progression. Furthermore, assessing the specific type of genetic variant in *COL4A3-A5* is crucial in determining the severity of Alport syndrome and evaluating the risk of CKD.
4. Regularly monitor blood pressure and proteinuria in individuals with Alport syndrome, and initiate RAAS blockade treatment, if necessary, as hypertension and proteinuria are identified as risk factors for CKD progression.
5. We recommend monitoring microalbuminuria and proteinuria in at-risk children with Alport syndrome from the age of 1 year or upon diagnosis, with annual follow-up tests, to detect and manage kidney impairment early.
6. Even mild forms of AS can lead to KF, so individuals with AS should be advised against kidney donation due to their own risk of developing kidney failure.
7. Initiate ACE inhibitors or ARB therapy at the time of genetic diagnosis for males with XLAS, individuals with ARAS, and individuals with digenic AS; consider ACE inhibitors or ARB therapy when proteinuria is detected in females with XLAS and individuals with ADAS (Fig. 25).

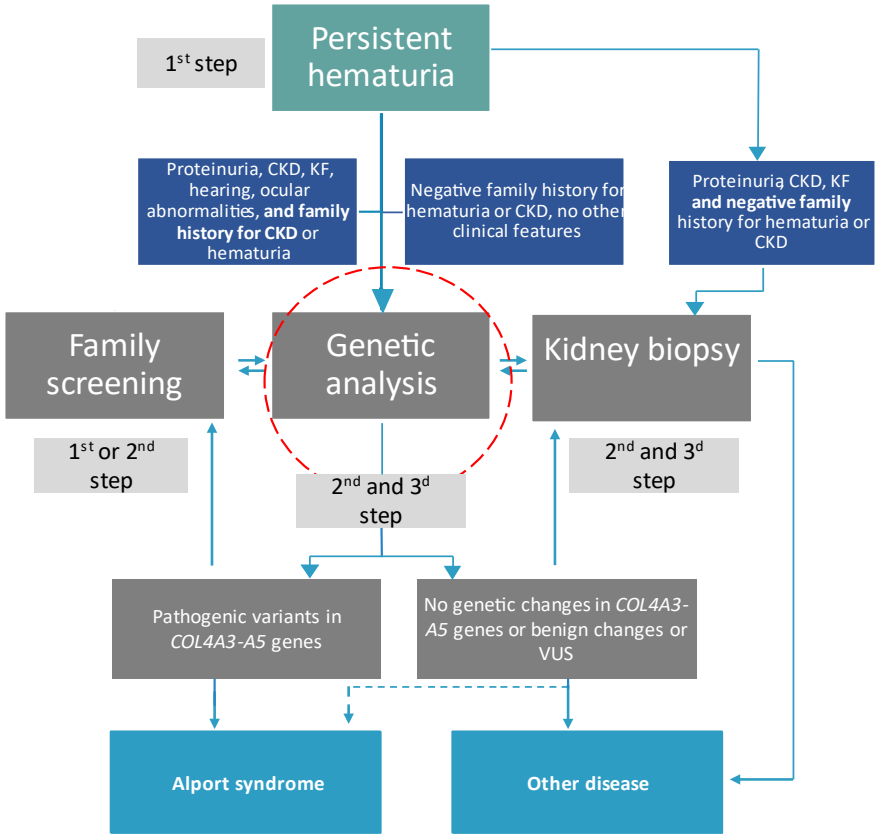


Figure 24. The diagnostic algorithm proposed by our study includes several steps.

The 1st step involves the identification of individuals with persistent hematuria, which is defined as more than three samples over a period of six months. Individuals with positive family history for CKD and additional clinical signs and symptoms, or individuals with negative family history but only hematuria, are referred for genetic analysis. Additionally, individuals with suspected AS, even without hematuria but with known AS cases in the family, may also be sent for genetic analysis. **The 2nd step** consists of genetic analysis, which can be initiated directly after the first step. Family screening is recommended following the identification of a new positive AS case in the family. In cases where individuals have a negative family history for CKD but exhibit persistent hematuria along with other features such as proteinuria, CKD, or KF, a kidney biopsy may be performed. **The 3d step** involves genetic analysis specific to AS findings in kidney biopsy, which may include unknown etiology FSGS, thickening and lamellation of the GBM, thinned GBM, and foot process effacement observed through electron microscopy. Individuals with negative genetic analysis or those with controversial results, such as variants of uncertain significance (VUS), particularly if other features such as proteinuria, CKD, or extrarenal manifestations are present, may also undergo kidney biopsy. AS is confirmed through molecular analysis by identifying pathogenic variants in the *COL4A3-A5* genes. A comprehensive inherited kidney disease panel, which includes the *COL4A3-A5* genes, is recommended. In the case of family screening, genetic analysis for all three *COL4A3-A5* genes is recommended, regardless of known familial variants.

Colour representing the risk of CKD	The risk of CKD progression	Management
	Males with XLAS, individuals with ARAS	Initiate ACE inhibitors or ARB therapy at the time of genetic diagnosis
	Females with XLAS, individuals with ADAS with proteinuria, hypertension, CKD <90 ml/min/1.73m^2 Individuals with digenic AS	Consider ACE inhibitors or ARB therapy when proteinuria is detected in females XLAS and individuals ADAS. Initiate ACE inhibitors or ARB therapy at the time of genetic diagnosis for digenic AS
	XLAS females, individuals with ADAS, with no clinical symptoms	No management needed. Annual follow-up of kidney function

Figure 25. Risk stratification and management for CKD progression in AS due to molecular analysis, proposed by our study. Red colour represents the highest risk, yellow – intermediate risk, and green – the lowest risk for CKD progression.

8. LIMITATIONS

Our study has several limitations. Firstly, the analysis was limited to a single centre, preventing us from estimating the prevalence of AS in Lithuania. Secondly, due to the small number of individuals experiencing kidney failure, we were unable to accurately determine kidney survival rates among different AS types. Additionally, limited data on eGFR values restricted our ability to choose alternative survival endpoints. Thirdly, the inclusion of children at the early stages of the disease may have introduced bias, as their true phenotypes may not yet be fully manifested. Fourthly, certain data, such as kidney biopsy results and information on hearing and ocular abnormalities, were missing and not applicable. Fifthly, some study participants were close relatives, which could lead to overlapping genotype-phenotype correlations. Lastly, not all genetic data provided by the genetic laboratory, including the specific location of genetic variants within exons, could be compared. To ensure unbiased results in future studies, it is recommended to separate paediatric and adult cohorts. Additionally, collaborating with additional foreign studies and local medical centres is advised to increase the number of patients and analyse their outcomes, such as kidney survival.

SANTRAUKA LIETUVIŲ KALBA

SANTRUMPOS

ADAS – autosominis dominantinis Alporto sindromas
ADPIL – autosominė dominantinė policistinė inkstų liga
AKFi – angiotenziną konvertuojančio fermento inhibitoriai
ARB – angiotenzino II receptorių blokatoriai
Arg – argininas
ARAS – autosominis recesyvinis Alporto sindromas
Asn – asparaginas
BM – bazinė membrana
COL4 – kolageno IV tipas
COL4A3-A5 – *COL4A3, COL4A4* ir *COL4A5* genai
DNR – deoksiribonukleorūgštis
eGFG – apskaičiuotas glomerulų filtracijos greitis
EM – elektroninė mikroskopija
GBM – glomerulų bazinė membrana
GD – genetinė diagnozė
Glu – glutamo rūgštis
Gln – glutaminas
Gly – glicinas
GV – genetinis variantas
IB – inkstų biopsija
LIL – lėtinė inkstų liga
LP – tikėtina patogeninis variantas
P – patogeninis variantas
RAAS – renino – angiotenzino – aldosterono sistema
St. – stadija
VUL SK – Vilniaus universiteto Santaros Klinikos
VUS – nežinomo patogeniškumo variantas
XLAS – su X chromosoma susijęs Alporto sindromas
ŽSGS – židininė segmentinė glomerulosklerozė

1. ĮVADAS

Kolageno IV α345 nefropatija, dar žinoma kaip Alporto sindromas (AS), yra antra pagal dažnumą paveldima inkstų liga, sudaranti apie 1–2% visų galutinės stadijos lėtinės inkstų ligos (LIL) atvejų (1,2). AS būdinga glomerulinė mikrohematurija, proteinurija ir progresuojanti LIL, ilgainiui sukelianti negrįžtamą inkstų funkcijos sutrikimą, neretai pasireiškiantį kartu su klausos pažeidimu ir specifiniais pokyčiais akyse (3).

Kolagenas IV yra svarbi inkstų glomerulų bazinių membranų (GBM), ausies sraigės, akių lęšiuko ir tinklainės struktūros dalis. AS atsiranda dėl kolageno IV grandines alfa-3, alfa-4, alfa-5 koduojančių genų, atitinkamai *COL4A3, COL4A4, COL4A5*, patogeninių pokyčių. Kolageno struktūra tampa nevisavertė, pakinta bazinės membranos laidumas. Patogeniniai variantai *COL4A5* gene sukelia su X chromosoma susietą Alporto sindromą (XLAS), o *COL4A3* ir *COL4A4* genų pokyčiai yra susiję su autosominiu dominantiniu (ADAS) arba autosominiu recesyviniu Alporto sindromu (ARAS) (4–6). Pokyčiai atsiradę dvejuose iš trijų *COL4A3-5* genų lemia digeninį AS (7).

Alporto sindromas buvo laikomas reta liga, pasireiškiančia 1 iš 5 000 arba 1 iš 53 000 gyventojų (8,9), tačiau remiantis naujausiais tyrimų duomenimis, AS yra kur kas dažnesnė liga, nei manyta iki šiol. Teigiama, jog XLAS pasireiškia 1 iš 2000 atvejų, tuo tarpu ADAS – 1 iš 100 gyventojų (10). AS klinikiniai simptomai varijuoja nuo izoliuotos besimptomės mikrohematurijos iki labai pažengusios LIL. Taigi daugelis asmenų, kuriems nėra akivaizdžių simptomų, gali sirgti AS to nežinodami (11). Inkstų ligos sunkumas, eiga ir kitų požymių (akių, klausos pažeidimo) atsiradimas priklauso ne tik nuo pažeisto *COL4A3-A5* geno ar jų derinio, bet ir nuo genetinio pokyčio tipo. Taškiniai (vienos amino rūgšties pakaitos, anglų k. *„missense“*) ir nonsense (anglų k. *„nonsense“)* variantai, delecijos, splaisingo (anglų k. *„splice site“*) ar rėmelio poslinkio (anglų k. *„frameshift“*) patogeniniai variantai lemia AS pasireiškimą ir galutinės stadijos LIL pradžią (12–17). Tyrimais įrodyta, jog ankstyvas renino-angiotenzino-aldosterono sistemą (RAAS) slopinančių vaistų skyrimas, reikšmingai pagerina inkstų funkciją ir sumažina LIL progresavimą AS sergantiesiems (18–20). Todėl ankstyva AS diagnostika pacientams, kuriems pasireiškia nuolatinė mikrohematurija ir proteinurija, svarbi tolimesnei ligos eigai ir prognozei. AS diagnozė patvirtinama atliekant *COL4A3-A5* genų molekulinę analizę (21). Įvairiose pasaulio šalyse trūksta duomenų apie AS, jo paplitimą bei genetinę įvairovę. Genetinių *COL4A3-A5* variantų nustatymas padeda įvertinti ligos pasireiškimą ir prognozę, nustatyti epidemiologinį paplitimą bei gydymo galimybes, o naujų genetinių variantų radimas papildo pasaulio genetines duomenų bazes ir padeda geriau

įsisąmoninti AS genotipo-fenotipo ryšį. Šiuo tyrimu siekiama įvertinti diagnostikos galimybes, genotipo ir fenotipo sąsajas, prognozę ir genetinį pasiskirstymą didelėje AS sergančių žmonių kohortoje. Tai yra pirmasis tokio pobūdžio AS tyrimas Lietuvoje. Tikimės, kad šio tyrimo rezultatai suteiks papildomų žinių apie *COL4A3-A5* genetinių variantų kintamumą, pasiskirstymą ir jų prognostinį poveikį AS sergantiems Lietuvos gyventojams ir padės palyginti mūsų sukauptą informaciją su kitomis šalimis bei papildyti pasaulio genetinių duomenų bazes naujais *COL4A3-A5* variantais. Tikimės, kad šis genetinių variacijų molekulinės patologijos analizės tyrimas reikšmingai prisidės kuriant tikslesnius molekulinius ir diagnostinius AS patikros algoritmus.

Tyrimo tikslas ir uždaviniai

Šio darbo tikslas – įvertinti kolageno IV α345 nefropatijų diagnostikos būdus, molekulines charakteristikas, genotipo-fenotipo koreliacijas ir prognostinius veiksnius Lietuvos kohortoje.

Uždaviniai:

1. Įvertinti Alporto sindromo diagnostikos galimybes ir jų reikšmę Lietuvos kohortoje.
2. Įvertinti *COL4A3-A5* genų molekulinius pokyčius ir nustatyti jų įtaką ligos progresavimui.
3. Išanalizuoti Lietuvos kohortoje rastų naujų *COL4A3-A5* variantų klinikinį pasireiškimą.
4. Įvertinti LIL progresavimo riziką pacientams, sergantiems skirtingu AS tipu.

Ginamieji teiginiai

1. Molekulinė *COL4A3-A5* genų analizė yra tinkamiausias būdas diagnozuoti AS ir įvertinti ligos progresavimo riziką.
2. *COL4A3-A5* gliciną pakeitusios amino rūgštys turi įtakos klinikiniam ligos sunkumui ir progresavimui sergant AS.
3. *COL4A3-A5* genų naujų variantų tipas ir dažnis Lietuvos šeimose skiriasi nuo kitose šalyse rastų variantų.
4. LIL progresavimo rizika yra susijusi su patogeniniais *COL4A3-A5* genų variantais ar jų deriniais bei skiriasi tarp genetinio pokyčio tipų ir lyties.

Daugėjant mokslinių tyrimų, susijusių su populiacine *COL4A3-A5* genų molekuline įvairove ir naujomis AS terapinėmis galimybėmis, išaugo AS diagnostikos poreikis Lietuvos populiacijoje. Nors šiuo metu dar nėra specifinio patogenetinio, ligos eigą modifikuojančio AS gydymo, tačiau mokslininkai ir farmacinės kompanijos vis daugiau skiria dėmesio žinomiems LIL sukeliantiems patogeniniams veiksniams – uždegimui ir fibrozei. Dauguma klinikinių tyrimų vertina gydymo galimybes, siekiant sumažinti šių veiksnių pasireiškimą ir LIL progresavimą. LIL, kurią sukelia AS, dažniausiai pasireiškia jauno amžiaus žmonėms, turintiems mažai gretutinių susirgimų, todėl norint tikslesnių, kitų ligų nepaveiktų „taikinio-terapijos“ rezultatų, AS sergantys žmonės gali tapti puikiais klinikinių tyrimų kandidatais (22).

Molekulinė *COL4A3-A5* analizė suteikia informacijos ne tik apie galimą AS pasireiškimą, ligos eigą, prognozę, riziką išsivystyti galutinės stadijos LIL, bet ir padeda įvertinti AS paveldimumą. Nors šis diagnostikos metodas nebėra laikomas novatorišku, genetinė analizė anksčiau netirtoje Lietuvos populiacijoje, suteikia naujų įžvalgų apie kolageno IV α345 nefropatijų molekulinius pokyčius, susijusius su jų aleliniu heterogeniškumu ir populiaciniu kintamumu. Šiame tyrime žinomų ir naujų *COL4A3-A5* variantų nustatymas plečia mūsų supratimą apie genotipo ir fenotipo koreliacijas, praturtina pasaulines genetinių duomenų bazes, skirtas mokslininkams ir farmacijos atstovams, siekiantiems atrasti naujus terapinius AS būdus. Naujų ir žinomų *COL4A3-A5* variantų nustatymas svarbus ir klinikinėje praktikoje, nustatant tikslią diagnozę, sumažinant įvairių invazinių procedūrų (pvz., inkstų biopsijos) poreikį, parenkant tinkamą ir ankstyvą gydymo strategiją bei identifikuojant riziką sirgti turinčius asmenis šeimoje. Genetinė AS diagnozė taip pat padeda identifikuoti simptomų neturinčius asmenius ir vertinti jų riziką inkstų donorystei. Be to, patvirtinta genetinė AS diagnozė svarbi planuojant šeimą, diskutuojant apie nėštumą, reprodukcines problemas ir vertinant AS perdavimo riziką vaikams.

2. TYRIMO METODIKA

Tyrimo planas ir dalyviai

Šis tyrimas buvo tarptautinio, daugiacentrio, stebėjimo, išilginio Alporto sindromo biožymenų tyrimo „BioAlport“ dalis (ClinicalTrials.gov Identifier: NCT02718027). Į analizę įtraukti tik Lietuvos pacientai. Dėl mažo pacientų, turinčių molekulinius *COL4A3-A5* pokyčius, skaičiaus, ir, siekiant reikšmingesnių darbo rezultatų analizuojant digeninį Alporto sindromą bei glicino pakaitos sukeltas molekulines charakteristikas, bendradarbiavome su kitomis tarptautinėmis AS darbo grupėmis, tokiomis kaip Australijos ir Didžiosios Britanijos AS darbo grupės bei Europos Retų Inkstų ligų Referentiniu Tinklu (*ERKNet, European Reference Network for Rare Kidney Diseases*).

Tyrimą patvirtino Lietuvos Vilniaus regioninis biomedicininių tyrimų etikos komitetas (BioAlport, Nr. 158200-16-857-367). Visi šio tyrimo dalyviai pasirašė informuotą sutikimą dalyvauti tyrime. Vaikų ir paauglių sutikimas buvo gautas iš jų tėvų ar globėjų.

Į tyrimo grupę buvo įtraukti asmenys iš visos Lietuvos, gydyti suaugusiųjų ar vaikų nefrologų Vilniaus universiteto ligoninėje Santaros klinikos (VUL SK) 2016–2022 metais. Dalyvių amžius svyravo nuo 2 mėnesių iki 82 metų. Šio tyrimo planas apėmė stebėjimo (aprašomojo pobūdžio) ir analitinius tyrimus. Tiriamieji, atitinkantys AS pirminės diagnostikos kriterijus (1 lentelė), pirmojo vizito metu buvo įvertinti suaugusių nefrologo (doktorantas) arba vaikų nefrologo.

1 lentelė. AS pirminės diagnostikos kriterijai

Diagnostikos kriterijus	Apibūdinimas
Klinikiniai požymiai	– Nuolatinė izoliuota mikroskopinė hematurija su dismorfiniais eritrocitais >6 mėn. – Nuolatinė hematurija su neaiškios etiologijos proteinurija – Nefrozinio lygio proteinurija arba steroidams atsparus nefrozinis sindromas – Klausos sutrikimai su nuolatine hematurija arba neaiškios etiologijos ŠKL – AS specifiniai akių pažeidimai (tinklainės dėmės, lentikonusas ir kt.)

Diagnostikos kriterijus	Apibūdinimas
	– Neaiškios etiologijos LIL arba KF, ypač jei yra teigiama ŠKL šeimos istorija, klausos sutrikimai arba nuolatinė hematurija
Inkstų biopsija	Putotos ląstelės, neaiškios etiologijos ŽSGS, plonos, supleišėjusios, sustorėjusios GBM, mažųjų podocitų kojyčių susiliejimas elektroninėje mikroskopijoje
AS atvejai šeimoje	Asmenys šeimoje, sergantys genetiškai patvirtintu AS

LIL – lėtinė inkstų liga; ŽSGS – židininė segmentinė glomerulosklerozė; GBM – glomerulų bazinė membrana; AS – Alporto sindromas

Pirmojo vizito metu pirminės diagnostikos kriterijus atitinkantiems tiriamiesiems buvo paimti kraujo mėginiai DNR ekstragavimui, taip pat surinkta išsami demografinė, klinikinė ir šeiminė anamnezė (2 lentelė).

2 lentelė. Retrospektyvieji tiriamųjų duomenys

Asmens duomenys	Renkamų asmens duomenų sąrašas
Demografiniai duomenys	Amžius, lytis, tautybė, gyvenamoji vieta
Asmens sveikatos duomenys	- Paciento amžius, kai buvo nustatyta hematurija ir proteinurija, galutinės stadijos LIL, arterinė hipertenzija. RAAS inhibitorių pradžia. - Inkstų biopsija: histologiniai radiniai (židininė segmentinė glomerulosklerozė (ŽSGS), mezangioproliferaciniai pokyčiai, suplonėjusios, supleišėjusios, sustorėjusios GBM). Inkstų biopsijos metu nustatytas glomerulų filtracijos greitis; proteinurija (vienkartiniame šlapime, g/L). - Klausos ir akių pažeidimai; gretutinės ligos
Šeiminė anamnezė	Šeiminė inkstų ligų, AS, hematurijos anamnezė.

Tiriamųjų DNR analizė atlikta dvejose genetinėse laboratorijose – Centogene laboratorijoje (n=178, Rostokas, Vokietija) ir VUL SK Medicininės Genetikos centre (n=43, Vilnius, Lietuva).

Į galutinį tyrimą buvo įtraukti tik tie asmenys, kuriems rasti kliniškai reikšmingi molekuliniai pokyčiai *COL4A3-A5* genuose (3 lentelė). Taip pat, į šią grupę buvo įtraukti asmenys tiriami dėl kitų genetinių ligų ir kuriems AS nustatytas atsitiktinai, aptikus *COL4A3-A5* genų pokyčius.

3 lentelė. Įtraukimo ir neįtraukimo į galutinį tyrimą kriterijai

Įtraukimo kriterijai
1. Asmenys, kuriems nustatyti patogeniniai (P), tikėtina patogeniniai (LP) arba potencialiai reikšmingi nežinomo patogeniškumo variantai (VUS) *COL4A3-A5* genuose, įskaitant: a. Asmenis, tirtus dėl AS, kurie atitiko AS pirminės diagnostikos kriterijus b. Asmenis, kuriems atsitiktinai nustatyti *COL4A3-A5* genų molekuliniai pokyčiai, įtariant kitą ligą 2. Abiejų lyčių asmenys, vyresni nei 2 mėnesiai („BioAlport“ tyrimo kriterijus) 3. Pasirašyta informuoto sutikimo dalyvauti tyrime forma
Neįtraukimo kriterijai
1. Asmenys, kuriems nustatyti gerybiniai, galimai gerybiniai variantai arba polimorfizmai *COL4A3-A5* genuose 2. Asmenys, kuriems įtariamas AS, bet genetiškai patvirtintos kitos genetinės ligos 3. Asmenys, turintys patogeninius *COL4A3-A5* genų pokyčius ir gretutines genetines patologijas

Asmenys, atitikę įtraukimo kriterijus buvo pakviesti antrajam vizitui pas suaugusių (doktorantė) arba vaikų nefrologą. Antrojo vizito metu buvo atliktas nefrologinis įvertinimas; visi asmenys buvo nukreipti specialistų, turinčių patirties ir specifinių žinių AS srityje, konsultacijoms, klausos ir akių vertinimui.

<u>Nefrologinis vertinimas ir tyrimų duomenys:</u>

1. Hematurijos nustatymas vienkartiniame šlapimo tyrime
2. Proteinurijos nustatymas biocheminiame šlapimo tyrime, naudojant vienkartinio šlapimo baltymo ir kreatinino santykį (B/K, mg/mmol) arba albumino/kreatinino santykį (A/K, mg/mmol)
3. Proteinurijos kiekio nustatymas, remiantis B/K santykiu (KDIGO, 2021):
    a. A1:B/K <15 mg/mmol (normalus baltymo kiekis ar pėdsakai)
    b. A2:B/K 15-49 mg/mmol (vidutinė proteinurija)
    c. A3:B/K 50-299 mg/mmol (sunki proteinurija)
    d. Nefrozinio lygio: B/K ≥300 mg/mmol suaugusiems ir ≥200 mg/mmol vaikams
4. Inkstų funkcijos nustatymas, remiantis glomerulų filtracijos greičio (eGFR) formule (4 lentelė), naudojant kreatininą (mkmol/L):
    a. Suaugusiems (CKD-EPI formulė, anglų k. *Chronic Kidney Disease Epidemiology Collaboration*)
    b. Vaikams (naujoji Švarco formulė, 2009)

5. Arterinės hipertenzijos (AH) buvimas ar nebuvimas (suaugusiems AH diagnozuota, jei rastas arterinio kraujo spaudimo (AKS) padidėjimas >130/90 mmHg, vaikams AKS vertintas standartizuotai pagal amžiaus, lyties ir ūgio standartinį nuokrypį: AH diagnozuota, jei sistolinis ir diastolinis AKS rastas didesnis negu 95 procentilė)

4 lentelė. Lėtinės inkstų ligos stadijos, remiantis eGFR (KDIGO, 2012) (111)

LIL stadija	eGFR (ml/min/1.73 m2)
1	≥90
2	60-89
3a*	45-59
3b*	30-44
4	15-29
5	<15

*3a ir 3b LIL stadija mūsų tyrime buvo skaičiuojamos kartu, nes tai neturėjo reikšmingos įtakos mūsų rezultatams ar tyrimo tikslams

Asmenims, kuriems buvo genetiškai patvirtinta AS diagnozė, buvo atliktas specifinis AS oftalmologinis vertinimas:

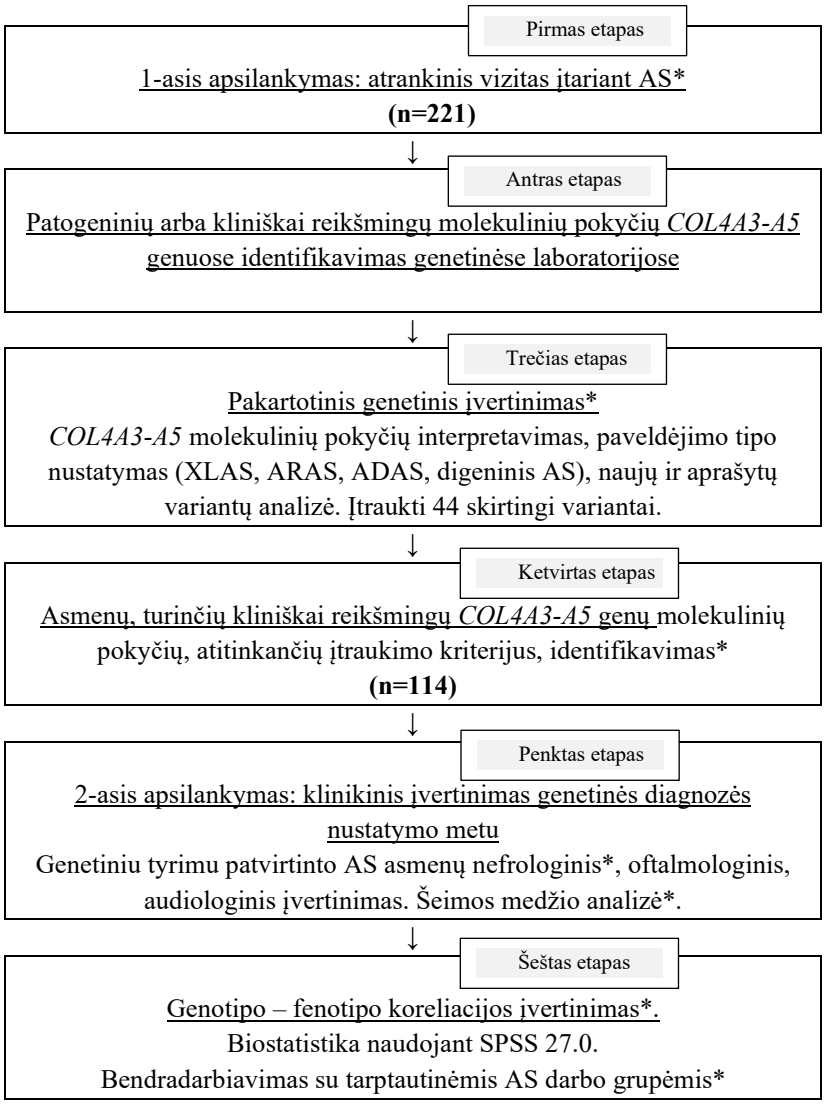
1. Ragenos anomalijų tyrimas plyšine lempa
2. Oftalmoskopija dėl perimakulinės dėmės retinopatijos
3. Retinoskopija dėl lentikonuso
4. Optinės koherencijos tomografija (OCT) dėl laikinojo tinklainės retėjimo

Aptikus specifinių pokyčių anksčiau aprašytas metodais buvo atlikta priekinio segmento ir dugno fotografija. Pacientas, turintis vieną ar kelis specifinius AS pokyčius akyse, buvo vertinamas kaip turintis Alporto oftalmopatiją.

Asmenys, kuriems buvo genetiškai patvirtintas AS, buvo siunčiami klausos vertinimui, atliekant audiogramas. Tik asmenys, turintys abipusį neurosensorinį klausos sutrikimą 2 000–8 000 Hz diapazone, buvo laikomi turinčiais AS specifinį klausos pažeidimą.

Šeimos nariai, turintys didelę riziką sirgti AS, buvo nukreipti molekulinei analizei ir tolesniam ištyrimui.

Tyrimo organizavimo etapai nurodyti 1 schemoje.



1 schema. Tyrimo organizavimo etapai. Iš 221 asmens, atitinkančių AS įtraukimo kriterijus, Alporto sindromas buvo diagnozuotas 114 asmenų, nustatyti 44 skirtingi *COL4A3-A5* genų pokyčiai. Šimtas septyni asmenys buvo neįtraukti į tyrimą dėl kliniškai nereikšmingų molekulinių pokyčių (gerybinių, galimų gerybinių ir polimorfizmo) *COL4A3-A5* genuose arba dėl su AS nesusijusių patogeninių pokyčių *NPHP1, CLCN5* arba *CFHR5* genuose. Šie pokyčiai buvo susiję su nefronoftize (n=1), Dento liga (n=2 nesusiję pacientai) arba CFHR5 nefropatija (n=1).*Doktorantės atsakomybė tyrime

Genetinė analizė kelių-keliasdešimties genų koduojančių sekų tyrimo naujos kartos sekoskaitos (NKS) metodu buvo atlikta dviejose genetikos laboratorijose – Centogene laboratorijoje (n=178, Rostokas, Vokietija) ir VUL SK Genetikos centro laboratorijoje (n=43, Vilnius, Lietuva). Genominė DNR fermentų pagalba buvo fragmentuota, regionai – „taikiai“ praturtinti naudojant DNR surinkimo zondus ir pradmenis, nukreiptus prieš koduojančius regionus ir žinomus patogeninius ar tikėtina patogeninius genų pokyčius. DNR bibliotekos buvo suskirstytos, naudojant Illumina platformą. Visas *COL4A5* kodavimo regionas*, COL4A3* ir *COL4A4* genai, įskaitant 10 bp besiribojančias intronines sekas, buvo amplifikuoti ir sekvenuoti. Siekiant ištirti trūkstamus NKS neapimtus DNR fragmentus, buvo taikytas Sangerio metodas. Genetinių duomenų analizė, anotacija ir interpretacija atlikta naudojant GRCh37/hg19 (ang. Genome Reference consortium Human Build 37/human genome 19) genomą. Visi tyrimai buvo atlikti, naudojant Centogene ar VUL SK genetinių laboratorijų patvirtintą programinę įrangą. VUL SK Medicininės Genetikos laboratorijoje Sangerio metodas buvo naudojamas ir šeiminiais atvejai, kai žinomas patogeninis *COL4A3-A5* variantas.

Duomenų bioinformacinė analizė ir atranka buvo atliekama atsižvelgiant į tikslinį genų surinkimą ir sekos nustatymą iš DNR mėginių. Kiekvieno nustatyto genetinio pokyčio patogeninio poveikio in-silico prognozė buvo įvertinta naudojant MutationTaster, PolyPhen-2 ir SIFT. Splaisingo (anglų k. „*splicing*“) variantai buvo išanalizuoti naudojant „Human Splicing Finder“ (//www.umd.be/HSF3/index.html) ir „MutationTaster“ analitinius įrankius. Patogeniniai pokyčiai, kurių nebuvo galima klasifikuoti naudojant nei vieną iš in-silico įrankių, buvo vertinami kaip neturintys duomenų (ang. *Not applicable*, N/a). Genetiniai *COL4A3-A5* pokyčiai doktoranto buvo patikrinti ir palyginti penkiose duomenų bazėse – Centogene, Franklin, ClinVar, InterVar ir Varsome (paskutinė peržiūra 2022 m. gruodžio mėn.), o patogeniškumas vertintas naudojant ACMG/AMP kriterijus, genetinius variantus suskirtant į penkias kategorijas – patogeniniai (P, 1 klasė), tikėtina patogeniniai (LP, 2 klasė), nežinomo patogeniškumo variantai (VUS, 3 klasė), tikėtina gerybiniai variantai (LBn, 4 klasė) arba polimorfizmai (Plm, 5 klasė).

Asmenys, kuriems buvo nustatytas vienas heterozigotinis arba hemizigotinis *COL4A5* geno pokytis, buvo vertinamai kaip turintys XLAS, o tiems kuriems buvo rasta po vieną heterozigotinį *COL4A3* arba *COL4A4* geno pokytį – ADAS. Du heterozigotiniai arba homozigotiniai variantai *COL4A3*

arba *COL4A4* genuose nulėmė *ARAS.* Jei pokyčiai buvo rasti dvejuose skirtinguose *COL4A3-A5* genuose, asmenims buvo nustatytas digeninis AS.

Literatūroje ADAS dar yra žinomas kaip plonųjų bazinių membranų liga, gerybinė šeiminė hematurija ar „autosominio recesyvinio AS nešiotojo statusas“. Nors vis dar nesutariama kaip turėtų būti vadinami vieną heterozigotinį *COL4A3* arba *COL4A4* variantą turintys asmenys, šiame tyrime nusprendėme naudoti terminą ADAS, vadovaudamiesi 2018 m. Kashtan ir kt. pasiūlyta AS klasifikacija (16).

Statistinė analizė

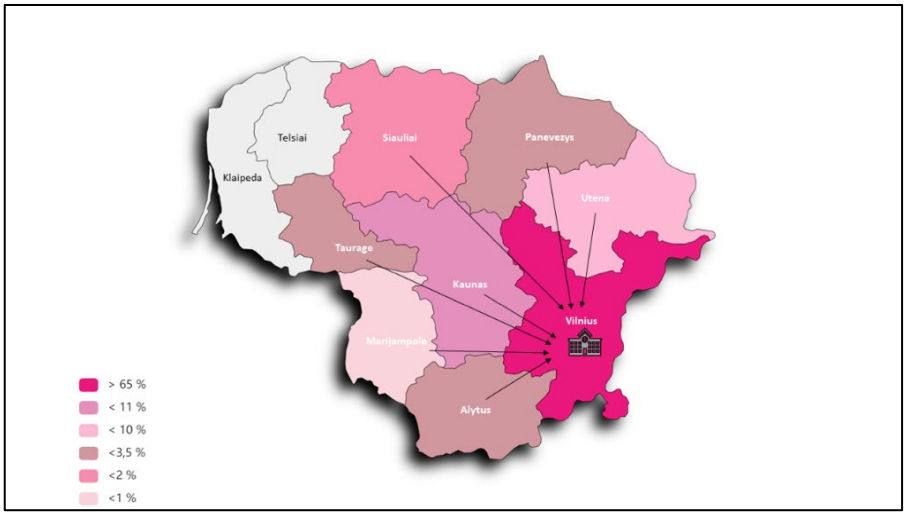
Kiekybiniams duomenims įvertinti buvo atlikta aprašomoji statistinė analizė, naudojant SPSS 29.0 (*ang. Statistical Package for the Social Sciences*) programinį paketą. Nuolatiniai kintamieji buvo pateikti kaip vidurkis (V) ± standartinis nuokrypis (SD) arba mediana (tarpkvartilis diapazonas [IQR]), priklausomai nuo jų pasiskirstymo. Kategoriniai duomenys buvo išreikšti dažniais ir procentais. Normalaus pasiskirstymo kiekybinėms reikšmėms palyginti buvo taikomi parametriniai kriterijai, o neparametriniai testai – nenormaliai paskirstytoms kiekybinėms reikšmėms ir eilės kintamiesiems. Pasirinktas pasitikėjimo lygis buvo 95 %, o statistiškai reikšmingi rezultatai buvo laikomi, jei $p \leq 0,05$. Nepriklausomi mėginių palyginimai buvo atlikti naudojant parametrinę dispersijos analizę (ANOVA) arba neparametrinį Dunn's Kruskal-Wallis testą. Chi kvadrato (x2) kriterijus buvo naudojamas įvertinti nepriklausomumą charakteristikų ir dažnių tarpusavio priklausomybę. Fisher testas buvo naudojamas kategoriškiems kintamiesiems palyginti, o Mann-Whitney testas – nuolatiniams skirtingų lyčių kintamiesiems ir *COL4A3-A5* ligą sukeliančio varianto buvimui vertinti.

Inkstų išgyvenamumas negalėjo būti įvertintas naudojant Kaplan-Meier testą dėl nedidelio V st. LIL sergančių asmenų skaičiaus.

3. REZULTATAI

3.1. Alporto sindromo pasiskirstymas

Visų tyrimo dalyvių pasiskirstymas pagal gyvenamą vietovę apėmė 16 Lietuvos miestų (1 pav.). Didžioji dalis asmenų gyveno Vilniaus regione, tuo tarpu Šiaurės Vakarų Lietuvoje pacientų nenustatyta.



1 pav. Tiriamos populiacijos (n=114) pasiskirstymas pagal Lietuvos miestus

3.2. Bendroji charakteristika

Genetinės analizės metu AS buvo diagnozuotas 114 asmenų, iš jų 53 (46,5 %) vyrams. I tiriamąją analizę įtraukti asmenys priklausė 63 skirtingoms šeimoms. Klinikiniai ir demografiniai į galutinį tyrimą įtrauktų dalyvių duomenys pateikti 5 lentelėje.

Asmenys iki 18 metų sudarė 20 % visų tyrimo dalyvių. Genetinės diagnozės nustatymo metu pacientų amžius svyravo nuo 1 iki 70 metų (amžiaus vidurkis buvo 32,5±18,4 metų) ir reikšmingai skyrėsi tarp lyčių. AS sergančių moterų genetinės diagnozės amžius buvo 35,64±18,65 metų, tuo tarpu AS vyrams buvo diagnozuotas ženkliai anksčiau – 28,82±17,6 metų (p=0,048).

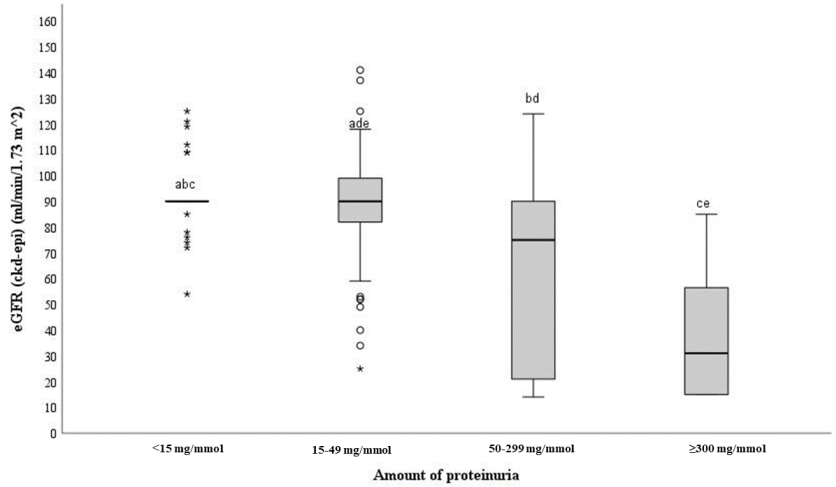
5 lentelė. Tyrimo dalyvių (n=114) demografinių ir klinikinių požymių charakteristika

Demografiniai ir klinikiniai požymiai	Iš viso asmenų, sergančių AS	Vyrai	Moterys	P vertė
Bendras asmenų skaičius, %	114 (100,0)	53 (46,5)	61 (53,5)	0,454
Probandų skaičius, %	60 (52,6)	32 (60,4)	28 (45,9)	0,123
Įtartas AS dėl šeiminės anam., %	54 (47,4)	21 (39,6)	33 (54,1)	0,123
Vidutinis GD amžius	32,5±18,4	28,8±17,6	35,6±18,6	**0,048**
Hematurija, %	114 (100,0)	53 (100,0)	61 (100,0)	-
Hematurijos nustatymo amžius	20,99±17,7	19.37±17.4	19,37±18,0	0,187
Proteinurija, %	81 (71,1)	36 (67,9)	45 (73,8)	0,492
Proteinurijos nustatymo amžius	26±17,7	24,0±19,5	27,5±16,2	0,381
Nefrozinio lygio proteinurija, %	11 (9,6)	5 (9,4)	6 (9,8)	0,942
LIL I st., %	69 (60,5)	34 (64,2)	35 (57,4)	$\times^2 = 7{,}321$, df = 4, p = 0,12
LIL II st., %	17 (14,9)	4 (7,5)	13 (21,3)	
LIL III st., %	15 (13,2)	7 (13,2)	8 (13,1)	
LIL IV st., %	3 (2,6)	3 (5,7)	0 (0,0)	
Galutinės st. LIL (LIL V st.), %	10 (8,8)	5 (9,4)	5 (8,2)	
Vidutinis LIL V st. pr. amžius	36,1±14,1	27,6±10,3	44,6±12,7	**0,048**
AH, %	53 (46,5)	29 (54,7)	24 (39,3)	0.101
RAAS inhibitorių vartojimas, %	66 (58,4)	33 (63,5)	33 (54,1)	0,314
Inkstų biopsija, %	49 (43,0)	23 (43,4)	26 (42,6)	0,934
Inkstų biopsijos atlikimo amžius	34,1±16,3	30,5±14,8	37,3±17,1	0,73
Klausos sutrikimai, %	31 (30,1)	19 (38,8)	12 (22,2)	0,067
Akių anomalijos, %	18 (18,2)	11 (22,9)	7 (13,7)	0,236

GD – genetinė diagnozė; anam. – anamnezė; AH – arterinė hipertenzija; LIL – lėtinė inkstų liga; st. – stadija; pr. – pradžia; RAAS – renino-angiotenzino-aldosterono sistema

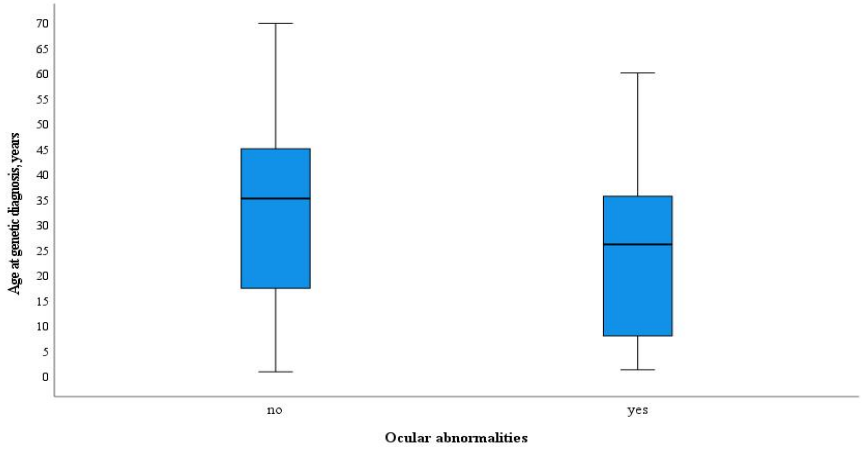
Arterinė hipertenzija dažniau buvo randama vyresniems nei 42 metų amžiaus asmenims ($p<0{,}001$). Skirtumo tarp lyčių rasta nebuvo. AH buvo nepriklausomas rizikos veiksnys, susijęs su blogesne inkstų veikla, lyginant su AH nesergančių asmenų inkstų funkcija ($r=-0.294$, $p=0.021$). Nei vienas vaikas nesirgo galutinės stadijos LIL, tuo tarpu suaugusiųjų grupėje eGFG <15 ml/min/1,73 m^2 pasireiškė atitinkamai 9,3 % ir 8,2 % suaugusių vyrų ir moterų. Moterims V st. LIL pasireiškė vėliau nei vyrams ($p=0{,}048$). Inkstų biopsija buvo atlikta 43% asmenų, sergančių AS. Vaikams inkstų biopsijų buvo atlikta reikšmingai mažiau nei suaugusiems ($p=0{,}009$).

Mikrohematurija pasireiškė visiems asmenims, nepriklausomai nuo amžiaus ar lyties. Proteinurija buvo nustatyta 71,1 % pacientų ir buvo susijusi su blogesne inkstų funkcija, lyginant su asmenimis, kuriems proteinurijos nebuvo ($p=0{,}001$, 2 pav.).



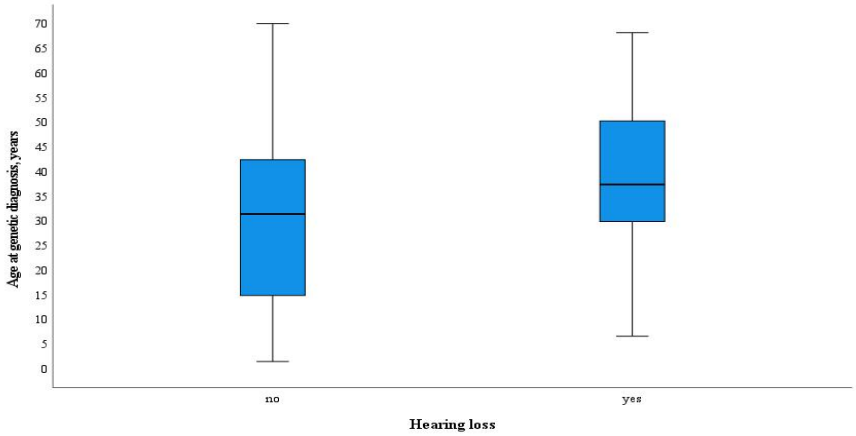
2 pav. AS sergančių asmenų eGFR ir proteinurijos ryšys (Kruskal-Wallis testas (×2= 26.732, df=3, p<0.001, abcde p<0.05)

Oftalmologinis įvertinimas buvo atliktas 86,8 % (99 iš 114) asmenų, iš kurių aštuoniolikai (24,6±18,3 metų amžiaus) buvo nustatyti AS specifiniai akių pažeidimai (3 pav.). Akių pokyčiai dažniau rasti vyresniems nei 32,5 metų asmenims (p=0,02, 95 proc. PI [1,23-11,62]) nepriklausomai nuo lyties (p=0,12).



3 pav. Asmenų, turinčių ir neturinčių akių pažeidimų, pasiskirstymas pagal amžių genetinės diagnozės nustatymo metu

Audiograma buvo atlikta 90,4 % (103 iš 114) asmenų, iš kurių 31 (38,6±17,1 metų amžiaus) nustatytas abipusis neurosensorinis prikurtimas (4 pav.). Klausos sutrikimai dažniau nustatyti vyresniems nei 20,4 metų asmenims (p=0,012, 95 % PI [1,4-13,2]) ir nesiskyrė tarp lyčių (p=0,114).



4 pav. Asmenų su klausos pažeidimais ir be klausos pažeidimų pasiskirstymas pagal amžių genetinės diagnozės nustatymo metu

3.3. *COL4A3-A5* genetinių pokyčių molekulinė charakteristika

Naujos kartos sekoskaitos metodu išanalizavus 114 asmenų *COL4A3*, *COL4A4* ir *COL4A5* genus, buvo identifikuoti 44 skirtingi genetiniai pokyčiai, iš kurių 28 (63,6 %) variantai buvo vertinami kaip nauji ir anksčiau neaprašyti (6 lentelė). Daugiau nei trečdalis rastų genetinių pokyčių buvo žinomi pasaulio duomenų bazėse, o septini iš jų buvo aprašyti ir kitų šalių populiacijose.

6 lentelė. *COL4A3-A5* rastų genetinių pokyčių bendroji charakteristika

Bendroji charakteristika	Bendras skč.	*COL4A3*	*COL4A4*	*COL4A5*
Skirtingų GV skč, %	44 (100,0)	12 (27,2)	16 (36,4)	16 (36,4)
Bendras asmenų, su GV, skč, %	114 (100,0)	21* (18,4)	39* (34,2)	61* (53,5)
Šeimų su GV skč*, %	63 (100,0)	14* (22,2)	35* (55,6)	30* (47,6)
Asmenų su 1 GV, skč., %	106 (100,0)	16 (15,1)	32 (30,2)	58 (54,7)
Asmenų, turinčių >1 GV, skč. %	8 (100,0)	3 (37,5)	6 (75,0)	3 (37,5)
Sudėtinių het. GV skč., %	4`ą (100,0)	2 (50,0)	2 (50,0)	Nėra
Šeimų su sudėtiniais GV skč, %	2 (100,0)	1 (50,0)	1 (50,0)	Nėra
Homozigotinių GV skč., %	1 (100,0)	Nėra	1 (100,0)	Nėra
Skirtingų digeninių GV skč., %	8 (100,0)	1 (12,5)	4 (50,0)	3 (37,5)
Asmenų su ≥2 GV skč.*, %	4* (100,0)	1* (25,0)	4 *(100,0)	3* (75,0)
Nauji GV, %	28 (100,0)	9 (32,1)	9 (32,1)	10 (35,7)
GV įtraukti į pasaulio DB, %	16 (100,0)	3 (18,8)	7 (43,8)	6 (37,5)
GV bendri su kt. šalimis, %	7 (100,0)	2 (28,6)	3 (42,9)	2 (28,6)

*Įskaitant tuos pačius asmenis, sergančius digeniniu AS arba ARAS (kryžminės grupės). GV-genetinis variantas; asm. – asmuo; het. – heterozigotinis; skč. – skaičius; DB – genetinės duomenų bazės, kt. – kitomis

Daugiau nei pusei asmenų (53,5 %) rasti *COL4A5* genetiniai pakitimai, tuo tarpu patogeninių *COL4A3* (18,4 %) ir *COL4A4* (34,2 %) variantų dažnis buvo reikšmingai mažesnis (p=0,042). Kai kuriems asmenims buvo nustatytas daugiau negu vienas pokytis tame pačiame arba kituose *COL4A3-A5* genuose. Skirtingų variantų dažnis kiekvienoje trijų *COL4A3* , *COL4A4* ir *COL4A5* genų grupėje buvo atitinkamai 27,2 %, 36,4 % ir 36,4 %. Tyrimo metu buvo nustatyti keturi skirtingi *COL4A3-A5* genetinių variantų tipai – vienos amino r. pakaitos (toliau, *missense*) variantai (75.0 %), rėmelio poslinko (9.1 %), splaisingo (13,6 %) ir nežinomo tipo (2.3 %) variantai. *Missense* variantai, įskaitant glicino pakaitas (n=25) ir kitų amino r. pakaitas (n=8), buvo dažniausiai pasitaikantis molekulinis *COLA4A3-A5* genų pokytis, kuris pasireiškė 76 % asmenų, sergančių AS. Tarp *COL4A3-A5* genų skirtingų *missense* variantų, dažniausiai buvo pakeista glicino amino rūgštis, kurią pakeitė tokios amino rūgštys kaip argininas (44 %, Arg), valinas (12 %, Val), glutamo rūgštis (12 %, Glu), serinas (16 %, Ser), asparto rūgštis (12 %, Asp) ir cisteinas (4 % Cis). Gliciną pakeitusių amino rūgščių pasiskirstymo dažnis kohortos asmenims su glicino pakaita (n=77) yra nurodytas 7 lentelėje.

7 lentelė. Gliciną pakeitusių amino rūgščių pasiskirstymo dažnis tarp asmenų

AR, pakeitusi gliciną	Bendras asm. skč.	*COL4A3*	*COL4A4*	*COL4A5*
Argininas, %	27 (35,1)	11 (68,8)	6 (37,5)	10 (22,2)
Valinas, %	5 (6,5)	2 (12,5)	0 (0,0)	3 (6,7)
Glutamo rūgštis, %	5 (6,5)	1 (6,3)	4 (25,0)	0 (0,0)
Serinas, %	10 (13,0)	2 (12,5)	3 (18,8)	5 (11,1)
Asparto rūgštis, %	27 (35,1)	0 (0,0)	0 (0,0)	27 (60,0)
Cisteinas, %	3 (3,9)	0 (0,0)	3 (18,8)	0 (0,0)
Bendras asm. skč., %*	77 (100,0)	16 (20,8)	16 (20,8)	45 (58,4)

*Asmenų skaičius vertintas atsižvelgiant į konkretaus geno pokyčio buvimą. Todėl asmenys, sergantys ARAS arba digeniniu AS, gali būti skaičiuojami du kartus

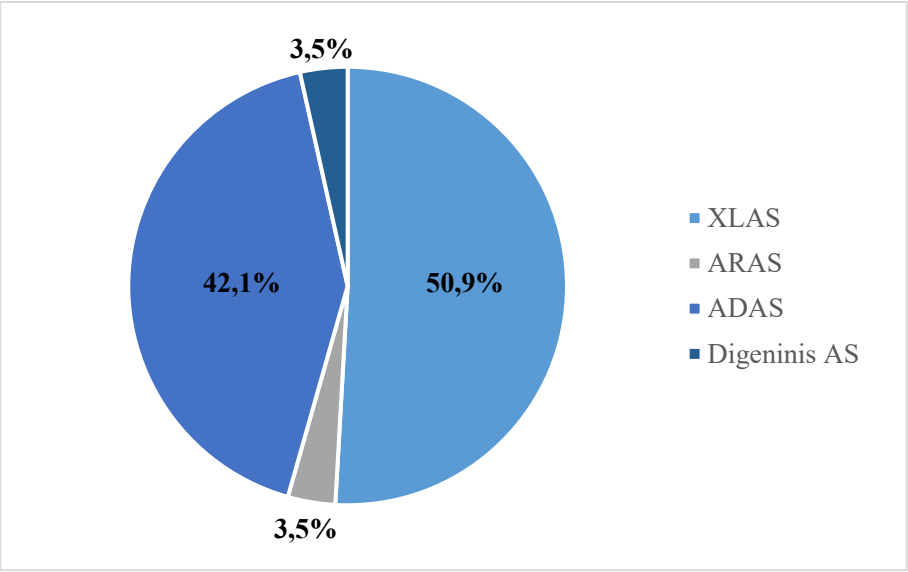
Tyrimo metu rastų 44 skirtingų *COL4A3-A5* genetinių variantų patogeniškumo pasiskirstymas parodytas 8 lentelėje.

8 lentelė. Skirtingų genetinių variantų (n=44) patogeniškumo pasiskirstymas

Patogeniškumas*	Bendras GV skč.	*COL4A3*	*COL4A4*	*COL4A5*
P (%)	6 (13,6)	1 (8,3)	1 (6,3)	4 (25,0)
LP (%)	27 (61,4)	8 (66,7)	9 (56,3)	10 (62,5)
VUS (%)	11 (25,0)	3 (25,0)	6 (37,5)	2 (12,5)
Bendras GV skč. (%)	44 (100,0)	12 (27,3)	16 (36,4)	16 (36,4)

P – patogeninis; LP – tikėtinas patogeniškumas; VUS – nežinomo patogeniškumo variantas; GV – genetinis variantas; skč – skaičius; * nustatytas genetinės laboratorijos

Tiriamojoje kohortoje identifikuoti keturi skirtingi AS tipai – XLAS, ARAS, ADAS ir digeninis AS (5 pav.).



5 pav. Įvairių tipų Alporto sindromo pasiskirstymas tiriamojoje populiacijoje

Bendradarbiaujant su Australijos kolegomis, atradome reikšmingą ryšį tarp *COL4A5* geno nestabilumo, atsiradusio dėl gliciną pakeitusių aminorūgščių, ir AS progresavimo. Patogeninio pokyčio vieta egzone didelės įtakos inkstų funkcijai neturėjo. Patogeniniai *COL4A5* variantai, susiję su glicino pakeitimu destabilizuojančiomis amino rūgštimis (Arg, Val, Glu, Asp, Trp), koreliavo su ankstyvesniu galutinės stadijos LIL išsivystymu ir klausos praradimu. Glicino pakaita netoli ne-kolageninio regiono *COL4A5* gene buvo susijusi su vėlesniu inkstų funkcijos blogėjimu, o *COL4A3* ir *COL4A4* genuose su mažesne hematurijos rizika [88]. Į LVOD duomenų bazę buvo įtraukti 9 skirtingi *COL4A3-A5* molekuliniai pokyčiai (n=45) iš Lietuvos kohortos.

3.3.1.Nauji molekuliniai variantai *COL4A3-A5* genuose

Nauji genetiniai variantai buvo nustatyti 60 asmenų iš 28 skirtingų šeimų. Bendrosios naujų variantų charakteristikos pateiktos 9, 10 ir 11 lentelėje. Naujų variantų dažnis iš visų rastų molekulinių pokyčių (n=44) *COL4A5, COL4A3* ir *COL4A4* genuose sudarė 62,5 % (10/16), 75,0 % (9/12) ir 56,3 % (9/16), atitinkamai, ir tarp genų reikšmingai nesiskyrė. Interpretuojant mūsų tyrimo metu identifikuotus naujus variantus, buvo remtasi skirtingų genetinių duomenų bazių (ClinVar, InterVar, Franklin, Centogene) apibrėžtais ACMG

kriterijais, patogeninių variantų analizės įrankių (PPT, SIFT, MT, Align-GVGD) rezultatais, klinikine atvejų eiga, V st. LIL atvejų šeimoje analize, inkstų biopsijos rezultatais, atsižvelgiant į lytį ir amžių. Klinikiniai požymiai, susiję su perklasifikavimu iš VUS į tikėtina patogeninius ar patogeninius variantus, buvo nefrozinio lygio proteinurija, proteinurija, kai eGFG <60 ml/min/1,73 m2, III-V st. LIL, inkstų funkcijos pakitimai kartu su klausos ir (arba) akių pažeidimais, LIL šeimos nariams, atsižvelgiant į jų amžių. Asmenys, sergantys gretutinėmis ligomis, kurios galėjo būti susijusios su inkstų funkcijos pablogėjimu, buvo neįtraukti į vertinimą.

9 lentelė. Naujų *COL4A5* variantų ACMG klasifikacija

COL4A5 geno GV	GV tipas (Nr.)	V/M skaičius	Skirtingų šeimų skč.	LAB ACMG	Mūsų interpretacija
c.3508G>C (p.Gly1170Arg)	Gly pakaita (3)	1V/2M	1	LP	P
3106+1G>A	Splaisingo GV(4)	1V/2M	1	LP	LP
c.1417_1418del (p.Val473Glufs*3)	Rėmelio poslinkio GV(6)	4M*	1	LP	P
c.2623G>A (p.Gly875Arg)	Gly pakaita (13)	2V/4M	2	LP	P
c.347delC (p.Pro116Glnfs*39)	Rėmelio poslinkio GV (14)	1V/1M	1	LP	LP
c.883G>A (p.Gly295Ser)	Gly pakaita (18)	1V	1	VUS	LP
c.2777G>T (p.Gly926Val)	Gly pakaita (21)	2V/1M	1	VUS	P
c.1374delinsTT (p.Pro459Serfs*6)	Rėmelio poslinkio GV (26)	1V/2M	1	LP	VUS
c.3554-2A>G	Splaisingo GV (33)	2M	1	LP	LP
c.466G>C (p.Gly156Arg)	Gly pakaita (34)	1M	1	LP	P

10 lentelė. Naujų *COL4A4* variantų ACMG klasifikacija

COL4A4 geno GV	GV tipas (Nr.)	V/M skaičius	Skirtingų šeimų skč.	LAB ACMG	Mūsų interpretacija
c.657+2dup	Nežinomas (5)	1M	1	LP	LBn
c.4151C>T (p.Ala1384Val)	Ne Gly pakaita (7)	2M*	2	VUS	LP*
c.594+1G>A	Splaisingo (9)	3V */3M	2	LP	LP*
c.1987G>C (p.Gly663Arg)	Gly pakaita (16)	2V	1	LP	LBn
c.4910G>A (p.Arg1637Gln)	Ne Gly pakaita (27)`	1M*	1	VUS	LP*
c.2756A>G (p.Glu919Gly)	Ne Gly pakaita (32)	1V/1M	1	VUS	LBn
c.4315G>A (p.Gly1439Ser)	Gly pakaita (36)	2V/1M	1	LP	LBn
c.3044G>A (Gly1015Glu)	Gly pakaita (40)	3V	1	LP	LBn
c.2347G>A (p.Gly783Arg)	Gly pakaita (41)	2V/1M	1	LP	LP

11 lentelė. Naujų *COL4A3* variantų ACMG klasifikacija

COL4A3 geno GV	GV tipas (Nr.)	V/M skaičius	Skirtingų šeimų skč.	LAB ACMG	Mūsų interpretacija
c.520G>A (p.Gly174Arg)	Gly pakaita (19)	1V/1M	1	LP	LP
c.4702C>T (p.Pro1568Ser)	Ne Gly pakaita (23)	2M*	1	VUS	P*
c.3247G>C (p.Gly1083Arg)	Gly pakaita (24)	2M*	1	VUS	P*
c.2711G>T (p.Gly904Val)	Gly pakaita (31)	1V	1	LP	LBn
c.4717G>A (p.Gly1573Ser)	Gly pakaita (35)	1V/1M	1	LP	LBn
c.416G>A (p.Gly139Glu)	Gly pakaita (37)	1M	1	LP	LP
c.1021C>T (p.Arg341Cys)	Ne Gly pakaita (38)	1V*	1	VUS	LP*
c.593G>T (p.Gly198Val)	Gly pakaita (39)	1V	1	LP	LP
c.2188G>C (p.Gly730Arg)	Gly pakaita (43)	1M	1	LP	LBn

* Įskaitant ARAS ir digeniniu AS sergančius asmenis. LAB – genetinė laboratorija, kurioje atliktas tyrimas; V – vyras; M – moteris; P – patogeninis; LP – tikėtina patogeninis; VUS – nežinomo patogeniškumo variantas; LBn – tikėtina gerybinis; Bn – gerybinis; GV – genetinis variantas; skč. – skaičius; Gly – glicinas; Nr. – numeris; pr. – pradžia

3.3.2. Bendri su pasaulio populiacija *COL4A3-A5* variantai

Siekdami padidinti statistinės ir aprašomosios analizės pagrįstumą, peržiūrėjome visus tyrime nustatytus variantus, norėdami nustatyti variantų naujumą (duomenų bazėse neaprašyti variantai) ir paplitimo dažnį, naudodami genetinėmis populiacijomis pagrįstas duomenų bazes, tokias kaip ClinVar, InterVar, Varsome, Franklin ir LVOD. Nustatyta, kad septyni mūsų lietuviškoje kohortoje nustatyti genetiniai variantai yra paplitę bendrojoje populiacijoje visame pasaulyje. Mūsų tyrimo metu nustatyti genetiniai variantai ir klinikinis jų patogeniškumo pasireiškimas pateikti 12 lentelėje.

12 lentelė. Pasaulinei populiacijai būdingų genetinių variantų pasiskirstymas Lietuvos tiriamojoje kohortoje

Genas	GV Nr.	GV	Tiriamojoje kohortoje paveldėtas AS tipas	Asmenų (šeimų) skaičius, V/M	ACMG LAB	Mūsų ACMG
COL4A3	44	p.Leu1474Pro (c.4421T>C)	ADAS	2V (2)	P	P
COL4A3	29	p.Gly1167Arg (c.3499G>A)	ADAS	2M (2)	P	LP
COL4A4	20	c.-101-4A>G	ADAS/ digeninis AS	1V*, 1M (2)	VUS	LBn
COL4A4	11	p.Gly999Glu (c.2996G>A)	ADAS/ digeninis AS	1M* (1)	VUS	LBn
COL4A4	10	p.Gly527Cys (c.1579G>T)	ADAS/ ARAS	3V*(2)	P	LP
COL4A5	12	p.Gly624Asp (c.1871G>A)	XLAS/ digeninis AS	10M*/12V (14)	P	P
COL4A5	17	p.Gly1229Ser (c.3685G>A)	XLAS	2V/2M (1)	P	P

GV – genetinis variantas; V – vyras; M – moteris; P – patogeninis; LP – tikėtina patogeninis; VUS – nežinomo patogeniškumo variantas; LBn – tikėtinas gerybinis; Bn – gerybinis

3.4. Alporto sindromo pirminės diagnostikos keliai

Septyniasdešimt procentų probandų buvo nukreipti genetiniam ištyrimui, radus specifinius AS būdingus pokyčius inkstų biopsijoje. Tuo tarpu šeiminė analizė ir genealoginio medžio sudarymas leido identifikuoti 54 asmenis. Trys procentai asmenų, įtrauktų į galutinę AS kohortą, buvo diagnozuoti atsitiktinai, juos tiriant dėl autosominės dominantinės policistinės inkstų ligos (ADPIL). Probandai tiriamojoje kohortoje sudarė 46,6 % XLAS, 75% ARAS, 54,2% ADAS ir 100 % digeninio AS atitinkamų grupių atvejų. Dažniausiai

Alporto sindromas XLAS (51,7 %) ir ADAS (45,8 %) grupėse buvo įtariamas atliekant šeimos patikrą. Moterims su XLAS statistiškai reikšmingai dažniau diagnozuotas AS atliekant šeimos patikrą, palyginus su vyrais (p=0,04; 95 % CI [1,6-14,9]), tuo tarpu vyrams AS dažniau įtartas dėl klinikinių simptomų ir specifinių radinių inkstų biopsijoje (p=0,04; 95% CI [0,01-1,04]). ARAS grupėje – trims asmenims (75 %) AS įtartas dėl specifinių klinikinių požymių arba atlikus inkstų biopsiją; vienos pacientės dvynei seseriai AS buvo diagnozuotas remiantis šeimine anamneze. Dauguma ADAS sergančių asmenų (46 %) buvo identifikuoti vykdant genealoginę analizę; dviems vyrams – atsitiktinai genetiškai tiriant dėl ADPIL. Trims asmenims, sergantiems digeniniu AS, buvo atlikta inkstų biopsija, leidusi įtarti AS; vienai moteriai atliktas molekulinis tyrimas dėl AS, atsiradus AS specifinių klinikinių požymių.

3.5. XLAS grupė: genotipo – fenotipo sąsajos

Iš viso tyrimo metu buvo nustatyti 58 asmenys, sergantys XLAS, o tai sudarė 51 % visų tyrimo dalyvių. XLAS grupėje AS diagnozuotas 27 (29,3±17,0 metų amžiaus) vyrams. Bendroji asmenų, sergančių XLAS charakteristika pateikta 13 lentelėje.

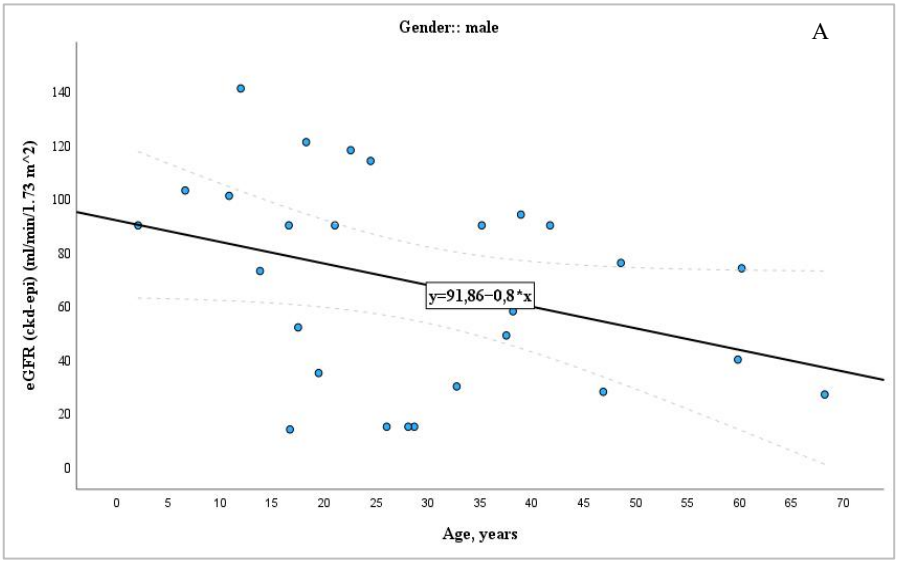
13 lentelė. Asmenų sergančių XLAS charakteristika pagal lytį (n=58)

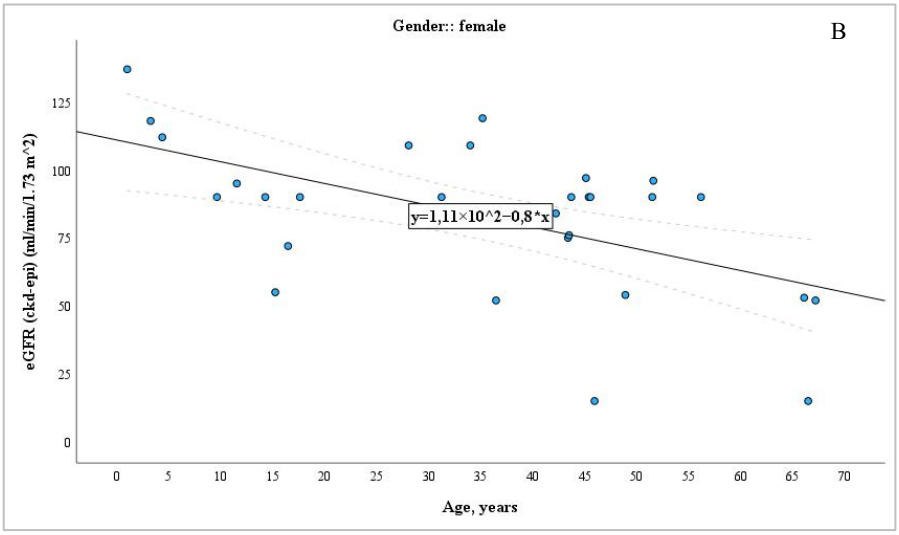
Demografiniai ir klinikiniai požymiai	Iš viso	Vyrai	Moterys	P vertė
Asmenų skaičius, %	58 (100,0)	27 (46,6)	31 (53,4)	>0,05
Vidutinis GD amžius	32,4±18,1	29,3±17,0	35,1±19,0	0,229
Probandų skč., %	27 (46,6)	18 (66,7)	9 (29,0)	**0,04**
Klinikinis įtarimas: Pož.+IB, %	19 (32,8)	11 (40,7)	8 (25,8)	0,227
Klinikinis įtarimas: tik Pož. %	7 (12,1)	6 (22,2)	2 (3,2)	**0,04**
Klinikinis įtarimas: Šeiminė an. %	31 (53,4)	9 (33,3)	22 (71,0)	**0,004**
Hematurija, %	58 (100,0)	27 (100,0)	31 (100,0)	>0,05
Hematurijos nustatymo amžius	17,4±16,4	14,0±15,2	20,4±17,0	0,139
Proteinurija, %	48 (82,8)	23 (85,2)	25 (80,6)	0,648
Proteinurijos nustatymo amžius	23,2±18,1	20,1±19,8	25,2±16,6	0,207
Nefrozinio lygio proteinurija, %	7 (12,0)	5 (18,5)	2 (6,5)	0,233
LIL I st., %	30 (51,7)	12 (44,4)	18 (58,1)	$x^2 = 4,7$ p=0,32 df .=4
LIL II st., %	9 (15,5)	3 (11,1)	6 (19,4)	
LIL III st., %	11 (19,0)	6 (22,2)	5 (16,1)	
LIL IV st., %	2 (3,4)	2 (7,4)	0 (0,0)	
LIL V st., %	6 (10,3)	4 (14,8)	2 (6,5)	
Vidutinis V st. LIL pr. amžius	32,8±16,0	23,5±5,4	51,5±12,0	0,06
AH, %	31 (53,4)	17 (63,0)	14 (45,2)	0,175
RAAS slopinimas, %	39 (67,2)	20 (74,1)	19 (61,3)	0,301

Demografiniai ir klinikiniai požymiai	Iš viso	Vyrai	Moterys	P vertė
Inksto biopsija, %	22 (37,9)	14 (51,9)	8 (25,8)	**0,041**
Inkstų biopsijos atlikimo amžius	26,8±15,1	26,4±14,2	27,6±17,5	0,785
Klausos sutrikimai, %	18 (31,0)	15 (60,0)	3 (11,5)	**<0,001**
Akių pažeidimai, %	7 (12,1)	6 (25,0)	1 (3,7)	**0,042**

GD – genetinė diagnozė; anam. – anamnezė; AH – arterinė hipertenzija; LIL – lėtinė inkstų liga; st. – stadija; pr. – pradžia; RAAS – renino-angiotenzino-aldosterono sistema; an. – anamnezė; skč. – skaičius; pož. – požymis; IB – inkstų biopsija

Hematurija buvo nustatyta visiems asmenims, sergantiems XLAS, proteinurija rasta daugiau nei 80 % atvejų. Galutinės stadijos LIL išsivystė 4 XLAS sergantiems vyrams ir 2 moterims, kurių vidutinis amžius buvo 23,5±5,4 (19–31) ir 51,5±12,0 metų, atitinkamai. Reikšmingo skirtumo tarp lyčių (p=0,064) ir ryšio tarp amžiaus, inkstų funkcijos ir vyriškos lyties (6 pav. A, n=27, p=0,063; r=-0,362) nenustatyta. Rastas atvirkštinis ryšys tarp inkstų funkcijos ir moterų amžiaus (6 pav. B, n=31, p=0,001; r=-0,553).





6 pav. Vyrų (A, n=27, p=0,063; r=-0,362) ir moterų (B, n=31, p=0,001; r=-0,553) amžiaus ir eGFG pasiskirstymo kreivė

Inkstų funkcija buvo reikšmingai blogesnė XLAS sergantiems asmenims, kuriems buvo rasta proteinurija, lyginant su tais, kuriems proteinurija nenustatyta (p=0,043). Rasta atvirkštinė koreliacija tarp vyresnio amžiaus ir mažesnio eGFG moterims, kurioms buvo proteinurija (n=25; r=-0,478; p=0,016, 16 pav.), skirtingai negu grupėje be proteinurijos (n=6, r=-0,429, p=0,397). Vyrų grupėje ryšio tarp vyrų amžiaus, eGFG ir proteinurijos nenustatėme.

Inkstų biopsija buvo atlikta 19 asmenų, sergančių XLAS. Vyrų amžiaus vidurkis inkstų biopsijos metu buvo 26,4±14,2 metų (mediana 21; [6–49]), o moterų – 27,6±17,5 metų (mediana 22, [9–55]). Vidutinis vyrų eGFG buvo 68,3±37,7 ml/min/1,73 m², moterų– 82,9±27,4 ml/min/1,73 m². Dažniausiai pastebėti EM požymiai buvo plonos GBM ir susiliejusios mažosios podocitų kojytės. Daugiau nei pusei asmenų, sergančių XLAS, pasireiškė GBM supleišėjimas arba sustorėjimas, kuris buvo susijęs su didesnėmis abiejų lyčių eGFG reikšmėmis, lyginant su ŽSGS grupe.

Iš 27 vyrų, sergančių XLAS, 55,6 % nustatyti klausos sutrikimai, tuo tarpu moterims abipusis neurosensorinis prikurtimas rastas kur kas rečiau (9,7 %). Vidutinis vyrų ir moterų amžius klausos sutrikimų nustatymo metu atitinkamai buvo 34,7±18,4 (mediana 32,7; [7-61]) ir 53,3±15,6 metai (mediana 56; [36,5-67,2]). Klausos sutrikimai vyrams pasireiškė anksčiau – 40,1 metų (95 % CI [31,6-48,6]), lyginant su moterimis 61,7 metų (95 % CI [53,8-69,4]) (p<0,001). Trims vyrams su abipusiu prikurtimu V st. LIL išsivystė 27,7 [26–29] metų amžiaus, tuo tarpu nei viena moteris nesirgo V st.

LIL, nors dviem iš jų buvo diagnozuota III st. LIL. Iš 27 vyrų, sergančių XLAS, akių pažeidimai nustatyti 25 % atvejų 29,1±16,6 metų amžiuje (mediana 27,0; [10,8–60,2]), tik vienai 56 metų amžiaus moteriai buvo rasti akių pažeidimai. Vyrams akių pažeidimai nustatyti anksčiau nei moterimis (p=0,016). Vidutinis amžius, kai nustatyti akių pažeidimai buvo 52,4 metai (95% CI [42,8-62,1]) vyrams ir 63,5 metai (95 % CI [57,4-69,4]) moterims. Koreliacijos tarp akių pažeidimo ir inkstų funkcijos nenustatėme abiejų lyčių grupėse.

Dažniausias *COL4A5* genetinių pokyčių tipas XLAS sergantiems vyrams ir moterims, buvo Gly pakaitos variantai ir pasireiškė atitinkamai 85,2 % ir 67,7 % visų XLAS atvejų. Abiejose grupėse kitų aminorūgščių pakaitų rasta nebuvo. Keturiems vyrams, sergantiems V st. LIL glicinas buvo pakeistas argininu ir valinu, tuo tarpu dviem moterims – argininu. Vyrų ir moterų genotipo ir fenotipo koreliacijos pateiktos 14-oje ir 15-oje lentelėse.

14 lentelė. XLAS sergančių vyrų genotipo ir fenotipo koreliacijos (n=27)

Požymis	Bendras vyrų skč.	Missense Gly GV	Rėmelio poslinkio GV	Splaisingo GV
Hematurija, %	27 (100,0)	23 (100,0)	2 (100,0)	2 (100,0)
Pro, %	23 (85,2)	19 (82,6)	2 (100,0)	2 (100,0)
Nefrozinė pro, %	5 (18,5)	5 (21,7)	0 (0,0)	0 (0,0)
LIL, I st. %	12 (44,4)	9 (39,1)	2 (100,0)	1 (50,0)
LIL, II st. %	3 (11,1)	2 (8,7)	0 (0,0)	1 (50,0)
LIL, III st. %	6 (22,2)	6 (26,1)	0 (0,0)	0 (0,0)
LIL, IV st. %	2 (7,4)	2 (8,7)	0 (0,0)	0 (0,0)
LIL, V st. %	4 (14,8)	**4 (17,4)**	0 (0,0)	0 (0,0)
Inkstų biopsija, %	14 (51,9)	12 (52,2)	1 (50,0)	1 (50,0)
PBM, %	10 (37,0)	8 (34,8)	1 (50,0)	1 (50,0)
Sustorėjusios ir supleišėjusios GBM, %	8 (29,6)	6 (26,1)	1 (50,0)	1 (50,0)
ŽSGS, %	5 (18,5)	5 (21,7)	0 (0,0)	0 (0,0)
Klausos sutrikimai, %	15 (55,6)	11 (47,8)	2 (100,0)	2 (100,0)
Akių pažeidimai, %	6 (22,2)	4 (17,4)	1 (50,0)	1 (50,0)
Iš viso skč., %	27 (100,0)	23 (85,2)	2 (7,4)	2 (7,4)

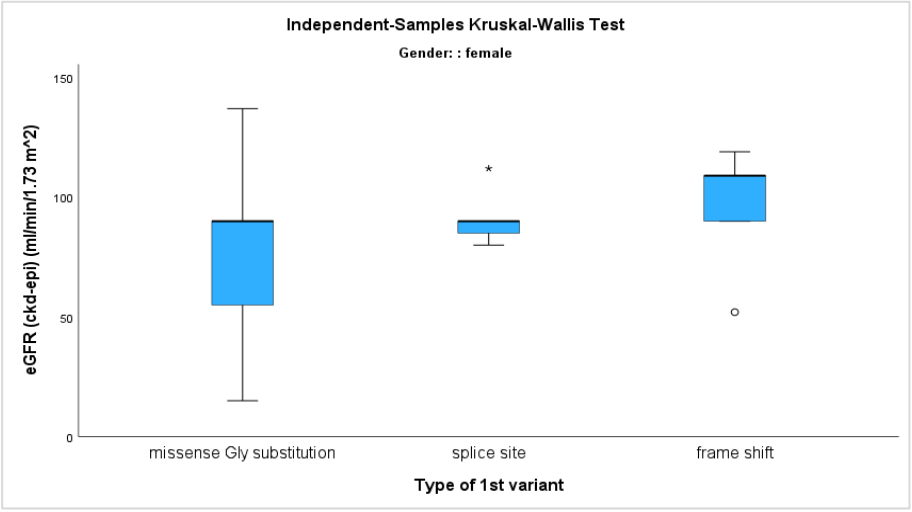
15 lentelė. XLAS sergančių moterų genotipo ir fenotipo koreliacijos (n=31)

Funkcijos	Bendras moterų skč.	Missense Gly GV	Rėmelio poslinkio GV	Splaisingo GV
Hematurija, %	31 (100,0)	21 (100,0)	5 (100,0)	5 (100,0)
Pro, %	25 (80,6)	18 (85,7)	3 (60,0)	4 (80,0)
Nefrozinė pro, %	2 (6,5)	1 (4,8)	0 (0,0)	1 (20,0)
LIL, I st. %	18 (58,1)	11 (52,4)	4 (80,0)	3 (60,0)

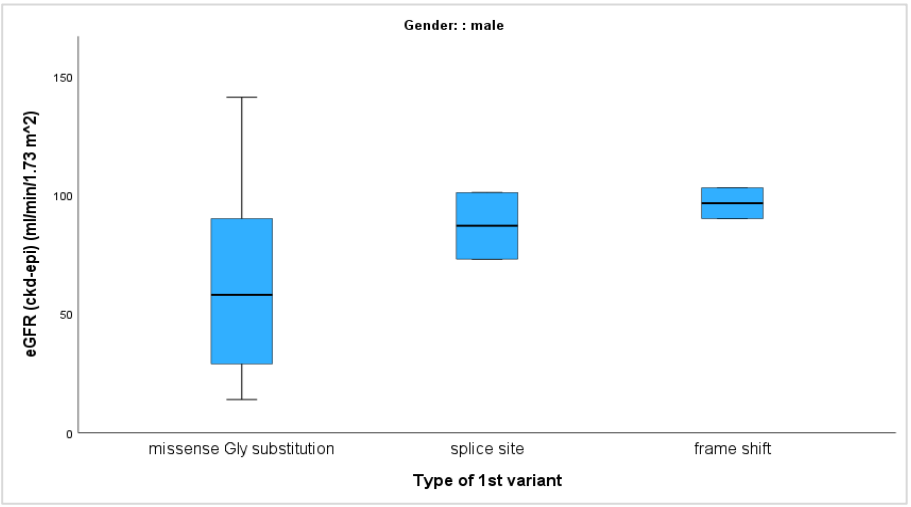
Funkcijos	Bendras moterų skč.	Missense Gly GV	Rėmelio poslinkio GV	Splaisingo GV
LIL, II st. %	6 (19,4)	4 (19,0)	0 (0,0)	2 (40,0)
LIL, III st. %	5 (16,1)	4 (19,0)	1	0 (0,0)
LIL, IV st. %	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,0)
LIL, V st. %	2 (6,5)	**2 (9,5)**	0 (0,0)	0 (0,0)
Inkstų biopsija, %	8 (25,8)	6 (28,6)	0 (0,0)	2 (40,0)
PBM, %	7 (22,6)	5 (23,8)	0 (0,0)	2 (40,0)
Sustorėjusios ir supleišėjusios GBM, %	4 (12,9)	2 (9,5)	0 (0,0)	2 (40,0)
ŽSGS, %	4 (12,9)	3 (9,7)	0 (0,0)	1 (20,0)
Klausos sutrikimai, %	3 (9,7)	2 (9,5)	1 (20,0)	0 (0,0)
Akių pažeidimai, %	1 (3,2)	1 (4,8)	0 (0,0)	0 (0,0)
Iš viso skč., %	31 (100,0)	21 (67,7)	5 (16,1)	5 (16,1)

Pro – proteinurija; LIL – lėtinė inkstų liga; GV – genetinis variantas; PBM – plona bazinė membrana inksto biopsijoje; GBM – glomerulų bazinė membrana; ŽSGS – židininė segmentinė glomerulosklerozė ; skč. – skaičius; st. - stadija

Ryšio tarp mažesnio eGFG ir skirtingo patogeninio varianto *COL4A3-A5* moterims (p=0,03; 7 pav.) ir vyrams (p=0,4; 8 pav.) nenustatyta.



7 pav. Moterų eGFG reikšmių pasiskirstymas priklausomai nuo skirtingų *COL4A5* genų tipų



8 pav. Vyrų eGFG reikšmių pasiskirstymas priklausomai nuo skirtingų *COL4A5* genų tipų

Klausos sutrikimai ir akių pažeidimai dažniau pasireiškė vyrams su Gly pakaitos genetiniu variantu, lyginant su kito tipo pokyčiu ($p<0,001$ ir $p=0,006$, atitinkamai). Reikšmingo skirtumo moterims tarp skirtingų genetinių pokyčių, susijusių su klausos anomalijomis ir akių pažeidimais, nenustatyta.

3.6. ARAS grupė: genotipo – fenotipo sąsajos

Iš viso tyrimo metu buvo identifikuoti keturi (3,5 %) asmenys, sergantys ARAS, tarp jų vienas vyras ir trys moterys. Trims asmenims nustatyti *COL4A3* arba *COL4A4* kombinuoti heterozigotiniai variantai (16 lentelė).

16 lentelė. Genetinė asmenų ARAS sergančių charakteristika (n=4)

ID	Šeimos; Asm. Nr., lytis	Amžius	Genas	GV Nr.	GV nukleotidų (baltymo) pokytis	ARAS tipas
A4/49716	7;62-I-V	7	*COL4A4*	9	c.594+1G>A[b]	Kombinuotas het.
			COL4A4	10	c.1579G>T (p.Gly527Cys)[a]	
A3/3232145	21-I-M	34	*COL4A3*	23	c.4702C>T (p.Pro1568Ser)[b]	Kombinuotas het.
			COL4A3	24	c.3247G>C (p.Gly1083Arg)[b]	
A3/3232146	21-II-M	34	*COL4A3*	23	c.4702C>T (p.Pro1568Ser)[b]	Kombinuotas het.
			COL4A3	24	c.3247G>C (p.Gly1083Arg)[b]	
A4/4/283867	38-I-M	32	*COL4A4*	28	c.1820C>T (p.Ala607Val)[a]	Homo

Nr. – numeris; het – heterozigotinis; homo – homozigotinis; M – moteris; V – vyras; GV – genetinis variantas; [b] – naujas GV; [a] – žinomas GV; ID – paciento identifikacijos kodas; amž. – amžius genetinės diagnozės metu

Vienai moteriai nustatytas ARAS, kurį sukėlė homozigotinis *COL4A4* p.Ala607Val variantas, susijęs su V st. LIL išsivystymu 32 metų amžiuje. Kita moteris V st. LIL pasiekė būdama 33 metų amžiaus, jai rasti du heterozigotiniai *COL4A3* p.Pro1568Ser ir p.Gly1083Arg variantai (17 lentelė). ARAS sergančių asmenų, grupėje klausos praradimas nustatytas visiems keturiems asmenims, jauniausiam buvo 7 metai. Asmenims, sirgusiems V st. LIL, klausos sutrikimai diagnozuoti vaikystėje.

17 lentelė. ARAS sergančių pacientų genotipo ir fenotipo koreliacijos (n=4)

ID	Amžius	Hematurija	Pro (kiekis)[0]	LIL stadija[0]	Klausos paž.	Akių paž.
A4/49716	7	+	+ (58)[6]	I	+	+
A3/3232145*	34	+	+ (350)[23]	V[33]	+	-
A3/3232146*	34	+	+ (300)[30]	IIIb	+	-
A4/4/283867	32	+	+ (423)[22]	V[32]	+	+

ID – paciento identifikacijos kodas; Pro – proteinurija; paž. – pažeidimas. *Dvynės seserys. [0] – parodo požymio pradžios amžių. Proteinurija vertinta pagal vienkartinio šlapimo baltymo-kreatinino santykį (mg/mmol): >300 mg/mmol vertinta kaip nefrozinio lygio proteinurija. Amžius vertintas genetinės diagnozės metu

Inkstų biopsija buvo atlikta 3 asmenims, sergantiems ARAS. Visiems asmenims rastos suplonėjusios, kai kur sustorėjusios ir supleišėjusios GBM bei mažųjų podocitų kojyčių susiliejimas. Tuo tarpu ŽSGS nustatyta tik 2 asmenims.

3.7. ADAS grupė: genotipo – fenotipo sąsajos

ADAS buvo diagnozuotas 48 (82,8 %) asmenims, turintiems vieną heterozigotinį *COL4A3* arba *COL4A4* variantą, iš jų – 24 vyrams. Vidutinis vyrų ir moterų diagnozės nustatymo amžius buvo atitinkamai 24±18,7 ir 37,7±20,3 metai. Bendras asmenų, turinčių vieną heterozigotinį *COL4A3* arba *COL4A4* variantą, skaičius nurodytas 18 lentelėje. Tyrimo metu reikšmingų AS pasireiškimo skirtumų tarp lyties (p=0,13) ir skirtingų *COL4A3* arba *COL4A4* genų nenustatyta (p=0,56), todėl tolimesnėje analizėje ADAS pasireiškimas buvo nagrinėjamas bendrai, neišskiriant pažeistų genų ar lyties.

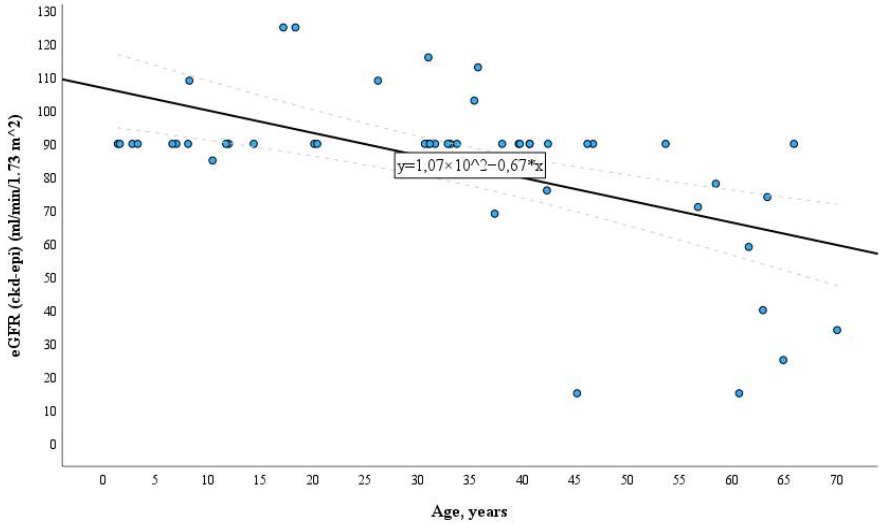
18 lentelė. Bendroji asmenų, sergančių ADAS, charakteristika, priklausomai nuo pažeisto geno (n=48)

Demografiniai ir klinikiniai požymiai	Bendras asmenų skč.	*COL4A*3het	*COL4A*4het	p vertė
Bendras asmenų skaičius, %	48 (100,0)	16 (33,3)	32 (66,7)	p>0,05
Vyrai, %,	24 (50,0)	8 (50,0)	16 (50,0)	1,000

Demografiniai ir klinikiniai požymiai	Bendras asmenų skč.	*COL4A3*het	*COL4A4*het	p vertė
Moterys, %,	24 (50,0)	8 (50,0)	16 (50,0)	1,000
Vidutinis GD amžius	33,2±19,8	33,3±18,9	33,2±21,0	0,984
Probandų skaičius, %	26 (54,2)	10 (62,5)	16 (50,0)	0,413
Klinikinis įt.: Pož.+IB, %	18 (37,5)	7 (43,8)	11 (34,4)	0,527
Klinikinis įt.: tik Pož. %	6 (12,5)	2 (12,5)	4 (12,5)	1000
Klinikinis įt.: Šeiminė an. %	22 (45,8)	6 (37,5)	16 (50,0)	0,413
Hematurija, %	48 (100,0)	16 (100,0)	32 (100,0)	p>0,05
Hematurijos dgn. amžius	27,0±18,3	23,4±16,3	28,8±19,2	0,344
Proteinurija, %	25 (52,1)	10 (62,5)	15 (46,9)	0,307
Proteinurijos dgn. amžius	23,0±17,3	25,1±14,2	37,9±17,8	0,70
Nefrozinio lygio pro, %	1 (2,1)	0 (0,0)	1 (3.1)	0,237
LIL I st., %	35 (72,9)	11 (68,8)	24 (75,0)	$x^2 = 4,093$ p=0,444 df .=4
LIL II st., %	7 (14,6)	4 (25,0)	3 (9,4)	
LIL III st., %	3 (6,3)	0 (0,0)	3 (9,4)	
LIL IV st., %	1 (2,1)	0 (0,0)	1 (3,1)	
LIL V st. %	2 (4,2)	1 (6,3)	1 (3.1)	
Vidutinis LIL V st. amžius	49,5±7,8	44,0±0	55,0±0	1,000
AH, %	18 (37,5)	6 (37,5)	12 (37,5)	1,000
RAAS slopinimas, %	19 (40,4)	8 (50,0)	11 (35,5)	0,337
Inkstų biopsija, %	21 (43,8)	9 (56,3)	12 (37,5)	0,217
IB atlikimo amžius	43,0±15,1	38,0±11,6	46,7±16,9	0,117
Klausos sutrikimai, %	8 (16,7)	4 (9,1)	4 (13,8)	0,414
Akių pažeidimai, %	8 (16,7)	1 (7,7)	7 (25,9)	0,236

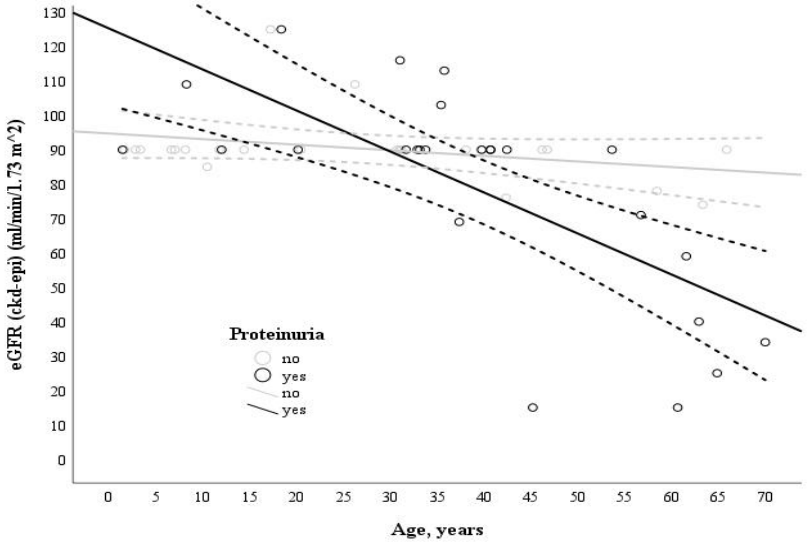
GD – genetinė diagnozė; anam. – anamnezė; AH – arterinė hipertenzija; LIL – lėtinė inkstų liga; st. – stadija; pr. – pradžia; RAAS – renino-angiotenzino-aldosterono sistema; an. – anamnezė; skč. – skaičius; pož. – požymis; IB – inkstų biopsija; dgn. – diagnozė; įt - įtariama

Hematurija nustatyta visiems 48 asmenims, sergantiems ADAS, o proteinurija daugiau nei pusei asmenų, kurių vienam rasta nefrozinio lygio proteinurija ir II st. LIL. Iš 26 asmenų, kuriems buvo nustatyta proteinurija, 62 % asmenų inkstų funkcija buvo normali. Du asmenys, kuriems pasireiškė vidutinio lygio proteinurija (B/K 15-49 mg/mmol), pasiekė V st. LIL būdami 55 ir 44 metų amžiaus. Trims asmenims, kuriems buvo nustatyta izoliuota hematurija, diagnozuota 2 st. LIL. Tyrime nustatytas reikšmingas ryšys tarp sumažėjusio eGFG ir vyresnio amžiaus (n=48, r=-0,542, p<0,001, 9 pav.).



9 pav. Asmenų, sergančių ADAS, (n=48) amžiaus ir eGFG reikšmių vieno matavimo metu koreliacijos grafikas (r=0,542, p<0,001)

Statistinė analizė atskleidė reikšmingą koreliaciją tarp vyresnio amžiaus ir sumažėjusio eGFG asmenims, kuriems rasta proteinurija (n=25; r=-0,737; p=0,013), tačiau reikšmingos koreliacijos tarp eGFG ir amžiaus asmenims be proteinurijos nenustatyta (n=23; r=-0,321, p=0,135, 10 pav.).



10 pav. Amžiaus ir eGFG ryšys vieno matavimo metu su proteinurija (n=25; r=-0,737; p=0,013) ir be proteinurijos (n=23; r=-0,321, p= 0,135) ADAS asmenų grupėje

ADAS grupėje dvidešimt vienam asmeniui (43,8 %) buvo atlikta inkstų biopsija. Daugiau nei 70 % asmenų inkstų biopsijos metu buvo rasta proteinurija. Vidutinis ADAS sergančių ADAS asmenų amžius inkstų biopsijos atlikimo metu buvo 43,0±15,1 (mediana, 36 [IQR, 17–69]) metų, o vidutinis eGFG buvo 85,3±30,9 ml/min/1,73 m^2. Proteinurijos dydis inkstų biopsijos metu svyravo nuo 0 iki 3,0 g/L (vidurkis, 0,6±0,9). Elektroninė mikroskopija buvo atlikta 90,5 % asmenų, dažniausiai rasti pokyčiai buvo suplonėjusios GBM ir mažųjų podocitų kojyčių susiliejimas. Sustorėjusi ir supleišėjusi GBM rasta 28,6 % asmenų (amžiaus vidurkis 49±17,4 metų), esant normaliai inkstų funkcijai.

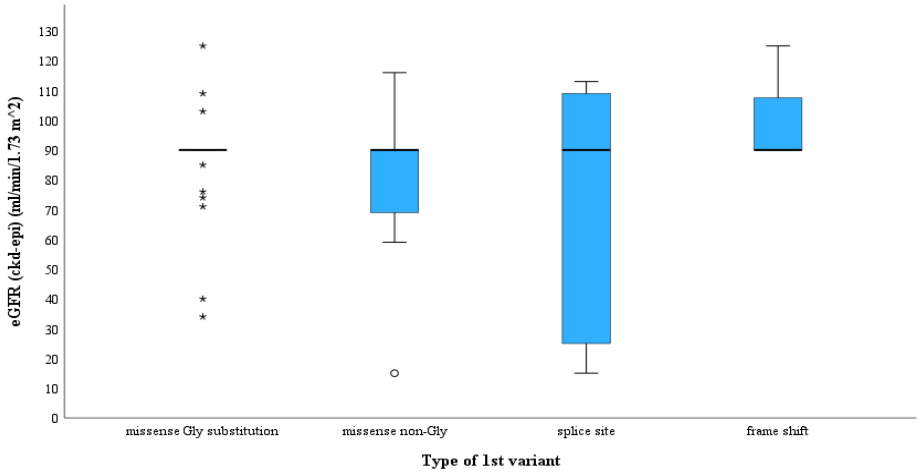
Galutinė LIL stadija išsivystė dviem asmenims - vienam jų rasta *missense* genetinis variantas, kai Leu buvo pakeistas į Pro (*COL4A3* p.Leu1474Pro (c.4421T>C)), kitam – patogeninis splaisingo variantas c.594+1G>A *COL4A4* gene. Genotipo-fenotipo koreliacijos nurodytos 19 lentelėje.

19 lentelė. ADAS sergančių pacientų genotipo ir fenotipo sąsajos (n=48)

Požymis	Asmenų sk. (%)	*Missense* Gly GV	Ne-Gly GV	Rėmelio posl. GV	Splaisingo GV	Nežinomas
Hematurija	48 (100)	27 (100,0)	10 (100,0)	4 (100,0)	6 (100)	1 (100)
Pro	25 (52,1)	11 (40,0)	7 (70,0)	2 (50,0)	5 (83,3)	0 (0,0)
Nefrozinė pro	1 (2,1)	0 (0,0)	1 (10,0)	0 (0,0)	0 (0,0)	0 (0,0)
LIL 1 st.	35 (72,9)	20 (74,1)	6 (60,0)	4 (100,0)	4 (66,7)	1 (100,0)
LIL 2 st.	7 (14,6)	5 (18,5)	2 (20,0)	0 (0,0)	0 (0,0)	0 (0,0)
LIL 3 st.	3 (6,3)	2 (7,4)	1 (10,0)	0 (0,0)	0 (0,0)	0 (0,0)
LIL 4 st.	1 (2,1)	0 (0,0)	0 (0,0)	0 (0,0)	1 (16,7)	0 (0,0)
LIL 5 st.	2 (4,2)	0 (0,0)	**1 (10,0)**	0 (0,0)	**1 (16,7)**	0 (0,0)
IB	21 (43,8)	7 (25,9)	8 (80,0)	2 (50,0)	3 (50,0)	1 (100)
PBM	18 (37,5)	7 (25,9)	7 (70,0)	2 (50,0)	2 (33,3)	1 (100)
SsSpGBM	6 (12,5)	2 (7,4)	3 (30.0)	0 (0,0)	1 (16.7)	0 (0,0)
ŽSGS	8 (16,7)	3 (11,1)	3 (30,0)	0 (0,0)	2 (33,3)	0 (0,0)
Klausos p.	8 (16,7)	4 (14,8)	2 (20,0)	0 (0,0)	1 (16,7)	1 (100)
Akių p.	8 (16,7)	5 (18,5)	0 (0,0)	0 (0,0)	3 (50,0)	0 (0,0)
Bendr. sk.	48 (100,0)	27 (56,3)	10 (20,8)	4 (8,3)	6 (12,5)	1 (2,1)

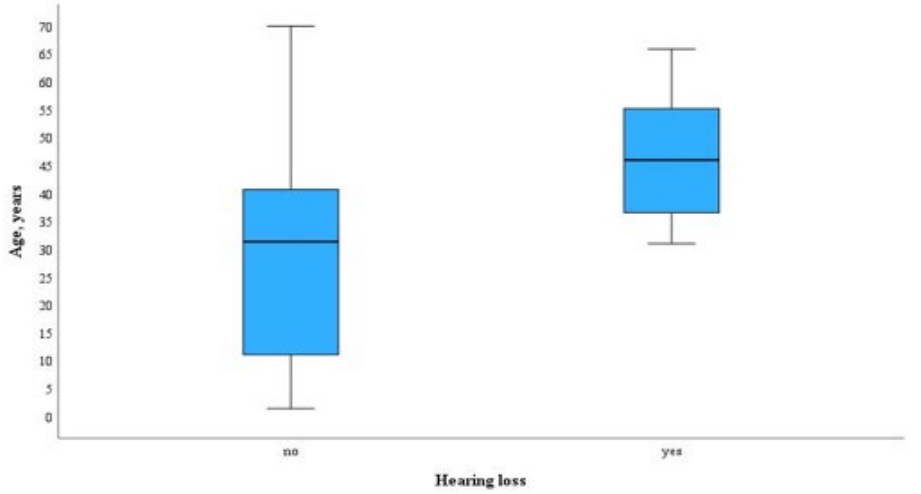
Pro – proteinurija; LIL – lėtinė inkstų liga; GV – genetinis variantas; PBM – plona bazinė membrana inksto biopsijoje; SsSpGBM – sustorėjusios ir supleišėjusios GBM; GBM – glomerulų bazinė membrana; ŽSGS – židininė segmentinė glomerulosklerozė ; st. – stadija; IB – inkstų biopsija; sk. – skaičius; p – pažeidimas; bendr. – bendras ; posl. – poslinkio

Reikšmingo skirtumo tarp inkstų funkcijos ir skirtingo genetinio varianto *COL4A3* arba *COL4A4* genuose, ADAS sergantiems asmenims, nenustatyta (11 pav.).



11 pav. Skirtingų tipų genetinių pokyčių *COL4A3-A4* genuose pasiskirstymas priklausomai nuo eGFG tarp ADAS sergančių asmenų (p=0,484)

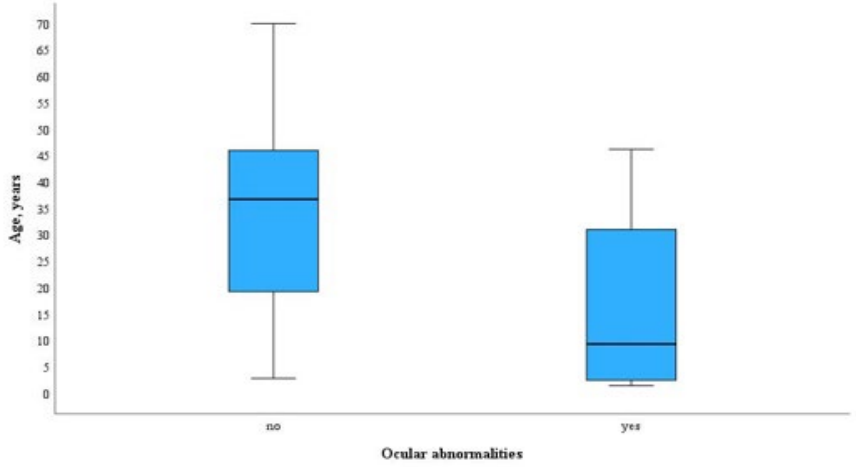
Klausos sutrikimai nustatyti aštuoniems (16.7 %) 46,5±11,8 metų amžiaus asmenims, sergantiems ADAS. Klausos sutrikimai dažniau nustatyti vyresniems negu 35,4 metų asmenims (p=0,02, 95 % CI [1,22-99,3]), 12 pav.



12 pav. Asmenų, sergančių ADAS, klausos sutrikimų pasiskirstymas. Mann-Whitney testas, p=0,02

Aštuoniems (16.7 %) asmenims (vidutinis amžius 16,6±17,3 metų), nustatyti akių pažeidimai. Mūsų tyrimo duomenimis akių anomalijos dažniau

pasireiškė vyresniems negu 6,6 metų asmenims (p=0,02, 95 % CI [1,6 - 216,1], 13 pav.). Reikšmingo skirtumo tarp skirtingų tipų molekulinių pokyčių ir klausos bei akių pažeidimų dažnio, nenustatyta.



13 pav. Akių anomalijų ir amžiaus pasiskirstymas. Mann-Whitney testas, p=0,02

3.8. Digeninio AS grupė: genotipo – fenotipo sąsajos

Iš viso buvo nustatyti keturi asmenys (3 moterys ir 1 vyras), sergantys digeniniu AS (20 lentelė).

20 lentelė. Genetinė asmenų sergančių ARAS charakteristika (n=4)

ID	Šeimos; Asm. Nr., lytis	Amž	Genas	GV Nr.	GV nukleotidų (baltymo) pokytis	Imituo-jamas tipas
A5/46512	5-I-M	14	*COL4A5*	6	c.1417_1418del (p.Val473Glufs*3)[b]	XLAS
			COL4A4	7	c.4151C>T (p.Ala1384Val)[b]	
A5/412818	8-I-M	39	*COL4A4*	11	c.2996G>A (p.Gly999Glu)[a]	XLAS
			COL4A5	12	c.1871G>A (p.Gly624Asp)[a]	
A5/4262450	24-I-M	26	*COL4A5*	26	c.1374delinsTT (p.Pro459Serfs*6)[b]	XLAS
			COL4A4	27	c.4910G>A (p.Arg1637Gln)[b]	
A3/438376	37-I-V	42	*COL4A3*	38	c.1021C>T (p.Arg341Cys)[a]	ADAS
			COL4A4	20	c.-101-4A>G[a]	

Nr. – numeris; het – heterozigotinis; homo – homozigotinis; M – moteris; V – vyras; GV – genetinis variantas; [b] – naujas GV; [a] – žinomas GV; ID – paciento identifikacijos kodas; amž. – amžius genetinės diagnozės metu. Imituojamas tipas – AS tipas, į kurį panašus digeninis AS, remiantis molekuliniais ir klinikiniais požymiais

Visiems asmenims pasireiškė hematurija ir proteinurija. Trijų asmenų (dviejų moterų ir vieno vyro) inkstų funkcija buvo normali, ir visiems

nustatyta nedidelė proteinurija (B/K 15-30 mg/mmol).Vienai moteriai rasta reikšminga proteinurija (B/K 60 mg/mmol/L) ir nustatyta II st. LIL. Nė vienas iš asmenų nepasiekė V stadijos LIL. Klausos sutrikimas ir specifiniai pokyčiai akyse rasti tik vienai 39 metų amžiaus moteriai, kuriai nustatyta II st. LIL (21 lentelė).

21 lentelė. Digeniniu AS sergančių pacientų genotipo ir fenotipo koreliacija (n=4)

ID	Amžius	Hematurija	Pro (kiekis)^{0}	LIL stadija^{0}	Klausos paž.	Akių paž.
A5/46512	14	+	+ (29)^{14}	I	-	-
A5/412818	39	+	+ (70)^{22}	II	+	+
A5/4262450	26	+	+ (23)^{16}	I	-	-
A3/438376	42	+	+ (18)^{35}	^{I}	-	-

ID – paciento identifikacijos kodas; Pro – proteinurija; paž. – pažeidimas. *Dvynės seserys. ^{0} – parodo požymio pradžios amžių. Proteinurija vertinta pagal vienkartinio šlapimo baltymo-kreatinino santykį (mg/mmol): >300 mg/mmol vertinta kaip nefrozinio lygio proteinurija. Amžius vertintas genetinės diagnozės metu

Digeninio AS asmenų grupėje inkstų biopsija buvo atlikta 3 asmenims. Visiems rastos suplonėjusios bazinės membranos, iš jų dviems moterims - sustorėjusios ir kai kur supleišėjusios GBM. Visiems asmenims buvo rastas mažųjų podocitų kojyčių susiliejimas. ŽSGS patvirtinta tik vienai moteriai, kuriai nustatytas *COL4A5* ir *COL4A4* digeninis variantas.

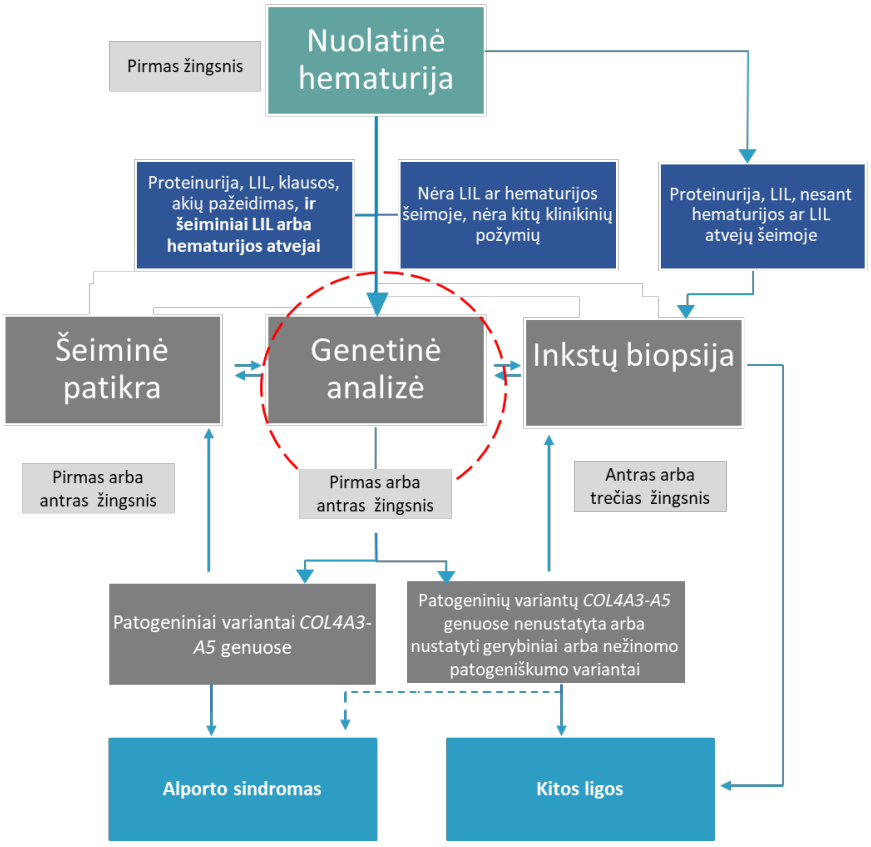
Bendradarbiaujant su Australijos darbo grupe ir analizuojant digeninio AS pasireiškimą, atradome, jog moterims, kurioms rastas digeninis AS, susijęs su *COL4A5* ir kitu *COL4A3* arba *COL4A4* variantu, buvo didesnė proteinurijos išsivystymo rizika, siejama su blogesne inkstų funkcija ateityje. Vyrams, turintiems patogeninių *COL4A5* variantų, papildomi variantai *COL4A4* arba *COL4A4* genuose įtakos ligos eigai neturėjo. Digeninio AS, paveldėto autosominiu būdu pasireiškimas imitavo ADAS arba ARAS ligos eigą ir buvo susijęs su proteinurija ir inkstų funkcijos blogėjimu [85].

4. IŠVADOS

1. Molekulinė *COL4A3-A5* genų analizė yra pirmo pasirinkimo diagnostikos metodas įtarus AS. Diagnozė buvo patvirtinta daugiau kaip pusei asmenų, kuriems buvo rasta nuolatinė mikroskopinė hematurija. Inkstų biopsija buvo atlikta 43% asmenų, sergančių AS, esant pažengusiai LIL. Vaikams inkstų biopsija buvo atliekama reikšmingai rečiau, negu suaugusiems.
2. Molekulinės savybės turi įtakos AS klinikiniam ligos pasireiškimui ir progresavimui. Patogeniniai *COL4A5* variantai, susiję su glicino pakeitimu destabilizuojančiomis amino rūgštimis (Arg, Val, Glu, Asp, Trp), koreliavo su ankstyvesniu galutinės stadijos LIL išsivystymu ir klausos praradimu. Glicino pakaita netoli ne-kolageninio regiono *COL4A5* gene buvo susijusi su vėlesniu inkstų funkcijos blogėjimu, o *COL4A3* ir *COL4A4* genuose su mažesne hematurijos atsiradimo rizika.
3. *COL4A3-A5* genų naujų variantų tipas ir dažnis Lietuvos šeimose skiriasi nuo kitose šalyse rastų variantų. NKS išanalizavus 221 asmenų, buvo identifikuoti 44 skirtingi *COL4A3-A5* genų molekuliniai pokyčiai, iš kurių 28 (63,6 %) variantai buvo vertinami kaip nauji. Genotipo ir fenotipo koreliacijos analizė parodė, kad kai kurie variantai pasireiškė fenotipine įvairove šeimoje. Daugiau nei trečdalis rastų genetinių pokyčių buvo bendri su pasaulio populiacija ir įrašyti į tarptautines genetinių duomenų bazes.
4. LIL progresavimo rizika yra susijusi su patogeniniais *COL4A3-A5* genų variantais ar jų deriniais bei skiriasi tarp genetinio pokyčio tipų ir lyties. Didžiausia LIL progresavimo rizika nustatyta XLAS sergantiems vyrams ir asmenims, sergantiems ARAS. Moterims, kurioms diagnozuotas XLAS ir asmenims, sergantiems ADAS ar digeniniu AS, lėtinė inkstų liga progresuoja lėčiau; progresavimo rizikos veiksnys yra proteinurija.

5. PRAKTINĖS REKOMENDACIJOS

1. Nustačius nuolatinę mikrohematuriją, rekomenduojama pacientus tirti dėl Alporto sindromo. Siūlomas AS diagnostikos algoritmas parodytas 14 paveiksle.
2. Rekomenduojama *COL4A3-4A5* genų molekulinę analizę įtraukti į bendrą inkstų panelę, skirtą genetinėms inkstų ligoms tirti, nes AS gali būti diagnozuojamas, net ir įtariant kitas genetines ligas (pvz. ADPIL).
3. Rekomenduojama atlikti visų *COL4A3-A5* variantų analizę, net jei žinomas tikslus šeiminis AS variantas, atsižvelgiant į galimą skirtingų AS tipų (pvz. digeninio AS) pasireiškimą toje pačioje šeimoje.
4. Nors VUS nustatymas *COL4A3-A5* genuose nepatvirtina Alporto sindromo, diagnozės patikslinimui rekomenduojama įvertinti klinikinę ligos eigą, šeimos anamnezę (LIL atvejai) ir apsvarstyti inkstų biopsiją, nes kai kurie VUS variantai gali būti patogeniniai ir susiję su progresuojančia ligos eiga.
5. Rekomenduojame reguliariai stebėti arterinį kraujospūdį ir vertinti proteinuriją asmenims, sergantiems AS, ir, jei reikia, pradėti gydymą RAAS blokatoriais, nes hipertenzija ir proteinurija yra LIL progresavimo rizikos veiksniai.
6. Rekomenduojame vertinti mikroalbuminuriją ir proteinuriją rizikos grupės vaikams, sergantiems Alporto sindromu nuo 1 metų amžiaus arba nustačius diagnozę, atliekant kasmetinius stebėjimo tyrimus, kad būtų galima anksti nustatyti ir skirti gydymą, mažinantį LIL progresavimą.
7. Net lengvos AS formos gali sukelti V stadijos LIL, todėl asmenims, sergantiems AS, rekomenduojam atsisakyti inkstų donorystės.
8. Rekomenduojama RAAS blokatoriais pradėti gydyti vyrus su XLAS ir asmenis, sergančius ARAS ir digeniniu AS. Apsvarstyti ARB arba AKF inhibitorių skyrimą moterims su XLAS arba ADAS pacientams, kai nustatoma proteinurija (15 pav.).



14 pav. Mūsų tyrimo pasiūlytas diagnostikos algoritmas.

Pirmas žingsnis: asmenų, kuriems nustatyta nuolatinė hematurija (daugiau nei trijuose mėginiuose per 6 mėn. laikotarpį), identifikavimas – dėl specifinių požymių (1) ar dėl šeiminės anamnezės (2). Genetinei analizei turėtų būti siunčiami šie asmenys: (1) turintys klinikinių požymių ir kurių šeimoje yra segančių LIL; (2) kuriems randama hematurija, tačiau šeimoje nėra LIL atvejų; (3) kuriems įtariamas AS dėl šeimoje esančių AS atvejų, nesant hematurijos. **Antras žingsnis**: molekulinė analizė dėl specifinių klinikinių požymių; šeiminės anamnezės; inkstų biopsijos. Šeimos patikra rekomenduojama nustačius naują AS atvejį šeimoje. Tais atvejais, kai šeimoje LIL atvejų nėra, tačiau pasireiškia nuolatinė hematurija ir kiti požymiai, pvz., proteinurija, LIL, prieš molekulinę analizę, gali būti atliekama inkstų biopsija. **Trečias žingsnis** apima genetinę analizę, kuri gali būti atliekama dėl specifinių pokyčių inksto biopsijoje (nežinomos etiologijos ŽSGS, suplonėjusi, sustorėjusi, supleišėjusi GBM ir podocitų kojyčių susiliejimas). Asmenims, kuriems genetiškai AS nepatvirtinamas, arba tiems, kurių rezultatai yra prieštaringi, pvz., nežinomos reikšmės variantai (VUS), ypač jei yra kitų požymių, tokių kaip proteinurija, LIL ar klausos ir regos pažeidimas, diferencine diagnostikai gali būti atliekama inkstų biopsija. AS patvirtinama atliekant molekulinę analizę, nustatant patogeninius *COL4A3-A5* genų variantus. Rekomenduojama atlikti paveldimų inkstų ligų panelę, apimančią *COL4A3-A5* genus. Šeimos patikros atveju rekomenduojama visų trijų *COL4A3-A5* genų molekulinė analizė, neatsižvelgiant į žinomus genetinius variantus šeimoje

Spalva, reiškianti LIL riziką	LIL progresavimo rizika	Valdymas
	Vyrai, sergantys XLAS; ARAS sergantys asmenys	Genetinės diagnozės metu pradėkite gydymą AKF inhibitoriais arba ARB
	Moterys, sergančios XLAS ir ADAS sergantys asmenys, kai kartu nustatoma albuminurija, hipertenzija, ar eGFG <90 ml/min/1,73 m^2 Digeniniu AS sergantys asmenys	Apsvarstykite galimybę skirti AKF inhibitorius arba ARB, moterims sergančioms XLAS ar asmenims su ADAS, kai nustatoma proteinurija. Rekomenduojama pradėti AKF inhibitorių arba ARB terapiją digeniniu AS sergantiems asmenims genetinės diagnozės metu
	Moterys, sergančios XLAS; ADAS sergantys asmenys kai nėra klinikinių simptomų	Gydymo nereikia. Kasmetinis inkstų funkcijos stebėjimas

15 pav. Mūsų tyrimo pasiūlytas LIL progresavimo rizikos stratifikavimas ir valdymas, remiantis molekulinio tyrimo rezultatais. Raudona spalva reiškia didžiausią riziką, geltona – vidutinę, o žalia – mažiausią LIL progresavimo riziką

6. TYRIMO TRŪKUMAI

Mūsų tyrimas turi keletą trūkumų. Pirma, analizė atlikta tik viename centre, todėl negalėjome įvertinti AS paplitimo Lietuvoje. Antra, dėl nedidelio V st. LIL turinčių asmenų skaičiaus negalėjome tiksliai nustatyti inkstų išgyvenamumo tarp skirtingų AS tipų. Be to, duomenų apie eGFG vertes trūkumas apribojo mūsų galimybes pasirinkti alternatyvius išgyvenamumo kriterijus. Trečia, vaikų įtraukimas ankstyvosiose ligos stadijose galėjo paveikti rezultatų pasiskirstymą, nes jų tikrieji fenotipai dar gali būti nevisiškai išreikšti. Ketvirta, ne visiems į tyrimą įtrauktiems asmenims buvo atlikti klausos ar akių diagnostiniai tyrimai, taip pat trūko kai kurių inkstų biopsijos duomenų (pvz., EM). Penkta, kai kurie tyrimo dalyviai buvo artimi giminaičiai, todėl genotipo ir fenotipo koreliacijos bendroje kohortoje galėjo sutapti ir keisti objektyvius tyrimo rezultatus. Galiausiai, ne visus genetinės laboratorijos pateiktus genetinius duomenis, įskaitant specifinę genetinių variantų vietą egzonuose, buvo galima palyginti.

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SUPPLEMENTS

Supp. Table 1. Genetic characteristics of heterozygous or hemizygous *COL4A3*, *COL4A4* and *COL4A5* variants

Gene	Nucleotide change	Protein change	No of var	Variant coordinates, SNP identifier	Type of var.	PP2	SIFT	MT	Align-GVGD
COL4A4	c.1579G>T [a]	p.Gly527Cys	10	NM_000092.4 (rs779930511)	Gly subs.	pd	dlt	dc	C0
	c.-101-4A>G [a]	-	20	NM_0000092.4 (rs147186690)	splice site	N/a	N/a	N/a	N/a
	c.1389del [a]	p.Asn464Thrfs Exon 21	25	M_000092.4	frameshift	N/a	N/a	N/a	N/a
	c.1820C>T [a]	p.Ala607Val Exon 25	28	NM_000092.4	missense	Pd	dlt	plm	C55
	c.3451G>A [a]	p.Gly1151Arg	42	NM_000092.4	Gly subs.	pd	dlt	dc	C15
	c.657+2dup [b]	-	5	NM_000092.4	unknown	N/a	N/a	N/a	N/a
	c.4151C>T [b]	p.Ala1384Val	7	NM_000092.4	missense	bng	tlt	plm	C0
	c.594+1G>A [b]	-	9	NM_000092.5	splice site	N/a	N/a	N/a	N/a
	c.5045G>A [a]	p.Arg1682Gln	15	NM_000092.5	missense	N/a	dc	dc	N/a
	c.1987G>C [b] 25 exon	p.Gly663Arg	16	NM_000092.5	Gly subs.	N/a	N/a	N/a	N/a
	c.2756A>G [b]	p.Glu919Gly	32	NM_000092.4	missense	bn	dlt	plm	C0
	c.4315G>A [b]	p.Gly1439Ser	36	NM_000092.4	Gly subs.	pd	dlt	dc	C0
	c.3044G>A [b]	p.Gly1015Glu	40	NM_000092.5	Gly subs.	pat	pat	pat	N/a
	c.2347G>A [b]	p.Gly783Arg	41	NM_000092.4	Gly subs.	pd	dlt	dc	C15
	c.4910G>A [b]	p.Arg1637Gln	27	NM_000092.4	missense	pd	tlt	dc	C0
	c.2996G>A [a]	p.Gly999Glu	11	NM_000092.4	Gly subs.	pd	pd	pd	pd
	c.898G>A[a]	p.Gly300Arg	22	NM_000091.3	Gly subs.	pd	dlt	dc	C65
	c.3499G>A [a] Exon 40	p.Gly1167Arg	29	NM_000091.3	Gly subs.	pd	dlt	dc	C25
	c.4421T>C [a]	p.Leu1474Pro	44	NM_000091.5	missense	N/a	pd	pd	N/a
	c.520G>A [b]	p.Gly174Arg	19	NM_000091.4	Gly subs.	pd	dlt	dc	C0

Gene	Nucleotide change	Protein change	No of var	Variant coordinates, SNP identifier	Type of var.	PP2	SIFT	MT	Align-GVGD
COL4A3	c.2711G>T [b]	p.Gly904Val	31	NM_000091.3	Gly subs.	pd	dlt	dc	C0
	c.4717G>A [b]	p.Gly1573Ser	35	NM_000091.3	Gly subs.	-	dlt	plm	C15
	c.416G>A [b]	p.Gly139Glu	37	NM_000091.3	Gly subs.	pd	dlt	dc	C15
	c.593G>T [b]	p.Gly198Val	39	NM_000091.3	Gly subs.	pd	dlt	dc	C65
	c.2188G>C [b]	p.Gly730Arg	43	NM_000091.3	Gly subs.	-	dlt	dc	C0
	c.4702C>T [b]	p.Pro1568Ser	23	NM_000091.3	missense	pd	dlt	dc	C65
	c.3247G>C [b]	p.Gly1083Arg	24	NM_000091.3	Gly subs.	pd	dlt	dc	C0
	c.1021C>T [b]	p.Arg341Cys	38	NM_000091.3	missense	pd	dlt	plm	C15
COL4A5	c.2297G>A [a]	p.Gly766Asp	1	NM_033380.2	Gly subs.	pd	pd	dc	N/a
	c.902G>A [a]	p.Gly301Asp	2	NM_033380.2	Gly subs.	pd	pd	pd	tlt
	c.4994+3A>G [a]	-	8	NM_033380.2	splice site	N/a	N/a	N/a	N/a
	c.1871G>A [a]	p.Gly624Asp Exon 25	12	NM_033380.1	Gly subs.	pd	dlt	dc	C65
	c.2623G>A [b]	p.Gly875Arg	13	NM_033380.2)	Gly subs.	pd	dlt	dc	C15
	c.3685G>A [a]	p.Gly1229Ser	17	NM_033380.1	Gly subs.	pd	dlt	dc	C55
	c.1032+1G>C [a] Intron 18	-	30	NM_033380.1	splice site	N/a	N/a	N/a	N/a
	c.3508G>C [b]	p.Gly1170Arg	3	NM_033380.2	Gly subs.	pd	pd	pd	C65
	3106+1G>A [b]	-	4	NM_033380.2	splice site	N/a	N/a	N/a	N/a
	c.1417_1418del [b]	p.Val473Glufs*3	6	NM_033380.1	frameshift	N/a	N/a	N/a	N/a
	c.347delC [b]	p.Pro116Glnfs*39	14	NM_033380.1	frameshift	N/a	N/a	N/a	N/a
	c.883G>A [b]	p.Gly295Ser	18	NM_033380.2	Gly subs.	N/a	N/a	N/a	N/a
	c.2777G>T [b]	p.Gly926Val	21	NM_033380.3	Gly subs.	N/a	N/a	N/a	N/a
	c.1374delinsTT [b]	p.Pro459Serfs*6	26	NM_033380.1	frameshift	N/a	N/a	N/a	N/a
	c.3554-2A>G [b]	-	33	NM_033380.1	splice site	dc	N/a	N/a	N/a
	c.466G>C[b]	p.Gly156Arg	34	NM_033380.1	Gly subs.	pd	dlt	dc	C65

AlignGVD: C0: least likely to interfere with function, C65: most likely to interfere with function; Gly subs – glycine substitution; Hemi- hemizygous; Het – heterozygous; pd-probably damaging; dlt – deleterious; dc – disease causing; plm – polymorphism; bn – benign variant; tlt – tolerated; pat – pathological; N/a – not applicable; chr – chromosome; PP2 – PolyPhen-2; MT – MutationTester; DB – database; [a]- known genetic variant; [b]- novel genetic variant

Supp. Table 2. ACMG classification of *COL4A3*, *COL4A4* and *COL4A5* variants

Gene	Nucleotide change	Protein change	No of var	ACMG ClinVar	ACMG InterVar	ACMG Cento	ACMG Franklin	Our study interpretation
COL4A4	c.1579G>T[a]	p.Gly527Cys	10	LP/VUS	VUS	P	LP	LP
	c.-101-4A>G[a]	-	20	LB/Bn/VUS	N/a	VUS	LB	LBn
	c.1389del[a]	p.Asn464Thrfs	25	P	VUS	P	P	LP
	c.1820C>T[a]	p.Ala607Val	28	VUS	VUS	LP	VUS	LP
	c.3451G>A[a]	p.Gly1151Arg	42	LP/VUS	N/a	LP	LP	LP
	c.657+2dup[b]	-	5	N/a	VUS	LP	N/a	LBn
	c.4151C>T[b]	p.Ala1384Val	7	N/a	VUS	VUS	VUS	LP
	c.594+1G>A[b]	-	9	LP	P	LP	P	LP
	c.5045G>A[a]	p.Arg1682Gln	15	P/VUS	VUS	N/a	P	LP
	c.1987G>C[b]	p.Gly663Arg	16	N/a	LP	N/a	VUS	LBn
	c.2756A>G[b]	p.Glu919Gly	32	VUS	VUS	VUS	VUS	LBn
	c.4315G>A[b]	p.Gly1439Ser	36	N/a	VUS	LP	VUS	LBn
	c.3044G>A[b]	p.Gly1015Glu	40	LP/VUS	VUS	N/a	LP	LBn
	c.2347G>A[b]	p.Gly783Arg	41	VUS	VUS	LP	VUS	LP
	c.4910G>A[b]	p.Arg1637Gln	27	N/a	VUS	VUS	VUS	LP
	c.2996G>A[a]	p.Gly999Glu	11	LP/VUS	Bn	VUS	Bn	LBn
COL4A3	c.898G>A[a]	p.Gly300Arg	22	P/LP/VUS	LP/VUS	LP	P	LP/LBn
	c.3499G>A[a]	p.Gly1167Arg	29	P	LP	P	P	LP
	c.4421T>C[a]	p.Leu1474Pro	44	LB-P	VUS	N/a	VUS	P
	c.520G>A[b]	p.Gly174Arg	19	P	VUS	LP	P	LP
	c.2711G>T[b]	p.Gly904Val	31	N/a	VUS	LP	VUS	LBn
	c.4717G>A[b]	p.Gly1573Ser	35	N/a	VUS	LP	VUS	LBn
	c.416G>A[b]	p.Gly139Glu	37	N/a	VUS	LP	VUS	LP
	c.593G>T[b]	p.Gly198Val	39	N/a	VUS	LP	VUS	LP
	c.2188G>C[b]	p.Gly730Arg	43	N/a	VUS	LP	VUS	LBn
	c.4702C>T[b]	p.Pro1568Ser	23	N/a	VUS	VUS	VUS	P

Gene	Nucleotide change	Protein change	No of var	ACMG ClinVar	ACMG InterVar	ACMG Cento	ACMG Franklin	Our study interpretation
	c.3247G>C[b]	p.Gly1083Arg	24	N/a	VUS	VUS	VUS	P
	c.1021C>T[b]	p.Arg341Cys	38	N/a	VUS	VUS	VUS	LP
COL4A5	c.2297G>A[a]	p.Gly766Asp	1	N/a	N/a	P	LP	P
	c.902G>A[a]	p.Gly301Asp	2	LP	LP	LP	P	P
	c.4994+3A>G[a]	-	8	VUS	N/a	LP	VUS	LP
	c.1871G>A[a]	p.Gly624Asp	12	LP/P	LP	P	P	P
	c.2623G>A[b]	p.Gly875Arg	13	N/a	LP	LP	LP	P
	c.3685G>A[a]	p.Gly1229Ser	17	P	LP	VUS	P	LP
	c.1032+1G>C[a]	-	30	P/LP	N/a	LP	P	LP
	c.3508G>C[b]	p.Gly1170Arg	3	N/a	LP	LP	LP	P
	3106+1G>A[b]	-	4	N/a	P	LP	LP	LP
	c.1417_1418del[b]	p.Val473Glufs*3	6	N/a	LP	LP	LP	P
	c.347delC[b]	p.Pro116Glnfs*39	14	N/a	P	LP	LP	LP
	c.883G>A[b]	p.Gly295Ser	18	N/a	VUS	VUS	LP	LP
	c.2777G>T[b]	p.Gly926Val	21	N/a	VUS	VUS	VUS	P
	c.1374delinsTT[b]	p.Pro459Serfs*6	26	N/a	LP	LP	LP	VUS
	c.3554-2A>G [b]	-	33	N/a	P	LP	LP	LP
	c.466G>C[b]	p.Gly156Arg	34	VUS	VUS	LP	LP	P

Cento – Centogene; var – variant; P- pathogenic; LP – likely pathogenic; VUS – variant of uncertain significance; Bn – benign; LBn – likely benign; N/a not applicable; [a]- known genetic variant; [b]- novel genetic variant

Supp. Table 3. General characteristics of 48 individuals with ADAS: heterozygous variants in *COL4A4* and *COL4A3*

ID	Family No	Member, gender	Age at diagnosis	Gene	Variant No	Nucleotide change	Protein change	Heterogeneity
A4106289	62	I-M	46	*COL4A4*	10	c.1579G>T	p.Gly527Cys	Missense Gly substitution
A4104793	47	I-M	20					
A4204591	45	I-F	32	*COL4A4*	20	c.-101-4A>G	-	Splice site
A4252247	22	I-M	18	*COL4A4*	25	c.1389del	p.Asn464Thrfs*7	Frameshift
A42555104	55	I-M	41					
A2555105		II-M	12					
A42555106		III-F	8					
A4282553	25	I-F	62	*COL4A4*	28	c.1820C>T	p.Ala607Val	Missense non-Gly substitution
A4283570	35	I-F	33					
A4424894	48	I-F	70	*COL4A4*	42	c.3451G>A	p.Gly1151Arg	Missense Gly substitution
A453671	63	I-F	66	*COL4A4*	5	c.657+2dup	-	Unknown
A476197	61	I-F	31	*COL4A4*	7	c.4151C>T	p.Ala1384Val	Missense non-Gly substitution
A49717	7	I-F	36	*COL4A4*	9	c.594+1G>A	-	Splice site
A49737		II-F	61					
A49738		III-M	26					
A49775		IV-F	12					
A494387	43	I-M	65					
A4151430	14	I-F	37	*COL4A4*	15	c.5045G>A	p.Arg1682Gln	Missense non-Gly substitution
A41558109	58	I-F	58					
A41654103	54	I-M	38	*COL4A4*	16	c.1987G>C	p.Gly663Arg	Missense Gly substitution
A41654111		II-M	7					
A4322957	29	I-F	41	*COL4A4*	32	c.2756A>G	p.Glu919Gly	Missense non-Gly substitution
A4322976		II-M	3					
A4363463	34	I-M	20	*COL4A4*	36	c.4315G>A	p.Gly1439Ser	Missense Gly substitution
A4363469		II-F	47					

ID	Family No	Member, gender	Age at diagnosis	Gene	Variant No	Nucleotide change	Protein change	Heterogeneity
A4363477		III-M	10					
A4404082	40	I-M	40	*COL4A4*	40	c.3044G>A	p.Gly1015Glu	Missense Gly substitution
A4404085		II-M	2					
A4404086		III-M	3					
A4414284	42	I-F	14	*COL4A4*	41	c.2347G>A	p.Gly783Arg	Missense Gly substitution
A44142100		II-M	40					
A44142110		III-M	63					
A3222042	20	I-F	42	*COL4A3*	22	c.898G>A	p.Gly300Arg	Missense Gly substitution
A3222043		II-F	17					
A3222044		III-M	8					
A3221872	18	I-M	31					
A3292654	26	I-F	33	*COL4A3*	29	c.3499G>A	p.Gly1167Arg	Missense Gly substitution
A32957108	57	I-F	56					
A3445098	50	I-M	34	*COL4A3*	44	c.4421T>C	p.Leu1474Pro	Missense non-Gly substitution
A34460114	60	I-M	45					
A3191736	17	I-F	35	*COL4A3*	19	c.520G>A	p.Gly174Arg	Missense Gly substitution
A3191774		II-M	7					
A3312856	28	I-M	31	*COL4A3*	31	c.2711G>T	p.Gly904Val	Missense Gly substitution
A3353268	32	I-F	1	*COL4A3*	35	c.4717G>A	p.Gly1573Ser	Missense Gly substitution
A3353296		II-M	31					
A3373665	36	I-F	63	*COL4A3*	37	c.416G>A	p.Gly139Glu	Missense Gly substitution
A339398	39	I-M	54	*COL4A3*	39	c.593G>T	p.Gly198Val	Missense Gly substitution
A3434995	49	I-F	42	*COL4A3*	43	c.2188G>C	p.Gly730Arg	Mis Gly substitution

Gly – glycine; F- female; M – male; No – number

Supp. Table 4. Genotype-phenotype correlation in 48 individuals with ADAS: heterozygous variants in *COL4A4* and *COL4A3*

Gene	Variant	Var No	Fam No., member, gender	Age at diagnosis	Hematuria	Pro, amount, (age at detection, y.o.)	CKD stage	KF- age at KF	Ocular ab.	Hearing ab.
COL4A4	p.Gly527Cys (c.1579G>T)	10	62-I-M	46	+	none	I	none	+	none
			47-I-M	20	+	none	I	none	none	none
	c.-101-4A>G	20	45-I-F	32	+	++ (27)	I	none	none	none
	p.Asn464Thrfs*7 (c.1389del)	25	22-I-M	18	+	+ (19)	I	none	none	none
			55-I-M	41	+	+ (36)	I	none	none	none
			55-II-M	12	+	none	I	none	none	none
			55-III-F	8	+	none	I	none	none	N/d
	p.Ala607Val (c.1820C>T)	28	25-I-F	62	+	+ (61)	IIIa	none	none	none
			35-I-F	33	+	+ (26)	I	none	none	none
	p.Gly1151Arg (c.3451G>A)	42	48-I-F	70	+	+ (62)	IIIb	none	none	none
	c.657+2dup	5	63-I-F	66	+	none	I	none	none	+
	p.Ala1384Val (c.4151C>T)	7	61-I-F	31	+	none	I	none	N/d	N/d
	c.594+1G>A	9	7-II-F	36	+	+ (29)	I	none	+	+
			7-III-F	61	+	++ (53)	V	+ (55)	none	none
			7-IV-M	26	+	none	I	none	none	none
			7-V-F	12	+	+ (12)	I	none	+	none
			43-I-M	65	+	+ (65)	IV	none	none	none
	p.Arg1682Gln (c.5045G>A)[a]	15	14-I-F	37	+	+++ (37)	II	none	N/d	+
			58-I-F	58	+	none	II	none	N/d	N/d
	p.Gly663Arg (c.1987G>C)	16	54-I-M	38	+	none	I	none	none	none
			54-II-F	7	+	none	I	none	+	none
	p.Glu919Gly (c.2756A>G)	32	29-I-F	41	+	+ (30)	I	none	none	none
			29-II-M	3	+	none	I	none	none	none
	p.Gly1439Ser (c.4315G>A)	36	34-I-M	20	+	+ (20)	I	none	none	none
			34-II-F	47	+	none	I	none	none	+
			34-III-M	10	+	none	II	none	none	none

Gene	Variant	Var No	Fam No., member, gender	Age at diagnosis	Hematuria	Pro, amount, (age at detection, y.o.)	CKD stage	KF- age at KF	Ocular ab.	Hearing ab.
	p.Gly1015Glu (c.3044G>A)	40	40-I-M	40	+	none	I	none	none	none
			40-II-M	2	+	none	I	none	+	none
			40-III-M	3	+	none	I	none	+	none
	p.Gly783Arg (c.2347G>A)	41	42-I-F	14	+	none	I	none	none	none
			42-II-M	40	+	+ (30)	I	none	none	none
			42-III-M	63	+	++ (61)	IIIb	none	N/d	N/d
COL4A3	p.Gly300Arg (c.898G>A)	22	20-I-F	42	+	none	II	none	none	none
			20-II-F	17	+	none	I	none	none	none
			20-III-M	8	+	+ (3)	I	none	none	none
			18-I-M	31	+	none	I	none	none	+
	p.Gly1167Arg (c.3499G>A)	29	26-I-F	33	+	+ (34)	I	none	N/d	none
			57-I-F	57	+	+ (37)	II	none	none	+
	p.Leu1474Pro (c.4421T>C)	44	50-I-M	34	+	+ (33)	I	none	none	none
			60-I-M	45	+	++ (30)	V	+ (44)	none	+
	p.Gly174Arg (c.520G>A)	19	17-I-F	35	+	++ (29)	II	none	none	none
			17-II-M	7	+	none	I	none	none	none
	p.Gly904Val (c.520G>A)	31	28-I-M	31	+	+ (15)	I	none	none	none
	p.Gly1573Ser c.4717G>A	35	32-I-F	1	+	+ (1)	I	none	+	none
			32-II-M	31	+	none	I	none	N/d	N/d
	p.Gly139Glu (c.416G>A)	37	36-I-F	63	+	none	II	none	N/d	none
	p.Gly198Val (c.593G>T)	39	39-I-M	54	+	+ (26)	I	none	none	+
	p.Gly730Arg (c.2188G>C)	43	49-I-F	42	+	++ (42)	I	none	none	none

Var – variant; No – number; Fam – family; M – male; F – female; Pro – proteinuria; y.o. – years of age; CKD – chronic kidney disease; KF – kidney failure; ab. – abnormalities; N/d – no data. Proteinuria: +: PCR 15-49 mg/mmol, ++: PCR 50-299 mg/mmol, +++: ≥300 mg/mmol for adults, ≥200 mg/mmol for children

Supp. Table 5. Kidney biopsy findings in individuals with ADAS: heterozygous *COL4A3* and *COL4A4* variants

Variant	Var No	Fam No., member, gender	KB (age at KB)	Interstitial foam cells	FSGS	Unspecific Ig M and C3 deposits in IF	IgA immunoglobulins in IF	Thin GBM	Thickened and lamellated GBM	FPE
***COL4A4* gene**										
p.Gly527Cys (c.1579G>T)	10	62-I-M	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
		47-I-M	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
c.-101-4A>G	20	45-I-F	+ (31)	-	-	+	-	+	-	+
p.Asn464Thrfs*7 (c.1389del)	25	22-I-M	+ (17)	-	-	-	-	+	-	+
		55-I-M	+ (36)	-	-	-	-	+	-	+
		55-II-M	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
		55-III-F	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
p.Ala607Val (c.1820C>T)	28	25-I-F	+ (61)	-	+	-	-	+	+	+
		35-I-F	+ (31)	-	-	-	-	+	-	+
p.Gly1151Arg (c.3451G>A)	42	48-I-F	+ (69)	-	+	-	+	+	+	+
c.657+2dup	5	63-I-F	+ (65)	-	-	-	-	+	-	+
p.Ala1384Val (c.4151C>T)	7	61-I-F	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
c.594+1G>A	9	7-II-F	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
		7-III-F	+ (53)	N/d	+	N/d	N/d	N/d	N/d	N/d
		7-IV-M	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
		7-V-F	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
		43-I-M	+ (64)	-	+	-	-	+	+	+
p.Arg1682Gln (c.5045G>A)	15	14-I-F	+ (36)	-	+	+	-	+	+	+
		58-I-F	+ (57)	-	-	-	-	+	-	+
p.Gly663Arg (c.1987G>C)	16	54-I-M	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
		54-II-F	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
p.Glu919Gly	32	29-I-F	+ (40)	-	-	-	-	+	-	+

Variant	Var No	Fam No., member, gender	KB (age at KB)	Interstitial foam cells	FSGS	Unspecific Ig M and C3 deposits in IF	IgA immunoglobulins in IF	Thin GBM	Thickened and lamellated GBM	FPE
(c.2756A>G)		29-II-M	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
p.Gly1439Ser (c.4315G>A)	36	34-I-M	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
		34-II-F	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
		34-III-M	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
p.Gly1015Glu (c.3044G>A)	40	40-I-M	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
		40-II-M	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
		40-III-M	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
p.Gly783Arg (c.2347G>A)	41	42-I-F	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
		42-II-M	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
		42-III-M	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
***COL4A3* gene**										
p.Gly300Arg (c.898G>A)	22	20-I-F	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
		20-II-F	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
		20-III-M	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
		18-I-M	+ (30)	-	-	-	-	+	-	+
p.Gly1167Arg (c.3499G>A)	29	26-I-F	+ (32)	-	-	-	-	+	-	+
		57-I-F	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
p.Leu1474Pro (c.4421T>C)	44	50-I-M	+ (33)	-	-	-	+	+	+	+
		60-I-M	+ (30)	N/d	+	-	-	N/d	N/d	N/d
p.Gly174Arg (c.520G>A)	19	17-I-F	+ (31)	-	-	-	-	+	+	+
		17-II-M	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
p.Gly904Val (c.520G>A)	31	28-I-M	+ (30)	-	-	-	-	+	-	+
p.Gly1573Ser c.4717G>A	35	32-I-F	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
		32-II-M	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
p.Gly139Glu (c.416G>A)	37	36-I-F	+ (61)	-	+	+	+	+	-	+

Variant	Var No	Fam No., member, gender	KB (age at KB)	Interstitial foam cells	FSGS	Unspecific Ig M and C3 deposits in IF	IgA immunoglobulins in IF	Thin GBM	Thickened and lamellated GBM	FPE
p.Gly198Val (c.593G>T)	39	39-I-M	+ (53)	-	+	-	-	+	-	+
p.Gly730Arg (c.2188G>C)	43	49-I-F	+ (42)	-	-	-	-	+	-	+

Var – variant; No – number; Fam – family; M – male; F – female; N/d – no data; FSGS – focal segmental glomerulosclerosis; IgM – immunoglobulin M; IgA – immunoglobulin A; KB – kidney biopsy; FPE – foot process effacement

Supp. Table 6. General characteristics of 4 individuals with ARAS: compound heterozygous and homozygous variants

ID	Family no	Member, gender	Age at diagnosis	Gene	Variant No	Nucleotide change	Protein change	Type of ARAS	Reported or novel variant
A4/49716	7; 62	I-M	7	*COL4A4*	9	c.594+1G>A	-	Compound het	Novel
				COL4A4	10	c.1579G>T	p.Gly527Cys		Reported
A3/3232145	21	I-F	34	*COL4A3*	23	c.4702C>T	p.Pro1568Ser	Compound het	Novel
				COL4A3	24	c.3247G>C	p.Gly1083Arg		Novel
A3/3232146		II-F	34	*COL4A3*	23	c.4702C>T	p.Pro1568Ser	Compound het	Novel
				COL4A3	24	c.3247G>C	p.Gly1083Arg		Novel
A4/4/283867	38	I-F	32	*COL4A4*	28	c.1820C>T	p.Ala607Val	Homo	Reported

No – number; ARAS – autosomal recessive Alport syndrome; het – heterozygous; homo – homozygous; F – female; M – male

Supp. Table 7. Genotype-phenotype correlation in 4 individuals with ARAS: compound heterozygous and homozygous variants

Gene	Variant	Var No	Fam No., member, gender, ID	Age at diagnosis	Hematuria	Proteinuria (age at detection, amount)	CKD stage	KF- age at kidney failure	Ocular ab.	Hearing ab.
COL4A4	c.594+1G>A	9	7-62-I-M (A4/49716)[y]	7	+	++ (6)	I	none	+	+
	p.Gly527Cys (c.1579G>T)	10								
	p.Ala607Val (c.1820C>T) x2	28	38-I-F A4/4/283867[xy]	32	+	+++ (22)	V	+ (32)	+	+
COL4A3	p.Pro1568Ser (c.4702C>T)	23	21-I-F A3/3232145	34	+	+++ (23)	V	+ (33)	none	+
			21-I-F A3/3232146	34	+	+++ (30)	IIIb	none	none	+
	p.Gly1083Arg (c.3247G>C)	24	21-I-F A3/3232145	34	Sharing phenotype with p.Pro1568Ser (c.4702C>T) variant					
			21-I-F A3/3232146	34	Sharing phenotype with p.Pro1568Ser (c.4702C>T) variant					

Var – variant, No – number, Fam – family, M – male, F – female, Pro – proteinuria, y.o. – years of age, CKD – chronic kidney disease, KF – kidney failure, ab. – abnormalities. Proteinuria: +: PCR 15-49 mg/mmol, ++: PCR 50-299 mg/mmol, +++: ≥300 mg/mmol for adults, ≥200 mg/mmol for children

Supp. Table 8. Kidney biopsy findings in individuals with ARAS: compound heterozygous and homozygous variants

Gene	Variant	Var No	Fam No., member, gender, ID	KB (age at KB)	Foam cells	FSGS	Unspecific Ig M and C3 deposits in IF	IgA in IF	Thin GBM	Thickened and lamellated GBM	FPE
COL4A4	c.594+1G>A	9	7-62-I-M (A4/49716)	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
	p.Gly527Cys (c.1579G>T)	10									
	p.Ala607Val (c.1820C>T) x2	28	38-I-F A4/4/283867	+ (26)	-	-	-	-	+	+	+

Gene	Variant	Var No	Fam No., member, gender, ID	KB (age at KB)	Foam cells	FSGS	Unspecific Ig M and C3 deposits in IF	IgA in IF	Thin GBM	Thickened and lamellated GBM	FPE
COL4A3	p.Pro1568Ser (c.4702C>T)	23	21-I-F A3/3232145	+ (31)	-	+	+	-	+	+	+
			21-II-F A3/3232146	+ (32)	+	+	-	-	+	+	+
	p.Gly1083Arg (c.3247G>C)	24	21-I-F A3/3232145	Sharing phenotype with p.Pro1568Ser (c.4702C>T) variant							
			21-II-F A3/3232146	Sharing phenotype with p.Pro1568Ser (c.4702C>T) variant							

Var – variant; No – number; Fam – family; M – male; F – female; N/d – no data; FSGS – focal segmental glomerulosclerosis; IgM – immunoglobulin M; IgA – immunoglobulin A; KB- kidney biopsy; FPE – foot process effacement

Supp. Table 9. General characteristics of 4 individuals with digenic AS

ID	Family no	Member, gender	Age at diagnosis	Gene	Variant No	Nucleotide change	Protein change	Type of Digenic AS	Reported or novel variant
A5/46512	5	I-F	14	COL4A5	6	c.1417_1418del	p.Val473Glufs*3 Frameshift	X-linked similar inheritance	Novel
				COL4A4	7	c.4151C>T	p.Ala1384Val		Novel
A5/412818	8	I-F	39	COL4A4	11	c.2996G>A	p.Gly999Glu	X-linked similar inheritance	Reported
				COL4A5	12	c.1871G>A	p.Gly624Asp		Reported
A5/4262450	24	I-F	26	COL4A5	26	c.1374delinsTT	p.Pro459Serfs*6	X-linked similar inheritance	Novel
				COL4A4	27	c.4910G>A	p.Arg1637Gln		Novel
A3/438376	37	I-M	42	COL4A3	38	c.1021C>T	p.Arg341Cys	ADAS similar inheritance	Novel
				COL4A4	20	c.-101-4A>G	-		Reported

No – number; ADAS – autosomal dominant Alport syndrome; F – female; M – male.

Supp. Table 10. Genotype-phenotype correlation in 4 individuals with digenic AS

Gene	Variant	Var. No	Fam No., member, gender, ID	Age at diagnosis	Hematuria	Pro (age at detection, amount)	CKD stage	KF- age at kidney failure	Ocular ab.	Hearing ab.
COL4A5	p.Val473Glufs*3 (c.1417_1418del)	6	5-I-F A5/46512[y]	14	+	+ (14)	I	none	none	none
COL4A4	p.Ala1384Val (c.4151C>T)	7								
COL4A4	p.Gly999Glu (c.2996G>A)	11	8-I-F A5/412818[xy]	39	+	++ (22)	II	none	+	+
COL4A5	p.Gly624Asp (c.1871G>A)	12								
COL4A5	p.Pro459Serfs*6 (c.1374delinsTT)	26	24-I-F A5/4262450[y]	26	+	+ (16)	I	none	none	none
COL4A4	p.Arg1637Gln (c.4910G>A)	27								
COL4A3	p.Arg341Cys (c.1021C>T)	38	37-I-M A3/438376	42	+	+ (35)	I	none	none	none
COL4A4	c.-101-4A>G	20								

Var – variant; No – number; Fam – family; M – male; F – female; Pro – proteinuria; y.o. – years of age; CKD – chronic kidney disease; KF – kidney failure; ab. – abnormalities. Proteinuria: +: PCR 15-49 mg/mmol, ++: PCR 50-299 mg/mmol, +++: ≥300 mg/mmol for adults, ≥200 mg/mmol for children

Supp. Table 11. Kidney biopsy findings in 3 of 4 individuals with digenic AS

Gene	Variant	Variant No	Fam No., member, gender, ID	KB (age at KB)	Foam cells	FSGS	Unspecific IgM and C3 deposits in IF	IgA in IF	Thin GBM	Thickened and lamellated GBM	FPE
COL4A5	p.Val473Glufs*3 (c.1417_1418del)	6	5-I-F A5/46512	+ (14)	-	+	-	-	+	+	+
COL4A4	p.Ala1384Val	7									

Gene	Variant	Variant No	Fam No., member, gender, ID	KB (age at KB)	Foam cells	FSGS	Unspecific IgM and C3 deposits in IF	IgA in IF	Thin GBM	Thickened and lamellated GBM	FPE
	(c.4151C>T)										
`COL4A4	p.Gly999Glu (c.2996G>A)	11	8-I-F A5/412818	+ (37)	+	-	-	+	+	+	+
COL4A5	p.Gly624Asp (c.1871G>A)	12									
COL4A5	p.Pro459Serfs*6 (c.1374delinsTT)	26	24-I-F A5/4262450	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
COL4A4	p.Arg1637Gln (c.4910G>A)	27									
COL4A3	p.Arg341Cys (c.1021C>T)	38	37-I-M A3/438376	+ (40)	-	-	-	-	+	-	+
COL4A4	c.-101-4A>G	20									

Var – variant; No – number; Fam – family; M – male; F – female; N/d – no data; FSGS – focal segmental glomerulosclerosis; IgM – immunoglobulin M; IgA – immunoglobulin A; KB- kidney biopsy; FPE – foot process effacement

Supp. Table 12. General characteristics of 58 individuals with XLAS: heterozygous or hemizygous variants in *COL4A5*

ID	Family no	Member, gender	Age at diagnosis	Gene	Variant No	Nucleotide change	Protein change	Type	Reported or novel variant
A5111	1	I-F	45	*COL4A5*	1	c.2297G>A	p.Gly766Asp	missense Gly	Reported
A5112		II-M	17						
A5113		III-M†	19						
A5224	2	I-M	38	*COL4A5*	2	c.902G>A	p.Gly301Asp	missense Gly	Reported
A5225		II-F	3						
A58615	6	I-M	14	*COL4A5*	8	c.4994+3A>G	-	splice site	Reported
A5121226	12	I-M	18						
A5121227		II-M	24						

ID	Family no	Member, gender	Age at diagnosis	Gene	Variant No	Nucleotide change	Protein change	Type	Reported or novel variant
A5121228		III-F	45	*COL4A5*	12	c.1871G>A	p.Gly624Asp	missense Gly	Reported
A5121229		IV-M	39						
A5122348	23	I-M	38						
A5122349		II-F	66						
A5122373		III-F	67						
A5123361	33	I-M	47						
A5123362		II-F	44						
A5123560	35	I-M	42						
A5123564		II-F	18						
A5124183	41	I-M	68						
A5124488	44	I-M	60						
A5124692	46	I-F	56						
A5125199	51	I-M	33						
A51252101	52	I-M	49						
A51253102	53	I-M	35						
A51256107	56	I-M	60						
A51259112	59	I-F	16						
A51259113		II-F	49						
A513919	9	I-F	1	*COL4A5*	13	c.2623G>A	p.Gly875Arg	missense Gly	Novel
A513920		II-M	26						
A513921		III-F	46						
A513922		IV-F†	66						
A5131379	13	I-M	2						
A5131380		II-F	31						
A5171531	15	I-F	43	*COL4A5*	17	c.3685G>A	p.Gly1229Ser	missense Gly	Reported
A5171532		II-M	17						
A5171533		III-M	12						

ID	Family no	Member, gender	Age at diagnosis	Gene	Variant No	Nucleotide change	Protein change	Type	Reported or novel variant
A5171534		IV-F	42						
A5302755	27	I-F	30	*COL4A5*	30	c.1032+1G>C	-	splice site	Reported
A5336	3	I-F	12	*COL4A5*	3	c.3508G>C	p.Gly1170Arg	missense Gly	Novel
A5337		II-F	43						
A5338		III- M	17						
A5449	4	I-M	11	*COL4A5*	4	3106+1G>A	-	splice site	Novel
A54410		II-F	4						
A54490		III-F	37						
A56511	5	I-F	36	*COL4A5*	6	c.1417_1418del	p.Val473Glufs*3	frame shift	Novel
A56513		II-F	34						
A56514		II-F	28						
A5141023	10	I-M	7	*COL4A5*	14	c.347delC	p.Pro116Glnfs*39	frame shift	Novel
A5141024		II-F	35						
A5181635	16	I-M	23	*COL4A5*	18	c.883G>A	p.Gly295Ser	missense Gly	Novel
A5211939	19	I-M	28	*COL4A5*	21	c.2777G>T	p.Gly926Val	missense Gly	Novel
A5211940		II-M	29						
A5211941		III-F	52						
A5262451	24	I-M	21	*COL4A5*	26	c.1374delinsTT	p.Pro459Serfs*6	frame shift	Novel
A5262452		II-F	52						
A5333058	30	I-F	10	*COL4A5*	33	c.3554-2A>G	-	splice site	Novel
A5333078		II-F	14						
A5343159	31	I-F	15	*COL4A5*	34	c.466G>C	p.Gly156Arg	missense Gly	Novel

No – number; ARAS – autosomal recessive Alport syndrome; het – heterozygous; homo- homozygous; F-female; M-male; † - deceased person

Supp. Table 13. Genotype-phenotype correlation of 58 individuals with XLAS: heterozygous or hemizygous variants in *COL4A5*

Variant	Var No	Fam No., member, gender	Age at diagnosis	Hematuria	Pro (age at detection, amount)	CKD stage	KF- age at kidney failure	Ocular ab.	Hearing ab.
COL4A5									
p.Gly766Asp (c.2297G>A)	1	1-I-F	45	+	+ (45)	I	none	none	none
		1-II-M	17	+	++ (15)	V	+(19)	none	none
		1-III-M†	19	+	++ (16)	IIIa	none	N/d	N/d
p.Gly301Asp (c.902G>A)	2	2-I-M	38	+	+++ (1)	IIIa	none	none	+
		2-II-F	3	+	+ (1)	I	none	none	N/d
c.4994+3A>G	8	6-I-M	14	+	++ (4)	II	none	none	+
p.Gly624Asp (c.1871G>A)	12	11-I-F	46	+	none	I	none	none	none
		12-I-M	18	+	none	I	none	none	none
		12-II-M	24	+	+ (29)	I	none	none	none
		12-III-F	45	+	+ (50)	I	none	none	none
		12-IV-M	39	+	+ (43)	I	none	none	none
		23-I-M	38	+	+ (7)	IIIa	none	none	+
		23-II-F	66	+	+ (30)	IIIa	none	none	none
		23-III-F	67	+	+ (67)	IIIa	none	none	+
		33-I-M	47	+	+++ (36)	IV	none	none	+
		33-II-F	44	+	++ (37)	I	none	none	none
		35-I-M	42	+	+ (40)	I	none	none	+
		35-II-F	18	+	+ (13)	I	none	none	none
		41-I-M	68	+	++ (67)	IV	none	none	+
		44-I-M	60	+	+ (34)	II	none	+	+
		46-I-F	56	+	++ (51)	I	none	+	+
		51-I-M	33	+	++ (15)	IIIb	none	none	+
		52-I-M	49	+	+(49)	II	none	none	none
		53-I-M	35	+	none	I	none	none	none
		56-I-M	60	+	+ (60)	IIIb	none	none	+

Variant	Var No	Fam No., member, gender	Age at diagnosis	Hematuria	Pro (age at detection, amount)	CKD stage	KF- age at kidney failure	Ocular ab.	Hearing ab.
		59-I-F	16	+	none	II	none	none	none
		59-II-F	49	+	none	IIIa	none	N/d	N/d
p.Gly875Arg (c.2623G>A)	13	9-I-F	1	+	+ (1)	I	none	none	N/d
		9-II-M	26	+	++ (14)	V	+ (20)	+	+
		9-III-F	46	+	++ (42)	V	+ (43)	none	none
		9-IV-F†	66	+	++ (20)	V	+ (60)	N/d	N/d
		13-I-M	2	+	none	I	none	N/d	N/d
		13-II-F	31	+	+ (29)	I	none	N/d	N/d
p.Gly1229Ser (c.3685G>A)	17	15-I-F	43	+	++ (25)	II	none	none	none
		15-II-M	17	+	none	I	none	none	none
		15-III-M	12	+	+ (11)	I	none	none	none
		15-IV-F	42	+	+ (25)	II	none	none	none
c.1032+1G>C	30	27-I-F	30	+	+++ (22)	II	none	none	none
p.Gly1170Arg (c.3508G>C)	3	3-I-F	12	+	+ (6)	I	none	none	none
		3-II-F	43	+	++ (25)	II	none	none	none
		3-III-M	17	+	+++ (7)	IIIb	none	none	none
3106+1G>A	4	4-I-M	11	+	+ (3)	I	none	+	+
		4-II-F	4	+	none	I	none	none	none
		4-III-F	37	+	+ (37)	II	none	none	none
p.Val473Glufs*3 (c.1417_1418del)	6	5-I-F	36	+	+ (21)	IIIa	none	none	+
		5-II-F	34	+	none	I	none	none	none
		5-III-F	28	+	+ (32)	I	none	none	none
p.Pro116Glnfs*39 (c.347delC)	14	10-I-M	7	+	++ (3)	I	none	N/d	+
		10-II-F	35	+	none	I	none	none	none
p.Gly295Ser (c.883G>A)	18	16-I-M	23	+	++ (13)	I	none	none	none
p.Gly926Val	21	19-I-M	28	+	+++ (6)	V	+ (31)	+	+
		19-II-M	29	+	+++ (2)	V	+ (24)	+	+

Variant	Var No	Fam No., member, gender	Age at diagnosis	Hematuria	Pro (age at detection, amount)	CKD stage	KF- age at kidney failure	Ocular ab.	Hearing ab.
(c.2777G>T)		19-III-F	52	+	+ (19)	I	none	none	none
p.Pro459Serfs*6 (c.1374delinsTT)	26	24-I-M	21	+	++ (5)	I	none	+	+
		24-II-F	52	+	+ (18)	I	none	none	none
c.3554-2A>G	33	30-I-F	10	+	+ (7)	I	none	-	none
		30-II-F	14	+	+ (14)	I	none	N/d	none
p.Gly156Arg (c.466G>C)	34	31-I-F	15	+	+++ (15)	IIIb	none	none	none

Var – variant; No – number; Fam – family; M – male; F – female; Pro – proteinuria; y.o. – years of age; CKD – chronic kidney disease; KF – kidney failure; ab. – abnormalities; N/d – no data. Proteinuria: +: PCR 15-49 mg/mmol, ++: PCR 50-299 mg/mmol, +++: ≥300 mg/mmol for adults, ≥200 mg/mmol for children

Supp. Table 14. Kidney biopsy findings in individuals with XLAS: heterozygous or hemizygous variants in *COL4A5*

Variant	Variant No	Fam No., member, gender	Kidney biopsy (age at kidney biopsy)	Foam cells	FSGS	Unspecific immunoglobulin M and C3 deposits in IF	IgA immunoglobulins in IF	Thin GBM	Thickened and lamellated GBM	Foot process effacement
COL4A5										
p.Gly766Asp (c.2297G>A)	1	1-I-F	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
		1-II-M	+ (17)	-	+	-	-	+	+	+
		1-III-M	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
p.Gly301Asp (c.902G>A)	2	2-I-M	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
		2-II-F	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
c.4994+3A>G	8	6-I-M	+ (11)	-	-	-	-	+	+	+
		11-I-F	+ (46)	N/d	+	-	-	N/d	N/d	N/d
		12-I-M	+ (15)	N/d	-	N/d	+	N/d	N/d	N/d
		12-II-M	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
		12-III-F	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
		12-IV-M	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
		23-I-M	+ (35)	+	+	-	-	+	+	+

Variant	Variant No	Fam No., member, gender	Kidney biopsy (age at kidney biopsy)	Foam cells	FSGS	Unspecific immunoglobulin M and C3 deposits in IF	IgA immunoglobulins in IF	Thin GBM	Thickened and lamellated GBM	Foot process effacement
p.Gly624Asp (c.1871G>A)	12	23-II-F	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
		23-III-F	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
		33-I-M	+ (46)	+	+	-	-	N/d	N/d	N/d
		33-II-F	+ (40)	-	+	-	-	+	-	+
		35-I-M	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
		35-II-F	+ (13)	-	-	-	-	+	-	+
		41-I-M	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
		44-I-M	+ (49)	-	-	-	-	+	-	+
		46-I-F	+ (55)	-	-	-	+	+	+	+
		51-I-M	+ (32)	-	+	-	-	+	+	+
		52-I-M	+ (47)	N/d	-	-	-	N/d	N/d	N/d
		53-I-M	+ (35)	-	-	-	-	+	-	+
		56-I-M	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
		59-I-F	+ (15)	-	-	-	-	+	-	+
		59-II-F	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
p.Gly875Arg (c.2623G>A)	13	9-I-F	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
		9-II-M	+ (18)	+	-	-	-	+	+	+
		9-III-F	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
		9-IV-F	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
		13-I-M	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
		13-II-F	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
p.Gly1229Ser (c.3685G>A)	17	15-I-F	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
		15-II-M	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
		15-III-M	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
		15-IV-F	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
c.1032+1G>C	30	27-I-F	+ (29)	-	-	+	+	+	+	+

Variant	Variant No	Fam No., member, gender	Kidney biopsy (age at kidney biopsy)	Foam cells	FSGS	Unspecific immunoglobulin M and C3 deposits in IF	IgA immunoglobulins in IF	Thin GBM	Thickened and lamellated GBM	Foot process effacement
p.Gly1170Arg (c.3508G>C)	3	3-I-F	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
		3-II-F	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
		3-III-M	+ (16)	+	+	-	-	+	+	+
3106+1G>A	4	4-I-M	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
		4-II-F	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
		4-III-F	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
p.Val473Glufs*3 (c.1417_1418del)	6	5-I-F	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
		5-II-F	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
		5-III-F	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
p.Pro116Glnfs*39 (c.347delC)	14	10-I-M	+ (6)	-	-	-	-	+	+	+
		10-II-F		N/d	N/d	N/d	N/d	N/d	N/d	N/d
p.Gly295Ser (c.883G>A)	18	16-I-M	+ (22)	-	-	-	-	+	+	+
p.Gly926Val (c.2777G>T)	21	19-I-M	+ (20)	+	-	-	-	N/d	N/d	N/d
		19-II-M	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
		19-III-F	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
p.Pro459Serfs*6 (c.1374delinsTT)	26	24-I-M	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
		24-II-F	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
c.3554-2A>G	33	30-I-F	+ (9)	-	+	-	-	+	+	+
		30-II-F	none	N/d	N/d	N/d	N/d	N/d	N/d	N/d
p.Gly156Arg (c.466G>C)	34	31-I-F	+ (14)	-	+	-	-	+	+	+

Var – variant; No – number; Fam – family; M – male; F – female; N/d – no data; FSGS – focal segmental glomerulosclerosis; IgM – immunoglobulin M; IgA – immunoglobulin A; KB- kidney biopsy; FPE – foot process effaceme

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Agnė Kerpauskienė (Čerkauskaitė) graduated from the Faculty of Medicine of Vilnius University in 2014 and obtained qualification of adult nephrology in 2018.

Dr. Kerpauskienė has been training at Nephrology Department at CHRU Claude Huriez Hospital in Lille, France, and Abdominal Surgery Department at Università degli Studi di Roma "La Sapienza" in Rome, Italy. In 2016 Agnė had her internship in basics of genetics at the University of Rostock, Centogene Disease Diagnostic Centre in Germany. From 2017 Dr. Kerpauskienė worked as a senior resident doctor in Paris, France, at the Centre for Genetics and Metabolic Diseases at the University of Versailles with prof. Dominique Germain.

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PUBLICATION LIST

Publications related to the results of the theses

1. Cerkauskaite A et al. Identification of 27 novel variants in genes COL4A3, COL4A4 and COL4A5 in Lithuanian families with Alport syndrome. Front Med (Lausanne). 2022 Mar 28; 9:859521. doi: 10.3389/fmed.2022.859521. ECollection 2022. Q1. IF 5.901.
2. Savige J, <...>, Cerkauskaite A et al. Digenic Alport syndrome – additional pathogenic heterozygous variants worsen X-linked disease in females and autosomal dominant disease. Clin J Am Soc Nephrol. 2022 Jun 8; CJN.03120322. doi: 10.2215/CJN.03120322.
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PRESENTATION LIST

Abstracts related to thesis topic

1. Cerkauskaite A et al. "Alport syndrome genotype-phenotype correlations in Lithuanian cohort" – RARD symposium, 19-22 June 2019, Bogota, Colombia
2. Cerkauskaite A et al. "Alport syndrome genotype-phenotype correlations in Lithuanian families" – international congress of American Society of Nephrology (ASN) 2019, 5-10 November 2019, Washington, USA.
3. Cerkauskaite A. et al. „Challenges in differential diagnosis of Alport syndrome" – international virtual conference „ XV Baltic nephrology congress" 11-12 December 2020. First place award for the best abstract https://www.lndta.lt/baltijos-saliu-xv-konferencijos-jaunuju-mokslininku-konkurso-nugaletojai/.
4. Janonyte K, Cerkauskaite A et al. „Identification of eighteen novel mutations in *COL4A3, COL4A4, COL4A5* genes in Lithuanian families with Alport syndrome "; International Health Sciences Conference, 29-30 April 2021, Kaunas, Lithuania (I conference abstract book: Kaunas, Lithuanian University of Health Sciences, 2021, p. 80-81).
5. Bagdonaviciute Karolina, Bieksaitė Emilija, Cerkauskaite Agne „Genotype – phenotype correlation in 37 Lithuanian families with Alport syndrome"; International Health Sciences Conference, 29-30 April 2021, Kaunas, Lithuania (I conference abstract book: Kaunas, Lithuanian University of Health Sciences, 2021, p. 80-81).
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1. Cerkauskaite A. „Genotype-phenotype correlation in Lithuanian population: one centre analysis "– ERKNet meeting for Expert of Alport syndrome: „Alport mutation meeting "6 Feb 2020, London, UK
2. Cerkauskaite A. „Challenges in differential diagnosis of Alport syndrome" – international virtual conference „ XV Baltic nephrology congress" 11-12 December 2020.
3. Cerkauskaite A. „Alport syndrome: a Lithuanian perspective "– new all-virtual, interactive international conference for the Alport syndrome community in Nordic-Baltic region "The 1st Alport Syndrome Club Meeting"10 June 2021. Vilnius, Lithuania.
4. Cerkauskaite A. „ News in glomerulopathies 2021: Alport syndrome world ": ERKNet, 20 December 2022
5. Agne Cerkauskaite-Kerpauskiene. "Importance of molecular analysis in collagen alpha-345 nephropathies." International young scientists congress for clinical and basic science in nephrology "Junger Niere," held on May 11-12, 2023. The research received the first-place award for the best scientific research.

AKNOWLEDGEMENTS

I would like to express my gratitude to my scientific supervisor, Prof. Dr. Arvydas Laurinavičius, for his guidance and support throughout my dissertation. I am also extremely grateful to my unofficial scientific consultant, Assoc. Prof. Dr. Edita Kazėnaitė, for enabling this research and providing limitless personal and academic support. Additionally, I would like to thank Prof. Dr. Marius Miglinas for our valuable professional conversations, and personal support in my academic journey. I am also thankful to my reviewers, Dr. Karolis Ažukaitis, Prof. Dr. Eglė Preikšaitienė, and Assoc. Prof. Dr. Justinas Besusparis, for their valuable feedback and recommendations.

I am grateful to my colleagues and the rest of the research team for providing me with the opportunity to conduct this research and for their resources and support. A special thanks goes to my kind mentor, Prof. Dr. Judy Savige and her team, and the UK Alport Syndrome Workshop organization for allowing me to be part of their incredible network of leaders and researchers.

I would like to express my sincere gratitude to my dear Mom, Prof. Dr. Rimantė Čerkauskienė, for her enormous personal and scientific support and for her words: “Never give up, be strong and go further,” and to my Dad Valdas, for his love and motivational words.

To my beloved husband Aurimas, I am deeply thankful for his love, patience, support, and unwavering happiness even throughout the challenging moments of this thesis process. I also extend my immense gratitude to my entire family and dear friends for their love and support, as without them, I would not have been able to complete this journey.

Finally, I would like to thank all the participants in my study for their invaluable contribution and willingness to share their experiences. Your involvement has greatly contributed to the success of this thesis. Thank you for your time and contribution.

I am grateful to everyone who has supported and guided me throughout this process. Your help has made this thesis possible.

NOTES

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