Annals of human genetics

Pseudodominant Alport syndrome caused by pathogenic homozygous and compound heterozygous COL4A3 splicing variants

Journal:	*Annals of Human Genetics*
Manuscript ID	AHG-SC-21-0399.R2
Wiley - Manuscript type:	Short Communication
Date Submitted by the Author:	16-Nov-2021
Complete List of Authors:	MOHAMED, MAHA; Newcastle University TELLEZ, JAMES; Newcastle University BERGMANN, CARSTEN; Albert-Ludwigs-Universitat Freiburg Medizinische Fakultat Gale, Daniel; University College London Department of Renal Medicine Sayer, John; Newcastle University OLINGER, ERIC; Newcastle University Faculty of Medical Sciences
Keywords:	Alport syndrome, COL4A3, chronic kidney disease, genetic counselling, NGS, haematuria

Pseudodominant Alport syndrome caused by pathogenic homozygous and compound heterozygous *COL4A3* splicing variants

MAHA MOHAMED[1], JAMES TELLEZ[2], CARSTEN BERGMANN[3], DANIEL P. GALE[4], JOHN A. SAYER[1,5,6] and ERIC OLINGER[5]

[1]Renal Services, Newcastle Upon Tyne Hospitals NHS Foundation Trust, Newcastle upon Tyne, NE7 7DN, United Kingdom

[2]Northern Genetics Service, Newcastle upon Tyne Hospitals NHS Foundation Trust, Central Parkway, Newcastle upon Tyne NE1 3BZ, United Kingdom

[3]Department of Medicine IV, Faculty of Medicine, Medical Center-University of Freiburg, Freiburg, Germany and Medizinische Genetik Mainz, Mainz, Germany

[4]Department of Renal Medicine, Royal Free Hospital, University College London, London, United Kingdom

[5]Translational and Clinical Research Institute, Faculty of Medical Sciences, Newcastle University, Central Parkway, Newcastle upon Tyne, NE1 3BZ, United Kingdom

[6]NIHR Newcastle Biomedical Research Centre, Newcastle upon Tyne, NE4 5PL, United Kingdom

Corresponding author:
Prof John A. Sayer, Professor of Renal Medicine
Translational and Clinical Research Institute, Faculty of Medical Sciences, Newcastle University, Central Parkway, Newcastle upon Tyne, NE1 3BZ, United Kingdom
Email: john.sayer@newcastle.ac.uk

Running title: Pseudodominant Alport syndrome

Abstract

Alport syndrome is a genetic disorder affecting the basement membranes of the kidney, ear and eye, and represents a leading cause of monogenic kidney disease. Alport syndrome is genetically heterogeneous with three key genes involved (*COL4A3-5*) and several transmission patterns, including monogenic X-linked, autosomal recessive/dominant and digenic. We report a consanguineous family where 13 individuals presented variable features of Alport syndrome including kidney failure on two generations and male-to-male transmission, suggesting autosomal dominant inheritance. *COL4A3-5* gene panel analysis surprisingly reveals two distinct, confirmed splice-altering variants in *COL4A3* (NM_000091.4: c.1150+5G>A and c.4028-3C>T) present in homozygous or compound heterozygous state in individuals with kidney failure. This adds a further mode of transmission for Alport syndrome where, in a consanguineous family, the independent segregation of two variants at the same locus may create a pseudodominant transmission pattern. These findings highlight the importance of a molecular diagnosis in Alport syndrome for genetic risk counselling, given the variable modes of inheritance, but also the pitfalls of assuming identity by descent in consanguineous families.

Keywords:

Alport syndrome, *COL4A3*, chronic kidney disease, genetic counselling, massively parallel sequencing, haematuria

Introduction

The implementation of massively parallel sequencing (MPS) in nephrology led to improvements in diagnosis, risk stratification, therapy and genetic counselling (Groopman et al. 2019). Paradigmatic for the impact of MPS on disease ontology is Alport Syndrome, a clinically and genetically heterogeneous nephropathy characterised by haematuria, proteinuria, progressive chronic kidney disease (CKD), sensorineural deafness and ocular abnormalities (Kashtan et al. 2018). Alport syndrome is the second most common cause of monogenic kidney disease affecting as much as 1 in 5,000-10,000 individuals (Groopman et al. 2019). It is caused by pathogenic variants in *COL4A3, COL4A4* and *COL4A5* that encode type IV collagen α3, α4, and α5 chains forming a triple helix that constitutes a critical structural component of basement membranes of the glomerulus, the inner ear, the lens and the retina (Kashtan et al. 2018). The genetics of Alport syndrome are complex with several modes of inheritance: semidominant X-linked Alport syndrome (XLAS, MIM# 301050) for *COL4A5* as well as autosomal recessive (ARAS, MIM# 203780) and dominant (ADAS) for both *COL4A3* (MIM# 104200) and *COL4A4*. Finally, digenic inheritance with variants in different *COL4A3-5* genes has been described (Furlano et al. 2021). XLAS is probably the most prevalent cause of kidney failure among Alport subtypes and males with XLAS or biallelic individuals with ARAS usually exhibiting more severe disease while heterozygous females with pathogenic *COL4A5* variants are generally more mildly affected (Savige et al. 2016). Monallelic pathogenic *COL4A3/COL4A4* variants lead to a wide spectrum of manifestations, ranging from completely asymptomatic, or isolated haematuria to progressive CKD with extrarenal manifestations, variously referred to as autosomal dominant thin basement membrane nephropathy or ADAS (Furlano et al. 2021; Gale 2013; van der Loop et al. 2000). The reasons for this phenotypic spectrum is unclear but modifying genetic or environmental factors have been proposed (Furlano et al. 2021). Pogression to kidney failure is less likely (and occurs later) than in XLAS or ARAS (Furlano et al. 2021). Autosomal and X-linked Alport syndrome manifest thus evidence for semidominant inheritance, where homozygotes, compound heterozygotes or hemizygotes for mutant

alleles are generally more severely affected than are heterozygotes. Additionally, MPS approaches identified *COL4A3-5* variants in individuals with bilateral renal cysts or FSGS with nephrotic range proteinuria (Gulati et al. 2020; Malone et al. 2014). This genetic and phenotypic heterogeneity explains the poor correlation between the *a priori* clinical suspicion for Alport syndrome and the final yield of molecular diagnoses. Furthermore, a reliable initial assessment of the mode of inheritance is often not possible (Fallerini et al. 2014; Groopman et al. 2019). Ergo, molecular diagnostics assessing all *COL4A3-5* genes play a paramount role in individuals suspected to have Alport syndrome, even when not fulfilling all clinical criteria, as they allow (i) a precise molecular diagnosis and genetic counselling, (ii) prognostic considerations and (iii) adequate early treatment and inclusion in clinical trials and they can sometimes replace invasive kidney and skin biopsy (Savige et al. 2013).

Here, we present a multiplex consanguineous family with features of Alport syndrome including kidney failure on 2 subsequent generations and male-to-male transmission where, surprisinlgy, MPS-based diagnostics revealed the correct diagnosis to be ARAS. This adds to the genetic heterogeneity of Alport syndrome and highlights multiple pitfalls when studying consanguineous families as well as the importance of a molecular diagnosis in Alport syndrome.

Results

We report a four generation family of Pakistani origin including 13 affected individuals with variable phenotypes including haematuria, proteinuria, CKD, kidney failure and severe hearing impairment (Figure 1A). At least two consanguineous marriages between first degree cousins occurred. Isolated haematuria was seen in three adult individuals, of both genders. The presence of haematuria, proteinuria, hearing impairment and CKD, including kidney failure, was present in 6 family members, 1 male and 5 females, from 2 consecutive generations. (Figure 1A and Table 1). Ophthalmological examination revealed mild changes in 3 family members (Table 1). Deceased individual III.3 was reported severely affected but the precise phenotypic details are unknown. One family member with CKD stage 3B (IV:4, at age 28 years) had a native kidney biopsy which showed focal segmental glomerulosclerosis and chronic parenchymal damage on light microscopy. Electron microscopy showed glomerular basement membrane thinning (Figure 1B) with occasional lamellation and splitting, consistent with Alport syndrome. Kidney failure in two consecutive generations with females and males equally affected, and male-to-male transmission, suggested autosomal dominant Alport syndrome. Disease manifestations in both mothers and fathers in generation III and the degree of consanguinity injected however some uncertitude into this assumption.

We performed MPS panel analysis of the *COL4A3-5* genes in individual IV:7 and identified two distinct variants in *COL4A3* (NM_000091.4): c.1150+5G>A and c.4028-3C>T. Both variants are rare and c.1150+5G>A, but not c.4028-3C>T, is predicted to affect splicing (Table 1 and Supplementary Figure 1). Segregation of the variants was performed in 10 additional family members using exon-PCR and Sanger sequencing and the genotype was inferred in three family members. For both pairs of parents in generation III, 1 spouse was homozygous (or inferred homozygous) for the variant c.1150+5G>A and the other spouse heterozygous for the variant c.4028-3C>T. Six of their eight collective offspring had compound heterozygous changes and 2 were heterozygous for c.1150+5G>A (Figure 1A). In total, six out of eight homozygous or compound heterozygous individuals presented all

four clinical features, i.e., haematuria, proteinuria, hearing impairment and CKD progressing to kidney failure in 5 among them. Onset of kidney failure ranged between 21 and 40 years. Conversely, 4 heterozygous carriers (2 for each allele) presented with isolated haematuria and in addition hearing impairment in 1 of them, but no evidence of progressive CKD (Figure 1C, Supplementary Figure 2 and Table 1).

We evaluated the effects on splicing of both variants using whole blood-derived RNA from individual IV.4 and were able to confirm the predicted skipping of exon 20 for variant c.1150+5G>A and could also demonstrate acceptor site loss and skipping of exon 46 for variant c.4028-3C>T. Both of these events lead to in-frame deletions within the collagenous domain of *COL4A3* (Figure 2 and Table 1). Evaluation of the two variants using the ACMG/AMG criteria as well as the modified criteria for Alport syndrome (Savige et al. 2021) conclude that both variants are pathogenic (Supplementary Table 1).

Discussion

Alport syndrome is genetically complex as 3 genes are implicated in different modes of inheritances: X-linked semidominant for *COL4A5*, autosomal recessive and dominant for *COL4A3* and *COL4A4*. In addition, phenotypic variability is partly due to more complex patterns such as digenic inheritance (Fallerini et al. 2017) or modifier non-*COL4* genes (Voskarides et al. 2012). Here we add to this heterogeneity by presenting a further inheritance pattern where the segregation of 2 different *COL4A3* splice-affecting variants in a consanguineous family creates an apparent dominant transmission of kidney failure of what is, in fact, an autosomal recessive trait in this family. Thereby, we highlight (i) the role of unbiased genetic testing taking into account more complex modes of transmission, (ii) the importance of a molecular diagnosis for individual genetic risk counselling in Alport syndrome, and (iii) the pitfalls of assuming identity by descent in consanguineous families.

Pseudodominance is defined as the occurrence of an autosomal recessive trait present in individuals in two or more generations of a family, thereby appearing to follow a dominant inheritance pattern. Pseudodominant inheritance in consanguineous families has been described in other recessive kidney diseases such as nephronophthisis (Hoefele et al. 2011) where heterozygotes are generally phenotypically unaffected. To the best of our knowledge, this is the first report of pseudodominant inheritence in Alport syndrome due to recessive variants in the same gene, indicating that this is either a rare or underreported event. Although transmission of kidney failure in this family is autosomal recessive, since heterozygotes exhibit isolated haematuria (and are likely to be at increased risk of renal impairment in later life (Gale 2013)) it was not possible to establish the correct mode of transmission without complete molecular information. In line, current guidelines for Alport syndrome recommend genetic testing to establish the correct mode of inheritance and to favor cascade genetic testing to assess at risk family members (Savige et al. 2019, 2021). It is important to notice that apparent dominant inheritance has been observed in digenic cases with *COL4A3* and *COL4A4* variants *in cis* (Fallerini et al. 2017). However, the risk of transmitting both variants together in this constellation is 50%, while there is minimal risk of

biallelic transmission to the next generation in our pedigree, provided that mating occurs with a *COL4A3* wildtype partner. Families where early (<50 years) onset of kidney failure with clinical and histological features of Alport syndrome are seen segregating as an autosomal dominant trait in multiple individuals need to be evaluated with great care before attributing this to a monoallelic disorder. Indeed, our report illustrates that risk to off-spring of affected Alport syndrome individuals is very different if the disease is actually biallelic.

We can only speculate about the origin of the 2 *COL4A3* alleles in our family. One scenario involves presence of both alleles at heterozygous state in the common ancestors from generation I. Alternatively, individuals II.1 and II.5 may be related, both introducing the allele c.4028-3C>T in the two branches of this family. Finally, we can infer that both parents II.3 and II.4 are heterozygous for c.1150+5G>A, that they therefore likely share a common ancestor that we are not aware of and thus introduce an additional loop of consanguinity leading to full phenotypic disease expression in generation III. Recent estimates suggest the presence of predicted pathogenic splice variants in 1:2000 south Asian individuals (Gibson et al. 2021) and no canonical splicing variant occurred more than twice in the gnomAD dataset. Therefore, the observation of multigeneration kidney failure in young adults associated with the presence of 2 distinct and independently segregating splice-affecting alleles is noteworthy and highlights the challenges and surprises when investigating consanguineous families for a genetically heterogeneous disease. Interestingly, a recent survey in the Genome Aggregation Database (gnomAD) estimated an overall population frequency of heterozygous predicted pathogenic variants in *COL4A3* or *COL4A4* of 0.94% (1:106) with highest prevalences in in people of Latino (in 1.58%), East Asian (1.34%) or African (1.28%) ancestries (Gibson et al. 2021). Given these high population prevalences, a pseudodominant inheritance pattern of two pathogenic variants might not be such a rare occurrence, especially in certain populations, and might account for some families that were labelled ADAS and where kidney failure occurred in several generations.

Finally, we also want to stress the importance of verifying potential disease-causing variants arising from MPS. *In silico* predictions stated that c.4028-3C>T was unlikely to affect

splicing. A variant affecting the some position (c.4028-3C>A) has been reported before in 3 heterozygous individuals, 2 with isolated haematuria and 1 with proteinuria and kidney failure, and its effect on splicing demonstrated (Bullich et al. 2015). Here, we could show that also c.4028-3C>T leads to exon 46 skipping. The second variant c.1150+5G>A is predicted to lead to exon 20 skipping and has been reported before in the Leiden Open Variation database as VUS. Using patient-derived RNA, we could confirm exon 20 skipping. In both instances, an inframe deletion of 42 amino acids (exon 46) or 12 amino acids (exon 20) within the collagenous domain is predicted to remove 12 and 4 Gly-X-Y repeats, respectively (Mariyama et al. 1994). Consistent with earlier reports of in-frame deletions within the collagenous domain (van der Loop et al. 2000), we provide evidence that both variants identified in our family are deleterious.

Ethical Approval

This study was approved by the North East - Newcastle & North Tyneside 1 Research Ethics Committee (18/NE/350). All included individuals provided informed and written consent.

Data Availability Statement

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials. Further phenotypic or sequencing data are available from the corresponding author (JAS), upon reasonable request. The identified genetic variant in *COL4A3* have been submitted to LOVD (#0000478196 and #0000478197).

Acknowledgements

We sincerely thank the affected individuals and members of this family for their support and informed written consent to publish this study.

Conflicts of Interest

CB holds a part-time faculty appointment at the University of Freiburg in addition to his employment with the Limbach Group for which he heads and manages Limbach Genetics GmbH. The other authors declare no conflicts of interest

Author Contributions

Conception of the study: MM, DPG, CB, JAS, EO; data acquisition: MM, JT, CB, DPG, JAS; data interpretation: MM, JT, JAS, EO; draft writing: MM, EO, JAS; manuscript editing: MM, JT, CB, DPG, JAS, EO. All authors approved the final version of the manuscript.

Funding

Maha Mohamed received funding and support from Health Education England (HEE) and NHS England Genomic education programme. Eric Olinger is supported by an Early

Postdoc Mobility Stipendium of the Swiss National Science Foundation (P2ZHP3_195181) and Kidney Research UK (Paed_RP_001_20180925).

CB's research lab receives support from the Deutsche Forschungsgemeinschaft (DFG) (BE 3910/8-1, BE 3910/9-1, and Collaborative Research Center SFB 1453) and the Federal Ministry of Education and Research (BMBF, 01GM1903I and 01GM1903G).

John Sayer is funded by Kidney Research UK and the Northern Counties Kidney Research Fund.

Daniel Gale is supported by the St Peter's Trust for Kidney, Bladder and Prostate Research

References

Bullich, Gemma, Daniel Trujillano, Sheila Santín, Stephan Ossowski, Santiago Mendizábal, Gloria Fraga, Álvaro Madrid, Gema Ariceta, José Ballarín, Roser Torra, Xavier Estivill, and Elisabet Ars. 2015. 'Targeted Next-Generation Sequencing in Steroid-Resistant Nephrotic Syndrome: Mutations in Multiple Glomerular Genes May Influence Disease Severity'. *European Journal of Human Genetics: EJHG* 23(9):1192–99. doi: 10.1038/ejhg.2014.252.

Fallerini, C., M. Baldassarri, E. Trevisson, V. Morbidoni, A. La Manna, R. Lazzarin, A. Pasini, G. Barbano, A. R. Pinciaroli, G. Garosi, E. Frullanti, A. M. Pinto, M. A. Mencarelli, F. Mari, A. Renieri, and F. Ariani. 2017. 'Alport Syndrome: Impact of Digenic Inheritance in Patients Management'. *Clinical Genetics* 92(1):34–44. doi: 10.1111/cge.12919.

Fallerini, C., L. Dosa, R. Tita, D. Del Prete, S. Feriozzi, G. Gai, M. Clementi, A. La Manna, N. Miglietti, R. Mancini, G. Mandrile, G. M. Ghiggeri, G. Piaggio, F. Brancati, L. Diano, E. Frate, A. R. Pinciaroli, M. Giani, P. Castorina, E. Bresin, D. Giachino, M. De Marchi, F. Mari, M. Bruttini, A. Renieri, and F. Ariani. 2014. 'Unbiased next Generation Sequencing Analysis Confirms the Existence of Autosomal Dominant Alport Syndrome in a Relevant Fraction of Cases'. *Clinical Genetics* 86(3):252–57. doi: 10.1111/cge.12258.

Furlano, Mónica, Victor Martínez, Marc Pybus, Yolanda Arce, Jaume Crespí, María Del Prado Venegas, Gemma Bullich, Andrea Domingo, Nadia Ayasreh, Silvia Benito, Laura Lorente, Patricia Ruíz, Vanesa López Gonzalez, Rosa Arlandis, Elisa Cabello, Ferran Torres, Lluis Guirado, Elisabet Ars, and Roser Torra. 2021. 'Clinical and Genetic Features of Autosomal Dominant Alport Syndrome: A Cohort Study'. *American Journal of Kidney Diseases: The Official Journal of the National Kidney Foundation* S0272-6386(21)00505-9. doi: 10.1053/j.ajkd.2021.02.326.

Gale, Daniel P. 2013. 'How Benign Is Hematuria? Using Genetics to Predict Prognosis'. *Pediatric Nephrology (Berlin, Germany)* 28(8):1183–93. doi: 10.1007/s00467-012-2399-y.

Gibson, Joel, Rachel Fieldhouse, Melanie Chan, Omid Sadeghi-Alavijeh, Leslie Burnett, Valerio Izzi, Anton Persikov, Daniel Gale, Helen Storey, and Judy Savige. 2021. 'Prevalence Estimates of Predicted Pathogenic COL4A3 - COL4A5 Variants in a Population Sequencing Database and Their Implications for Alport Syndrome'. *Journal of the American Society of Nephrology*. doi: 10.1681/ASN.2020071065.

Groopman, Emily E., Maddalena Marasa, Sophia Cameron-Christie, Slavé Petrovski, Vimla S. Aggarwal, Hila Milo-Rasouly, Yifu Li, Junying Zhang, Jordan Nestor, Priya Krithivasan, Wan Yee Lam, Adele Mitrotti, Stacy Piva, Byum H. Kil, Debanjana Chatterjee, Rachel Reingold, Drew Bradbury, Michael DiVecchia, Holly Snyder, Xueru Mu, Karla Mehl, Olivia Balderes, David A. Fasel, Chunhua Weng, Jai Radhakrishnan, Pietro Canetta, Gerald B. Appel, Andrew S. Bomback, Wooin Ahn, Natalie S. Uy, Shumyle Alam, David J. Cohen, Russell J. Crew, Geoffrey K. Dube, Maya K. Rao, Sitharthan Kamalakaran, Brett Copeland, Zhong Ren, Joshua Bridgers, Colin D. Malone, Caroline M. Mebane, Neha Dagaonkar, Bengt C. Fellström, Carolina Haefliger, Sumit Mohan, Simone Sanna-Cherchi, Krzysztof Kiryluk, Jan Fleckner, Ruth March, Adam Platt, David B. Goldstein, and Ali G. Gharavi. 2019. 'Diagnostic Utility of Exome Sequencing for Kidney Disease'. *The New England Journal of Medicine* 380(2):142–51. doi: 10.1056/NEJMoa1806891.

Gulati, Ashima, Angel M. Sevillano, Manuel Praga, Eduardo Gutierrez, Ignacio Alba, Neera K. Dahl, Whitney Besse, Jungmin Choi, and Stefan Somlo. 2020. 'Collagen IV Gene Mutations in Adults With Bilateral Renal Cysts and CKD'. *Kidney International Reports* 5(1):103–8. doi: 10.1016/j.ekir.2019.09.004.

Hoefele, Julia, Ahmet Nayir, Moumita Chaki, Anita Imm, Susan J. Allen, Edgar A. Otto, and Friedhelm Hildebrandt. 2011. 'Pseudodominant Inheritance of Nephronophthisis Caused by a Homozygous NPHP1 Deletion'. *Pediatric Nephrology (Berlin, Germany)* 26(6):967–71. doi: 10.1007/s00467-011-1761-9.

Kashtan, Clifford E., Jie Ding, Guido Garosi, Laurence Heidet, Laura Massella, Koichi Nakanishi, Kandai Nozu, Alessandra Renieri, Michelle Rheault, Fang Wang, and Oliver Gross. 2018. 'Alport Syndrome: A Unified Classification of Genetic Disorders of Collagen IV A345: A Position Paper of the Alport Syndrome Classification Working Group'. *Kidney International* 93(5):1045–51. doi: 10.1016/j.kint.2017.12.018.

van der Loop, F. T., L. Heidet, E. D. Timmer, B. J. van den Bosch, A. Leinonen, C. Antignac, J. A. Jefferson, A. P. Maxwell, L. A. Monnens, C. H. Schröder, and H. J. Smeets. 2000. 'Autosomal Dominant Alport Syndrome Caused by a COL4A3 Splice Site Mutation'. *Kidney International* 58(5):1870–75. doi: 10.1111/j.1523-1755.2000.00358.x.

Malone, Andrew F., Paul J. Phelan, Gentzon Hall, Umran Cetincelik, Alison Homstad, Andrea S. Alonso, Ruiji Jiang, Thomas B. Lindsey, Guanghong Wu, Matthew A. Sparks, Stephen R. Smith, Nicholas J. A. Webb, Philip A. Kalra, Adebowale A. Adeyemo, Andrey S. Shaw, Peter J. Conlon, J. Charles Jennette, David N. Howell, Michelle P. Winn, and Rasheed A. Gbadegesin. 2014. 'Rare Hereditary COL4A3/COL4A4 Variants May Be Mistaken for Familial Focal Segmental Glomerulosclerosis'. *Kidney International* 86(6):1253–59. doi: 10.1038/ki.2014.305.

Mariyama, M., A. Leinonen, T. Mochizuki, K. Tryggvason, and S. T. Reeders. 1994. 'Complete Primary Structure of the Human Alpha 3(IV) Collagen Chain. Coexpression of the Alpha 3(IV) and Alpha 4(IV) Collagen Chains in Human Tissues'. *The Journal of Biological Chemistry* 269(37):23013–17.

Savige, Judy, Francesca Ariani, Francesca Mari, Mirella Bruttini, Alessandra Renieri, Oliver Gross, Constantinos Deltas, Frances Flinter, Jie Ding, Daniel P. Gale, Mato Nagel, Michael Yau, Lev Shagam, Roser Torra, Elisabet Ars, Julia Hoefele, Guido Garosi, and Helen Storey. 2019. 'Expert Consensus Guidelines for the Genetic Diagnosis of Alport Syndrome'. *Pediatric Nephrology (Berlin, Germany)* 34(7):1175–89. doi: 10.1007/s00467-018-3985-4.

Savige, Judy, Deb Colville, Michelle Rheault, Susie Gear, Rachel Lennon, Sharon Lagas, Moira Finlay, and Frances Flinter. 2016. 'Alport Syndrome in Women and Girls'. *Clinical Journal of the American Society of Nephrology: CJASN* 11(9):1713–20. doi: 10.2215/CJN.00580116.

Savige, Judy, Martin Gregory, Oliver Gross, Clifford Kashtan, Jie Ding, and Frances Flinter. 2013. 'Expert Guidelines for the Management of Alport Syndrome and Thin Basement Membrane Nephropathy'. *Journal of the American Society of Nephrology: JASN* 24(3):364–75. doi: 10.1681/ASN.2012020148.

Savige, Judy, Helen Storey, Elizabeth Watson, Jens Michael Hertz, Constantinos Deltas, Alessandra Renieri, Francesca Mari, Pascale Hilbert, Pavlina Plevova, Peter Byers, Agne Cerkauskaite, Martin Gregory, Rimante Cerkauskiene, Danica Galesic

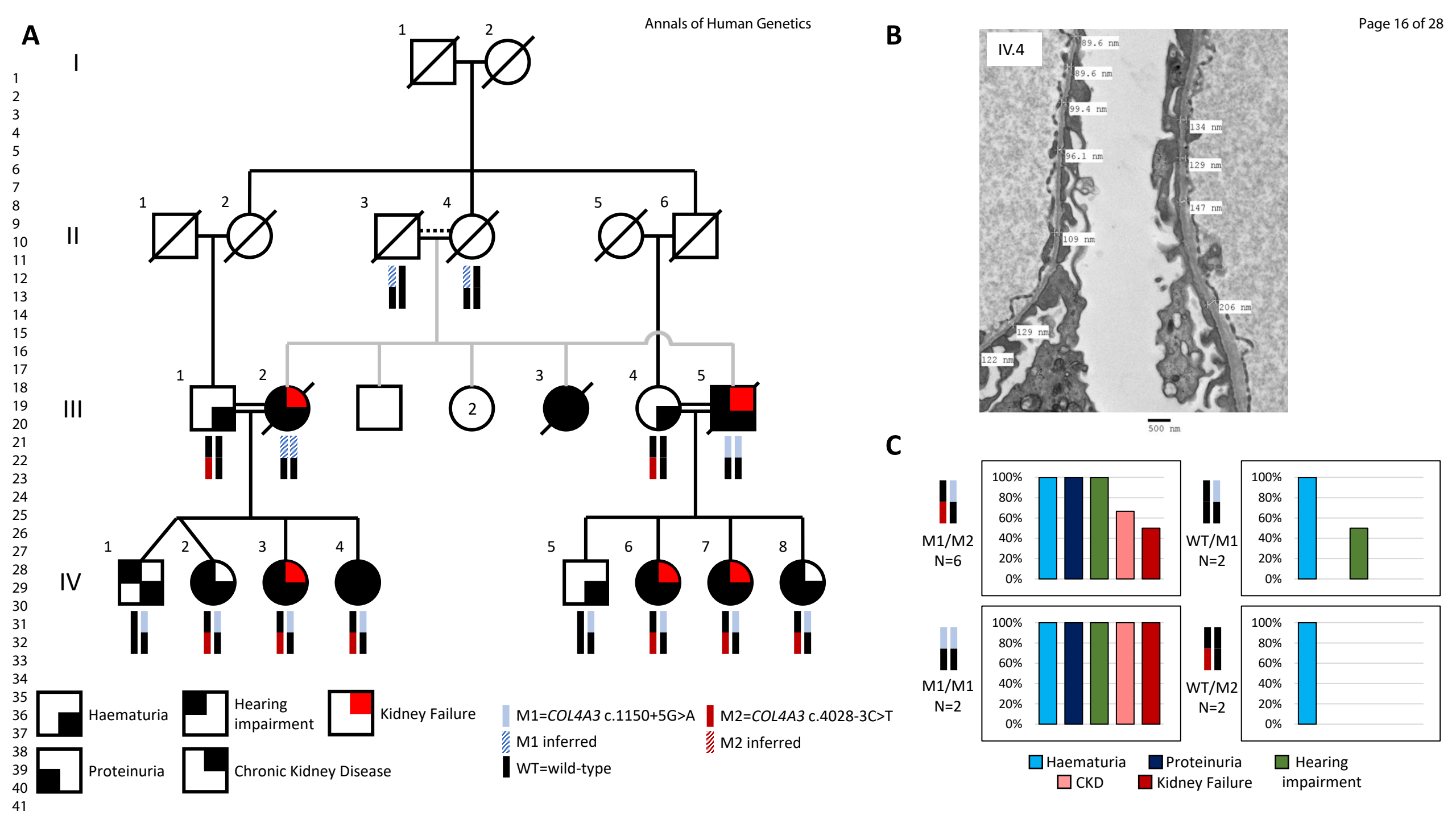
Ljubanovic, Francesca Becherucci, Carmela Errichiello, Laura Massella, Valeria Aiello, Rachel Lennon, Louise Hopkinson, Ania Koziell, Adrian Lungu, Hansjorg Martin Rothe, Julia Hoefele, Miriam Zacchia, Tamara Nikuseva Martic, Asheeta Gupta, Albertien van Eerde, Susie Gear, Samuela Landini, Viviana Palazzo, Laith Al-Rabadi, Kathleen Claes, Anniek Corveleyn, Evelien Van Hoof, Micheel van Geel, Maggie Williams, Emma Ashton, Hendica Belge, Elisabeth Ars, Agnieszka Bierzynska, Concetta Gangemi, and Beata S. Lipska-Ziętkiewicz. 2021. 'Consensus Statement on Standards and Guidelines for the Molecular Diagnostics of Alport Syndrome: Refining the ACMG Criteria'. *European Journal of Human Genetics: EJHG* 29(8):1186–97. doi: 10.1038/s41431-021-00858-1.

Voskarides, Konstantinos, Maria Arsali, Yiannis Athanasiou, Avraam Elia, Alkis Pierides, and Constantinos Deltas. 2012. 'Evidence That NPHS2-R229Q Predisposes to Proteinuria and Renal Failure in Familial Hematuria'. *Pediatric Nephrology (Berlin, Germany)* 27(4):675–79. doi: 10.1007/s00467-011-2084-6.

Figure legends

Figure 1. Clinical and genetic analysis of multiplex consanguineous family with Alport syndrome. (A) Pedigree diagram displaying females (circles) and males (squares) presenting various features of Alport syndrome as indicated by symbol filling. *COL4A3* allele status is indicated below the symbols. Please note that genotypes for individuals II.3, II.4 and III.2 are inferred from offspring and that the consanguineous marriage between II.3 and II.4 is inferred from offspring genotype. (B) Native renal biopsy for individual IV.4. Electron microscopy shows glomerular basement thinning down to 90 nanometres with occasional glomerular basement lamellation and splitting consistent with Alport syndrome. (C) Distribution of phenotypes according to individual genotype. Please note that 100% of heterozygous individuals present haematuria, while none of them showed proteinuria, chronic kidney disease (CKD) or kidney failure. Conversely, among homozygous or compound heterozygous individuals, 8/8 (100%) presented proteinuria, 6/8 (75%) CKD and 5/8 (62.5%) kidney failure.

Figure 2. Identified *COL4A3* variants lead to exon skipping. (A) Genomic DNA Sanger sequencing chromatogram extract confirms heterozygous *COL4A3* (NM_000091.5) c.1150+5G>A variant in individual IV.3. (B) RT-PCR product electrophoresis and amplicon sequencing confirm aberrant splicing with *COL4A3* exon 20 skipping in individual IV.3. (C) Genomic DNA Sanger sequencing chromatogram extract confirms heterozygous *COL4A3* (NM_000091.5) c.4028 -3C>T variant in individual IV.3. (D) RT-PCR product electrophoresis and amplicon sequencing confirm aberrant splicing with *COL4A3* exon 46 skipping in individual IV.3.


A
I
II
III
IV
Haematuria
Hearing impairment
Kidney Failure
Proteinuria
Chronic Kidney Disease
M1=COL4A3 c.1150+5G>A
M2=COL4A3 c.4028-3C>T
M1 inferred
M2 inferred
WT=wild-type
B
IV.4
89.6 nm
89.6 nm
99.4 nm
96.1 nm
109 nm
129 nm
122 nm
134 nm
129 nm
147 nm
206 nm
500 nm
C
M1/M2
N=6
M1/M1
N=2
WT/M1
N=2
WT/M2
N=2
100%
80%
60%
40%
20%
0%
Haematuria
Proteinuria
Hearing impairment
CKD
Kidney Failure

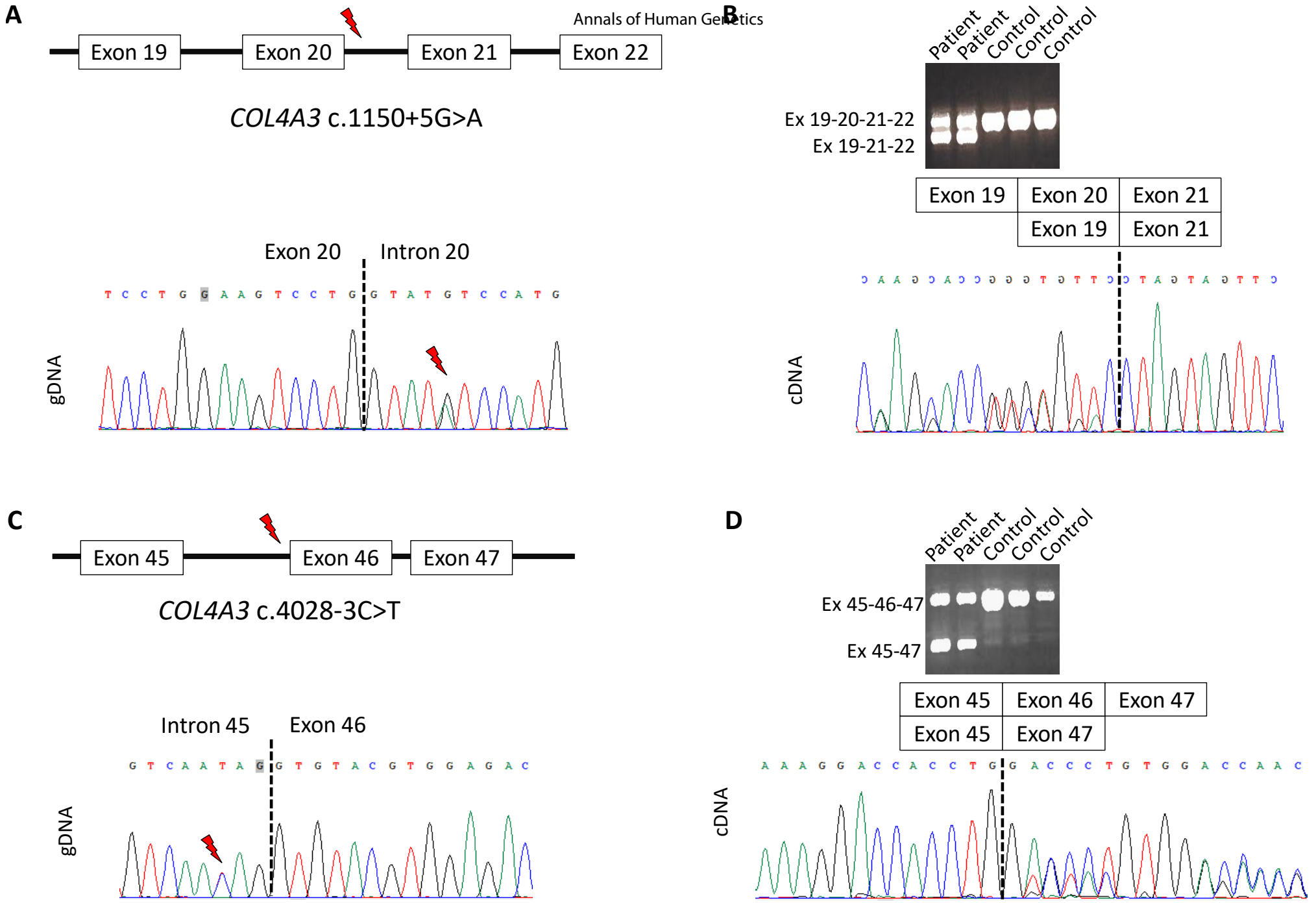

A
Exon 19
Exon 20
Exon 21
Exon 22
COL4A3 c.1150+5G>A
Exon 20
Intron 20
gDNA
B
Patient
Patient
Control
Control
Control
Ex 19-20-21-22
Ex 19-21-22
Exon 19
Exon 20
Exon 21
Exon 19
Exon 21
cDNA
C
Exon 45
Exon 46
Exon 47
COL4A3 c.4028-3C>T
Intron 45
Exon 46
gDNA
D
Patient
Patient
Control
Control
Control
Ex 45-46-47
Ex 45-47
Exon 45
Exon 46
Exon 47
Exon 45
Exon 47
cDNA

Table 1. Clinical characteristics of family members and description of *COL4A3* alleles

Family member	*COL4A3* Allele 1	*COL4A3* Allele 2	Gender	Age range (years)	Haematuria	Proteinuria	CKD stage (years)	Hearing impairment	Eye exam
III:1	WT	*c.4028-3C>T*	Male	60-65	Yes	No	1	No	N/A
III.2	inferred c.1150+5G>A	inferred c.1150+5G>A	Female	50-55	Yes	Yes	5 (Age 40)	Yes	N/A
III:4	*c.4028-3C>T*	WT	Female	60-65	Yes	No	1 (Age 61)	No	N/A
III:5	*c.1150+5G>A*	*c.1150+5G>A*	Male	? (50-55)	Yes	Yes	5 (Age 35)	Yes	N/A
IV:1	*c.1150+5G>A*	WT	Male	30-35	Yes	N/A	N/A	Yes	N/A
IV:2	*c.1150+5G>A*	*c.4028-3C>T*	Female	30-35	Yes	Yes	1	Yes	N/A
IV:3	*c.1150+5G>A*	*c.4028-3C>T*	Female	40-45	Yes	Yes	5 (Age 21)	Yes	N/A
IV:4	*c.1150+5G>A*	*c.4028-3C>T*	Female	30-35	Yes	Yes	3B	Yes	Mottled retinal epithelial pigmentation, myopia
IV:5	*c.1150+5G>A*	WT	Male	40-45	Yes	N/A	N/A	No	N/A
IV:6	*c.1150+5G>A*	*c.4028-3C>T*	Female	40-45	Yes	Yes	5 (Age 37)	Yes	N/A
IV:7	*c.1150+5G>A*	*c.4028-3C>T*	Female	36-40	Yes	Yes	5 (Age 30)	Yes	Flecks in both maculars, dots in the peripheral retina
IV:8	*c.1150+5G>A*	*c.4028-3C>T*	Female	30-35	Yes	Yes	1	Yes	Myopia, angioid streaks

COL4A3 variant (*NM_000091.5*)	Genomic coordinates (GRCh38)	gnomAD	HSF impact prediction	MaxEnt	SpliceAI	Patient RNA studies
c.1150+5G>A; p.(Gly372_Pro383del)	chr2:227261122:G:A	1/249,146/0	DS alteration, most probably affecting splicing	DS: 8.14 -> 3.72: -54.3%	Delta score for donor loss: 0.61	in-frame skipping of exon 20, silent (ggu -> gga)
c.4028-3C>T; p.(Gly1343_Leu1384del)	chr2:227304016:C:T	Not reported	No significant impact on splicing signals	AS: 9.87 -> 8.83: -10.5%	Delta score for acceptor loss: 0.36	in-frame skipping of exon 46, silent (ggu -> gga)

Transcript used: RefSeq NM_000091.5

Abbreviations: AS, acceptor splice site; CKD, chronic kidney disease - stage 1: eGFR >90ml/min/1.73m^2, stage 2: eGFR 60-89ml/min/1.73m^2, stage 3A: eGFR 45-59ml/min/1.73m^2, stage 3B: eGFR 30-44ml/min/1.73m^2, stage 4: eGFR 15-29ml/min/1.73m^2, stage 5: eGFR <15ml/min/1.73m^2 or initiation of renal replacement therapy or kidney transplantation; DS, donor splice site; GnomAD, Genome Aggregation Database (displayed as allele counts/total alleles/homozygotes); HSF, Human Splicing Finder; N/A, not available. HSF impact predictions and MaxEnt Donor Site variation score were generated in 08/2021 through https://hsf.genomnis.com. SpliceAI was generated via Ensembl Variant Effect Predictor.

Supplementary Information

Pseudodominant Alport syndrome caused by pathogenic homozygous and compound heterozygous *COL4A3* splicing variants

Methods

Web resources

Ensembl VEP: https://www.ensembl.org/info/docs/tools/vep/index.html

GnomAD v2.1.1: https://gnomad.broadinstitute.org/

HGMD®: http://www.hgmd.cf.ac.uk/ac/index.php

Human Splice Finder: https://hsf.genomnis.com/login (Desmet et al. 2009)

Leiden Open Variation Database - *COL4A3* gene homepage:

https://databases.lovd.nl/shared/genes/COL4A3

NCBI ClinVar: https://www.ncbi.nlm.nih.gov/clinvar/

ProteinPaint: https://pecan.stjude.cloud/proteinpaint (Zhou et al. 2016)

Varsome: https://varsome.com/ (Kopanos et al. 2019)

Ethical approvals and patient assessment

This study was approved by the North East - Newcastle & North Tyneside 1 Research Ethics Committee (18/NE/350). Detailed phenotyping was undertaken by the recruiting physicians and authors of this study and clinical data regarding family relation, gender, kidney function (haematuria, proteinuria, chronic kidney disease, kidney failure), hearing loss and ocular lesions were recorded. Following informed and written consent, DNA was obtained from all affected individuals.

Genomic DNA isolation, amplification, sequencing and variant annotation

An automated genomic DNA extraction method from blood samples was used, utilizing the QIA symphony DNA kit (#931236, Qiagen, Germany). Affected individuals had their DNA tested using a targeted predesigned MPS Alport syndrome gene panel (ALPORT MASTR Multiplicom, MRC-Holland, the Netherlands), which specifically amplified and sequenced the entire coding exons for the *COL4A3, COL4A4* and *COL4A5* genes (Gillion et al. 2018). After quality control, samples were sequenced using an MPS illumina Mi Seq® platform (Illumina, San Diego, CA, USA) with paired end sequencing using the sequencing by synthesis

technology. Data analysis was performed using Nextgene® software (Softgenetics LLC, PA, USA).

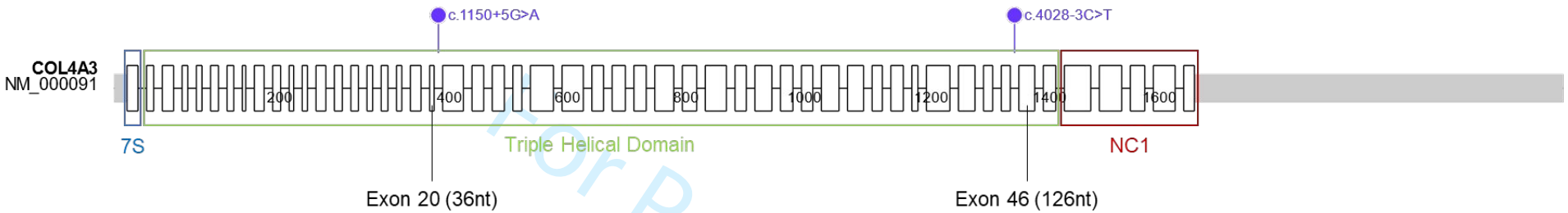
Determining pathogenicity of sequence variants

Variant pathogenicity was determined in accordance with guidelines from the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (Richards et al. 2015). Online disease databases were screened to determine if variants were reported previously (LOVD, ClinVar, HGMD) and to determine allele frequencies (gnomAD). We utilized *in silico* splice prediction software tools Human Splicing Finder (Desmet et al. 2009), SpliceAI (Jaganathan et al. 2019) as well as MaxEntScan (Yeo and Burge 2004). Sanger sequencing was utilized to confirm variants of interest and their segregation. Variants were described according to the *COL4A3* reference transcript (NM_000091.4) using the nomenclature recommended by the Human Genome Variation Society.

RNA preparation and RT-PCR

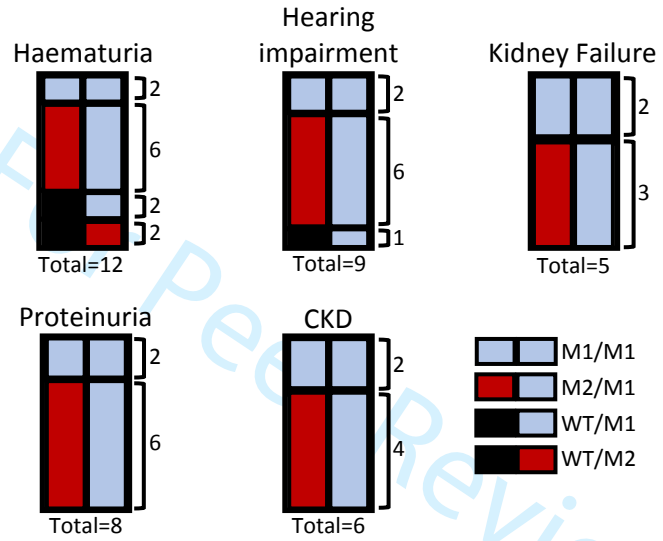
Total RNA from peripheral blood lymphocytes was isolated using RNeasy mini kit (Qiagen) according to the manufacturer's instructions and quantified using a NanoDrop 2000 spectrophotometer. 1µg RNA was reverse-transcribed using an oligo-dT primer and SuperScript III Reverse Transcriptase (Thermo Fisher Scientific). The resulting cDNA was diluted 1 in 10 and used in PCR reactions using GoTaq® DNA Polymerase (Promega) and primers targeting *COL4A3* exons 19 & 22 and exons 45 & 47 respectively. Product electrophoresis was performed on a 1% agarose gel. Sanger sequencing of RT-PCR products was carried out by the Northern Molecular Genetics Service.

Supplementary Figure 1. Identified *COL4A3* variants



COL4A3 RNA (RefSeq NM_000091) and exon structure with UTR in grey and annotated with domains (N-terminal 7S domain, triple helical domain, and C-terminal non-collagenous (NC1) domain as in (Braunisch et al. 2018). Variants c.1150+5G>A and c.4028-3C>T painted above RNA structure. Exon and protein domain visualization performed using ProteinPaint (Zhou et al. 2016).

Supplementary Figure 2. Distribution of genotypes according to phenotype



Schematic display of the contribution of the different genotypes to the renal and extra-renal manifestations in the reported family. Please note that only homozygous or compound heterozygous individuals presented with proteinuria, chronic kidney disease or kidney failure.

Supplementary Table 1. ACMG/AMP criteria for detected *COL4A3* variants

NM_000091.4: c.1150+5G>A	**ACMG/AMP criteria** (Richards et al. 2015)	**ACMG/AMP criteria adaptations for Alport syndrome** (Savige et al. 2021)
PS3	Well-established (robust and reproducible) *in vitro* or *in vivo* functional studies supportive of a damaging effect on the gene or gene product	Due to their complexity, functional assays are mostly used for research and not for diagnostic utility. The findings must be specific for the variant being tested and not simply be true for the gene.
PM2	Absent from controls (or at extremely low frequency if recessive) in gnomAD, ESP, 1000 Genomes or ExAC	Monoallelic pathogenic variants in *COL4A5* affect at least one in 5000 of the population, and heterozygous pathogenic *COL4A3* and *COL4A4* variants one in 100, which means some pathogenic variants are present in large reference databases of normals
PM3	For recessive disorders, detected in trans with a pathogenic variant	
PP1	Co-segregation with disease in multiple affected family members in a gene definitively known to cause the disease	
PP4	Patients' phenotype or family history is highly specific for a disease with a single genetic aetiology	Applicable in families with history of microscopic haematuria, hearing loss and renal failure. At least 80% of individuals with inherited haematuria can be demonstrated to have a pathogenic variant in one or more of the *COL4A3–COL4A5* genes
PP3	Multiple lines of computational evidence support a deleterious effect on the gene or gene product (conservation, evolutionary, splicing impact, etc.) (impact on splicing predicted by HSF, MaxEnt, SpliceAI, see Table 1)	
Pathogenic (1 strong + 2 moderate + 3 supporting)		
NM_000091.4: c.4028-3C>T	**ACMG/AMP criteria** (Richards et al. 2015)	**ACMG/AMP criteria adaptations** (Savige et al. 2021)
PS3	Well-established (robust and reproducible) *in vitro* or *in vivo* functional studies supportive of a damaging effect on the gene or gene product	Due to their complexity, functional assays are mostly used for research and not for diagnostic utility. The findings must be specific for the variant being tested and not simply be true for the gene.

PM2	Absent from controls (or at extremely low frequency if recessive) in gnomAD, ESP, 1000 Genomes or ExAC	Monoallelic pathogenic variants in *COL4A5* affect at least one in 5000 of the population, and heterozygous pathogenic *COL4A3* and *COL4A4* variants one in 100, which means some pathogenic variants are present in large reference databases of normals
PM3	For recessive disorders, detected in trans with a pathogenic variant	
PP1	Co-segregation with disease in multiple affected family members in a gene definitively known to cause the disease	
PP4	Patients' phenotype or family history is highly specific for a disease with a single genetic aetiology	Applicable in families with history of microscopic haematuria, hearing loss and renal failure. At least 80% of individuals with inherited haematuria can be demonstrated to have a pathogenic variant in one or more of the *COL4A3–COL4A5* genes
BP4	Benign computational verdict based on 1 benign prediction from DANN vs no pathogenic predictions and the position is not strongly conserved (CSH phyloP100way = -0.264 is less than 5) (Varsome, (Kopanos et al. 2019)). Furthermore, no significant impact on splicing predicted by HSF, MaxEnt, SpliceAI (see Table 1)	
Pathogenic (1 strong + 2 moderate + 2 supporting)		

Supplementary References

Braunisch, Matthias C., Maike Büttner-Herold, Roman Günthner, Robin Satanovskij, Korbinian M. Riedhammer, Pierre-Maurice Herr, Hanns-Georg Klein, Dagmar Wahl, Claudius Küchle, Lutz Renders, Uwe Heemann, Christoph Schmaderer, and Julia Hoefele. 2018. 'Heterozygous COL4A3 Variants in Histologically Diagnosed Focal Segmental Glomerulosclerosis'. *Frontiers in Pediatrics* 6:171. doi: 10.3389/fped.2018.00171.

Desmet, François-Olivier, Dalil Hamroun, Marine Lalande, Gwenaëlle Collod-Béroud, Mireille Claustres, and Christophe Béroud. 2009. 'Human Splicing Finder: An Online Bioinformatics Tool to Predict Splicing Signals'. *Nucleic Acids Research* 37(9):e67. doi: 10.1093/nar/gkp215.

Gillion, Valentine, Karin Dahan, Jean-Pierre Cosyns, Pascale Hilbert, Michel Jadoul, Eric Goffin, Nathalie Godefroid, Martine De Meyer, Michel Mourad, Yves Pirson, and Nada Kanaan. 2018. 'Genotype and Outcome After Kidney Transplantation in Alport Syndrome'. *Kidney International Reports* 3(3):652–60. doi: 10.1016/j.ekir.2018.01.008.

Jaganathan, Kishore, Sofia Kyriazopoulou Panagiotopoulou, Jeremy F. McRae, Siavash Fazel Darbandi, David Knowles, Yang I. Li, Jack A. Kosmicki, Juan Arbelaez, Wenwu Cui, Grace B. Schwartz, Eric D. Chow, Efstathios Kanterakis, Hong Gao, Amirali Kia, Serafim Batzoglou, Stephan J. Sanders, and Kyle Kai-How Farh. 2019. 'Predicting Splicing from Primary Sequence with Deep Learning'. *Cell* 176(3):535-548.e24. doi: 10.1016/j.cell.2018.12.015.

Kopanos, Christos, Vasilis Tsiolkas, Alexandros Kouris, Charles E. Chapple, Monica Albarca Aguilera, Richard Meyer, and Andreas Massouras. 2019. 'VarSome: The Human Genomic Variant Search Engine'. *Bioinformatics (Oxford, England)* 35(11):1978–80. doi: 10.1093/bioinformatics/bty897.

Richards, Sue, Nazneen Aziz, Sherri Bale, David Bick, Soma Das, Julie Gastier-Foster, Wayne W. Grody, Madhuri Hegde, Elaine Lyon, Elaine Spector, Karl Voelkerding, Heidi L. Rehm, and ACMG Laboratory Quality Assurance Committee. 2015. 'Standards and Guidelines for the Interpretation of Sequence Variants: A Joint Consensus Recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology'. *Genetics in Medicine: Official Journal of the American College of Medical Genetics* 17(5):405–24. doi: 10.1038/gim.2015.30.

Savige, Judy, Helen Storey, Elizabeth Watson, Jens Michael Hertz, Constantinos Deltas, Alessandra Renieri, Francesca Mari, Pascale Hilbert, Pavlina Plevova, Peter Byers, Agne Cerkauskaite, Martin Gregory, Rimante Cerkauskiene, Danica Galesic Ljubanovic, Francesca Becherucci, Carmela Errichiello, Laura Massella, Valeria Aiello, Rachel Lennon, Louise Hopkinson, Ania Koziell, Adrian Lungu, Hansjorg Martin Rothe, Julia Hoefele, Miriam Zacchia, Tamara Nikuseva Martic, Asheeta Gupta, Albertien van Eerde, Susie Gear, Samuela Landini, Viviana Palazzo, Laith Al-Rabadi, Kathleen Claes, Anniek Corveleyn, Evelien Van Hoof, Micheel van Geel, Maggie Williams, Emma Ashton, Hendica Belge, Elisabeth Ars, Agnieszka Bierzynska, Concetta

Gangemi, and Beata S. Lipska-Ziętkiewicz. 2021. 'Consensus Statement on Standards and Guidelines for the Molecular Diagnostics of Alport Syndrome: Refining the ACMG Criteria'. *European Journal of Human Genetics: EJHG* 29(8):1186–97. doi: 10.1038/s41431-021-00858-1.

Yeo, Gene, and Christopher B. Burge. 2004. 'Maximum Entropy Modeling of Short Sequence Motifs with Applications to RNA Splicing Signals'. *Journal of Computational Biology: A Journal of Computational Molecular Cell Biology* 11(2–3):377–94. doi: 10.1089/1066527041410418.

Zhou, Xin, Michael N. Edmonson, Mark R. Wilkinson, Aman Patel, Gang Wu, Yu Liu, Yongjin Li, Zhaojie Zhang, Michael C. Rusch, Matthew Parker, Jared Becksfort, James R. Downing, and Jinghui Zhang. 2016. 'Exploring Genomic Alteration in Pediatric Cancer Using ProteinPaint'. *Nature Genetics* 48(1):4–6. doi: 10.1038/ng.3466.