Frequent *COL4* mutations in familial microhematuria accompanied by later-onset Alport nephropathy due to focal segmental glomerulosclerosis

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Transparency Declaration

None to declare

ABSTRACT

Familial microscopic hematuria (FMH) is associated with a genetically heterogeneous group of conditions including the collagen-IV nephropathies, the heritable C3/CFHR5 nephropathy and the glomerulopathy with fibronectin deposits. The clinical course varies widely, ranging from isolated benign familial hematuria to end-stage renal disease (ESRD) later in life. We investigated 24 families using Next Generation Sequencing (NGS) for five genes: *COL4A3, COL4A4, COL4A5, CFHR5* and *FN1*. In 17 families (71%), we found 15 pathogenic mutations in *COL4A3/A4/A5*, nine of them novel. In five families patients inherited classical AS with hemizygous X-linked *COL4A5* mutations. Even more patients developed later-onset Alport-related nephropathy having inherited heterozygous *COL4A3/A4* mutations that cause thin basement membranes. Amongst 62 heterozygous or hemizygous patients, eight (13%) reached ESRD, while 25% of patients with heterozygous *COL4A3/A4* mutations, aged >50-yrs, reached ESRD. In conclusion, *COL4A* mutations comprise a frequent cause of FMH. Heterozygous *COL4A3/A4* mutations predispose to renal function impairment, supporting that thin basement membrane nephropathy is not always benign. The molecular diagnosis is essential for differentiating the X-linked from the autosomal recessive and dominant inheritance. Finally, NGS technology is established as the gold standard for the diagnosis of FMH and associated collagen-IV glomerulopathies, frequently averting the need for invasive renal biopsies.

Key words: next generation sequencing; *COL4A3/COL4A4/COL4A5*; familial microscopic hematuria; thin basement membrane nephropathy (TBMN); focal segmental glomerulosclerosis (FSGS); end stage renal disease (ESRD); Alport Syndrome; later-onset Alport-related nephropathy (LOAN)

Introduction

Familial microscopic hematuria (FMH) is a common presenting feature of glomerular disease and it can be associated with later progression to proteinuria and reduced glomerular filtration rate (GFR), depending on the specific disease or gene at fault. During the past three decades the correlation between molecular genetics and hematuria of glomerular origin has been well described. Six genes (*COL4A3, COL4A4, COL4A5, CFHR5, MYH9, FN1*) have been identified, which when mutated cause hereditary forms of microscopic hematuria (MH) (1). In most cases mutations are found in *COL4A3, COL4A4* or *COL4A5* genes that code for the respective alpha chains of the trimeric collagen IV, the most important structural component of the glomerular basement membrane (GBM) (2-4).

Glomerular MH is the earliest presenting sign in males with AS and hemizygous mutations in the X-linked *COL4A5* gene and in males or females with autosomal recessive AS (ARAS) who inherit homozygous or compound heterozygous mutations in the *COL4A3* or *COL4A4* genes. Glomerular MH is also the cardinal presenting finding in patients with thin basement membranes, a condition known with the descriptive term of thin basement membrane nephropathy (TBMN), which has an estimated population prevalence of 0.3-1%. About 40-50% of these patients inherit heterozygous mutations in either of the *COL4A3/A4* genes, thus actually representing the heterozygous carriers of the ARAS (5-7). Most patients with AS will progress to ESRD at some point in their early life, usually before the age of 30-yrs, while in patients with heterozygous *COL4A3/A4* mutations the long term clinical outcome is largely unpredictable (8).

Until recently, TBMN caused by heterozygous *COL4A3/A4* mutations and commonly diagnosed in early life, was considered as a rather benign condition with excellent prognosis for life, with patients demonstrating mostly isolated MH or MH plus added low grade proteinuria of no particular clinical significance. However, work on a large Greek-Cypriot cohort and subsequent studies from several

other groups around the world, have established that an important percentage of patients with thin basement membranes, will develop clinically significant proteinuria later in their lives, usually accompanied by hypertension and a variable rate of GFR decline, up to ESRD. They are rarely associated with hearing loss (9-12). Work on the same Greek-Cypriot cohort linked *COL4A3/A4* heterozygous mutations with a severe outcome, usually after the 5th decade of life due to focal segmental glomerulosclerosis (FSGS) (10, 13). In fact, it is now well clarified that patients with heterozygous mutations in the *COL4A3/A4* genes can be misdiagnosed with hereditary FSGS (8, 14, 15). Consequently, many patients with thin basement membranes follow a clinical course reminiscent to a milder or later-onset Alport-related nephropathy (LOAN).

Herewith, we aimed at establishing the frequency of mutations in patients of 24 families from Greece presenting with FMH, most of them originating from the island of Crete. The molecular investigation was performed with the use of high throughput Next Generation Sequencing (NGS). We found 15 clearly pathogenic mutations, nine novel, in 17 families.

Materials and Methods

Patients and families

We studied 24 Greek families presenting with glomerular FMH that were consecutively referred to our centers. They included 237 subjects, of whom 177 were affected patients. DNA was available for 121 patients. Inclusion criteria required that all families had a minimum of three affected subjects with isolated MH or MH plus proteinuria and reduced GFR. Other non-glomerular causes of hematuria were excluded. Renal biopsies were available for patients in 14/24 (58.8%) families. In the participating clinical units, biopsies are not routinely performed in the presence of isolated MH. Nine out of the 14 families with available biopsies had at least one biopsy that showed thin membranes on electron microscope (EM). Two out of these nine also had signs of AS and four had additional signs of FSGS.

For the remaining families, EM study was not available, but a number of them had evidence of FSGS on light microscopy. In five families, one or more patients had hearing loss.

All clinical and biological material was accessible through the University of Cyprus Biobank (16). This research project was approved by the Cyprus National Bioethics Committee.

Next generation sequencing (NGS), Sanger DNA sequencing and mutation assessment

DNA from peripheral blood leucocytes of patients was isolated by a salting out procedure (17) or by using the Qiagen Blood Midi Kit (Qiagen, Hilden, Germany). A DNA sample of one patient from each family was submitted for NGS analysis. *COL4A3, COL4A4* and *COL4A5* targeted sequencing was performed using a custom Ampliseq panel on the Ion Torrent PGM sequencer (Life Technologies, Carlsbad, CA, USA) and results were analyzed as previously described (7). Briefly, by considering all processed samples, the mean depth of coverage achieved by targeted NGS was 180x. BAM files were submitted to the European Nucleotide Archive (ENA) under the accession number (PRJEB21296).

Variants were validated by Sanger DNA sequencing that was performed using the ABI BigDye Terminator v1.1 Cycle Sequencing Kit and the ABI PRISM 3130xl genetic analyzer (Applied Biosystems, Carlsbad, CA, USA). The oligonucleotide sequences were adopted from previous publications (10). Sanger DNA re-sequencing or restriction enzymes were used for examination of additional family members and healthy controls (Supplementary Table 1). Pathogenicity of each DNA variant was assessed based on segregation in the family, resolving to available mutation databases and testing a number of healthy controls. Also, in silico assessment of variants was performed by utilizing a number of available algorithms including SIFT (18), SNPs3D (19), Grantham scoring (20) and Polyphen2 (21) (Table 1), with cutoff values of pathogenicity assessment of missense variants as follows: SIFT predicts for every position that aminoacid substitutions are damaging with score less than 0.05, otherwise if the score is equal to or greater than 0.05 the particular change is considered tolerated. In SNPs3D a positive score indicates a variant classified as non-deleterious, and a negative

score indicates a deleterious variant. The Grantham score has a minimum score 0 and the maximum is 215. Higher scores correspond to higher differences in chemical properties, thus increase the probability of a substitution to be damaging. Polyphen 2 scores range from 0 to 1 and higher values indicate an increased probability of the amino acid substitution to be damaging.

Results

Mutations identified and their clinical context

We identified 15 clearly pathogenic mutations, nine of them novel, in 17 families. Three mutations were in the *COL4A3*, nine mutations in the *COL4A4* and five in the *COL4A5* gene (Table 1). Two mutations are expected to affect splicing, as they involve the invariable splicing consensus sequences and one is a single nucleotide deletion in the coding sequence, causing a translation frameshift. Fourteen mutations are single amino acid substitutions, 12 of them involving glycine residues in the collagenous domain, which are absolutely conserved and indispensable in every third position, in order for the triple helix to form normally. Mutation *COL4A5*-p.C1567R, involves one of the 12 highly conserved cysteines in the NC1 domain, which is important for chain recognition during trimer and helix formation (22).

Our NGS gene panel included also *CFHR5* and *FN1* but no pathogenic mutations were detected in either of them. *CFHR5* is included because a mutation of exon 2-3 duplication is endemic among Cypriots, where our lab is located (23). *FN1* is included because when mutated, it is a known rare cause of FMH (24). None of the 9 novel missense mutations was present in any of at least 35 normal controls, nor were they included in mutation databases, including the HGMD Professional, LOVD and ARUP. Certain families are quite extended; nonetheless DNA was not available from all affected individuals. Important findings in specific families are described below and in Tables 2 & 3.

Family CR-5316

COL4A4-p.G948Dfs2* (c.2843delG) was found in homozygosity in two young individuals, a girl and a boy, who developed severe AS (See Supplementary Figure 1 for all pedigrees). Reportedly, the homozygosity in this family could not be attributed to conspicuous parent consanguinity, although they all lived in a very remote and isolated village on the island of Crete. This may suggest that the mutation had first occurred a long time ago. The boy reached ESRD at age 23-yrs and the girl at age 19-yrs and was transplanted towards the end of 2013, at age 22. Their biopsy documented FSGS by light microscopy and ultrastructural features of AS by EM. Both children and their mother had hearing loss. Of eight heterozygous individuals, three had only MH, four had MH plus proteinuria and one developed renal impairment at 53-yrs. Additionally, the patients had variant *COL4A4*-p.L30F *in cis* configuration with *COL4A4*-p.G948Dfs2*. Aminoacid residue p.L30 is located on the 7S domain of collagen IV. This variant was not found in more than 100 control samples of the general population. It is more likely that the causal mutation is p.G948Dfs2*, with p.L30F being a rare variant of unclassified significance (VUS). This variant was recently registered independently under rs758174051 [(found in 4/120780 samples) - ExAc Consortium, Broad Institute, Cambridge, MA, USA)]. *In silico* analysis indicated that it is pathogenic (Table 1). Interestingly, in the same family, variant *COL4A4*-p.P222L was identified in two individuals, mother and daughter. Reportedly, the mother, who also carries the pathogenic mutation *COL4A4*-p.G948Dfs2* *in trans*, does not have AS. The daughter, who carries only *COL4A4*-p.P222L, did not show MH on one urine test. Additionally, this variant was not found in 35 normal controls of the general population of Crete, or in more than 100 Greek-Cypriot samples tested by NGS. Therefore, excluding the possibility of non-penetrance, variant *COL4A4*-p.P222L is most probably a rare polymorphism, unlikely to be pathogenic.

Families CR-5408 and CR-5424

In Cretan families CR-5408 and CR-5424 (Figure 1A and 1B) mutations *COL4A4*-p.G774R and *COL4A4*-p.G1465D were found *in cis* configuration in five out of the six patients. One patient in family CR-5408 carries only the *COL4A4*-p.G774R mutation (previously reported by (25), most

probably due to a recombination event. *COL4A4*-p.G1465D was reported previously as a probably damaging variant (26). This suggests the primary mutation is *COL4A4*-p.G774R and that *COL4A4*-p.G1465D is either neutral or has a milder effect, although this is a hypothesis to be tested in the future. *In silico* analysis is highly predictive for it being a pathogenic mutation, but for now it remains as VUS (Table 1). A biopsy was available from patient IV:2 (MH plus proteinuria at age 19-yrs), showing thin basement membranes and focal sites of podocyte effacement, in EM (Figure 2A and 2B). Biopsy was available from patient IV:4 of family CR-5424 (Figure 2C) showing FSGS, but unfortunately EM analysis was not available.

Other families studied

Overall, in this study there was variable clinical information for 62 patients (24M/38F) at ages ranging from 16-81 years (Table 2 and Table 3). All mutations identified were invariably associated with MH, while 11/42 (26%) of patients with autosomal mutations in *COL4A3/A4*, had added proteinuria without renal impairment and another 9/42 (21%) had progressed to kidney function decline. Six out of the 17 families studied had at least one biopsy with proven thin basement membranes. Out of 17 carriers of heterozygous *COL4A3/A4* mutations, older than 50-yrs in this cohort, at least 7 (41%) developed renal impairment, while 5 of all 42 (12%) reached ESRD. Importantly, FSGS was reported in patients from four of eight families with thin basement membranes and three of five AS families that had biopsies performed. Hearing loss was present in patients with X-linked AS, specifically family CR-4245 (*COL4A5*-p.G365V), CR-4246 (*COL4A5*-p.G743V) and CR-4244 (*COL4A5*-p.G1036R) (Table 3). Also hearing loss was evident in a family with ARAS (family CR-5316; mutation *COL4A4*-p.G948Dfs2*), as well as in a patient with later-onset Alport related nephropathy (family GR-5499; mutation *COL4A4*-c.489+1G>C) (Table 2).

Four patients in family GR-5473 presented exclusively with isolated MH. DNA was available from two out of four patients that carried the mutation *COL4A4*-p.G143V. This mutation was previously found in a Greek-Cypriot unrelated family, perhaps as a recurrent event, although unknown genetic relationship

cannot be excluded (7). Each of mutations *COL4A4*-p.G748S, *COL4A4*-p.G774R & p.G1465D was a common finding between two seemingly unrelated families (Table 2) and probably represent founder effects on the island of Crete.

Altogether in this cohort (Tables 2 & 3), eight patients (13%, 7M/1F) had progressed to ESRD at ages 23-65 years. Three of them reached ESRD at an unusually young age: a) A female in family CR-4244 at age 23-yo (mutation *COL4A5*-p.G1036R), b) a male in family CR-5363 at age 23-yo (mutation *COL4A4*-p.G748S) and c) a male in family CR-4246 at age 25-yo (mutation *COL4A5*-p.G743V). In the latter case of family CR-4246, rapid progression to ESRD is explained by the very fact that this male patient is a misdiagnosed X-linked AS case, supported in further by his reported hearing loss. Furthermore, ESRD in case (b) can be attributed to the subject's extreme obesity, while in case (a) a likely explanation could be the skewed inactivation of the X chromosome, as it is a female with a heterozygous *COL4A5* pathogenic mutation. Her mother and sister with the same mutation present a benign phenotype so far. The probability of digenic inheritance is largely excluded as the sample was analysed for all *COL4* related genes by NGS technology. The role of unknown modifier genes predisposing to a more severe outcome is of course another possible explanation.

Mutation *COL4A3*-p.G1251S was identified in all affected members of CR-5333, the largest family studied in this work with 24 reported patients. Unfortunately, access to only a small number of them was possible. Among carriers, three males reached ESRD at ages 55, 61 and 65-yrs. Renal biopsy showed thin basement membranes with FSGS in one male and two females. Another male patient showed FSGS, while no EM was available.

Phenocopies and incomplete penetrance

In each one of families GR-5310, CR-4244 and CR-4246 (mutations *COL4A4*-p.G1154V, *COL4A5*-p.G1036R and *COL4A5*-p.G743V respectively), one phenocopy patient was identified as he presented with MH without carrying the respective mutation (27). It is postulated that MH in these patients was

due to a different cause. Contrastingly, in family GR-5433 where a splicing defect was found (*COL4A3*-c.765+2T>C), a mutation female carrier appeared completely healthy, a sign of incomplete penetrance. Interestingly, her sister died on dialysis (no DNA sample was available), thus showing the existence of extreme phenotypes within the same family. Equally important is that a young girl in this family, presented in 2013 at age 11-yrs with MH and proteinuria of 500mg/day, accompanied by thin basement membranes. She is already on treatment with ACE inhibitors and her kidney function remains normal.

In the same context, in family CR-4245 four patients with MH do not carry the *COL4A5*-p.G365V mutation. We suspect that a second genetic etiology exists for these patients, since married-in individuals (DNA sample not available), also had MH (Supplementary Figure 1).

Coding non-pathogenic variants

We identified 51 polymorphic variants in the coding sequences of all five genes, with some of them being novel. Importantly, 39 were non-synonymous, one of which involved a glycine in the 7S domain of *COL4A3*, p.G43R (Table 4). The identification and recording of non-pathogenic variants facilitates classification of findings in future investigations, saving time and money (28).

Discussion

We investigated patients from 24 families with MH from Greece for mutations in five genes and found mutations in 17 (71%). Consider that another elegant publication had found mutations in 80% of patients of familial hematuric nephropathies using NGS technology (29). This is the largest genetic study performed for FMH in Greece. Heterozygous mutations in *COL4A3/A4* genes are known to be responsible for FMH, due to thin basement membranes. It is well established that a significant percentage of these patients with develop later in life added proteinuria and renal function decline, while about 30% will progress to ESRD by the age of 70-yrs, according to previous results (30) (and

refs therein). Several authors use the term autosomal dominant AS to characterize patients bearing heterozygous *COL4A3/A4* mutations, who develop severe disease symptoms that include ESRD and rarely hearing loss (26, 31, 32). During the recent International Workshop on AS at Gottingen, Germany, September 2015, it was discussed that the term TBMN, which in older literature is associated with familial benign hematuria, be negated and substituted by another terminology that would assist in consolidating that benign FMH is a misnomer (33).

Perhaps a better term to describe this condition, reminiscent to, but different from, classical AS, is autosomal dominant later-onset Alport-related nephropathy (LOAN); it combines the name of Alport, which is a collagen IV nephropathy and the documented later age at onset of serious clinical symptoms and ultrastructural or extrarenal findings, if and when they occur (34). Due to the high population frequency of LOAN, genetics clinics and laboratories examine more patients who progress to chronic renal failure or ESRD because of inheritance of LOAN than patients who inherit classical AS. Similar findings concerning the high risk of LOAN patients progressing to renal function decline of variable degree, have been reported by several groups, who referred to these patients as having inherited TBMN (11, 35-37).

In a previous publication of our group we reported on 14 families with 54 patients who had solely manifested isolated FMH of glomerular origin and we found no mutations in the *COL4* genes. Presumably these patients have inherited mutations in other, yet unknown, less deleterious genes (7). This is another important reason that unknown genes, which when mutated may cause real isolated "benign" FMH, should be identified and studied. The molecular diagnosis will be indicative of the good prognosis of such patients. Patients from such families may be attracting the attention of researchers less frequently or with less interest and escape detailed genetic studies.

It is important to note that in the current study we focused on deciphering the mutation load of patients presenting glomerular FMH, with or without further progression. In five of 24 families there was clear segregation of X-linked AS, with mutations in the *COL4A5* gene. In the remaining 19 families,

mutations in the *COL4A3/A4* genes were documented in 12, representing 63%. This is somewhat higher than the previously reported 40-50% in biopsy-documented TBMN families (38). This slight discrepancy might be explained by the relatively small number of families included here or by the structure of the Cretan population which favors founder phenomena, as demonstrated also here, where non-related families carried the same mutation. In papers reporting on FMH, mutations in the *COL4A3/A4* genes were identified in 36%, 38% and 17.5% of 22, 21 and 40 families respectively. Also, in another population of Greek-Cypriots we found pathogenic mutations in 27/68 families, representing 39.7% (7, 25, 38, 39).

Although we had limited clinical data for 5/42 patients with identified heterozygous mutations in the *COL4A3A/A4* genes (Table 2), useful results can be extracted with regards to the disease progression. ESRD was observed in 5 patients (12%), at ages of 23, 55, 61, 63 and 65-yrs. Three of them belonged to a large Cretan family, CR-5333 (mutation *COL4A3*-p.G1251S), suggesting that a genetic modifier might be co-inherited, predisposing them to adverse outcome at later ages. In addition, by taking into account only patients older than 50-yrs, the percentage of reduced GFR is 41% (7/17). This fact supports the use of LOAN as an alternative term to describe patients with thin basement membranes who can be complicated by proteinuria and decline of kidney function on long follow-up, perhaps with the effect of genetic modifiers (40-43). For three such putative modifiers [two in the *NPHS2* gene (podocin) and one in the *NEPH3* gene (filtrin)] co-inherited with a *COL4A3* mutation, functional cell culture studies were also supportive (42, 43). In the present cohort none of the patients examined carried a high risk modifier allele, most probably due to their low population frequency.

The ages at onset of ESRD in severe LOAN, usually in the 50s-60s, appear to overlap with the ages at onset of ESRD in patients with milder X-linked AS, which sometimes may be misdiagnosed as TBMN, especially if the pedigree structure is misleading (44-48). This certainly reflects the significant phenotypic heterogeneity, which can be partly attributed to the great allelic heterogeneity and partly to the likely role of genetic modifiers. Incomplete penetrance also was revealed in this cohort, as some

family members remained healthy even though they have inherited the causative mutations. Phenocopies are occasional observations that require caution, especially if they happen to be the samples chosen for the molecular investigation, thus providing erroneous results of no mutation findings.

Conclusions

1. Patients with FMH should be investigated molecularly for all three *COL4* genes. The diagnosis of X-linked AS in 5 out of 17 families was only documented through genetic analysis, without having information to satisfy classical criteria (49). The pedigree structure is not always indicative of the mode of inheritance, which is required to provide proper genetic counselling and risk assessment for family planning. Consider also, that EM biopsy results at young age cannot differentiate between classical AS and TBMN or LOAN. The molecular analysis is the gold standard for the diagnosis and the biopsy should be used in more demanding or complicated cases for guiding medical treatment.

2. It is worth emphasizing that patients with *COL4* mutations lacking a molecular diagnosis, can be mistakenly diagnosed with primary FSGS upon a positive renal biopsy, thereby undergoing erroneous treatments (8, 10, 14, 15). The consensus demands genetic analysis by NGS technology as a first line of investigation in most cases of isolated FMH or MH plus added proteinuria before resolving to invasive procedures (50). Patients who are positive for heterozygous mutations should be on closer follow-up and never left unattended due to the risk for hypertension and later-onset progressive disease. The close clinical care and timely institution of therapy upon the appearance of hypertension or proteinuria will prolong the kidney function.

3. Current technologies have revealed hundreds of DNA variants, often of unknown significance. Variant analysis includes the extensive screening of online databases (ARUP, LOVD, HGMD, Ensembl, dpSNP), while unrecorded findings are systematically evaluated using a number of prediction

software for scoring (see Material and Methods), and their frequency is examined in a representative sample of the general population.

4. Equally important is to maintain and curate an in-house database of variants, pathogenic or neutral, for easy access during genetic investigations. It is the norm that every population has its own repertoire of DNA variants with varying frequencies, especially when serving island populations with strong founders like the Cretan, the Cypriot and others.

5. The term "familial benign hematuria" should be avoided all-together as a misnomer and an alternative term, perhaps "Later-Onset Alport-related Nephropathy" could be adopted to describe patients with thin basement membranes due to heterozygous *COL4* mutations, with Alport-like adverse outcomes at older ages. We understand that some authors who find also ultrastructural glomerular features pathognomonic for AS, or even extra renal manifestations (although very rare), prefer the use of autosomal dominant AS, although this term has not been globally accepted.

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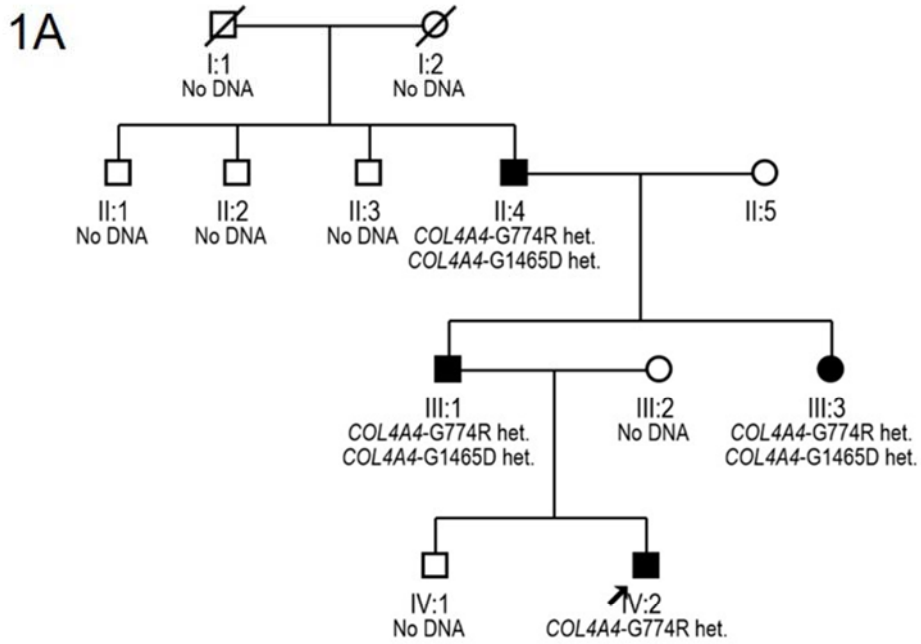
Figure legends

Figures 1A & 1B: Pedigrees of families CR-5408 and CR-5424, with patients that carry two mutations, *COL4A4*-p.G774R and *COL4A4*-p.G1465D *in cis*. Judging from the fact that patient IV-2 in CR-5408 carries only mutation *COL4A4*-p.G774R, this is definitely pathogenic. Patients in these families had features of progressive disease, with biopsies featuring podocyte effacement and focal segmental glomerulosclerosis.



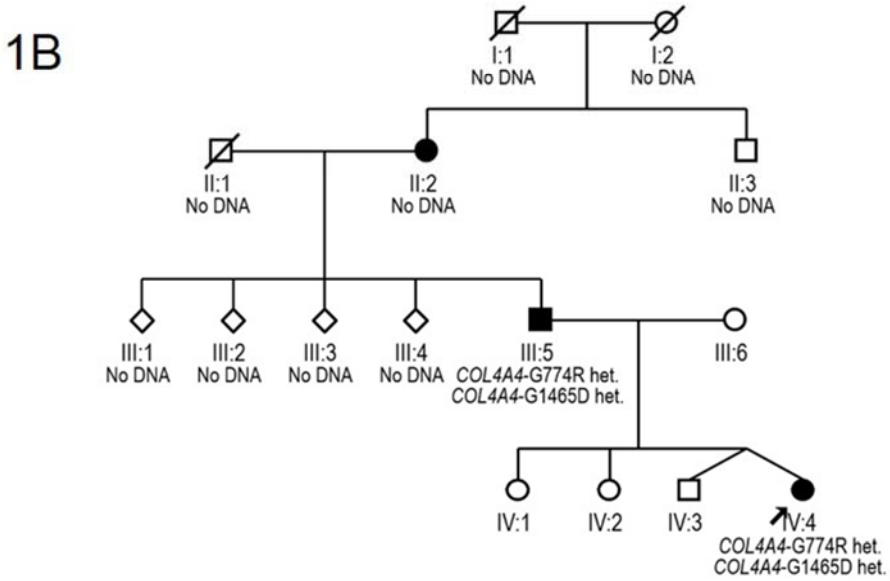
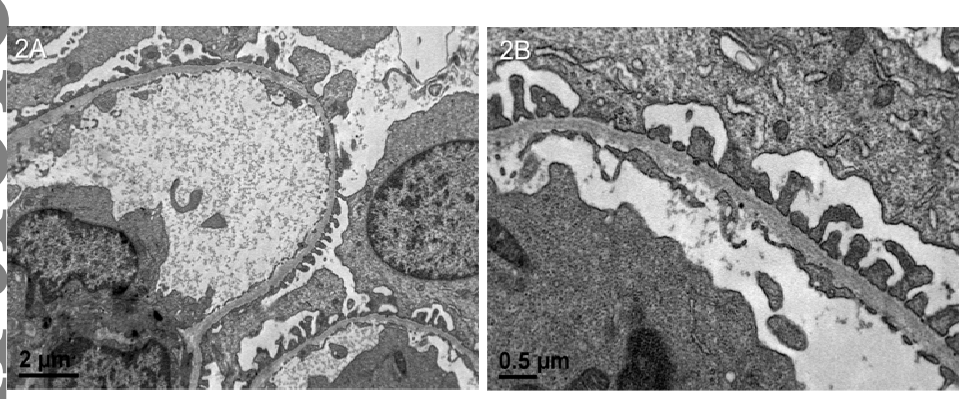


Figure 2A & 2B: Electron microscope of biopsy from patient IV-2, family CR-5408. There is thinning of the glomerular basement membrane, average thickness of 190 nm, with regions of podocyte foot process effacement.



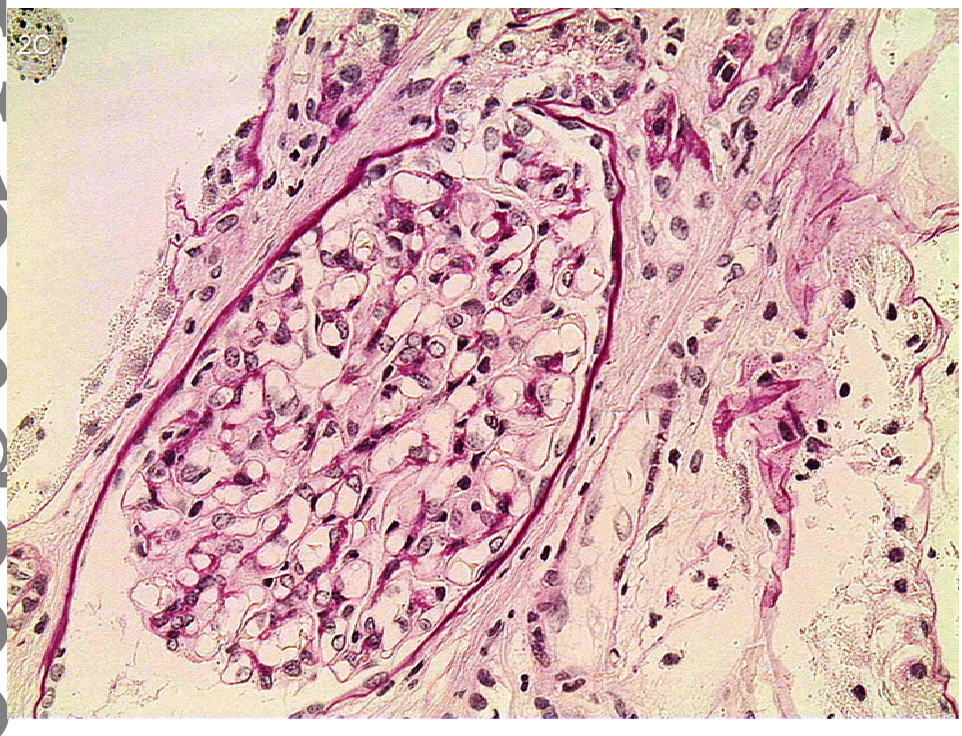


Figure 2C: Light microscope of biopsy from patient IV-4, family CR-5424. Thin basement membranes, glomerular tuft synechia close to the uric pole and relative glomerulomegaly for patient's age. PASx400

Table 1: Information on the 15 pathogenic mutations identified here, plus the two variants of unclassified significance (VUS: *COL4A4*-p.L30F and *COL4A4*-p.G1465D). Underlined are the nine novel mutations identified here. NA: Not Available

Gene	Coding	Protein	dbSNP entry	MAF	SIFT	SNPs3D	Grantham	Polyphen-2	Reference
COL4A3	c.765+2T>C		rs869025326	NA	NA	NA	NA	NA	(Kovacs, Kalmar et al. 2016)
COL4A3	c.1006G>T	p.G336C	NA	NA	0.00	-2.74	159	1.000	(Fallerini, Dosa et al. 2014)
COL4A3	c.3751C>A	p.G1251S	NA	NA	0.00	-2.70	56	1.000	Novel – this study
COL4A4	c.88T>C	p.L30F	rs758174051	A=0.00003/4 (ExAC)	0.06	NA	22	0.976	NA
COL4A4	c.428G>T	p.G143V	NA	NA	0.00	-3.71	109	1.000	(Papazachariou, Demosthenous et al. 2014)
COL4A4	c.489+1G>C		NA	NA	NA	NA	NA	NA	Novel – this study
COL4A4	c.2242G>A	p.G748S	rs762139460	T=0.00004/5 (ExAC)	0.00	-2.29	56	0.954	Novel – this study
COL4A4	c.2320G>C	p.G774R	rs569681869	G=0.00009/11 (ExAC)	0.00	-4.07	125	1.000	(Slajpah, Gorinsek et al. 2007)
COL4A4	c.2843delG	p.G948Dfs2*	NA	NA	NA	NA	NA	NA	Novel – this study
COL4A4	c.3044G>A	p.G1015E	rs764323652	T=0.00005/6 (ExAC)	0.01	-3.23	98	1.000	Novel – this study
COL4A4	c.3461G>T	p.G1154V	NA	NA	0.00	-4.68	109	1.000	Novel – this study
COL4A4	c.4393G>A	p.G1465D	rs533297350	T=0.00009/11 (ExAC)	0.06	-2.61	94	0.999	(Fallerini, Dosa et al. 2014)
COL4A5	c.1094G>T	p.G365V	NA	NA	0.00	-3.07	110	1.000	Novel – this study
COL4A5	c.2228G>T	p.G743V	NA	NA	0.00	-4.37	109	1.000	Novel – this study
COL4A5	c.3106G>A	p.G1036R	NA	NA	0.00	-4.39	125	1.000	(Hertz 2009)
COL4A5	c.4060G>A	p.G1354S	NA	NA	0.03	-3.38	56	1.000	Novel – this study

COL4A5	c.4699T>C	p.C1567R	rs104886288	NA	0.00	-3.13	180	1.000	(Knebelmann, Breillat et al. 1996)

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Table 2: Clinical and pathologic data for the families with a heterozygous pathogenic mutation in the *COL4A3* or *COL4A4* gene. All families had been referred for genetic investigation because of the presence of familial microscopic hematuria. Some families had many more subjects than DNA available for these studies.

Family	Mutation carriers (molecularly confirmed)			Biopsy Results	Mutation	MH Only (age)	MH+ Proteinuria, Normal GFR (age)	Impaired renal function OR ESRD (age)	Not clinically tested (age)	ESRD (age)
	Total	♂	♀							
CR-4214	1	0	1	mesangio-hyperplastic GN, focal sclerosis, IgM+, C3+, weak *COL4A5* in IHC (1♀)	*COL4A4*-p.G748S	0	1♀ (56)	0	0	0
GR-5310	1	0	1	TBMN (1♀)	*COL4A4*-p.G1154V	0	1♀ (78)	0	0	0
CR-5316*	8	5	3	*FSGS (no EM) (1♀)	*COL4A4*-c.2843delG (p.G948Dfs2*) /*COL4A4*-p.L30F	3♂ (22, 29, 39)	2♂ (50, 81) 2♀ (42, 56)	1♀ (53)	0	0
CR-5319	4	1	3	ND	*COL4A3*-p.G336C	1♂ (16) 1♀ (52)	0	0	2♀ (21, 88)	0
CR-5333	9	4	5	TBMN-FSGS (1♂, 2♀,) FSGS (no EM) (1♂)	*COL4A3*-p.G1251S	1♀ (22)	2♀ (46, 57)	3♂ (64, 70, 76)	1♂ (?) 2♀ (31, ?)	3♂ (65, 61, 55)
CR-5363	4	2	2	TBMN-FSGS (1♂) FSGS, IgM+, C3+ (no EM) (1♂)	*COL4A4*-p.G748S	1♀ (38)	0	2♂ (34, 72) 1♀ (46)	0	1♂ (23)
CR-5408	4	3	1	TBMN (1♂)	*COL4A4*-p.G774R / *COL4A4*-p.G1465D	2♂ (47, 69) 1♀ (45)	1♂ (19)**	0	0	0
GR-5416	1	1	0	ND	*COL4A4*-p.G1015E	0	0	1♂ (55)	0	0
CR-5424	2	1	1	TBMN-Alport (1♀)	*COL4A4*-p.G774R / *COL4A4*-p.G1465D	1♂ (62) 1♀ (20)	0	0	0	0
GR-5433	3	0	3	TBMN-FSGS (2♀)	*COL4A3*-c.765+2 T>C	1♀ (67 asympt.)	2♀ (12, 41)	0	0	0
GR-5473	2	0	2	ND	*COL4A4*-p.G143V	2♀ (7, ?)	0	0	0	0
GR-5499	3	2	1	ND	*COL4A4*-c.489+1 G>C	1♂ (42) 1♀ (39)	0	1♂ (75)	0	1♂ (63)
SUM (%)	**42 100%**	**19 45%**	**23 55%**	**13**	**12**	**8♂/9♀ 40,5%**	**3♂/8♀ 26%**	**7♂/2♀ 21%**	**1♂/2♀ 7%**	**5♂ 12%**

*Two patients of this family have Alport Syndrome due to homozygosity (one biopsy proven) and are not included in this table.
**This patient is carrier only for the *COL4A4*-p.G774R mutation.
? Not known

ESRD, End-stage renal disease; EM, Electron Microscope; FSGS, Focal segmental glomerulosclerosis; MH, Microscopic hematuria; ND, Not done or not determined with certainty; TBMN, Thin basement membrane nephropathy; CR, Cretan origin; GR, Greek origin (not Cretan)

Table 3: Clinical and pathologic data for the families with a pathogenic mutation in the X-linked *COL4A5* gene. All families had been referred for genetic investigation because of the presence of familial microscopic hematuria and not Alport syndrome. Some families had many more subjects than DNA available for these studies.

Family	**Mutation carriers (molecularly confirmed)**			**Biopsy Results**	**Mutation**	**MH Only (age)**	**MH+ Proteinuria, Normal GFR (age)**	**Impaired renal function OR ESRD (age)**	**ESRD (age)**
	Total	♂	♀						
GR-4242	4	1	3	Immuno-deposits (1♀)	*COL4A5*-p.G1354S	1♂ (14) 2♀ (39, 47)	0	1♀ (59)	0
GR-4243	3	1	2	ND	*COL4A5*-p.C1567R	1♂ (8) 1♀ (10)	1♀ (37)	0	0
CR-4244	3	0	3	FSGS (no EM) (1♀) MePGN-FSGS (1♀)	*COL4A5*-p.G1036R	0	2♀ (34, ?)	1♀ (32)	1♀ (23)
CR-4245	5	1	4	FSGS (no EM) (2♂)	*COL4A5*-p.G365V	4♀ (17, 18, 19, 21)	0	1♂ (49)	1♂ (28)
CR-4246	5	2	3	FSGS (no EM) (1♂)	*COL4A5*-p.G743V	2♀ (14, 48)	1♂ (14) 1♀ (14)	1♂ (46)	1♂ (25)
SUM (%)	**20 100%**	**5 25%**	**15 75%**	**6**	**5**	**11 55%**	**1♂/4♀ 25%**	**2♂/2♀ 20%**	**2♂/1♀ 15%**

? Not known

CRF, Chronic renal failure; ESRD, End-stage renal disease; EM, Electron Microscope; FSGS, Focal segmental glomerulosclerosis; MH, Microscopic hematuria; ND, Not done or not determined with certainty; TBMN, Thin basement membrane nephropathy; CR, Cretan origin; GR, Greek origin (not Cretan); MePGN, membrano-proliferative glomerulonephritis

Table 4: Non-pathogenic coding variants found by NGS in *COL4A3*, *COL4A4*, *COL4A5*, *CFHR5* and *FN1* genes. Two variants, *CFHR5*-p.Y565C and *FN1*-p.T700=, have not been found in any of the databases looked for (Ensembl, ClinVar, HGMD, LoVD) and remain as variants of unclassified significance.

	Gene	Change in coding sequence	Change in protein sequence	SNP code	MAF
1	COL4A3	c.127G>C	p.G43R	rs13424243	C-23%
2	COL4A3	c.172G>A	p.G58S	rs184730597	A-0.02%
3	COL4A3	c.222G>T	p.P74=	rs187950806	T<0.01%
4	COL4A3	c.373T>C	p.C125R	This study	N/A
5	COL4A3	c.422T>C	p.L141P	rs10178458	C-20%
6	COL4A3	c.485A>G	p.E162G	rs6436669	A-20%
7	COL4A3	c.976G>T	p.D326Y	rs55703767	T-12%
8	COL4A3	c.1195C>T	p.L399=	rs10205042	C-23%
9	COL4A3	c.1223G>A	p.R408H	rs34505188	A-10%
10	COL4A3	c.1352A>G	p.H451R	rs11677877	G-11%
11	COL4A3	c.1452G>A	p.G484=	rs34019152	A-11%
12	COL4A3	c.1721C>T	p.P574L	rs28381984	T-40%
13	COL4A3	c.3258G>A	p.G1086=	rs147085074	A-0.3%
14	COL4A3	c.3807C>A	p.D1269E	rs57611801	A-4%
15	COL4A3	c.4494C>G	p.T1498=	rs200454769	G-0.06%
16	COL4A4	c.665C>T	p.P222L	rs773533313	T-0.02%
17	COL4A4	c.1413A>G	p.K471=	This study	N/A
18	COL4A4	c.1444C>T	p.P482S	rs2229814	C-45%
19	COL4A4	c.1634G>C	p.G545A	rs1800516	C-0.02%
20	COL4A4	c.3011C>T	p.P1004L	rs1800517	C-45%
21	COL4A4	c.3594G>A	p.G1198=	rs10203363	A-50%
22	COL4A4	c.3684G>A	p.K1228=	rs2229812	A-50%
23	COL4A4	c.3979G>A	p.V1327M	rs2229813	A-49%
24	COL4A4	c.4080G>A	p.P1360=	rs2228556	A=49%
25	COL4A4	c.4207T>C	p.S1403P	rs3752895	T-47%
26	COL4A4	c.4548A>G	p.V1516=	rs2228555	A-36%
27	COL4A4	c.4932C>T	p.F1644=	rs2228557	T-46%
28	COL4A5	c.2896A>G	p.K966E	This study	N/A
29	COL4A5	c.3513A>G	p.Q1171=	rs2273051	G-17%
30	COL4A5	c.3519T>G	p.G1173=	rs61735627	G-0.03%
31	CFHR5	c.136C>T	p.P46S	rs12097550	T-1%
32	CFHR5	c.432A>T	p.K144N	rs181511327	T<1%
33	CFHR5	c.576T>G	p.F192L	rs151134004	G<1%
34	CFHR5	c.1067G>A	p.R356H	rs35662416	A-2%
35	CFHR5	c.1694A>G	p.Y565C	This study	N/A
36	FN1	c.44A>T	p.Q15L	rs1250259	T-14%
37	FN1	c.378C>T	p.I126I	rs2289202	T-14%
38	FN1	c.1070G>A	p.G357E	rs140926439	A<1%
39	FN1	c.1110G>A	p.T369T	rs149123808	A<1%
40	FN1	c.2091C>T	p.F697F	rs149878788	T<1%
41	FN1	c.2100C>T	p.T700T	This study	N/A
42	FN1	c.2442T>A	p.P814P	rs7596677	A-47%
43	FN1	c.2449A>C	p.T817P	rs2577301	A<1%
44	FN1	c.2489G>A	p.R830H	rs775576039	A<1%
45	FN1	c.3111A>C	p.G1037G	rs7589580	C-29%
46	FN1	c.3156A>C	p.P1052P	rs1053238	C-29%
47	FN1	c.4725A>G	p.E1484E	rs13652	G-13%
48	FN1	c.5691A>T	p.G1897G	rs1132741	T-32%
49	FN1	c.6634A>G	p.I2212V	rs17449032	G-16%
50	FN1	c.6781G>A	p.V2261I	rs1250209	G<1%
51	FN1	c.7161T>C	p.Y2387Y	rs11651	G-28%