

**Collagen type IV-related nephropathies in Portugal:
pathogenic *COL4A3* and *COL4A4* mutations and
clinical characterization of 25 families**

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Abstract

Pathogenic mutations in genes *COL4A3/COL4A4* are responsible for autosomal Alport syndrome (AS) and thin basement membrane nephropathy (TBMN). We used Sanger sequencing to analyze all exons and splice site regions of *COL4A3/COL4A4*, in 40 unrelated Portuguese probands with clinical suspicion of AS/TBMN. To assess genotype-phenotype correlations, we compared clinically relevant phenotypes/outcomes between homozygous/compound heterozygous and apparently heterozygous patients. Seventeen novel and four reportedly pathogenic *COL4A3/COL4A4* mutations were identified in 62.5% (25/40) of the probands. Regardless of the mutated gene, all patients with ARAS manifested chronic renal failure (CRF) and hearing loss, whereas a minority of the apparently heterozygous patients had CRF or extrarenal symptoms. CRF was diagnosed at a significantly younger age in patients with ARAS. In our families, the occurrence of *COL4A3/COL4A4* mutations was higher, while the prevalence of XLAS was lower than expected. Overall, a pathogenic *COL4A3/COL4A4/COL4A5* mutation was identified in >50% of patients with fewer than three of the standard diagnostic criteria of AS. With such a population background, simultaneous next-generation sequencing of all three genes may be recommended as the most expedite approach to diagnose collagen IV-related glomerular basement membrane nephropathies.

Key words: Alport syndrome, *COL4A3*, *COL4A4*, *COL4A5*, Thin Basement Membrane Nephropathy.

Introduction

Alport syndrome (AS) is a hereditary hematuric glomerulopathy associated with sensorineural hearing loss (SNHL) and ocular abnormalities, caused by pathogenic mutations in the genes encoding the $\alpha 3$ (*COL4A3*), $\alpha 4$ (*COL4A4*) and $\alpha 5$ (*COL4A5*) chains of collagen type IV (1, 2), which are major structural components of the glomerular basement membrane (GBM). Although rare in the general population, AS is one of the most common genetic disorders leading to advanced chronic renal failure (CRF) and end-stage renal disease (ESRD) in young adults. The majority of affected families have X-linked AS (XLAS; MIM#301050), due to mutations in *COL4A5*. Homozygous or compound heterozygous mutations in *COL4A3* or *COL4A4* cause autosomal recessive AS (ARAS; MIM#203780), which is diagnosed in about 15% of cases. Autosomal dominant AS (ADAS; MIM#104200) is an uncommon and clinically less severe form of the disease, segregating with heterozygous mutations in *COL4A3* or *COL4A4*. Heterozygosity for pathogenic mutations in these genes can also be identified in up to 40-50% of the families with thin basement membrane nephropathy (TBMN; MIM#141200), clinically characterized by persistent or recurrent hematuria, but lacking the extrarenal complications of AS. However, some of these patients develop significant proteinuria, hypertension and progressive chronic kidney disease (CKD), even reaching ESRD later in life (2).

The heterotrimeric association of $\alpha 3(\text{IV})/\alpha 4(\text{IV})/\alpha 5(\text{IV})$ in the GBM and the spectrum of overlapping clinical manifestations associated with pathogenic mutations in any of their genes, ranging from isolated hematuria to ESRD, is the rationale for the nosological concept of collagen IV-related GBM nephropathies (3). Comprehensive clinical investigation of probands and their relatives, including screening for renal and extrarenal signs of AS in family members, along with thorough pedigree analysis, are key steps in the diagnostic process of collagen IV-related GBM nephropathies (4).

Mutational analysis of *COL4A3* and *COL4A4* can be used to confirm the diagnosis of autosomal collagen IV-related GBM nephropathies, perhaps averting the need for kidney biopsy (2). Screening of asymptomatic at-risk relatives, proper counseling of living-related donors for kidney transplantation, and preimplantation or prenatal diagnosis for prevention of disease recurrence in the progeny of at-risk couples, become much easier in families where the causative mutation(s) has(have) been identified.

The purpose of this study was to estimate the prevalence of pathogenic *COL4A3* and *COL4A4* mutations among Portuguese patients with collagen IV-related GBM nephropathies, in order to implement a cost-effective laboratory approach to their genetic diagnosis.

Material and Methods

Details of the study design, data collection protocol, patient inclusion criteria and ethics clearance are reported elsewhere (manuscript submitted to Clinical Genetics). In brief, 40 apparently unrelated probands diagnosed with AS/TBMN, either with a family history suggestive of autosomal inheritance of kidney disease (n=5) or without detectable pathogenic mutations in *COL4A5* (n=35), as screened by Sanger sequencing and multiplex ligation-dependent probe amplification, were selected for molecular analysis of *COL4A3* and *COL4A4*.

Genomic DNA was extracted from leukocytes. Polymerase chain reaction (PCR) products covering the entire coding sequence and splice regions of the two genes were generated, purified and automatically sequenced by capillary electrophoresis either at the Molecular Genetics Laboratory, Viapath, London, United Kingdom (n=18), the Center for Nephrology and Metabolic Diseases, Weisswasser, Germany (n=12), or through Gendia, Antwerp, Belgium (n=10). The confirmatory assays in probands and genetic testing of at-risk relatives, targeted to the pathogenic mutation(s) in each family, were carried out at the

Department of Genetics, Faculty of Medicine, University of Porto, Porto, Portugal (PCR conditions provided upon request).

For the genotype-phenotype correlation analyses, relevant outcomes were statistically compared between homozygotes and compound heterozygotes, clinically corresponding to patients with ARAS, and the (apparently) heterozygous individuals, irrespective of their clinical phenotypes.

Results

Three previously reported (5–7) and 10 novel (likely) pathogenic *COL4A3* mutations were identified in 18 apparently unrelated families (Table S1, Supporting Information). The pathogenic mutation p.(Gly407Arg) was found in 8 families from the same geographic region, segregating with the same *COL4A3* intragenic polymorphisms. This mutation caused ARAS when present together with a second *COL4A3* pathogenic mutation and, in heterozygotes, was associated with a wide array of age-dependent clinical phenotypes, ranging from absence of microscopic hematuria, to isolated microscopic hematuria, to ESRD. The recurrently described p.(Leu1474Pro) variant of uncertain significance (VUS) (3, 5–10) was identified in two female probands, in compound heterozygosity with different pathogenic *COL4A3* mutations. As these two patients had early progression to ESRD, they were included with the other homozygotes and compound heterozygotes for the genotype-phenotype correlation analyses.

One previously reported (10, 11) and seven novel (likely) pathogenic *COL4A4* mutations were identified in 8 families (Table S1, Supporting Information). The recurrently described p.(Gly999Glu) VUS (7, 12, 13) was found, apparently in heterozygosity, in a 37-year-old female who had been on renal replacement therapy (RRT) since age 18 years. Mutational screening of *COL4A3* and *COL4A5* did not show additional sequence variants,

but since this *COL4A4* VUS is of questionable pathogenicity, reaching polymorphic frequency in the Slovenian population (13), this proband was not included in the genotype-phenotype correlation analyses.

Genetic screening of at-risk relatives allowed identification of three compound heterozygotes with pathogenic *COL4A3* mutations and 31 heterozygotes for a pathogenic *COL4A3* (n=20) or *COL4A4* (n=11) mutation. In 11 families with *COL4A3* mutations and five families with *COL4A4* mutations, the disease phenotype segregated with the identified mutation(s) in at least one relative of the proband.

The diagnosis of ARAS was genetically confirmed in 12 probands and in three of their relatives. Five probands were apparently homozygous for *COL4A3* (n=3) or *COL4A4* (n=2) mutations, the other seven being compound heterozygotes for *COL4A3* mutations. The higher prevalence of *COL4A3* mutations in probands with ARAS was statistically significant (p=0.02). Although the heterozygous condition of their parents could be genetically confirmed only in two cases, the phenotypes of the probands, and their family histories, were compatible with clinical diagnosis of ARAS in all cases. Parental consanguinity was recognized in two of the ARAS probands, including a compound heterozygote for *COL4A3* mutations.

Thirteen probands were apparently heterozygous for pathogenic *COL4A3* (n=7) or *COL4A4* (n=6) mutations. Besides microscopic hematuria, 12 of these patients also manifested proteinuria and seven had developed CKD stage ≥ 2 , with mutations *COL4A3* p.(Gly407Arg) and *COL4A4* p.(Asp191Glyfs*29) being associated with risk of progression to ESRD. Among the prospectively identified heterozygotes of whom the relevant laboratory data were available, 25% (6/24; age range: 24-78 years) had no manifestations of kidney disease.

The median ages and gender distribution in the subcohort of homozygous/compound heterozygous patients, and in the subcohort of apparently heterozygous patients, was similar (Table 1). The diagnosis of microscopic hematuria was made at a significantly younger median age in homozygous/compound heterozygous patients (12 vs. 26 years; $p=0.006$). As compared with (apparently) heterozygous patients, the prevalence of proteinuria, hypertension and CRF were significantly higher in the former, while median ages at diagnosis were significantly lower. All homozygous/compound heterozygous patients, but only 14% of (apparently) heterozygous patients, had already started RRT ($p<0.001$); however, the difference in the median ages at start of RRT between the two groups did not reach statistical significance. Subjective hearing loss, anterior lenticonus and dot-and-fleck retinopathy were significantly higher in homozygous/compound heterozygous patients. Diagnostic kidney biopsies were performed at significantly younger ages in homozygous/compound heterozygous patients, but the prevalence of distinctive GBM ultrastructural features of AS did not differ between the two groups (data not shown).

In patients who underwent complete assessment of the standard AS diagnostic criteria (14), a highly significant linear trend ($p<0.01$) was observed between the number of criteria identified in each patient and the probability of detecting at least one pathogenic mutation in any of the *COL4A3*/*COL4A4*/*COL4A5* genes (Table 2). The probability of identifying a pathogenic *COL4A3*, *COL4A4* or *COL4A5* mutation in patients with microscopic hematuria and two additional diagnostic criteria was 57%.

Discussion

We have identified four previously reported and 17 novel (likely) pathogenic *COL4A3* and *COL4A4* mutations in 25 of 40 (62.5%) apparently unrelated Portuguese probands with the clinical suspicion or diagnosis of an autosomal collagen IV-related GBM nephropathy.

The relative frequencies of the different types of pathogenic *COL4A3*/*COL4A4* mutations identified do not statistically differ from those reported in the Human Gene Mutation Database (HGMD®; <http://www.hgmd.cf.ac.uk/>, accessed on March 1, 2014). Small deletions/duplications constituted 42.9% (9/21) of the total and presumably affect the mRNA reading frame in all but one case. Missense mutations, all involving glycine substitutions, accounted for 28.6% (6/21). However, since large deletions/duplications and deep intronic mutations were not screened in any of the genes, the overall prevalence of pathogenic *COL4A3*/*COL4A4* mutations in our cohort may have been slightly underestimated.

The *COL4A3* glycine substitution p.(Gly407Arg) was identified in several apparently unrelated families that probably shared a common ancestor, suggesting that it might be a founder mutation in the Portuguese population. Individuals carrying this mutation in (apparent) heterozygosity exhibited a wide range of renal disease manifestations, from asymptomatic microscopic hematuria to ESRD, but the majority (5/6; 83%) also had audiological confirmed SNHL.

Among the (apparently) heterozygous individuals for a *COL4A3* or *COL4A4* pathogenic mutation, at least 11% developed ESRD and at least 22% showed SNHL on audiological examination. Although these cases might represent true instances of ADAS in families with incomplete or late-onset penetrance of ESRD, it cannot be excluded that some of these cases have ARAS with the second pathogenic mutation escaping detection, or even that the hearing loss has a distinct cause. Alternatively, superimposed environmental risk factors for CKD or the co-inheritance of genetic risk variants in other *loci* may contribute to the progression to ESRD in probands with more severe renal phenotypes (2).

Whatever the biological explanation for the phenotypic variability of heterozygous *COL4A3* or *COL4A4* mutations, the incomplete penetrance of clinical manifestations implies that genotyping of at-risk individuals is the only reliable way of determining their genetic

status, identifying those who need specific medical follow-up and reproductive genetic counseling. Because the progression of renal disease in heterozygous carriers of pathogenic *COL4A3* or *COL4A4* mutations is largely unpredictable, periodic clinical surveillance is warranted, even in individuals who are asymptomatic at baseline evaluation, and early institution of pharmacological renin-angiotensin system blockade, to delay the onset of CRF and progression to ESRD, should be properly considered (4).

Overall, the two-stepped molecular analyses of *COL4A5* and *COL4A3/COL4A4* in our cohort established the diagnosis of a collagen IV-related GBM nephropathy in 72.3% of 65 apparently unrelated families; however, only 46.8% of those families with a genetically confirmed diagnosis had XLAS (manuscript submitted to Clinical Genetics). The lower than expected prevalence of XLAS in our cohort, and the high *COL4A3/COL4A4/COL4A5* mutation detection rate in patients with fewer than three of the standard diagnostic criteria of AS, are in line with the results of recently published Italian (15) and French (16) studies which have used next-generation sequencing (NGS) to simultaneously screen *COL4A5/COL4A4/COL4A3* for pathogenic mutations. The proportion of families (27.7%; 18/65) in which our diagnostic approach failed to identify a pathogenic mutation was significantly lower than in the Italian study.

NGS is a promising method for detecting pathogenic mutations in genetically heterogeneous disorders like the collagen IV-related GBM nephropathies. Particularly in populations where XLAS is not predominant, like the Portuguese, NGS may be cost-effective as first-tier approach to the genetic diagnosis of patients with clinical suspicion of AS/TBMN.

References

1. Kashtan CE. Alport Syndrome and Thin Basement Membrane Nephropathy. In: Pagon RA, Adam MP, Ardinger HH, Bird TD, Dolan CR, Fong CT, Smith RJH, Stephens K, editors. Seattle (WA): University of Washington: 1993-2014: GeneReviews® [Internet; updated 2013 Feb 28].
2. Deltas C, Pierides A, Voskarides K. Molecular genetics of familial hematuric diseases. *Nephrol Dial Transplant* 2013; 28 (12): 2946–2960.
3. Longo I, Porcedda P, Mari F et al. *COL4A3/COL4A4* mutations: from familial hematuria to autosomal-dominant or recessive Alport syndrome. *Kidney Int* 2002; 61 (6): 1947-1956.
4. Savige J, Gregory M, Gross O, Kashtan C, Ding J, Flinter F. Expert guidelines for the management of Alport syndrome and thin basement membrane nephropathy. *J Am Soc Nephrol* 2013; 24 (3): 364–375.
5. Heidet L, Arrondel C, Forestier L, et al. Structure of the human type IV collagen gene *COL4A3* and mutations in autosomal Alport syndrome. *J Am Soc Nephrol* 2001; 12 (1): 97–106.
6. Badenas C, Praga M, Tazon B, et al. Mutations in the *COL4A4* and *COL4A3* genes cause familial benign hematuria. *J Am Soc Nephrol* 2002; 13 (5): 1248-1254.
7. Wang YY, Rana K, Tonna S, Lin T, Sin L, Savige J. *COL4A3* mutations and their clinical consequences in thin basement membrane nephropathy (TBMN). *Kidney Int* 2004; 65 (3): 786–790.
8. Lemmink HH, Mochizuki T, van den Heuvel LP, et al. Mutations in the type IV collagen alpha 3 (*COL4A3*) gene in autosomal recessive Alport syndrome. *Hum Mol Genet* 1994; 3 (8): 1269–1273.
9. van der Loop FT, Heidet L, Timmer ED, et al. Autosomal dominant Alport syndrome caused by a *COL4A3* splice site mutation. *Kidney Int* 2000; 58 (5): 1870–1875.

10. Tazon Vega B, Badenas C, Ars E, et al. Autosomal recessive Alport's syndrome and benign familial hematuria are collagen type IV diseases. *Am J Kidney Dis* 2003; 42 (5): 952-959.
11. Lemmink HH, Nillesen WN, Mochizuki T, et al. Benign familial hematuria due to mutation of the type IV collagen alpha4 gene. *J Clin Invest* 1996; 98 (5): 1114-1118.
12. Buzza M, Dagher H, Wang YY, et al. Mutations in the *COL4A4* gene in thin basement membrane disease. *Kidney Int* 2003; 63 (2): 447-453.
13. Slajpah M, Gorinsek B, Berginc G, et al. Sixteen novel mutations identified in *COL4A3*, *COL4A4*, and *COL4A5* genes in Slovenian families with Alport syndrome and benign familial hematuria. *Kidney Int* 2007; 71 (12): 1287-1295.
14. Flintner FA, Cameron JS, Chantler C, Houston I, Bobrow M. Genetics of classic Alport's syndrome. *Lancet* 1988; 2 (8618): 1005-1007.
15. Fallerini C, Dosa L, Tita R, et al. Unbiased next generation sequencing analysis confirms the existence of autosomal dominant Alport syndrome in a relevant fraction of cases. *Clin Genet* 2013; doi: 10.1111/cge.12258 (in press).
16. Moriniere V, Dahan K, Hilbert P et al. Improving Mutation Screening in Familial Hematuric Nephropathies through Next Generation Sequencing. *J Am Soc Nephrol* 2014; pii:ASN.2013080912 (in press).

Table 1. Comparison of clinical and demographic characteristics presented by Portuguese individuals with pathogenic *COL4A3* and *COL4A4* mutations according to their mutation status — homozygotes and compound heterozygotes versus heterozygotes.

Demographic and clinical features	Homozygotes and compound heterozygotes (N=15)	Heterozygotes (N=44)	p-value
Index-cases: % (n _o /n _t)	80 (12/15)	30 (13/44)	0.001
Male gender: % (n _o /n _t)	53 (8/15)	32 (14/44)	0.137
Age at enrolment: median [interquartile range]; (n)	42 [17]; (15)	46 [26.8]; (44)	0.296
Renal disease manifestations			
History of macroscopic hematuria: % (n _o /n _t)	55 (6/11)	12 (3/25)	0.012
Age at diagnosis: median [range]; (n)	8 [15]; (3)	17 [12]; (3)	0.343
History of microscopic hematuria: % (n _o /n _t)	100 (9/9)	77 (27/35)	0.175
Age at diagnosis: median [interquartile range]; (n)	12 [14.3]; (6)	26 [20.5]; (26)	0.006
History of proteinuria: % (n _o /n _t)	100 (12/12)	70 (23/33)	0.042
Age at diagnosis: median [interquartile range]; (n)	16 [13.5]; (9)	25 [19.5]; (22)	0.002
History of hypertension: % (n _o /n _t)	92.3 (12/13)	52 (17/33)	0.016
Age at diagnosis: median [interquartile range]; (n)	18 [14.8]; (8)	42 [18.5]; (13)	0.000
History of CKD stage 2 or higher: % (n _o /n _t)	100 (14/14)	51 (18/35)	0.001
Age at diagnosis: median [interquartile range]; (n)	20 [10]; (9)	46 [19.5]; (17)	0.000
History of renal replacement therapy : % (n _o /n _t)	100 (15/15)	14 (5/36)	0.000
Age at onset: median [interquartile range]; (n)	23 [12]; (15)	36 [22]; (5)	0.042
Glomerular basement membrane structural abnormalities			
Age at kidney biopsy: median [interquartile range]; (n)	17.5 [7.8]; (6)	34 [19]; (9)	0.004
Hearing loss			
Self-noticed or subjective: % (n _o /n _t)	93.3 (14/15)	35 (12/34)	0.000
Age first noticed: median [interquartile range]; (n)	9 [19]; (9)	36 [32*]; (3)	0.086
Hearing loss confirmed by audiogram: % (n _o /n _t)	90 (9/10)	53 (10/19)	0.098
Age at diagnosis: median [interquartile range]; (n)	32 [22]; (7)	44 [26.5]; (9)	0.009
Ocular anomalies			
Anterior lenticonus: % (n _o /n _t)	33 (3/9)	0 (0/25)	0.014
Age at diagnosis: median [interquartile range]; (n)	30 (-); (1)	-	-
Maculopathy: % (n _o /n _t)	63 (5/8)	9.1 (3/33)	0.003
Age at diagnosis: median [interquartile range]; (n)	34 [25]; (3)	40.5 [35]; (2)	0.610
Cataracts: % (n _o /n _t)	83 (5/6)	12 (3/25)	0.002
Age at diagnosis: median [range]; (n)	42.5 [17]; (2)	58 [15]; (3)	0.207

The following statistics were used: the chi-square or the Fisher's exact tests for comparisons of proportions; survival analysis for comparisons of time to events; non-parametric tests to compare selected continuous clinical outcomes. The data were analyzed with the software package PASW® Statistics 18 (SPSS Inc., Chicago, IL, USA).

N: total number of subjects in each subcohort; n: number of cases in whom the age of onset of a particular phenotypic manifestation or outcome could be assessed; n_o/n_t: number of subjects in whom a particular phenotypic feature or outcome was observed / number of subjects that were assessed for the particular phenotypic feature or outcome.

Ages are expressed in years.

CKD: chronic kidney disease, classified according to the guidelines of the National Kidney Foundation [New York, NY, USA; http://www.kidney.org/professionals/kdoqi/guidelines_ckd/p4_class_g1.htm];

eGFR: estimated glomerular filtration rate, calculated from serum creatinine levels using the CKD-EPI equation [http://www.kidney.org/professionals/kdoqi/gfr_calculator.cfm], expressed in ml/min/1.73m².

* Indicates the range instead of the interquartile range.

^a Statistics not computed for this variable because the proportions in the two groups are a constant.

Table 2. *COL4A3*, *COL4A4* and *COL4A5* mutation detection rate per number of diagnostic criteria met, among genetically tested Portuguese probands with complete or only partial assessment of the standard diagnostic criteria for Alport syndrome (14).

Diagnostic criteria met	<i>COL4A5</i> mutation-positive probands	<i>COL4A3</i> or <i>COL4A4</i> mutation-positive probands	<i>COL4A</i> (3,4,5) mutation-negative probands	Number of probands with each number of diagnostic criteria	<i>COL4A</i> (3,4,5) mutation detection rate per number of diagnostic criteria met
Probands with complete assessment of the standard diagnostic criteria					
1	0/2 (0%)	0/2 (0%)	2/2 (100%)	2	0/2 (0%)
2	2/7 (28.6%)	2/7 (28.6%)	3/7 (42.8%)	7	4/7 (57%)
3	1/9 (11.1%)	7/9 (77.8%)	1/9 (11.1%)	9	8/9 (89%)
4	4/5 (80%)	1/5 (20%)	0/5 (0%)	5	5/5 (100%)
<i>totals</i>	7	10	6	23	*p = 0.02
Patients with complete or partial clinical assessment of the standard diagnostic criteria					
1	4/13 (30.8%)	4/13 (30.8%)	5/13 (38.5%)	13	8/13 (62%)
2	10/28 (35.7%)	10/28 (35.7%)	8/28 (28.6%)	28	20/28 (71%)
3	4/19 (21.1%)	11/19 (57.9%)	4/19 (21.1%)	19	15/19 (79%)
4	4/5 (80%)	1/5 (20%)	0/5 (0%)	5	5/5 (100%)
<i>totals</i>	22	26	17	65	*p = 0.37

* The chi-square test for trend was used to assess the statistical association between the numbers of diagnostic criteria of Alport syndrome identified in each patient and the probability of finding pathogenic mutation(s) in any of the *COL4A3*/*COL4A4*/*COL4A5* genes. The data were analyzed with the software package GraphPad Prism® 5.0 (GraphPad Software, Inc.; La Jolla, CA, USA).