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Original Article

COL4A4 gene study of a European population: description of new mutations causing autosomal dominant Alport syndrome

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Abstract: Background: Autosomal forms of Alport syndrome represent 20% of all patients (15% recessive and 5% dominant). They are caused by mutations in the COL4A3 and COL4A4 genes, which encode α -3 and α -4 collagen IV chains of the glomerular basement membrane, cochlea and eye. Thin basement membrane nephropathy may affect up to 1% of the population. The pattern of inheritance in the 40% of cases is the same as autosomal dominant Alport syndrome: heterozygous mutations in these genes. The aim of this study is to detect new pathogenic mutations in the COL4A4 gene in the patients previously diagnosed with autosomal Alport syndrome and thin basement membrane nephropathy in our hospital. Methods: We conducted a clinical and genetic study in eleven patients belonging to six unrelated families with aforementioned clinical symptoms and a negative study of COL4A3 gene. The molecular study was made by conformation of sensitive gel electrophoresis (CSGE) and direct sequencing of the fragments that show an altered electrophoretic migration pattern. Results: We found two pathogenic mutations, not yet described: IVS3 + 1G > C is a replacement of Guanine to Cytosine in position +1 of intron 3, in the splicing region, which leads to a pathogenic mutation. c.4267C > T; p.P1423S is a missense mutation, also considered pathogenic. We also found seven new polymorphisms. Conclusions: We describe two new pathogenic mutations, responsible for autosomal dominant Alport syndrome. The other families of the study were undiagnosed owing to problems in the method employed and the possibility of mutations in other genes, giving rise to other diseases with similar symptoms.

Keywords: Autosomal alport syndrome, COL4A4 gene, spicing, missense

Introduction

Thin basement membrane nephropathy (TBMN) and Alport syndrome [1] are the two major clinical entities associated with disorders of the glomerular basement membrane occurring with haematuria. TBMN affects up to 1% of the population and is defined by a thinning of the glomerular basement membrane associated with haematuria and minimal proteinuria, with normal kidney function. It is inherited in an autosomal dominant pattern [1, 2].

Alport syndrome (AS) is a hereditary nephropathy characterized by progressive glomerulonephritis resulting in kidney failure, with typical ultrastructural changes in the glomerular basement membrane and haematuria, which is

often associated with sensorineural hearing loss and damage to the eye [2].

Both entities may be caused by mutations in COL4A3 or COL4A4 genes, which encode α 3(IV) and α 4(IV) collagen chains [3], which are present in the glomerular basement membrane, the cochlea and the eye. Autosomal recessive Alport syndrome (ARAS) (15% of cases of AS) is a consequence of homozygous mutations in COL4A3 and COL4A4 gene, or a result of compound heterozygous mutations in these genes. Autosomal dominant Alport syndrome (ADAS) and TBMN (5% of cases of AS) result from heterozygous mutations in these genes [4]. ARAS carriers and those affected by the dominant forms are included in the term “collagen type IV (α 3- α 4) nephropathy” [2].

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Table 1. Distribution and age of onset of symptoms studied

Clinical symptoms	Number of patients	Onset (years)
Chronic kidney disease	8 (72.7%)	39.5 (30-58, 25)
End-stage chronic kidney disease	4 (36.4%)	45.5 (24-52, 75)
Haematuria	10 (90.9%)	29 (8, 25-44)
Proteinuria	10 (90.9%)	39 (17, 5-56)
Hypertension	8 (72.7%)	41.5 (23, 25-52, 25)
Hearing loss	8 (72.7%)	8 (5-61)
Macular flecks	1 (9.1%)	48 (48-48)

The main indication of the disease is haematuria, but it can also cause proteinuria. The result is the development of chronic renal failure and end stage renal disease. The impairment of renal function is rapid and severe in both men and women with the recessive form, while symptoms for patients with ADAS may be milder and more gradual, which means the kidney disease may only become apparent around the 7th decade [5, 6].

We searched mutations in the COL4A4 gene in 6 families who suffer from autosomal forms of the disease, which had no pathogenic mutations in the COL4A3 gene. We describe seven, as yet unreported polymorphisms and two new pathogenic mutations in the COL4A4 gene associated with ADAS.

Material and methods

Eleven patients who belonged to six unrelated families with clinical symptoms of ARAS or collagen type IV ($\alpha3$ - $\alpha4$) nephropathy were selected. The clinical diagnosis of Alport syndrome was established when the index patient met at least two of the following criteria [7]: (1) Positive family history of macro or microscopic hematuria with or without progression to end-stage chronic kidney disease; (2) Progressive sensorineural high tone hearing loss; (3) Characteristic ocular injuries (lenticonus or perimacular retinopathy); (4) Compatible anatomopathological injuries.

The COL4A3 gene was previously analysed in all index patients and we did not find any pathogenic mutation. We made a descriptive analysis of the clinical data of the families in order to determine the characteristics of disease presentation. Results are expressed as percentages, median and interquartile range. The variables studied are listed in **Tables 1, 2**.

Molecular analysis

High molecular weight DNA was extracted from peripheral blood in all index patients. Coding regions and flanking intronic sequences of the COL4A4 gene were studied after amplification by polymerase chain reaction with Master Mix (Promega), under standard conditions (**Table S1**). The samples obtained were analysed by heteroduplex with acrylamide gels to

detect the possible mutations in these regions. Direct sequencing of the fragments that showed an altered electrophoretic migration pattern was performed in both directions, using an ABI 3100 Genetic Analyzer. Reading and processing of automatic sequences were performed with the 4Peaks 1.7.2 (1.7.1) program for Mac OS X (<http://www.mekentosj.com/science/4peaks>).

Results

We studied 11 patients, five males (45.5%) and six females (54.5%). Analysis of family trees (**Figure 1**) showed that seven patients (63.6%) had an autosomal dominant mode of inheritance. Five of them had clinical ADAS (family 6) and the remaining two patients suffered from BHF (family 1). Three patients (27.3%) suffered from ARAS and had a history of consanguinity (family 2) and the last patient (9.1%) had no family history, so the mode of inheritance could not be determined. The median age of clinical diagnosis was 51.5 years (19.75 to 55.75).

First family: The index patient was a 23-year-old woman who had an episode of microhematuria and proteinuria of 0.55 mg/24 h at the age of 14, which remained stable. Since that time she suffered from sporadic episodes of microhematuria. There were no signs of arterial hypertension, impairment of renal function, hearing loss, lenticonus or macular flecks. The complete kidney study and the renal biopsy did not reveal any conclusive results.

Her mother was a 48-year-old woman who was diagnosed with an isolated episode of microhematuria at the age of 42. Any previous episodes had not been reported. She did not show arterial hypertension, proteinuria, kidney failure or hearing or visual impairment. The maternal grandparents of the index case did not show any clinical symptom.

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Table 2. Distribution of the symptoms in each family

Family	Symptom	1	2	3	4	5	6
Hamaturia		Yes	Yes	Yes	Yes	Yes	Yes
Proteinuria		No	Yes	Yes	Yes	Yes	Yes
Chronic kidney disease		No	Yes	Yes	Yes	Yes	Yes
End-stage chronic kidney disease		No	Yes	Yes	No	No	Yes
Hearing loss		No	Yes	Yes	Yes	Yes	Yes
Macular flecks		No	No	Yes	No	No	No
Macrothrombocytopenia		No	No	No	Yes	No	No
Laminated glomerular basement membrane		No	Not studied	Not studied	Yes	Not studied	Yes

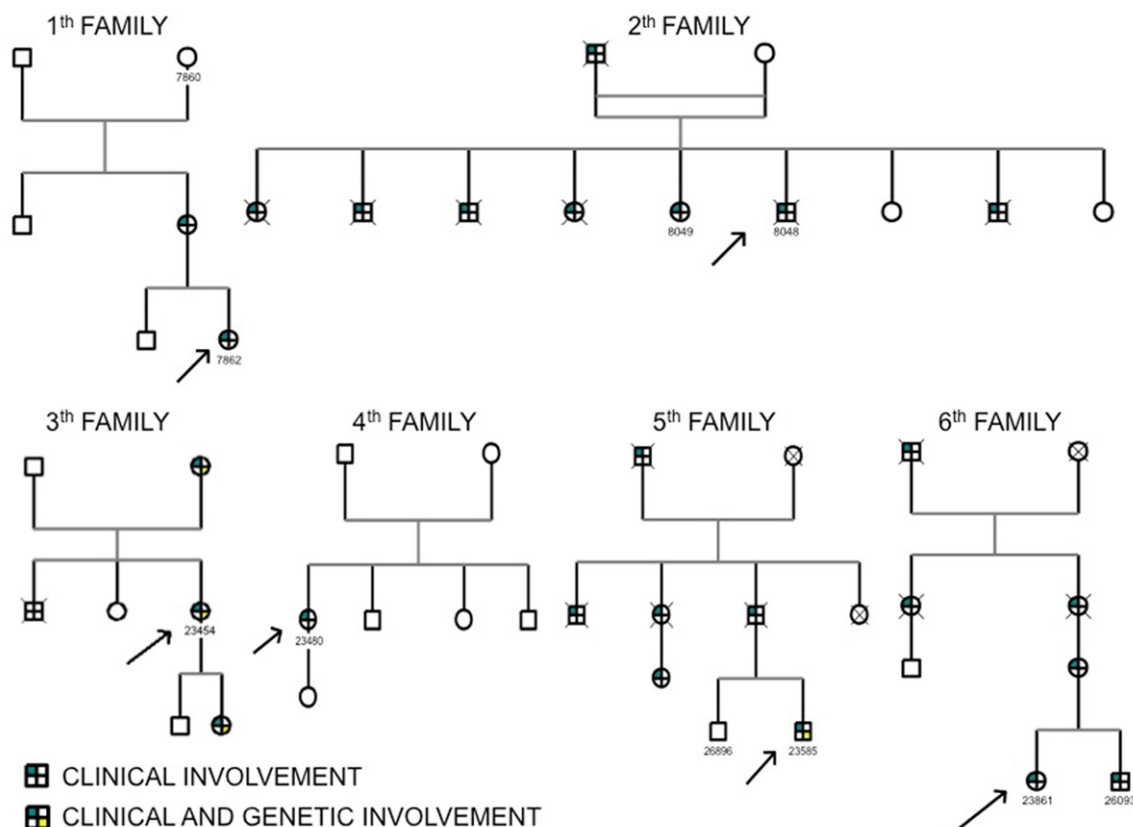


Figure 1. Genealogic trees of the studied families. Family 2 shows an autosomal recessive pattern of inheritance. The rest of them have an autosomal dominant one.

The personal and family record led us to suspect TBMN, but we did not find any pathogenic mutation after sequencing 25 exons of the COL4A4 gene.

Second family: The index case was a 54-year-old man. He had high tones sensorineural hearing loss from the age of 5. At the age of 50, he suffered from arterial hypertension, microhematuria and proteinuria of 1.9 g/24 h, with chronic kidney disease, which resulted in the

need of dialysis two years later. No renal biopsy was performed because he suffered from severe kidney impairment at the beginning of clinical monitoring. He was never diagnosed with visual impairment.

The patient came from a family of 9 children and his parents were related.

The father had been deaf since childhood and suffered from chronic kidney disease, which did

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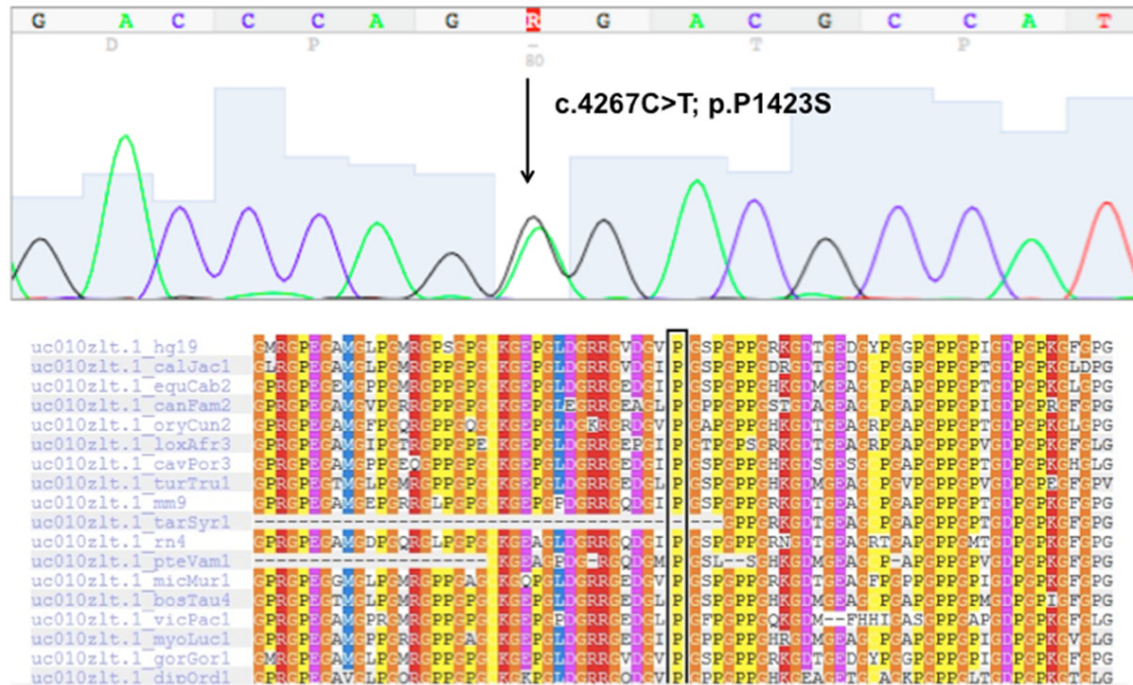


Figure 2. Genetic sequence of the c.4267C > T; p.P1423S mutation and the polypheny multiple sequence alignment, in which we can see the high degree of conservation of proline among different species. This fact leads us to think that the change of proline to serine could have a critical effect on the resulting protein.

not evolve to an end-stage one. The existence of hematuria, proteinuria or ocular injuries like lenticonus or macular flecks could not be studied.

The mother only suffered from type 2 diabetes.

The eldest sister had suffered from high tones sensorineural hearing loss since childhood. She died at the age of 50 due to a cerebral hemorrhage. She did not have renal disorders, visual impairment or arterial hypertension.

The next three siblings, two males and one female, suffered from sensorineural hearing loss and died during childhood due to unknown causes. The fifth sister was a 68-year-old woman who had had arterial hypertension and type 2 diabetes mellitus since she was 53. At 61 years old, she was diagnosed with high tones sensorineural hearing loss as well as chronic kidney disease, with creatinine clearance around 50 ml/min, which remained stable since that time. At the age of 62, a urinalysis showed microscopic hematuria and proteinuria (0.2 g/24 h). She refused to undergo a renal biopsy. She did not suffer ocular impairment.

The eighth brother had had high tones sensorineural hearing loss from the age of 4. At 39, he was diagnosed with nephrotic range proteinuria (3.9 g/24 h), macroscopic hematuria and chronic kidney disease, which evolved to end-stage kidney disease at the age of 53. It was then he started hemodialysis. He died two years later due to complications derived from the aforementioned kidney disease. He never suffered from hypertension or ocular injuries.

The two remaining sisters did not show any significant symptoms or abnormalities on testing.

The main symptom of the family was sensorineural hearing loss. However, we did not have any specific data regarding the continued development of this condition as most of the patients died during childhood and the information was not included in their medical records.

The clinical and familiar data suggested the presence of ARAS, but no pathogenic mutation was found after sequencing 20 exons of the studied gene.

Third family: The index case was a 49-year-old woman who, at the age of 6 was diagnosed with

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Table 3. Mutations found in our patients

Mutation	Meaning	Bibliography	Families
IVS 3 + 1 G > C	Splicing	This work	5
IVS10-39 T > C	Polimorfism	This work	2
IVS 12-58 A > G	Polimorfism	This work	4
IVS 12 + 30 G > A	Polimorfism	This work	3
IVS 17 + 24 A > T	Polimorfism	This work	6
IVS 19 + 21 C > A	Polimorfism	This work	4
IVS 37-61 G > T	Polimorfism	This work	2
IVS 38 + 40 C > A	Polimorfism	This work	2
c.3011C > T; p.P1004L	Polimorfism	[8]	6
c.3594G > A; p.G1198G	Polimorfism	[15]	1
c.3684G > A; p.K1228K	Polimorfism	[16]	1
c.3979G > A; p.V1327M	Polimorfism	[17]	1
c.4080G > A; p.P1360P	Polimorfism	[11]	1
c.4207T > C; p.S1403P	Polimorfism	[8]	5, 6
c.4267C > T; p.P1423S	Missense	This work	3
c.4932C > T; p.F1644F	Polimorfism	[17]	4, 5, 6

hearing loss from the age of 79. He did not show visual impairment.

The paternal family had a significant history of sensorineural hearing loss, as can be seen in the family tree. These patients could not be studied to determine the presence of other components of AS, as some of them had already passed away and others did not live in the city where the study was conducted.

The data of this family suggested the presence of ADAS. We found, in COL4A4 gene, the mutation IVS3 + 1G > C (c.192 + 1C in HGVS nomenclature) which had not been previously described. It should be considered pathogenic since the canonical sequence in RNA is affected. The absence of RNA prevented us from performing a functional study. However, the index case's brother who had no AS symptoms did not have it.

Sixth family: The index case was a 41-year-old woman who had her first episode of macrohematuria when she was 3 years old, after a tonsillar infection. From that time on, she suffered almost constantly from microhematuria, with episodes of macrohematuria due to different kinds of infections. At the age of 6, proteinuria of 0.35 g/24 h was diagnosed, which increased progressively until nephrotic range at 36 years old. A renal biopsy was performed at 22 years

old, which showed the lamination of the glomerular basement membrane.

She developed arterial hypertension and chronic kidney disease when she was pregnant (aged 36) which evolved rapidly and she was included in a hemodialysis program 3 years later.

The presence of high tones sensorineural hearing loss was established when she was 8 years old. She never showed any ocular impairment. Her brother was a 33-year-old who had had macrohematuria and nephrotic range proteinuria from the age of 9 months. He developed chronic kidney disease at 14 with a progressive worsening of his creatinine clearance. He began a program of peritoneal dialysis 5 years later. He never underwent renal biopsy. He was diagnosed with sensorineural hearing loss

when he was one year old and, like his sister did not show any visual impairment.

The mother of both patients suffered from microhematuria, non-nephrotic proteinuria, arterial hypertension, grade III chronic kidney disease and sensorineural hearing loss since adolescence. The patient's maternal grandfather died when he was 20 years old. He suffered from sensorineural hearing loss and chronic kidney disease, as did his two daughters (the patient's grandmother and aunt). The existence of hematuria, proteinuria or ocular impairment in these patients could not be determined because they had all passed away.

The family data suggested the existence of ADAS, but we could not find any pathogenic mutation after sequencing 21 exons of the COL4A4 gene.

Besides the two new pathogenic mutations, we have also found seven new intron variants (**Table 3**) in the different families. These variants are also present in healthy controls.

Discussion

In our work, we have searched pathogenic mutations in COL4A4 gene in families with phenotypic signs of ARAS, ADAS and TBMN, which had a previous diagnosis of absence of mutations in COL4A3 gene.

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The study of family 3 led us to the discovery of a missense mutation, not yet described. Mutation c.4267C > T; pP1423S involves the change of a proline by a serine in a region in which there has been no specific protein domain described (**Figure 2**). However, the $\alpha 4$ chain has 26 sequences Gly-Xaa-Yaa, wherein Xaa and Yaa is often proline or hydroxyproline. This confers flexibility to the protein [8]. Proline 1423 is located in a sequence Glycine-Valine-Proline. So this change could reduce that flexibility.

Although it was not possible to perform a functional study (due to the lack of availability in our laboratory) we can, however, speculate, from the population study in silico data and family segregation, that there is a high probability that this is the causative mutation of ADAS in this family.

The phenotype caused by this mutation is variable, since some members have mild disease (hearing loss and mild impairment of renal function) and other relatives have an aggressive form, with the need for renal replacement therapy early in life. The possible reason for this phenotypic heterogeneity may be the presence of pathogenic mutations in genes encoding other proteins of the glomerular basement membrane (integrins, metalloprotease, Laminins or podocin), and, by modulating the COL4A4 mutation, promote the onset of the kidney impairment [9, 10].

In family 5 (who suffer from ADAS) a mutation in intron 3 of the COL4A4 gene was found. Although most of the mutations described so far in the COL4A3 and COL4A4 genes are missense mutations, intronic mutations that modify the RNA maturation (*splicing*) have also been described [11]. The mutation IVS3 + 1G > C of the COL4A4 gene changes RNA processing, which affects a canonical sequence processing. The absence of RNA from the proband prevented us from determining whether the mutation involves the incorporation of intron 3 or loss of exon 3 in the coding sequence. Regardless, a change in the reading frame and a premature stop codon, which would produce a protein unable of adequately trimerizing, would likely occur. **Figure 3** shows the reading phase change if exon 3 is lost, and the premature stop codon that is produced in the case of the inclusion of intron 3 in RNA, which generates a truncated protein.

In this family, the mutation generates a mild phenotype, in which chronic kidney disease appears at an advanced age and there is no development to end stage chronic kidney disease. This phenotype coincides with the one traditionally described for ADAS [1, 2].

The main limitation of our study is that the technique used to detect mutations (PCR amplification of all exons, detection of changes in their electrophoretic mobility in heteroduplex and sequencing these exons) does not detect 100% of mutations [12]. This fact could explain why in families 2 and 6, with a clear clinic diagnosis of ARAS and ADAS, no pathogenic mutation was detected.

However, in families 1 and 4, this lack of diagnosis may be due to the technique's shortcomings or because of other diseases that may present with similar symptoms.

Family 1 had the diagnostic of TBMN, and only 40% of patients with this disease are heterozygous carriers of mutations in genes COL4A3/A4 [6], but the remaining members do not present any impairment in these genes. It has recently been reported that mutations in the CFHR5 gene, which encodes a complement protein, are associated with glomerulopathy with haematuria and risk of progressing to chronic kidney disease [13].

In the index case of family 4, the absence of COL4A3/COL4A4 gene mutations may also be related to a different clinical entity than AS. Macrothrombocytopenia has been associated with microhematuria, secondary to mutations in the MYH9 gene. There are three clinical entities: the May-Hegglin, Fechtner and Epstein syndromes, which share ultrastructural lesions with AS [14] and may be associated with hearing impairment and mimic this syndrome.

The seven undescribed intronic variants detected have been considered as population polymorphisms, because they are located in intronic regions, far from the splicing sites, which are shared by different families, as well as from randomly selected healthy individuals.

Conclusions

We describe two new pathogenic mutations in the COL4A4 gene, responsible for autosomal dominant Alport syndrome and 7 new intronic variants, considered as demographic polymorphisms.

Genetic diagnosis of autosomal Alport syndrome is hampered by the large presence of polymorphisms [8, 15-17] (both in the exons and in the intronic regions), and by the possibility that other genes are involved in generating very similar clinical syndromes.

Disclosure of conflict of interest

None.

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Table S1. Primers and amplification conditions of the COL4A4 gene

Exon	Secuency 5'-3'	Anealing Temperature	Size
2F	TCTGGAAGAGAAGACTGGCA	55 °C	152 pb
2R	AAGCAGGCAATCACACTGA		
3F	TGTTTAAATTAATCTGCGTT	52 °C	105 pb
3R	GCAACCAGAGCTAGTG		
4F	CGATGAGTACTGGTATACTA	55 °C	152 pb
4R	ATGCTGCCCATGTTGGTCTT		
5F	ACCCCATTTCTTTTAAATC	55 °C	208 pb
5R	GGTGAGTCTTTCATGTGAAT		
6F	TCTCTTGTGTTTTATTTCTG	50 °C	127 pb
6R	GATGAGTACTTCTGCCTTTT		
7F	TTTCGCAAAAATGCTTCACT	55 °C	211 pb
7R	CCACAGGGCCTGTCACTTA		
8F	TACTGAAATGGTAATACGCT	55 °C	184 pb
8R	CATGGGCTTACCTATTTGGA		
9F	TGTGTGGACTTAAAGCGATG	51 °C	96 pb
9R	TAGAGCCTGCTCAGGAGACT		
10F	TTGGGTAACAGATGCACTGA	55 °C	129 pb
10R	AAGGGATCACATCAGCAGTG		
11F	TTGTGTTTTTTCTCCCTTG	49 °C	110 pb
11R	TTTCATTGTTCAAGGCTCTA		
12F	AGCCAGAAGTCTTAATTGCT	55 °C	156 pb
12R	TCACCATTGCTCCTCAGAG		
13F	GGGTGGAACCTTCAAACA	55 °C	179 pb
13R	TACTTTCCAAGGTGACATAT		
14F	GGAGATGGAATTCAGTATGT	55 °C	198 pb
14R	AAAGACCATGAGAAATAACA		
**			
15F	CCCCTCTAAATGTTGTCATC	57 °C	180 pb
15R	TTTGAGCTTGTGGGACTACT		
16F	AATGATGCACTGAGCTGGTT	57 °C	201 pb
16R	GCACGCAACAGTACAACCTC		
17F	ATTTGTCACCCCGTCACTTT	57 °C	200 pb
17R	GAATGATTCCTGGCAATACT		
18F	CCAGGCAACATGAGTAAAT	55 °C	155 pb
18R	TGGAGGAACTGAATAGGAAC		
**			
19F	TGCACATACCATTGTTTAT	55 °C	175 pb
19R	CCAGGGCACATCAGGGCATC		
**			
20F	TTCTTCTACAGAGACGTTT	50 °C	259 pb
20R	TGCTAATGGATATGAATAAG		
21F	TATAGAAGACAGTCAGAAAA	50 °C	181 pb
21R	TAGAAATCTACCTTTGGTG		
22F	AAATATGACAAATCTGCCAT	55 °C	227 pb
22R	GGAAAGATGACTGGTAAGAG		
**			

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23F	TGATCCATCACAATTAACCT	51 °C	149 pb
23R	CAGGGAGTTAAGTGATTGAT		
24F	ACTTTACCCTCTGCTGATAA	55 °C	227 pb
24R	GGGAAATAGTTGTTTGTATG		
**			
25F	GACATTCAGTGGTTGGTAAT	55 °C	281 pb
25R	TAAACACTTGATCCCAAAG		
**			
26F	TCAGTTATGTGAATGCCGCT	51 °C	146 pb
26R	TGGGAAGTATATAAGACAGT		
27F	TGAGTCTGTGTTTTGTTTT	47 °C	161 pb
27R	AAAAAAAAAACCTCAC		
**			
28F	ATTGTTCTATACTTGCACA	55 °C	309 pb
28R	TCTATGCACCAAAGGACAG		
**			
29F	TGGGCCATCTGTATAGTTTT	52 °C	269 pb
29R	TAATAGTAAGTAGGGTAAGC		
30F	GCCTTCACACACTGTGGTCA	55 °C	240 pb
30R	ATGGGAGGACATCATGGAAA		
*, **			
31F	TCCTAAAACTTTATGCTCTC	50 °C	221 pb
31R	TCAAATACCAGAAACAAATG		
32F	CCTGTTCATTTTGTTCTTGC	52 °C	187 pb
32R	TGTCAACTTATTTGATATGG		
33F	TTTCAGCAGAGACCTGTAAC	52 °C	271 pb
33R	AAGAACAGAAAGGTTTTATT		
34F	GTTGTGCATGTGCCATTTGT	55 °C	154 pb
34R	GATGGCTTCTGTATCTCC		
35F	TGAGACCAAATTAATTGTC	55 °C	211 pb
35R	TCATTGCCAGCTAGAAGTAA		
36F	CAAACGGCAACTCTGATGTT	52 °C	183 pb
36R	AGTGCTCAGGAAGTCTCCAG		
37F	TATCTGGCCATCTGCAAAAC	55 °C	173 pb
37R	TTGTGGGATGGGCTTCATT		
38F	GCGTTTGTGGCTAGAGTGAG	55 °C	189 pb
38R	GAACCATGGACTGAAGCTCAG		
39F	AGGCACTATAACAGGGACAAA	54 °C	420 pb
39R	CATCCTTTGTCATGATTCTCTC		
40F	ACCTTCAAATGCAATGAGG	52 °C	184 pb
40R	CATCCTTTGTCATGATTCTCTC		
41F	TTTTGTCTCTTCTCTGTGG	42 °C	218 pb
41R	AGTTATTCACATATTACTTA		
*			
42F	GCCCTCATTTTTATGTTTTG	52 °C	189 pb
42R	GTTGGAAGCTCACCTGGAAG		
43F	GACTGGCCTCGTTTG	49 °C	178 pb
43R	TTAATATCCTTACAGCACCC		
44F	ATTACACAAGCGGTGATTCC	54 °C	213 pb
44R	AGAATTCATTCAGCAATA		

COL4A4 gene in European population

45F	CACCAGCATCATAAACTT	49 °C	186 pb
45R	AGGTTTACAGTGTCAGAGAA		
46F	AGTGCCAGAACAGAGGTGCT	55 °C	297 pb
46R	GGAGATGGGCGATCCTGTA		
47F	ACACCAGCTGTCTTCTTC	55 °C	353 pb
47R	TGAATGAGCCAGGGTTT		
48F	GTGTGTGTCTGAGCCCTAAT	55 °C	323 pb
48R	TGGTGAATTCGCATTCT		

Investigators: Consolación Rosado, Elena Bueno, Rogelio González-Sarmiento. In this table we show the amplification conditions of the primers of the COL4A4 gene in our laboratory. *0.5 mL MgCl₂ **0.3 mL DMSO.