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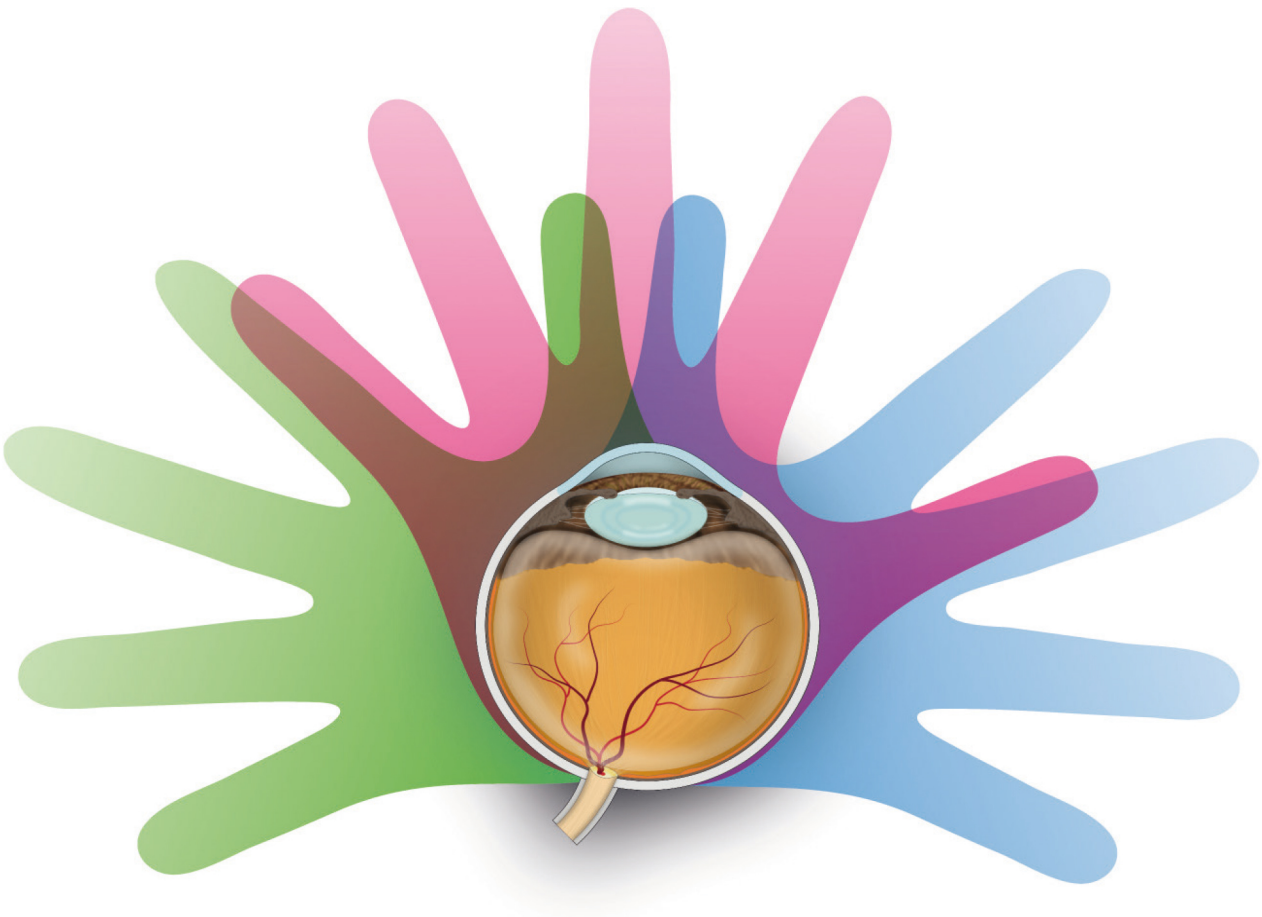
New genes involved in rare diseases in ophthalmology

Jaume Crespí Vilimelis



UAB
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HOSPITAL DE LA SANTA CREU I SANT PAU

THESIS

NEW GENES INVOLVED IN RARE DISEASES IN OPHTHALMOLOGY

Jaume Crespí Vilimelis

Faculty of Medicine, Autonomous University of Barcelona
Surgery Department and Surgical Specialties

Directors:

Dr José Antonio Buil Calvo, Director of the Ophthalmology service at the Hospital de la Santa Creu i Sant Pau. Associate Professor at the Autonomous University of Barcelona.

Prof José García - Arumí, Director of the Ophthalmology service at the Hospital Vall d'Hebron. Professor of the Autonomous University of Barcelona.

C/Modolell 47 Át
08021 Barcelona
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J66436817
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Jose Buil Antoni Calvo, Head of the Ophthalmology Department of the Santa Creu i Sant Pau and associate professor in the UAB and José García-Arumi, Head of the Ophthalmology Department of Valle de Hebrón and Professor at the UAB

CERTIFY

That Jaume Crespí Vilimelis, Graduate in Medicine and Surgery by the University of Barcelona, a specialist in Ophthalmology of the Hospital de la Santa Creu i Sant Pau, Barcelona, working under their direction, has carried out the studies that make up this thesis entitled:

New genes involved in rare diseases in Ophthalmology.

Work that finishes on the date below mentioned, with all its utilisation, having been revised by the undersigned and agreeing with its presentation in order to be evaluated as DOCTORAL THESIS

We resolve to any extent and consequences, signing this certificate in Barcelona, 26th June 2015.

José Antonio Buil Calvo

José García-Arumí

To MY FAMILY…

To Markus, Niklas and Erik…for the stolen hours of soccer and park.

To Elina…for leaving everything to be by my side.

To my parents…for never letting me fall.

"Your genetics load the gun. Your lifestyle pulls the trigger"

Mehmet Oz

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INDEX OF ABBREVIATIONS

RD: Rare diseases

fRAT: Familial Retinal Arteriolar Tortuosity

RP: Retinitis Pigmentosa

ERG: Electroretinogram

ARRP: Autosomal Recessive Retinitis Pigmentosa

ADRP: Autosomal Dominant Retinitis Pigmentosa

XLRP: X-linked Retinitis Pigmentosa

LCA: Leber congenital amaurosis

MD: macular distrophy

OMIM: Online mendialian inheritance in man

RPE: retinal pigment epithelium

MFRP: membrane Frizzled transmembrane proteins

AL: axial length

OCT: optical coherence tomography

AS-OCT: anterior segment OCT

PM: posterior microphthalmos

hESC-dPRE: human embryonic stem cell-derived retinal pigment epithelium

WM: Wyburn-Mason Syndrome

VHL: Von Hippel Lindau

BIH: Benign idiopathic intracranial hypertension

FD: Fabry disease

CNS: central nervous system

ICH: intracranial haemorrhage

HD: Huntington's disease

NGS: next-generation DNA sequencing

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Rationale

1. RATIONALE

Rare diseases (RD) are minority diseases affecting one of every 2000 people. In total, more than 7000 thousand rare diseases affecting 7% of the world's population have been identified. In Spain, it is estimated to affect 3 million people. Although globally they are suffered by a significant number of people, the RD valued separately only affect a small proportion, sometimes only dozens of them. This complicates enormously to define the characteristics of the disease, to identify the causal mechanisms and to recruit patients for clinical trials destined to their treatment. Despite these difficulties, thanks to the current process of globalization and technological progress, which include improvements in global communication (social networking) and genomics, we can better investigate these diseases. **Approximately 80% of the minority diseases have a genetic origin. The majority are caused by mutations in a single gene,** as for example an enzyme deficiency (such as the α-galactosidase A in Fabry disease). Other diseases, such as Charcot-Marie -Tooth disease, can have multiple genetic causes. In either case, knowing the genetic mechanisms that cause them is essential for developing an effective treatment.

At present, more than 900 inherited diseases in which the eye is affected in a unique way or in the context of a systemic disease appear registered. One of the first and most relevant genes that were discovered was the Rb1, the first, and prototypical, tumour suppressor gene discovered. As many discoveries in medicine,

Rb was discovered studying a rare disease, the retinoblastoma. This Rb1 gene was discovered in 1986 in the chromosome 13, and is one of the tumour suppressor most frequently mutated in cancer. The protein that produces Rb1 is one of the most important factors that control the development of the cell cycle. During the three following decades the first oncogenes and tumour suppressor genes were discovered, which led to the development of a new field, the genetics of cancer. Since then, a specialty such as Ophthalmology, which was mainly based on clinical diagnosis and funduscopic findings, was transformed into a field where retinopathies, that seemed to be identical in the fund of the eye, were caused by mutations in different genes. In other cases, ocular diseases that seemed to be completely different between them were due to mutations in the same gene.

The purpose of this thesis is to identify the genetic basis, to date not known, of two rare diseases in Ophthalmology: the Retinitis Pigmentosa associated to Microphthalmos and Familial Retinal Arteriolar Tortuosity (fRAT). **Although they are two minority illnesses with barely about twenty described cases, the discovery of the causative gene, just as with the Rb1, could open the door to understand basic mechanisms of vision and ocular angiogenesis.** In addition, the affectation in the patients is severe: In the RP associated to microphthalmos almost all patients result in legal blindness. In the case of fRAT, they can present vascular aneurysms of the central nervous system with a high risk of hemorrhage and morbidity/mortality associated. Therefore, it is necessary to know and understand the genetic basis of these diseases, both for their current application (genetic counselling and prenatal diagnosis) as for the development of treatments in the near future (gene therapy).

BACKGROUND
RP- NANOPHTALMOS

BACKGROUND
RP- NANOPHTALMOS

2.1 RETINITIS PIGMENTOSA

The Retinitis Pigmentosa (RP; OMIM 26800) are a group of hereditary retinal diseases, genetically and clinically heterogeneous, characterized by a loss of function of the photoreceptors (rods and cones) that involve nyctanopia (night blindness), loss of visual field (tunnel vision) and decreased visual acuity. In the ophthalmologic examination, this translates into pigmentation of the retina (bony spicules), attenuation of retinal vessels, and pallor of the optic nerve, associated with diminished or abolished Electroretinogram´s response (ERG). **The RP represents the single most frequent cause of inherited blindness in adults,** with a prevalence of approximately 1 in 4000 people, and affects 1.5 million individuals throughout the world. [1]

Approximately between 50 and 60% of the cases of RP are autosomal recessive inheritance (ARRP), 30-40% are autosomal dominant inheritance and 10-15% are linked to the X, with some rare cases of mitocondrial or digenic inheritance[1]. The RP is also classified as non-syndromic or syndromic (associated with extra ocular clinical demonstrations). **The RP is considered a heterogeneous disease: more than 50 genes involved with almost 3100 mutations in these genes have been described.** The syndromic forms are equally heterogeneous: mutations in 12

genes can cause the Usher syndrome and in 17 genes the Bardet-Biedl syndrome (between the two syndromes, over 1200 mutations have been described).[2,3,4] In addition to this *genetic heterogeneity* (many genes may cause the same phenotype), the RP also presents *allelic heterogeneity* (there can be many different mutations that cause disease in each gene), *phenotypic heterogeneity* (different mutations in the same gene can cause different diseases) and finally *clinical heterogeneity* (the same mutation in different individuals can produce a different clinical manifestation, even among members of the same family).

In spite of this complexity, a significant progress has been carried out in recent years in the identification of new genes involved and in the screening of patients for pathological mutations. Special mention should be made to the creation of a biological database (**RetNet**) that provides information with scientific end on the genes and loci of the hereditary retinal diseases, among them the retinitis pigmentosa. From this database RetNet (http://www.sph.uth.tmc.edu/retnet/) and that of the Human Gene Mutation Database (http://www.hgmd.cf.acuk/) 56 genes involved in the non-syndromic RP have been found, fact that is summarized in Table 1.

If we include the genes involved in the systemic forms and Leber's congenital amaurosis (LCA), they are nearly 100 genes involved in the RP.

	Symbol	Location	Protein	Type of RP	Other Diseases	Mutations
1	ABCA4	1p22.1	ATP-binging transporter-retinal	AR	Recessive macular dystrophy Fundus flaivimaculatus, and cone. rod dystrophy	680
2	BEST1	11q12.3	Bestrophin 1	AD / AR	Dominant vítreo-retino-choroidopathy recessive bestrophinopathy; dominant Best type macular dystrophy	232
3	C2ORF71	2p23.2	Crs 2 ORF71	AR		13

	Symbol	Location	Protein	Type of RP	Other Diseases	Mutations
4	C8ORF37	8q22.1	Crs 8 ORF37	AR	Recessive cone-rod dystrophy	4
5	CA4	17q23.2	carbonic anhydrase IV	AD		6
6	CERKL	2q31.3	ceramid kinase-like protein	AR	Recessive cone-rod dystrophy with inner retinopathy	8
7	CLRN1	3q25.1	Clarin-1	AR	Recessive Usher syndrome	23
8	CNGA1	4p12	Rod cGMP-gated channel α subunit	AR		8
9	CNGB1	16q13	Rod cGMP-gated	AR		6
10	CRB1	1q31.3	Crumbs homolog 1	AR	Recessive LCA, dominant pigmented paravenous chorioretinal atrophy	183
11	CRX	19q13.32	Cone-Rod otx-like photoreceptor homebox transcription factor	AD	Recessive, dominant and de novo LCA, dominant cone-rod dystrophy	51
12	DHDDS	1p36.11	Dehydrodolichyl diphophate synthetase	AR		1
13	EYS	6q12	Eyes shut / spacemaker	AR		1
14	FAM161A	2p15	Family with sequence similarity 161 member A	AR		6
15	FSCN2	17q25.3	Retinal fascin homolog 2, actin bundling protein	AD	Dominant macular dystrophy	1
16	GUCA1B	6p21.1	Guanylate cyclase activating protein 1B	AD	Dominant macular Dystrophy	3
17	IDH3B	20q13	NAD(+)-specific isocitrate dehydrogenase 3 beta	AR		2
18	IMPDH1	7q32.1	Inosine monophosphate dehydrogenase 1	AD	Dominant LCA	14

	Symbol	Location	Protein	Type of RP	Other Diseases	Mutations
19	IMPG2	3q12.3	Interphotore-ceptor matrix proteoglycan 2	AR		10
20	KLHL7	7p15.3	Kelch-likle proetin	AD		3
21	LRAT	4q32.1	Lecithin retinol acyltranferase	AR	Recessive LCA	10
22	MAK	2q13	c-mer protooncogene receptor tyrosine kinase	AR		9
23	MERTK	2q13	c-mer protoon-cogene receptor tyrosine kinase	AR		27
24	NR2E3	15q23	Nuclear receptor subfamily group E3	AD/AR		45
25	NRL	14q11.2	Neural retina lucine zipper	AD/AR	Recessive RP	14
26	OFD1	Xp22.2	Oral-facial-digital syndrome protein	Xlinked	Orofaciodigital syndrome1, Simpson-Golabi-Behmel Sd	127
27	PDE6A	5q33.1	cGMP phophodiesterase subunit	AR		16
28	PDE6B	4p16.3	Rod cGMP PD β subunit	AR	Dominant congenital stationary blindness	39
29	PDE6G	17q25.3	PD 6G cGMP-specific rod	AR		1
30	PRCD	17q25.1	Progressive rod-cone degene-ration protein	AR		2
31	PROM1	4p15.32	Prominin 1	AR	Dominant Stargart-like and bulls eye macular dystrophy; Dominant cone-rod dystrophy	9
32	PRPF3	1q21.2	Human homolog of yeast pre-mRMN splicing factor 3	AD		3

	Symbol	Location	Protein	Type of RP	Other Diseases	Mutations
33	PRPF6	20q13.33	Human homolog of yeast pre-mRMN splicing factor 3	AD		2
34	PRPF31	17p13.3	Human homolog of yeast pre-mRMN splicing	AD		2
35	PRPF31	19q13.42	Human homolog of yeast pre-mRMN splicing	AD	Recessive LCA	65
36	PRPH2	6p21.1	Peripherin 2	AD/ digenic	Dominant macular dystrophy	123
37	RBP3	10q11.22	Retinol binding protein 3	AR		2
38	RDH12	14q24.1	Retinol dehydrogenase 12	AR/AD		66
39	RGR	10q23.1	RPE-Retinal G protein-coupled receptor	AR	Dominant choroidal sclerosis	7
40	RHO	3q22.1	Rhodopsin	AD/AR	Dominant congenital stationary night blindness	161
41	RLBP1	15q26.1	Retinaldehyde-binding protein 1	AR	Recessive Bothnia dystrophy; recessive retinitis punctata albescens; recessive Newfoundland rod-cone dystrophy	20
42	ROM1	11q12.3	Retinal outer segment membrane protein 1	AD/ digenic with PRPH2		11
43	RP1	8q12.1	RP1 protein	AD/AR		67
44	RP2	Xp11.23	Retinitis pigmentosa 2 (x-linked)	X-linked		76
45	RP9	7p14.3	RP9 protein or PIM1- kinase associated prot	AD		2
46	RPE65	1p31.2	Retinal pigment epithelium-specific 65KDa protein	AD/AR	Recessive LCA	134
47	RPGR	Xp11.4	RP GTPase regulator	x-linked	x-linked cone dystrophy 1 x-linked atrophic macular dystrophy	151

	Symbol	Location	Protein	Type of RP	Other Diseases	Mutations
48	SAG	2q37.1	Arrestin (s-antigen)	AR	Recessive Oguchi disease	11
49	SEMA4A	1q22	Semaphorin 4A	AD	Dominant cone-rod dystrophy	3
50	SNRNP200	SNR-NP200	Small nuclear ribonucleoprotein	AD		7
51	SPATA7	14q31.3	Spermatogenesis associated protein 7	AR	Recessive LCA	15
52	TOPORS	9p21.1	Topoisomerase I binding arg/ser rich protein	AD		8
53	TTC8	14q32.11	Tetratricopeptide repeat dominant 8	AR	Recessive Bardet-Biedl Sd	14
54	TULP1	6p21.31	Tubby-like protein 1	AR	Recessive LCA	31
55	USH2A	1q41	Usherin	AR	Recessive Usher Syndrome	392
56	ZNF513	2p23.3	Zinc finger protein 513	AR		1

Table 1. Genes involved in Retinitis Pigmentosa.

Due to the extensive genetic variability of the RP, the detection of mutations with the classic techniques of molecular biology turns out to be expensive and laborious. In recent years, screening of mutation techniques based on the technology *first arrayed extension* (APEX) [5] has been developed, which allow the detection of multiple mutations in the same patient. Initially, the **microarrays APEX** were developed for screening of the entire ABCA4 gene. This gene is involved in several retinal dystrophies including LCA, which is much less heterogeneous than the RP. More recently, microarrays have been developed for Usher syndrome [6] and for the detection of mutations in 17 genes of the ARRP [7]. Another procedure that has been developed in recent years is the **genetic chip**, which in addition to screen for known mutations also analyses the variants around genetic sequences (up to 30kb) [8], having potential thus to detect new mutations.

These new advances in the genetic diagnosis have the potential to improve our understanding of the pathogenesis and to establish a better correlation genotype-phenotype in a disease as genetically heterogeneous as the RP. In addition, they help to improve the clinical management of patients and their families.

2.2 NANOPHTHALMOS - MICROPHTHALMOS

Nanophthalmos is a rare congenital eye disorder (15/100,000 births) in which an alteration in the ocular development is produced that translates into a reduction in the total axial length (between 14 and 20.5 mm) accompanied by a high hyperopia (between + 8.00 and + 25.00 diopters).[9] In general, there are no other major ocular malformations associated and the scleral thickness is increased. An ultrastructural study of the sclera of these patients revealed alterations in the interbreeding of collagen fibers, absence of elastic fibers and an increase in fibronectin [10]. The nanophthalmic eyeball should lodge the neural retina that will have a normal development and size, while the sclera and choroid are abnormally thickened and cover less than half of their normal area [10] (**Figure 1**). Thus, the nanophthalmic eyes have an increased risk of uveal effusion, exudative retinal detachment and angle-closure glaucoma [11].

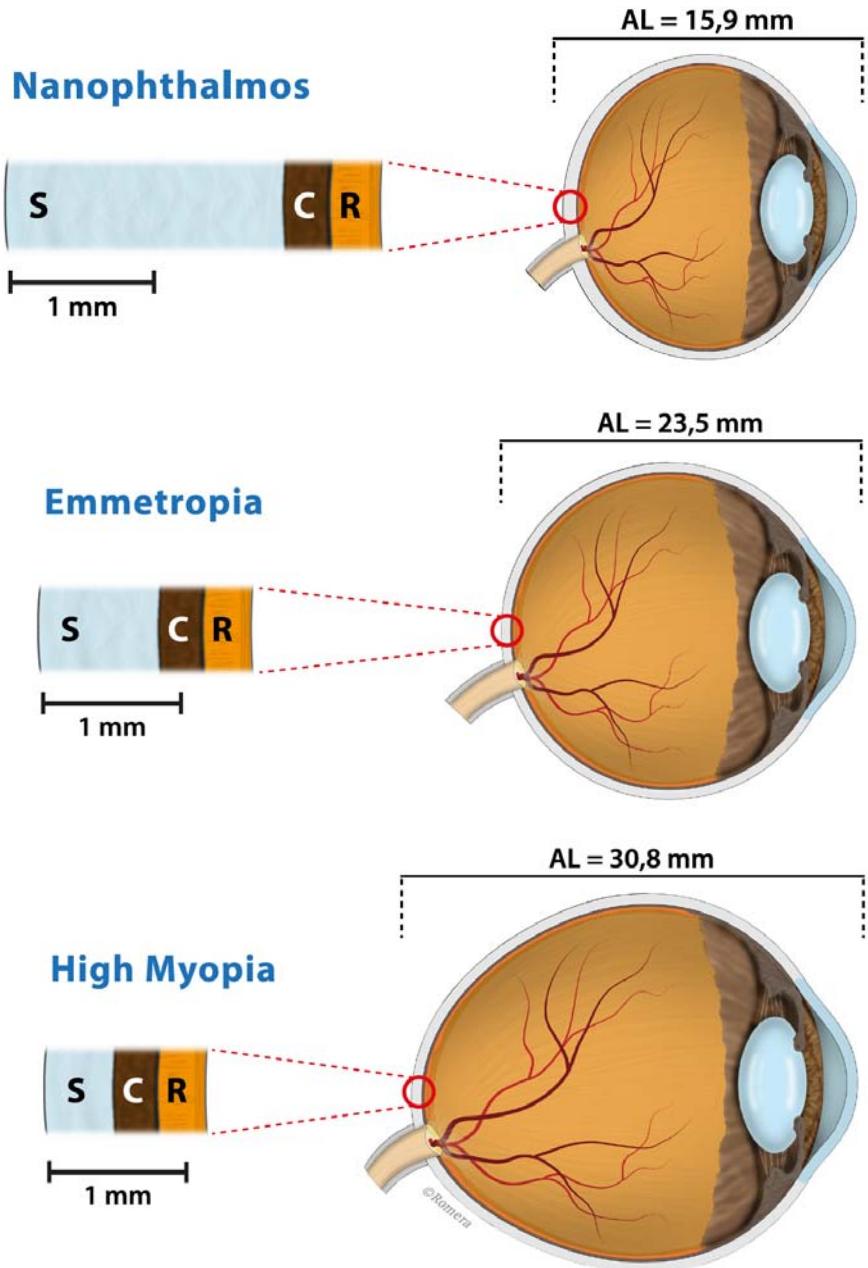

Fig 1. Emmetrope eye, myopic eye and with nanophthalmos diagram.

A term that is commonly used as a synonym in the medical literature is **microphthalmos.** Microphthalmos is considered a structural malformation of the eyeball in which the axial length is at least two standard deviations lower than

similar controls by age [12] or, in general, an antero-posterior diameter less than 20 mm in adults[13].

This malformation is classified in **simple microphthalmos** if it is not accompanied by any other greater malformation and **complex microphthalmos** when other ocular abnormalities are identified[14]. In the literature, the term "simple microphthalmos" is sometimes used to refer to nanophthalmos.

By the clinical and genetic data that we have, we can say that the microphthalmos and its more severe form, **anophthalmos**, are different manifestations of the same clinical spectrum of ocular malformations. Approximately 80% of subjects with microphthalmos/anophthalmos present associated systemic malformations[15], and in total, in the literature more than 100 syndromic entities that include microphthalmos/anophthalmos are described[16]. Nevertheless, we must clarify that the term anophthalmos has been incorrectly used in the medical literature. The **primary or true anophthalmos** is incompatible with life; in these cases, the primary optic vesicle has stopped its development and this abnormal development also includes major malformations of the central nervous system, which are incompatible with life. In the majority of published cases, the term anophthalmos is used as a synonym for what should be more correctly called "extreme microphthalmia" or "clinical anophthalmia" [13].

Finally, let us define what a **posterior microphthalmos is:** It is a rare subtype of microphthalmos that is defined by having a reduction in the total axial length with a normal corneal diameter. This condition has been associated with high hyperopia and retinal folds in the papillomacular bundle [17, 18, and 19]. The posterior microphthalmos must be distinguished from the nanophthalmos, a structural anomaly that combines microphthalmos and microcornea, **while in the posterior microphthalmos the corneal diameter is normal.** [20]

GENES ASSOCIATED WITH NANOPHTHALMOS/ MICROPHTHALMOS

Although in most cases it is a malformation that occurs sporadically, the autosomal recessive inheritance is the most likely way of transmission when it is hereditary [17].

A review of all the genes involved in simple nanophthalmos/microphthalmos (without other systemic malformations associated) is featured below. Microphthalmos designates a heterogeneous group of ocular malformations which, as has been said above, involve a reduction of the anterior-posterior diameter of the eyeball, high hyperopia, decreased corneal diameter and anterior chamber, while the lens is normal or increased by age [21].

A) ISOLATED/ SIMPLE MICROPHTHALMOS (SPORADIC OR AR)

Mutations in 8 genes located in different chromosomes that have given rise to the following phenotypes of isolated microphthalmos have been described to date: MCOP1, MCOP2, MCOP3, MCOP4, MCOP5, MCPO6, MCOP7 y MCOP8.[22] (omim.org/entry/251600)

Phenotype	Location	Genes / Locus	OMIM number
MCOP1	14q 32	MCOP1	251600
MCOP2	14q24.3	CHX10	610093
MCOP3	18q21.3	RAX	611038
MCOP4	8q22.1	GDF6	613094
MCOP5	**11q23**	**MFRP**	**611040**
MCOP6	2q37.1	PRSS56	613517
MCOP7	12p13.1	GDF3	613704
MCOP8	15q26	ALDH1A3	615113

B) SIMPLE MICROPHTHALMOS (INHERITANCE AD)

In the same manner as in AR simple microphthalmos, AD it is characterized by an eyeball of small size, a relationship lens/eye increased and an increased incidence of angle-closure glaucoma [23]. To date, 4 implied phenotypes have been described, although the genes are not completely known [24]: (omim.org/entry/600165)

Phenotype	Location	Genes / Locus	OMIM number
NNO1	11p	NNO1	600165
NNO2	11q23.3	NNO2, **MFRP**	606227
NNO3	2q11-q14	NNO3	611897
NNO4	17q11.2	NNO4, **TMEM98**	615949

The latter case described above has been recently published. Awadallah et al. identified mutations of the TMEM98 gene in a region of chromosome 17 in a family (5 generations) with nanophthalmos of autosomal dominant inheritance. The mutations were not found neither in the non-affected members of the family nor in 285 controls[25].

In general, all these malformations derive genetic mutations that cause alterations in early embryonic stages during the formation of the optic vesicle. **The few genes known so far that are involved in the pathogenesis of microphthalmos are transcription factors that play a key role in the initial period of eye development that takes place in the first two months of development**[26].

2.3 OCULAR SYNDROMES ASSOCIATED WITH THE MFRP GENE

The general problem of how the organs regulate their size during the development has its better example of precision in the human eye: during the postnatal growth the human eye maintains the distance between the cornea and the fovea within the optimal focal length, with a margin of error of 2%[27]. Studies on birds and mammals have established that this process is guided/governed by the visual experience. The images' focus quality is locally evaluated in the retina, which enables or disables signals in adjacent tissues to promote or restrict the antero-posterior growth of the eye[28, 29].

A) PHENOTYPE 1: MFRP- NANOPHTHALMOS

Sundin et al. published for the first time the association between nanophthalmos with a gene mutation in 2005 [29]. They had previously made studies of genetic linkage in *Amish families* in the US (with nanophthalmos previously described by Cross and Yoder in 1976 [30]) that resulted in a single locus 11q23.3. Subsequently, 4 mutations in the gene MFRP were identified. **The MFRP gene does not have a similar relative in the human genome. It is a gene that expresses itself selectively in the eye** and encodes for a glycosylated transmembrane protein. This protein has counterpart domains of other genes related to intercellular signalling, endocytosis and proteolysis [31]. Thus for example, its C-terminal domain is related to the transmembrane proteins Frizzled family. The Frizzled proteins are receptors of the Wingles (Wnts) a cell-cell signposting molecules

family that mediate in the regulation of the growth and differentiation during the development[31]. **MFRP is specifically expressed in the RPE and the ciliary body.** Although its function is not known exactly, the authors established that the MFRP gene is not critical to the functioning of the retina, since the patients that lack completely of MFRP can retain a good visual acuity with correction and have a normal electroretinogram[31]. **Subsequent studies suggest that the gene encodes for the protein MFRP which does not express during the formation of the optic vesicle, but it does in the EPR during the sub-sequent embryonic and post-natal stage thus regulating the growth of the eyeball** [32]. The authors establish that the eyeball of patients with *MFRP nanophthalmos* - /- have an average axial length (AL) at the time of the birth of 14.05 mm. In adulthood, these same patients have an average AL of 16.1 mm, estimating that the postnatal growth is only 2.05mm. In addition, these 2 mm could be due only to the growth of the anterior segment and lens (that have normal dimensions) and do not present growth of the vitreous cavity from birth[32].

This growth is minimal compared to the 6.5 mm that requires a normal eye to arrive at an average AL in adulthood of 23.55 mm. In this way, in these patients with mutations of the gene MFRP, the volume of the eyeball in adulthood is barely 25% of the average in normal adults. In fact, **these eyes are so small that even their average AL in adulthood is smaller than the AL of a normal newborn** [32].

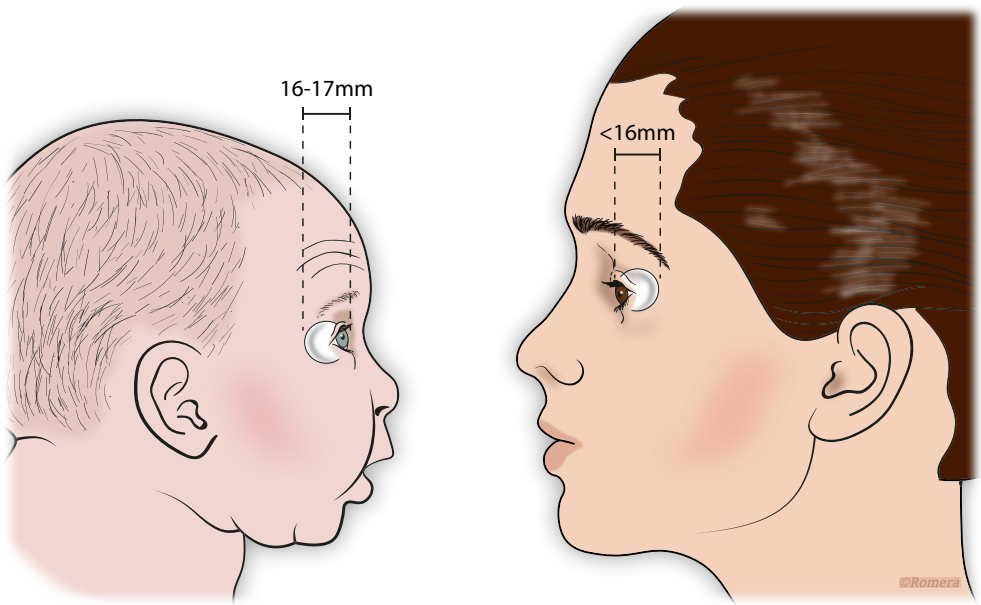

Fig 2. Example of MFRP related Microphtalmos

B) PHENOTYPE 2: MFRP-NANOPHTHALMOS-RETINITIS PIGMENTOSA-FOVEOSCHISIS- OPTIC DISK DRUSEN

Ayala-Ramirez et al.[33] (2006) described a female patient of 49 years, of consanguineous family, that presented a progressive decline of both the night vision and the visual acuity beginning at the age of 24. In the exploration, the visual acuity was 20/200 in OD and 20/100 in OS, she had normal corneal diameters with an axial length decreased, increased scleral thickness, optic disk drusen, abolished ERG and atrophy of the RPE in midperipheral retina with bone spikes (Fig 2). The OCT showed a diffuse macular thickening, with schisis of the outer layers of the retina, absence of foveal depression and evidence of intraretinal cysts. The patient had 3 siblings affected, all of them presented similar symptoms of loss of visual acuity and in the exploration they showed the same ophthalmologic findings. None of them presented any systemic abnormality, mental retardation or deafness. As these were symptoms which had not been previously described in the medical literature and there was an autosomal recessive inheritance, a diagnostic approach through analysis of candidate genes was carried out: the MFRP gene and the CHX10 gene which is described that cause autosomal recessive forms of nanophthalmos (NN02, 609549) and microphthalmos (e.g. MCOPCB3, 610092), respectively, were analysed. In the 4 affected members of the family, the authors identified of homozygous form a deletion of a 1-bp (498delC) that resulted in the complete functional loss of the protein. The two parents were heterozygous for this mutation. No mutation was detected for the CHX10 gene[33].

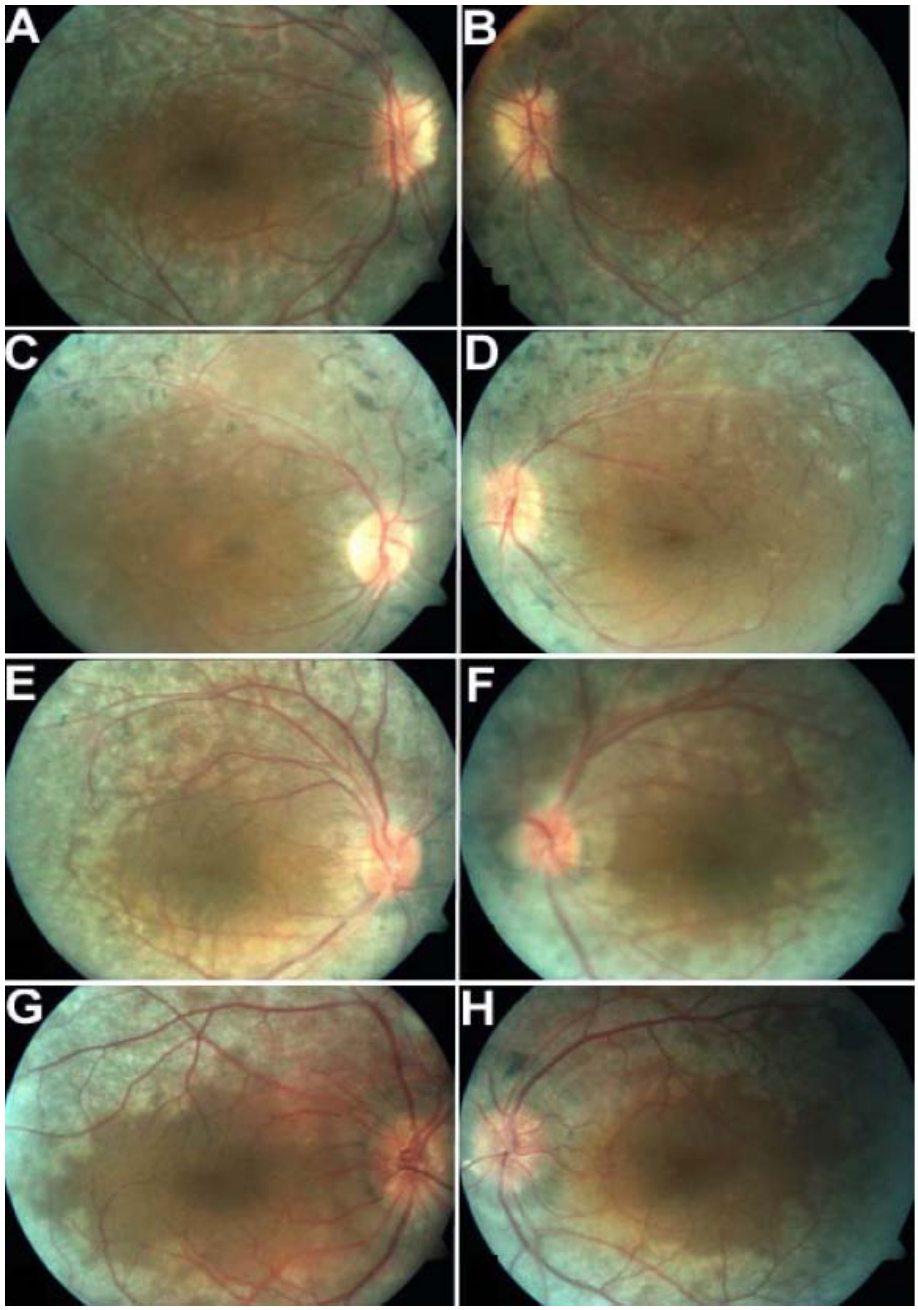

Fig 3. Retinographies of the 4 affected brothers with RP-microphthalmos[33].

1. *Confirmation of the candidate gene MFRP and of the ocular syndrome:*

The confirmation of the candidate gene must be performed with screening of mutations of the gene in different patients familiarly unrelated. This is done using DNA samples of affected patients and control subjects. As a general rule, if the candidate gene is correct, the group of patients with different mutations in the DNA samples and the control group will not present the same mutations. Therefore, the phenotype disease would result from the loss of function of that gene.

In the case of the Nanophthalmos-RP syndrome described above (phenotype 2) the involvement of the MFRP gene must be confirmed with other affected patients not related to the same family where it was described. **In genetics, as in the rest of medicine, a single case described requires more evidence to confirm that this is a new syndrome**. In the accompanying article in this thesis, we published the case of three Spanish brothers affected with the same ocular phenotype that presented homozygous mutations in exon 5 of the MFRP gene being confirmed thus, as the responsible for the RP-Nanophthalmos and establishing an emerging relationship genotype-phenotype for this syndrome.

Summarizing, the phenotypes associated with mutations of the MFRP gene are two (OMIM, * 606227):

1. The previously described by Sundin et al. [31]: **Nanophthalmos AR without other major funduscopic findings** or alterations in the ERG that indicate retinal dystrophy. (NNO2; OMIM 609549).
2. A second phenotype described in the first article of this thesis encompasses: **Posterior Microphthalmia, Retinitis Pigmentosa, Foveoschisis and Optic Disk Drusen**. (MCOP5, OMIM 611040).

In the attached article from the American Journal of Ophthalmology, we confirmed the gen **MFRP as the cause of this new syndrome that we call Nanophthalmos-Retinitis Pigmentosa-Foveoschisis - Optic Disk Drusen**. **In addition, we hypothesize that the MFRP gene not only would have a role in the development and growth of the eyeball, but it would also have a key role in the maintenance of the photoreceptors. This hypothesis was subsequently confirmed by Jungyeon et al. in experimental models with genetically modified mice for the *Mfrp gene***[32] . In this study, the MFRP gene seems to be necessary for the normal development of the microvilli of the EPR. The mutation of this *Mfrp* gene in mice, leads to a reduction of the initial number of apical microvilli. **These defects in the RPE would contribute to the abnormal development of outer segments of photoreceptors (OS) and/or of the phagocytosis of the RPE finally giving rise to a degeneration of the photoreceptors**[32]. Currently, the MFRP gene alone is already considered as the cause of Retinitis Pigmentosa.

PUBLISHED STUDIES

A NOVEL MUTATION CONFIRMS MFRP AS THE GENE CAUSING THE SYNDROME OF NANOPHTHALMOS-RETINITIS PIGMENTOSA-FOVEOSCHISIS-OPTIC DISK DRUSEN

American Journal of Ophthalmology 12/2008

Impact Factor: 4.02 - 1st Quartile (Ophthalmology)

A Novel Mutation Confirms *MFRP* as the Gene Causing the Syndrome of Nanophthalmos–Renititis Pigmentosa–Foveoschisis–Optic Disk Drusen

JAUME CRESPÍ, JOSÉ A. BUIL, FRANCISCA BASSAGANYAS, JOSÉ I. VELA-SEGARRA, JESÚS DÍAZ-CASCAJOSA, RAUL AYALA-RAMÍREZ, AND JUAN C. ZENTENO

• PURPOSE: To describe the clinical and genetic characteristics of the second family with a recently described recessive syndrome characterized by posterior microphthalmos, retinitis pigmentosa, foveoschisis, and optic disk drusen.

• DESIGN: Observational case report.

• METHODS: Three affected subjects and one healthy sibling from a consanguineous marriage from Spain were studied. Complete ophthalmologic examinations including A- and B-mode ultrasonography (US), electroretinography (ERG), fluorescein retinal angiography (FA), and optical coherence tomography (OCT) were performed in each individual. Genetic analysis included polymerase chain reaction amplification and direct nucleotide sequencing of the complete *MFRP* gene.

• RESULTS: All three affected siblings had bilateral shortening of the posterior ocular segment associated with high hyperopia and normal anterior segment dimensions. Best-corrected visual acuity ranged from 20/200 to 20/60. Funduscopy, ERG, and FA were compatible with retinitis pigmentosa, and B-mode ultrasound showed optic disk drusen. OCT analysis revealed outer retinal layer schisis with absence of foveal pit. Inheritance of this syndrome followed an autosomal recessive pattern. Molecular analysis revealed a novel homozygous 1-bp deletion (c.498delC) in exon 5 of *MFRP*, predicting a prematurely truncated protein (P166fsX190). A healthy sister demonstrated to be a carrier of the mutation.

• CONCLUSIONS: We confirmed that the syndrome of posterior microphthalmos, retinitis pigmentosa, foveoschisis, and optic disk drusen constitutes a distinct autosomal recessive entity. The novel frameshift mutation identified in the family described here validates *MFRP* as the gene responsible for this particular disease, which characteristically involves structures located at the posterior segment of the eye. (Am J Ophthalmol 2008; 146:323–328. © 2008 by Elsevier Inc. All rights reserved.)

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From the Department of Ophthalmology, University of Barcelona, Hospital de Sant Pau y de la Santa Creu, Barcelona, Spain (J.C., J.A.B., F.B., J.I.V.-S., J.D.-C.); and the Departments of Retina (R.A.-R.) and Genetics-Research Unit (J.C.Z.), Institute of Ophthalmology "Conde de Valenciana," Mexico City, Mexico.

Inquiries to Juan C. Zenteno, Department of Genetics, Institute of Ophthalmology "Conde de Valenciana," Chimalpopoca 14, Col. Obrera CP 06800, Mexico City, Mexico; e-mail: jczenteno@institutodeoftalmologia.org

MICROPHTHALMOS IS A DEVELOPMENTAL OCULAR defect that can occur as an isolated anomaly or in conjunction with other ocular or extraocular anomalies producing well-defined Mendelian syndromes.[1,2] The term "posterior microphthalmos" is applied to eyes combining a reduced axial length with a normal-sized cornea.[3] Posterior microphthalmos is related to nanophthalmos, an anomaly characterized by microphthalmos, microcornea, and a tendency toward spontaneous or postsurgical uveal effusions.[4] Typically, eyes with posterior microphthalmos or with nanophthalmos have axial lengths of 13.0 mm to 18.5 mm, exhibit shallow anterior chamber and thickening of both the choroidal vascular bed and sclera,[5] and are extremely hyperopic with refractive errors ranging from +8.00 to +25.00 diopters. Eyes with posterior microphthalmos, in contrast with nanophthalmic eyes, have a normal anterior chamber depth (ACD).

The clinical association of microphthalmos/nanophthalmos with retinitis pigmentosa has been recognized in several subjects.[6–12] Although most cases with this clinical combination have been sporadic,[8,10–12] there is evidence that the association can be transmitted either as an autosomal dominant[6] or an autosomal recessive trait.[7,9] Recently, Ayala-Ramirez and associates[13] described a Mexican family with a novel autosomal recessive syndromic entity encompassing posterior microphthalmos, retinitis pigmentosa, foveoschisis, and optic nerve head drusen. In this consanguineous, four-affected-member family, the authors demonstrated that the disease was caused by a homozygous frame shifting mutation in *MFRP*, a gene located at chromosome 11q23 and encoding the Membrane-type *F*rizzled-*R*elated *P*rotein.[13]

In this article, the clinical and molecular analysis of a consanguineous Spanish family with the syndrome of posterior microphthalmos–retinitis pigmentosa–foveoschisis–optic disk drusen is described. The identification of a novel frame-shift mutation in three affected brothers from this family validates *MFRP* as the responsible gene for this unusual hereditary ocular syndrome affecting selectively posterior structures of the eye.

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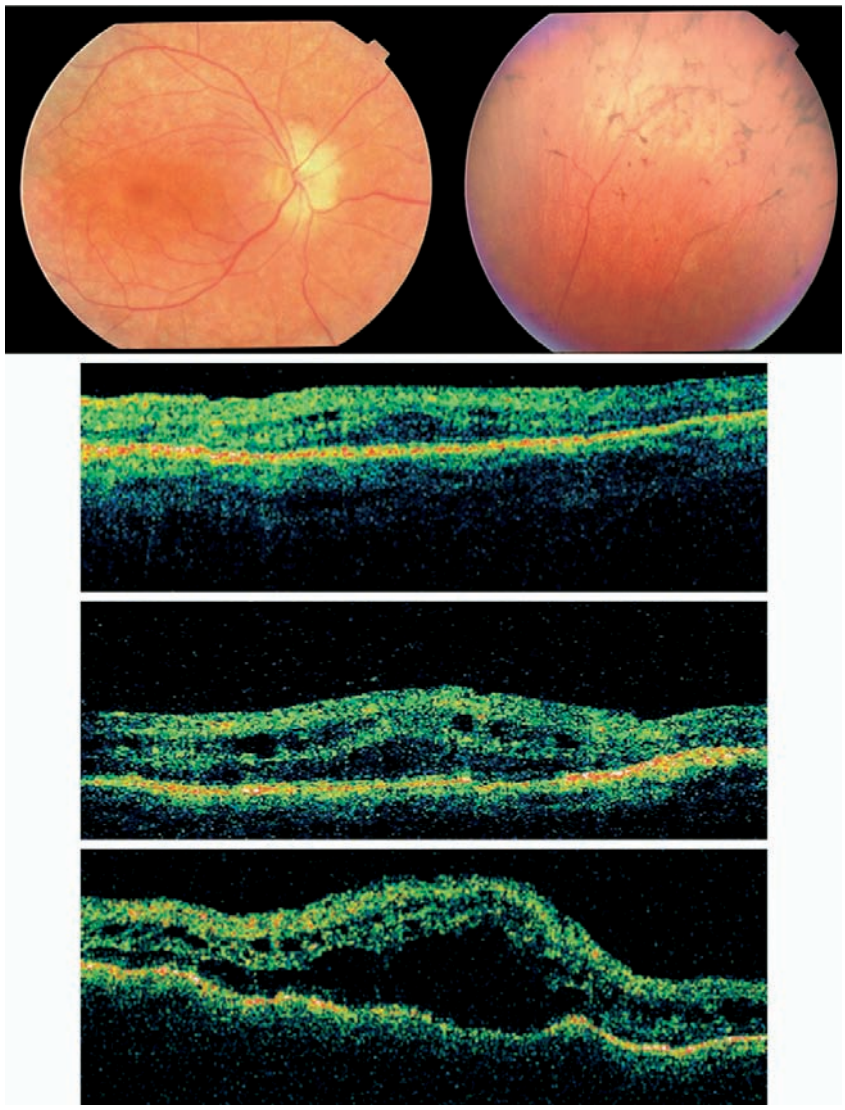

FIGURE 1. Phenotypic appearance of subjects with the syndrome of nanophthalmos–retinitis pigmentosa–foveoschisis–optic disk drusen. Funduscopic images showing optic disk drusen, patchy areas of hypopigmentation, and blunted macular reflex in Patient 1 OD [right eye] (Top left). Midperipheral bone spicule-like pigment clumping and vascular attenuation are also evident in Patient 1 OD (Top right). Retinal optical coherence tomography (OCT) demonstrated localized foveal schisis in Patients 1 (Second panel, top to bottom), 2 (Third panel, top to bottom), and 3 (Bottom). Note that the older subject (Patient 3) has a more severe degree of retinal schisis.

METHODS

THE STUDY INCLUDED THREE AFFECTED BROTHERS FROM A Spanish consanguineous family (parents were first cousins). They have six unaffected siblings and no prior familial antecedents of ocular or extraocular malformations. An unaffected sister was included in the study. Patients 1 and 3 have a history of ocular surgical interventions for treatment of elevated intraocular pressure, with development of blinding malignant glaucoma in Patient 3's left eye. In all patients, ophthalmologic examinations included determination of best-corrected visual acuity (BCVA), slit-lamp and dilated fundus inspection, applanation tonometry, fundus photography, A-mode and B-mode ultrasound (US), electroretinogram (ERG), fluorescein retinal angiography (FA), and optical coherence tomography (OCT).

TABLE. Clinical Features of Three Siblings With the Syndrome of Nanophthalmos–Retinitis Pigmentosa–Foveoschisis–Optic Disk Drusen

Patient No.	Age (y)	Visual Acuity (logMAR)	Corneal Diameter (mm)	Axial Length (mm)	Refractive Error (D)	IOP (mm Hg)	Posterior Segment Findings	OCT Findings
1	40	0.4 OD	11.3 mm OD	16.04 OD	+16.0 OD	16 OD	Optic disk drusen, retinitis	Foveoschisis; macular
		0.3 OS	11.5 mm OS	16.08 OS	+16.5 OS	16 OS	pigmentosa	thickness: 250 μ
2	54	0.1 OD	10.9 mm OD	14.72 OD	+19 OD	20 OD	Optic disk drusen, retinitis	Foveoschisis; macular
		0.1 OS	10.5 mm OS	14.80 OS	+19 OS	28 OS	pigmentosa	thickness: 480 μ[a]
3	60	0.05 OD	11.2 mm OD	15.31 OD	+18 OD	18 OD	Optic disk drusen, retinitis	Foveoschisis; macular
		NLP OS	11.1 mm OS	15.43 OS	-------	18 OS	pigmentosa	thickness: 650 μ[a]

D = diopters; IOP = intraocular pressure; logMAR = logarithm of minimal angle of resolution; NLP = no light perception; OD = right eye; OS = left eye; y = years.
[a]Macular edema.

Genomic DNA was obtained in each subject from peripheral blood lymphocytes according to standard methods. The 13 exons and exon/intron boundaries of the *MFRP* gene were amplified by polymerase chain reaction (PCR) using pairs of primers derived from the published sequences of the gene (Ensembl Transcript ID ENST00000360167). Primer sequences and annealing temperatures for PCR are available on request. Direct nucleotide sequencing of PCR-amplified products was performed using the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California, USA). Samples were run in an ABI Prism 310 Genetic Analyzer (Applied Biosystems). DNA from parents was not available.

RESULTS

THE TABLE SUMMARIZES THE OPHTHALMOLOGIC FEATURES of the patients. All of them demonstrated an identical bilateral phenotype characterized by decreased eye axial length with normal corneal diameters (posterior microphthalmos), retinal dystrophy, optic disk drusen, and localized foveoschisis. Data obtained on funduscopy (Figure 1), ERG, FA, and campimetry were fully compatible with advanced stages of rod-cone dystrophy (retinitis pigmentosa). OCT imaging revealed outer retinal layer schisis with absence of the foveal pit in all subjects (Figure 1), in addition to macular edema in Patients 2 and 3. Fundoscopic examination showed optic disk drusen, which was confirmed by B-mode US and autofluorescence. Although the ocular phenotype was remarkably similar in the three brothers, the oldest patients (2 and 3) exhibited a more severe degree of both foveoschisis and visual function impairment (Table).

Mutation analysis of the *MFRP* gene in DNA from the three affected brothers demonstrated a novel homozygous 1-bp (cytosine) deletion at position 498 (designated c.498delC) in exon 5. The mutation originates a shift of the open reading frame from residue proline 166 and predicts a premature truncation of the protein, 25 codons downstream (P166fsX190). The predicted truncated protein lacks almost all functional domains of MFRP, most likely leading to a complete loss of functional protein. The mutation was confirmed in each subject by sequencing the exon 5 antisense strand. No additional deleterious mutations were observed in any of the remaining *MFRP* exons in DNA from the three patients. Sequence analysis in DNA from the unaffected sister revealed the mutation to be present in heterozygous state (Figure 2).

DISCUSSION

MICROPHTHALMOS IS A DEVELOPMENTAL DEFECT THAT results from reduction in prenatal eye growth and can be divided into nanophthalmos, an anomaly characterized by microphthalmos, microcornea, and a tendency toward spontaneous or postsurgical uveal effusions; and posterior microphthalmos, defined by the combination of posterior segment shortening and normal corneal diameter. Although classically nanophthalmos has been distinguished from posterior microphthalmos based on the presence of normal corneal size and normal ACD in the latter, the growing clinical and genetic data indicate that these two malformations can be considered distinct clinical manifestations of the same anomaly.[14] Although corneal diameters were normal, two subjects from this family developed angle-closure glaucoma, so the term *nanophthalmos* appears to be more appropriate for describing the ocular malformation of this pedigree.

Nanophthalmos can occur as an isolated anomaly or in conjunction with additional ocular or extraocular malformations.[7,9,15] Recently, a distinct autosomal recessive clinical entity characterized by posterior microphthalmos, retinitis pigmentosa, localized foveoschisis, and optic disk drusen was identified in a family of Mexican origin.[13] Using a candidate gene approach, the authors demonstrated that a mutation in the *MFRP* gene was responsible

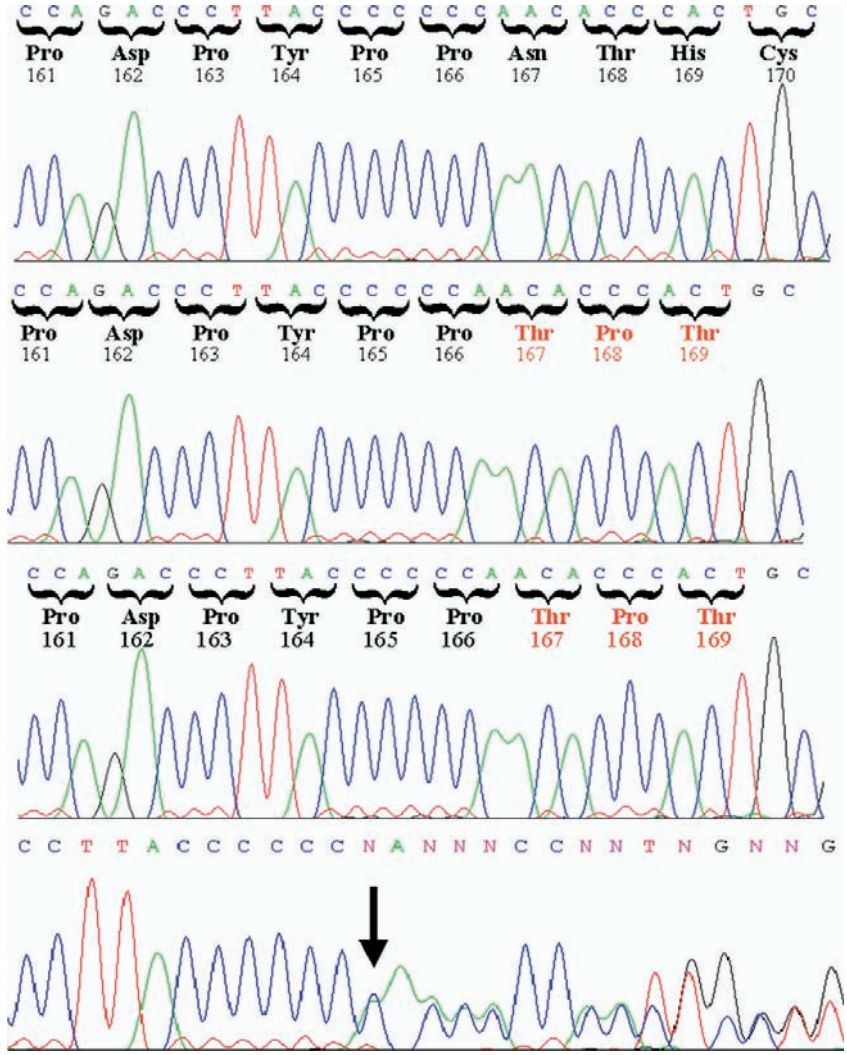

FIGURE 2. Sequence analysis in exon 5 of the *MFRP* gene in the syndrome of nanophthalmos–retinitis pigmentosa–foveoschisis–optic disk drusen. Normal sequence from a control DNA is shown in the top panel. A homozygous C deletion at nucleotide position 498 (codon 166) in two affected individuals is shown in the second and third panels (Top to Bottom). Amino acid numbers are indicated; frame-shifted residues are in red. The mutation predicts a truncated MFRP protein (P166fsX190). The unaffected sister carried the mutation in heterozygous state (Bottom panel; arrow indicates the sequence frame-shift).

for this new disease.[13] Here, the second family with this particular phenotype is reported, validating the disease as a discrete autosomal recessive entity.

The two families described so far with the syndrome of nanophthalmos–retinitis pigmentosa–foveoschisis–optic disk drusen exhibit a strikingly similar phenotype, characteristically involving structures located at the posterior segment of the eye. Angle-closure glaucoma, an absent finding in affected individuals from the previous family, was recorded in two subjects from the present pedigree. This trait appears not to be related to patient age because patients in the present family did develop glaucoma during adolescence. According to these observations, patients with this syndrome should be informed of a potential prognosis of glaucoma and they should be carefully followed for early detection of this complication. Papillomacular retinal folds (and, more infrequently, retinoschisis) have been reported in eyes with posterior microphthalmos, presumably arising from a disparity in growth between the sclera and retina.[16] In affected subjects from the two families described with the syndrome, localized foveal schisis has invariably been found, suggesting that a specific

mechanism originates splitting in this particular retinal site.

The novel frame-shift mutation identified in the family described here validates *MFRP* as the gene responsible for the disease. *MFRP* is predominantly expressed in the apical membrane of the retinal pigment epithelium and encodes a transmembrane protein that has a C-terminal domain related to the Wnt-binding domain of frizzled (Fz) proteins. Wnts includes a family of secreted-type glycoproteins with 22~24 conserved cysteine residues that act as cell-cell signaling molecules to mediate cell fate determination during development. MFRP mutations were previously identified in some familial and sporadic cases of nanophthalmos with high hyperopia but, notably, without funduscopic or electroretinographic evidence of retinal dysfunction.[17] In contrast, MFRP has an important role in mouse retinal function as the recessive retinal degeneration mutation *rd6*, a splicing mutation in the orthologous *Mfrp* gene, results in the skipping of *Mfrp* exon 4 and originates a phenotype characterized by small, white retinal spots, and progressive photoreceptor degeneration resembling human flecked retinal dystrophies like Stargardt disease and fundus flavimaculatus.[18] Remarkably, eyes of *Mfrp*^{rd6} are of normal axial length.[19] Based on these observations, Pauer and associates[20] analyzed the entire *MFRP* gene in 152 patients with inherited retinal degenerations including retinitis pigmentosa, Leber congenital amaurosis, and Stargardt macular dystrophy, but no pathogenetic mutations were identified in any case. In contrast with these data, our results indicate that some MFRP mutations can originate photoreceptor dysfunction leading to a syndromic form of retinitis pigmentosa.[13] As discussed below, these differences could be related to the specific type of MFRP mutation. Several lines of evidence for the participation of molecules of the Wnt signaling pathway in inherited retinal degenerations have been previously established.[21]

It is interesting to note that the P166fsX190 mutation described in the present family is very similar to the P166fsX199 mutation described in the original family with the syndrome of posterior microphthalmos–retinitis pigmentosa.[13] In both cases, the predicted mutant proteins lacked the two cubilin-related, the two low-density lipoprotein receptor–related, and the C-terminal cysteine rich-frizzled–related domains (CRD). Despite having distinct mutations, both families exhibited a strikingly similar phenotype that includes shortened posterior segment, retinitis pigmentosa, foveoschisis, and optic disk drusen. These observations suggest that certain MFRP domains play an important role in maintaining the normal structure and function of posteriorly located eye structures. Notably, a similar truncating mutation, Q175X, was described by Sundin and associates[17] in a 9-year-old patient with isolated nanophthalmos, high refractive error, and no retinal pigmentary anomalies. Comparing the two MFRP P166fs mutations observed in the two unrelated microphthalmos–retinitis pigmentosa families with the Q175X mutation associated to isolated nanophthalmos,[17] it is tempting to hypothesize that the tract of 10 amino acids from position 166 to position 175 (P-N-T-H-C-V-W-H-I-Q) could play a role in the etiology of this syndromic form of microphthalmos–retinitis pigmentosa. Alternatively, the aberrant frame-shifted amino acids could confer novel properties to the protein.

In conclusion, we present clinical and molecular evidence confirming that the association of microphthlamos–retinitis pigmentosa–foveoschisis–optic disk drusen constitutes a discrete autosomal recessive condition caused by homozygous truncating mutations in the *MFRP* gene. Additional studies are needed to identify why some MFRP truncating mutations cause only nanophthalmos whereas others result in this complex ocular syndrome affecting preferentially structures located at the posterior segment of the eye.

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CORRIGENDUM

Crespí J, Buil JA, Bassaganyas F, et al. A Novel Mutation Confirms *MFRP* as the Gene Causing the Syndrome of Nanophthalmos–Renititis Pigmentosa–Foveoschisis–Optic Disk Drusen. *Am J Ophthalmol* 2008:146(2):323–328.

In the June 2008 issue, an article published in this journal listed the first author Jaume Crespí as a graduate of the University of Barcelona. The institution should be correctly listed as the Autonomous University of Barcelona.

The authors regret this error.

2.5 DISCUSSION AND CONCLUSIONS

Since our publication confirming the syndrome of Nanophthalmos-RP, more than **thirty indexed publications have cited our article** (PubMed / Researchgate). Also, the database for Mendelian diseases **OMIM** (edited by the Johns Hopkins University School of Medicine) **has recognized the syndrome and its molecular basis linked to the MFRP gene and has been awarded a number that recognizes it as a unique clinical and genetic entity (MCOP5): #611040**. In this link all of the publications related to the syndrome and its genetic findings are described. Any author or doctor who has patients with a similar phenotype can access this information free of charge through the PubMed or directly in OMIM (omim.org/entry/611040), which facilitates the diagnosis and diffusion of this syndrome. As we've discussed in the introduction, this online database OMIM, is one of those tools that has been a huge help for Rare or Minority Diseases as it greatly facilitates the work of the researchers. **The final objectives of the identification of the causative gene in these minority diseases is mainly, to facilitate the diagnosis of new cases establishing a correlation genotype-phenotype and to understand the molecular basis of the disease to develop treatment strategies (**e.g. gene therapy). This, in sum, is what has happened in the years following our publication with the ocular syndrome related to MPFR and which is summarized in the following articles:

1) GENOTYPE-PHENOTYPE CORRELATION: Alberto Neri et al.[34] in 2012 published a sporadic case of a patient with this syndrome and a monitoring of 30 months. In this article, the autors **review all the published cases to date** of Nanophthalmos-RP with mutation of the MFRP (17) and stablish a **correlation**

genotype-phenotype for this disease. A summary of the 17 known cases is made and establishes that the phenotype syndrome, expressivity and age of onset vary between the affected families and between members of the same family. The degree of affectation of RP is also variable, from cases that presented a slight alteration of the EPR in adulthood or others that present an advanced form of RP in childhood. The majority of cases (11/17) show the "complete" phenotype that includes PM, RP, drusen of ON, and foveoschisis. The rest (6/17) have an "incomplete" phenotype as they do not present foveoschisis and drusen of ON (4 cases) or only drusen of ON (two cases). Interestingly, **all patients with homozygous mutation c.492delC (alternatively named c.498delC) presented the complete phenotype.**

They also stress that the closed-angle glaucoma is not a characteristic feature of these patients and that only two cases presented during adolescence **(these are the cases presented in our accompanying article, due to this, in our publication we talk about nanophthalmos and not about posterior microphthalmos)**. As discussed earlier, the anterior segment in these patients is usually normal or has the lens increased by age, which is illustrated in the following figure of the article **(Fig.4):**

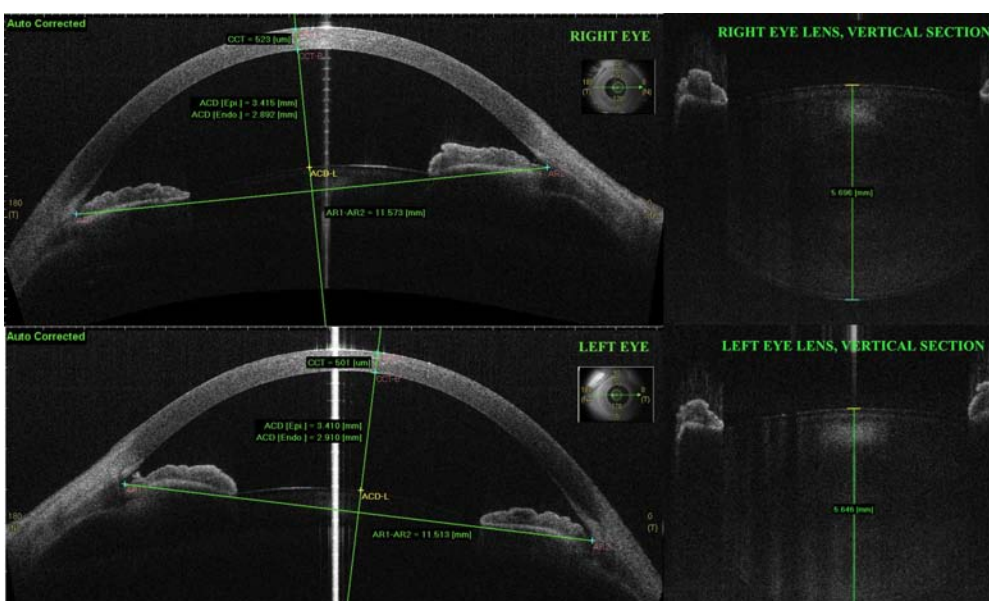

Fig. 4. Analysis of the anterior segment with AS-OCT in a patient with MFRP-syndrome.

2) GENE THERAPY: To carry it out, should be initially established animal models simulating the human disease and that allow a better understanding of the molecular mechanisms of the disease that is meant to be treated. In a similar way to what has happened with the Leber's Congenital Amaurosis (RPE65-LCA), there is also an animal model described for the MFRP-Retinitis Pigmentosa disease that serves to carry out preclinical studies for treatment (*proof-of-concept studies*). Jungyeon Won et al.[32] (2008) designed genetically modified mice (*Mfrp* $^{rd6/rd6)}$ that presented progressive retinal degeneration. These showed that the outer segments of photoreceptors are not properly formed, that their phagocytosis is altered and that the number of apical microvilli of RPE is decreased. Dinculescu A. et al.[35] published in 2012 the first preliminary work with gene therapy in Retinitis Pigmentosa caused by mutations in MFRP. They conducted a study with genetically modified mice for this disease (*Mfrp* $^{rd6/rd6)}$ and injected a modified AAVV8 vector (AND733F) containing the *Mfrp gene* of normal mice (*wild-type)*. The vector was introduced in the subretinal space 14 days after the birth of the mice to prevent retinal degeneration. Subsequently, the function of the retina was studied with ERG and the expression of MFRP with immunohistochemistry. In all the rd6 mice treated, the ERG was found to be within the normal parameters as opposed to the non-treated that presented a reduction of the progressive amplitude from the 25th day of birth. As for the expression of MFRP in treated *rd6* mice, an expression in the EPR similar to the one produced in normal *wild-type* mice was detected. In *non-treated rd6 mice* no MFRP protein in the RPE or in the ciliary body was detected. **Therefore, these preclinical results suggest that the application of gene therapy (with certain modifications) in humans is viable.**

Finally, in the last few years great progress has been made in the development of stem cells for use in retinal degenerative diseases. An example of this progress is the publication of The Lancet by Schwartz et al.[36] on the use of hESC-dPRE

(human embryonic stem cell-derived retinal pigment epithelium) in patients with Stargardt disease and macular degeneration, where they demonstrated efficacy and safety after their subretinal injection. In this line, **a study of gene therapy and stem cells as a preclinical model for treatment of Retinitis Pigmentosa associated with MFRP has also been carried out.** Li Y et al. [37] injected an AAV8 vector (Y733F) that expresses MFRP in human iPS-EPS cells of patients with mutation of the MFRP. As a result, the cells treated with this vector recovered pigmentation and transepithelial resistance, suggesting thus their potential use in subsequent clinical trials in humans. **In addition, this article, is the first in the medical literature that successfully used human cells iPS-EPS as a gene therapy receiver.**

In conclusion, we can say that the enclosed publication in this thesis has served to confirm the MFRP gene as the cause of this new ocular syndrome and has laid the foundations for later research and development of possible gene or cellular therapies. We have also contributed to the clarification of the role of the MFRP gene and the description of its key role in the growth of the eyeball and in the subsequent maintenance of the photoreceptors.

BACKGROUND FRAT

3. BACKGROUND fRAT

3.1 FAMILIAL RETINAL ARTERIOLAR TORTUOSITY

The familial retinal arteriolar tortuosity (fRAT) is a rare or minority disease with a hundred cases described that is characterized by a marked tortuosity of the arteries of second and third order without affectation of the arteries of first order and the venous system 38. The disease was first described by Beyer in 1958 39 that described this syndrome in a father of 43 years and his two children aged 17 and 12 respectively. The father and eldest child also presented a foveal hemorrhage. Werner and Gafner40 subsequently published in 1961 the same case in a father of 47 years and his three children. Caigianut and Werner (1968)41 observed 4 persons in one family with arteriolar retinal tortuosity and recurrent hemorrhages. In 1972, Goldberg et al. 42 described a family with 12 affected members, suggesting the possibility of autosomal dominant inheritance. Wells and Kalina43 published in 1985 three families with fRAT with an autosomal dominant pattern of inheritance and spontaneous retinal hemorrhages. All hemorrhages were resolved without affecting the visual acuity. The retinal arterial tortuosity was not present in general in childhood when showed hemorrhages, but became more apparent during adolescence and adulthood. Some members of the family showed isolated hemorrhages without tortuosity. These authors concluded that the tortuosity could

be acquired in a progressive way instead of being a static or congenital disorder. Clearkin et al. (1986) 44 reported a family with fRAT affecting 6 members over 3 different generations. Three members developed tortuosity during adolescence and four had hemorrhages. One of the patients suffered decreased visual acuity caused by an anterior optic neuropathy. Sears et al. 45 reported in 1998 a family with typical features of the disorder.

More recently, **Sutter FK and Helbig H [46] (2003) conducted a review of the entire literature and obtained the following conclusions:** 1) they confirmed that it is a disease of autosomal dominant inheritance **without causal gene known.** 2) It is characterized by a **pathognomonic pattern** of progressive tortuosity in arteries of second and third order in the macular and peripapillary area that develop during childhood and increases during adulthood. 3) This vascular alteration may be accompanied by **hemorrhages** intra- or preretinal that can occur spontaneously or during a physical exertion. 4) **It is not accompanied by** other disorders **or systemic malformations**.

The conclusions of this review are the ones that have prevailed to the present time to define this syndrome.

Although the authors state that they have not been accompanied by systemic alterations we find publications in the literature (both prior to and subsequent to 2003) where fRAT accompanies itself by some systemic alterations, although in almost all cases are sporadic or isolated. **Anyway, it is now considered, as a minimum debatable, the fact that fRAT is not associated with systemic alterations [47]**. Below, we shall summarize these associations:

Prior to 2003 [46]

- Malformation in the Kieselbach nasal septum.
- Vascular mass in the spinal cord.
- VI nerve palsy.
- Simultaneous conjunctival hemorrhage.
- Conjunctival telangiectasias.

Subsequent to 2003:

- Association with tortuosity of the nail bed capillaries. [47]
- Association with internal carotid artery aneurysm. [48]
- Association with Factor VII deficiency. [49]
- Association with hematuria, muscle cramps and arrhythmia. [50]

In the cases subsequent to 2003, with the exception of the case associated with aneurysm of the carotid artery, the other findings have been described in several members of the families published by each author, so it seems plausible to think that they are part of a systemic hereditary syndrome. On the contrary, these same associations are not presented in all the fRAT published cases, so it is questionable that they are part of a unique ocular clinical entity previously described. In any case, as of 2003, the association with other systemic disorders has been relatively frequent and the topic remains under debate.

Especially interesting is the work that associates fRAT with tortuosity of the subungual capillaries. The nailbed capillaroscopy is a simple and non-invasive method for the detailed study of the microcirculation in a wide range of diseases and is considered a mirror of the systemic vascular processes. It has a high degree of correlation and exceptional predictive and prognostic value in diseases such as: diabetes mellitus, scleroderma, primary chronic polyarthritis, and systemic lupus erythematosus and especially with ocular capillaries and glaucoma. [51] The main capillaroscopic alterations of the nail bed that can be found are:

1. Tortuosities.
2. Increase of capillary diameter.
3. Neoangiogenesis.
4. Hemorrhages or thrombosis.
5. Reduced capillary density.

Regarding **Tortuosities,** it is important to mention that a modest number of them can be observed in healthy people of advanced age. The presence of an important tortuosity of the capillary (greater than 20 %) has been documented in various diseases such as systemic lupus erythematosus, Behçet's disease and scleroderma[52]. In Gekeler et al. [47] publication, the patients (a father of 62 years and his two daughters 19 and 28 years) presented the typical fRAT traits in the fundus of the eye and the nail bed capillaroscopy was normal except for the very marked capillary tortuosity (more than 30% of capillary loops in the three patients). **Fig 5 and 6.** This degree of isolated tortuosity is exceptional and has been documented in very few cases [52].

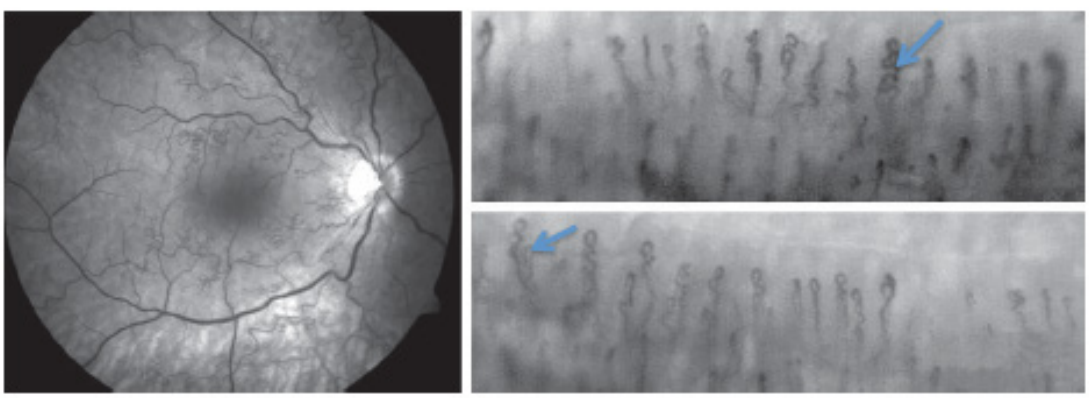

Fig 5. Patient with fRAT and nail bed capillaroscopy with vascular loops.

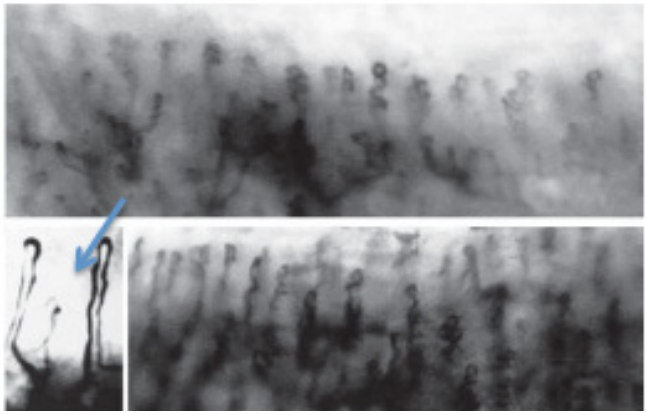

Fig 6. Capillaroscopy in patient with fRAT compared with normality (Blue arrow).

This study demonstrates, in the author's opinion, that in the etiopathogenesis of fRAT underlies a systemic disorder that should be more widely studied. This family was finally re-studied and reclassified as HANAC Syndrome in 2010 by Plaisier and Gekeler [76] (syndrome that we will describe later).

The assumption that it is a question of a systemic disease is consistent with the fact that it is a genetic disease and therefore could have extraocular involvement if the gene is expressed in other parts of the body. **The problem to continue studying this syndrome is that we do not know the gene that causes it and therefore it is difficult to fully understand its pathogenesis and the systemic extent.** On the contrary, their clinical diagnosis is relatively easy, as the funduscopic image with tortuosity of arteries of second- and third-order is **pathognomonic of the disease** (i.e. no other disease presents this specific type of tortuosity) and by the fact that additional complementary examinations to diagnose it are not required.

In conclusion, we are faced with a **rare or minority disease in ophthalmology (approximately 100 cases described [46]), of autosomal dominant inheritance, classified in OMIM with the number % 180000,** but with the distinctive "%", which indicates that its molecular basis is unknown. **In the attached work in this thesis we describe for the first time in the literature the association of a family with fRAT (a father and two daughters) with a gene, the COL4A1.**

3.2 OTHER CAUSES OF RETINAL VASCULAR TORTUOSITY

Although there are no chapters of books or review articles in the medical literature on the specific topic of the vascular tortuosity, we have made a self-review through the bibliography available (PubMed/ OMIM/ Retina eBook, Ryan MD) and we have classified all the causes of vascular tortuosity in the retina in four groups:

1. Vascular malformations / Tumours
2. Ophthalmological Diseases
3. Systemic diseases with ocular affectation
4. Hereditary small-vessel disease of the CNS *(small-vessel disease)*

1. *Vascular malformations or tumours*: in this section the phacomatoses are mainly comprised: Wyburn-Mason Syndrome (WM) of unknown cause that produces arteriovenous malformations (**Fig. 7 and 8**) and the Von Hippel-Lindau disease (VHL) that is caused by high penetrance mutations in the VHL tumour suppressor gene (OMIM 193300). In general, it is about vascular malformations of great size (WM) or vascular tumours (VHL). In fact, in these cases there is no "primary" tortuosity, but that we would call it "secondary" as they derive from arteriovenous malformations that produce a hypertrophy of the afferent artery and dilatation of the efferent venous system. These changes will result in a secondary level, both tortuosity of the arteries and the veins involved.

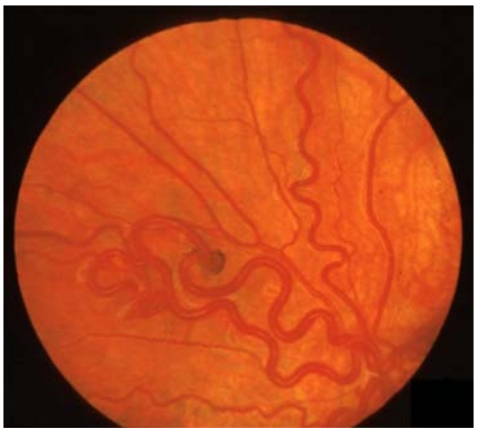
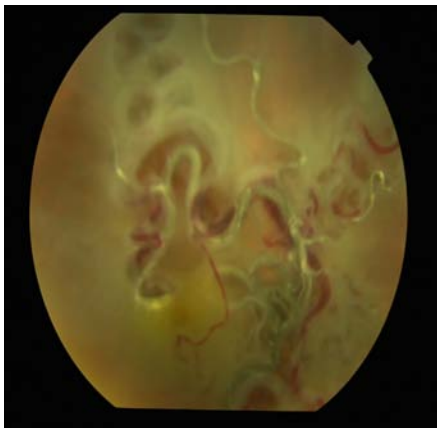

Fig. 7 and 8. Wyburn-Mason syndrome and AV malformation and post-therapeutic microsurgical clipping by cavernous fistula associated in WMS.

2. *Ophthalmological Diseases:* this is a heterogeneous group of diseases that have no relationship between them and in which vascular tortuosity occurs for two main reasons: 1) Due to ischemia or hypoxia 2) Due to flow obstruction.

   Among these diseases we will only highlight as examples:

- **PLUS Disease** in retinopathy of prematurity (ROP): this is characterised by an arterial tortuosity and venous dilation of the posterior pole in two or more quadrants. Although its cause is not entirely clear, it is believed that these changes are due to the ischemia/hypoxia in the ROP and in fact their presence indicates severity of the impairment [53]. (**Fig 9**).

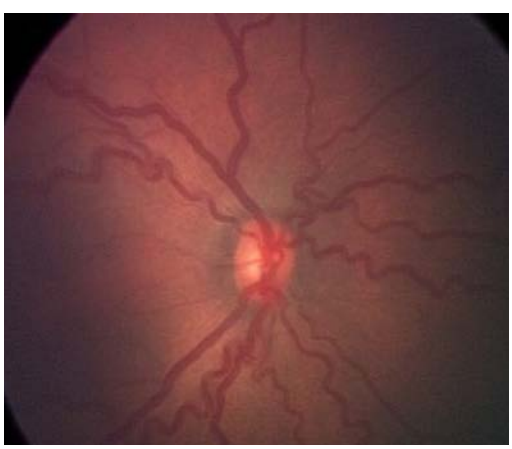
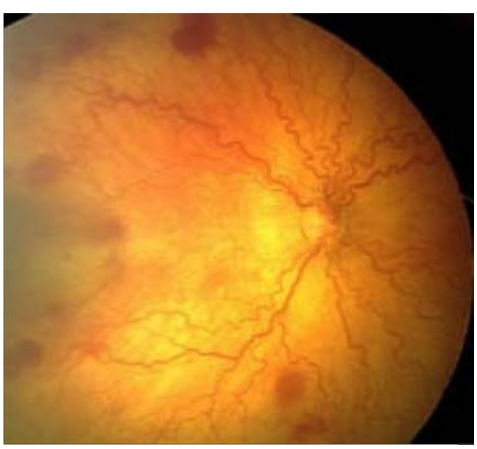

Fig.9. PLUS Disease in the context of ROP.

- **Venous obstructions:** An obstruction of the retina's venous flow occurs in them. The veins, in contrast to the arteries, lack smooth muscle cells and to a resistance in the forward flow, initially increase of caliber and subsequently become tortuous. (**Fig.10**).

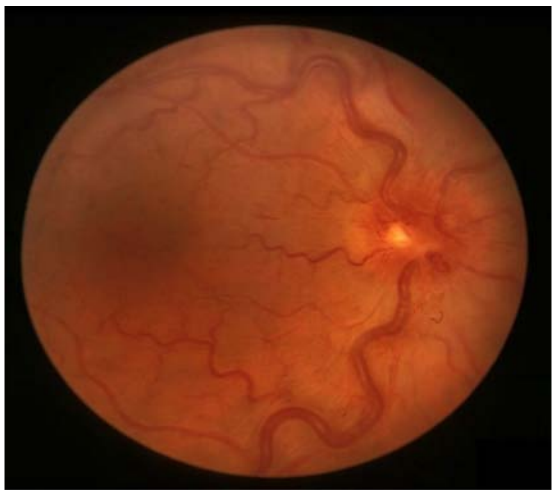

Fig 10. Obstruction of central retinal vein with venous tortuosity.

In this group of ophthalmological diseases we should also encompass fRAT, as genetic cause of arteriolar tortuosity of hereditary cause, without the presence of hypoxia/ischemia or flow obstruction. As we have discussed in the previous chapter, these patients present arterial tortuosity only in the arteries of second and third order without affecting the venous system (**Fig 11**).

This type of tortuosity is pathognomonic since in general, the tortuosity in the diseases of the retina is venous and when it affects the arteries, it usually does to the major ones.

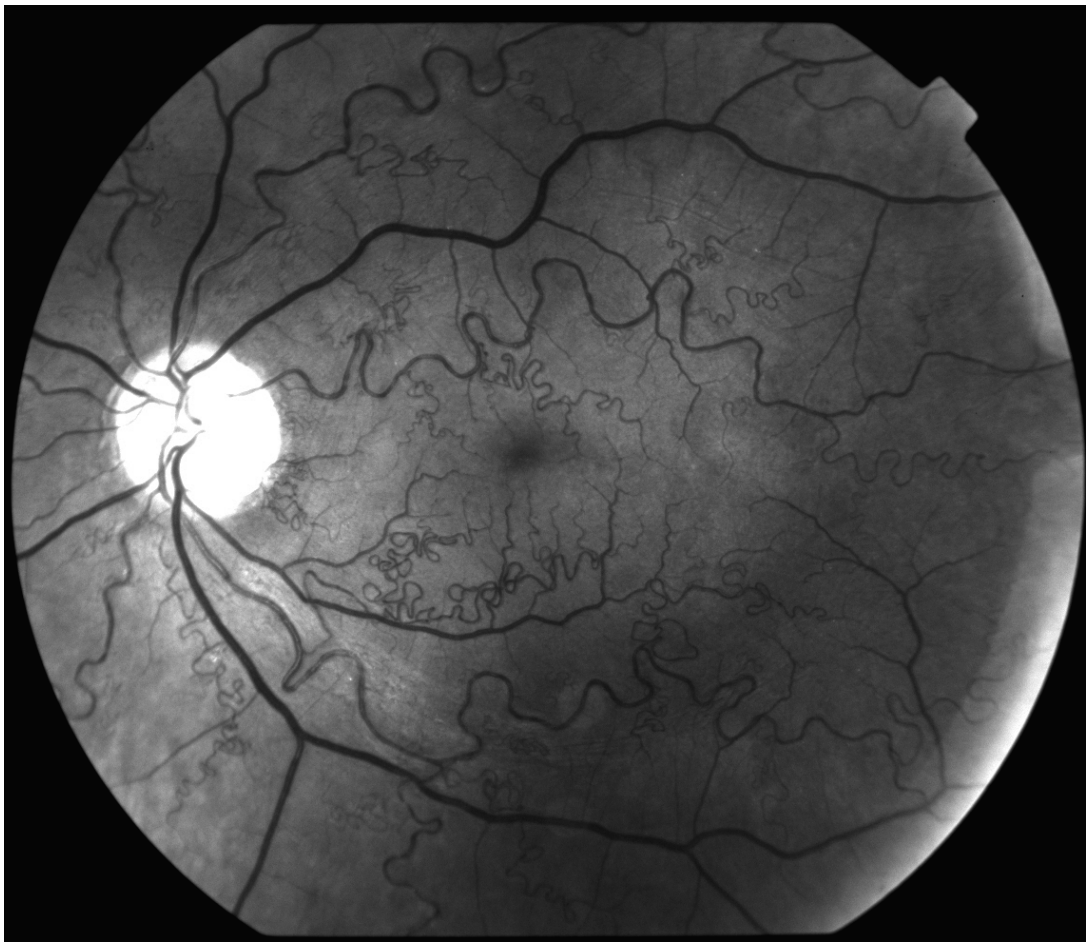

Fig 11. Female patient of 58 years with fRAT (red-free retinography).

3. *Systemic diseases with ocular involvement*: although many systemic diseases can give a certain tortuosity of the retinal vascular tree (in particular the diabetes mellitus and essential hypertension) in this section three groups for their high degree of tortuosity or peculiarity will only be highlighted:

    - *Hematologic Diseases*: Polycythemia Vera, Sickle Cell Anemia, Waldenstrom's Macroglobulinemia. In general, they are diseases which cause a continued venous stasis and that can present venous tortuosity without necessarily occurring a complete obstruction of the central retinal vein.

- *CNS diseases*: Carotid-cavernous fistula, benign or idiopathic intracranial hypertension (BIH). They are illnesses that hamper the extraocular venous return and in a retrograde way can cause an increase in the resistance retinal venous flow and therefore increase the tortuosity. **(Fig. 12**)

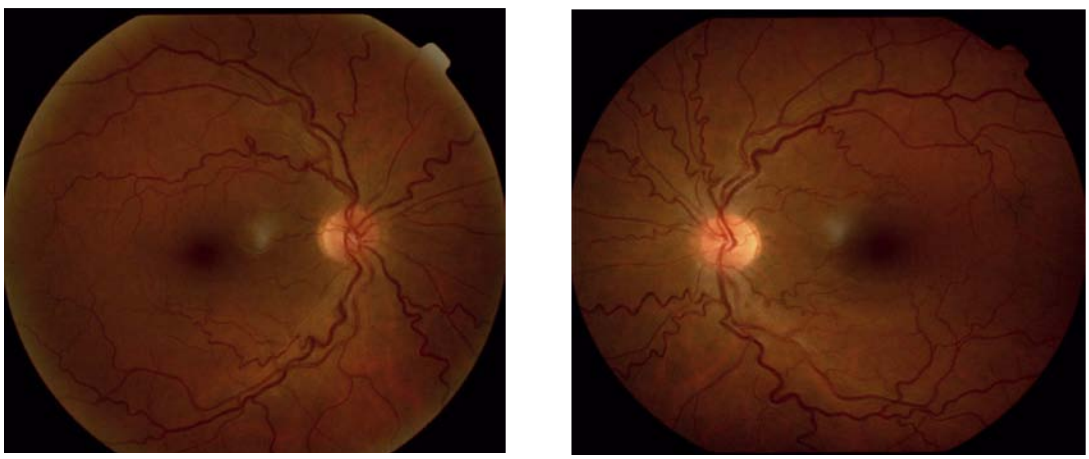

Fig. 12. Female patient, 32 years with BIH.

- *Fabry disease (FD)*: this is a rare or minority disease, underdiagnosed, which can potentially be fatal and that presents some typical features simply identifiable in a routine ophthalmological examination. It is a disease linked to the X chromosome that affects the lysosomal storage by mutations in the gene that encodes the enzyme a -galactosidase (OMIM 301500). The ocular manifestations of Fabry Disease are: Cornea verticillata, cataract, tortuosity or aneurysms of the vessels of the bulbar conjunctiva and retinal arteriolar tortuosity [54] (**Fig. 13 and 14**).

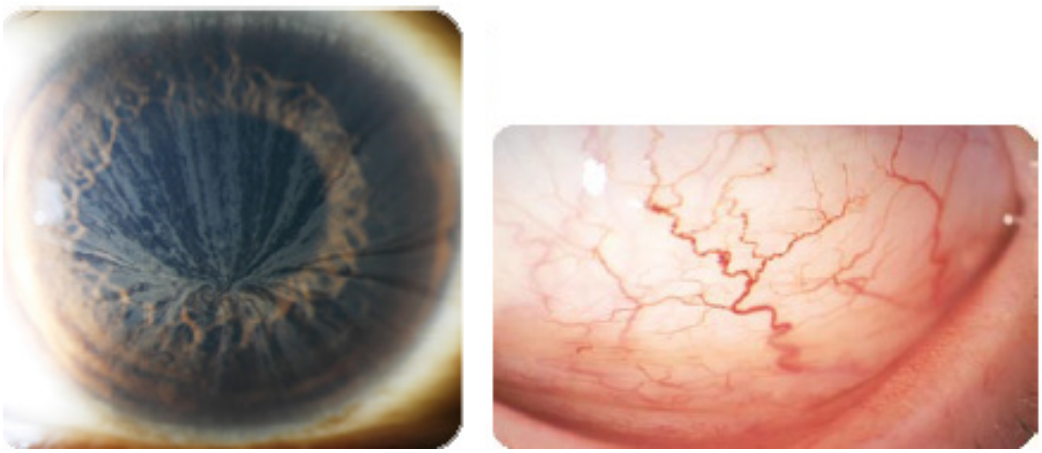

Fig 13. Cornea verticillata and conjunctival aneurysms in a patient with FD.

Note that the vascular tortuosity is more frequently seen in those patients with greater severity of the disease and that increases with age. On the contrary, we can observe cornea verticillata in not diagnosed asymptomatic patients[54]. Therefore, the screening of the ophthalmologist is basic to detect cases at an early stage or not diagnosed, as an efficient enzyme replacement therapy is now available: FABRAZYME (algasidasa beta, GENZYME).[55]

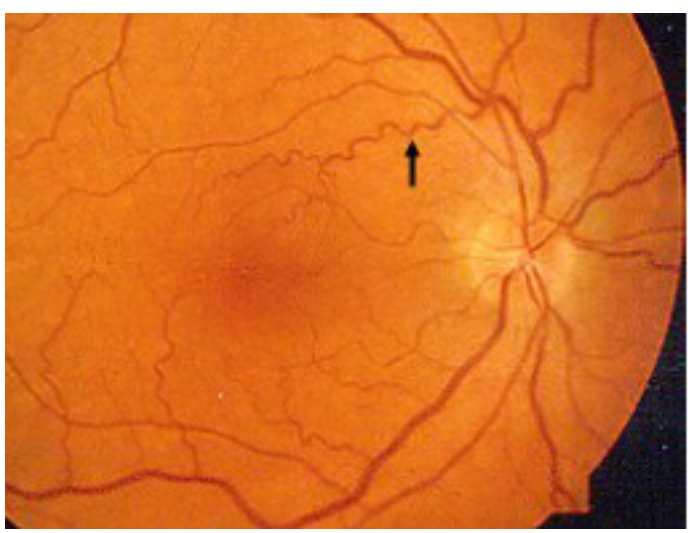

Fig 14. Arteriolar tortuosity in patient with FD. Published bay Sodi et al[54]

4. *Hereditary Diseases of the CNS*: Are a group of genetic diseases of recent description that imply affectation of the microcirculation of the CNS (small vessel disease) and often also have alterations of retinal vessels. Although they are rare or minority diseases, it is now believed that they may be responsible for up to 30% of the cerebrovascular ischemic accidents (CVA) in young patients 56. We can classify them in 4 entities with their corresponding OMIM numbers, their gene responsible and location (**Table 2**). Only the first and fourth can present arteriolar tortuosity, although the other two syndromes can also affect the retinal vascular tree. We will make a brief description of these entities:

4.1 Retinal vasculopathy with cerebral leukodistrophy (RVCL)

4.2 Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL)

4.3 Cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL)

4.4 Brain vessel disease with or without ocular anomalies (BSVD).

RVCL	#192315	Gene: TREX 1	Location 3p21.31
CADASIL	#125310	Gene: NOTCH3	Location 19p13.12
CARASIL	#600142	Gene: HTRA1	Location 10q26.13
BSVD	#607595	Gene: COL4A1	Location 13q34

Table 2. OMIM classification for small vessel disease

RVCL: This is an autosomal dominant disease that is characterized by loss of vision, CVA and dementia that begins in young adults, and that implies death in the majority of the cases in 5-10 years from the onset[57]. It is usually associated with Raynaud's phenomenon, migraine, micronodular cirrhosis and renal impairment (glomerular dysfunction). The visual loss is due to a retinal vasculopathy

characterized by telangiectasias, microaneurysms and capillary obliteration with yuxtamacular onset (**Fig.15**) that can trigger complete occlusion of the main artery branches and areas of peripheral retinal ischemia [58]. Eventually, it can also be seen proliferative retinopathy. In tests of CNS image are usually frequent the presence of "pseudotumors" with perilesional edema and central necrosis [59].

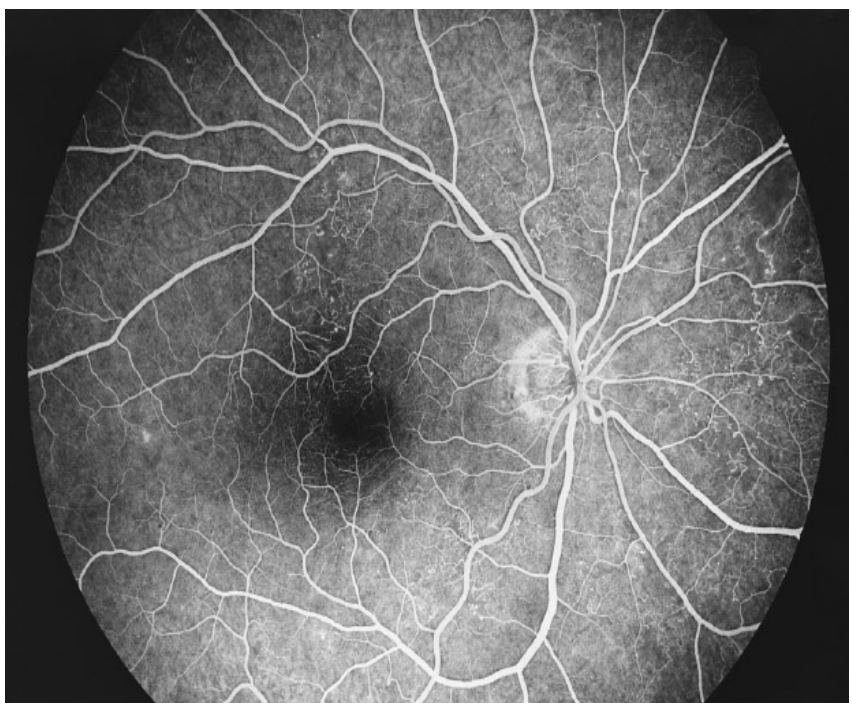

Fig 15. Angiography of patient with RVCL

The loss of exonuclease function of the TREX1 gene would lead to an alteration and premature loss of endothelial cells that eventually, would trigger the retinal and brain microangiopathy that characterizes this syndrome [59].

CADASIL: It is an autosomal dominant genetic disease that causes migraine, multiple CVA in younger age groups and finally subcortical vascular dementia. It presents a generalized arteriopathy both of the arterioles of the retina and the arterioles of the cerebral cortex (which are similar to those of the retina). In contrast to what happens with RVCL, this arteriopathy causes a great variety of

changes in the arterioles of the retina **without affecting its function,** therefore, it does not usually present visual alterations [60]. Rufa et al.[61] described a patient with CADASIL whose first symptom was a nonarteritic anterior ischemic optic neuritis, although the fact that the ocular involvement is rare was highlighted. In general these arterioles of the retina are usually thinned and elongated (anti-tortuosity) by the effect of a likely vasoconstriction related to the etiopathogenesis of the disease [60]. (**Fig. 16**)

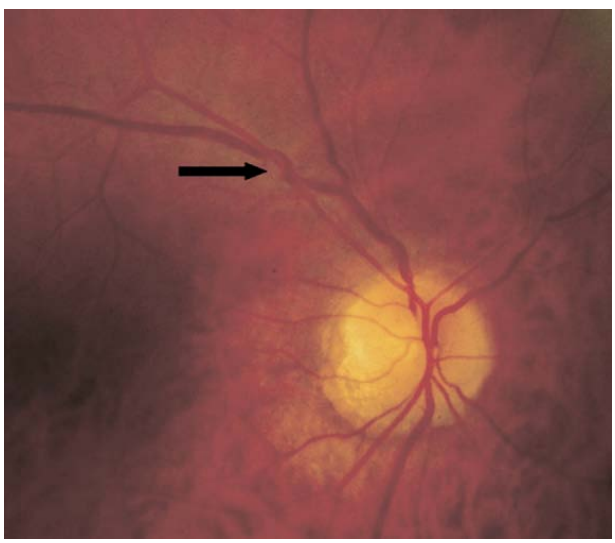

Fig 16. Arterial narrowing and elongation in patient with CADASIL.

CARASIL: It is a rare AR inheritance disease that courses with subcortical brain infarcts, alopecia, spondylosis, progressive motor paralysis and dementia. In general, it appears in the second or third decade of life. Although it shares clinical features with CADASIL, it is a much rarer and infrequent disease, with only 50 cases described to date [62]. Atherosclerotic changes in small size arteries in the white substance of the brain, heart and kidneys have been described. The protease activity of HTRA1 is necessary for the inhibition of signalling of the TGF- b molecules. The mutant proteins (CARASIL) were unable to suppress TGF-b activity, and increased expression TGFB1 was observed in the tunica

media of affected small arteries causing end-stage vascular fibrosis [62]. Curiously a SNP of the HTRA1 gene (associated to high expression of this protein) is one of the genetic risk factors with greater association with neovascular AMD [63]. In these patients with CARASIL (low expression of HTRA1) AMD has not been detected and affectation of the retinal arterioles is not described.[62] This suggests that the signalling of the TGF- b perhaps is not completely inhibited in the retina in these patients, because for this to happen, other factors should also intervene such as the type of cell where is expressed and the extracellular matrix. Anyway, no publication has been found in the literature (PubMed search) that studies specifically ophthalmological changes in patients with CARASIL.

BSVD: This is a genetic disease that also affects the CNS small vessel and/or ocular structures. In most cases, leukoencephalopathy is associated with various ophthalmologic alterations/malformations (which include arterial tortuosity). It is caused by mutations of the COL4A1 gene [64]. This syndrome and others linked to mutations of this gene are described below in a specific chapter.

3.3 SYNDROMES LINKED TO THE COL4A1 GENE

The **COL4A1 gene** encodes the α_1 **subunit** of type IV collagen. There are six α subunits (α^1 to α^6) that are assembled producing three different networks of type IV collagen. **These three networks of collagen comprise the largest component of basal membranes of the human body**: $\alpha_1\alpha_1\alpha_2$ (IV), $\alpha_3\alpha_4\alpha_5$ (IV) and $\alpha_5\alpha_5\alpha_6$ (IV). **The network $\alpha_1\alpha_1\alpha_2$ (IV) is widely expressed in many tissues of the human body**, whereas $\alpha_3\alpha_4\alpha_5$ (IV) and $\alpha_5\alpha_5\alpha_6$ (IV) have a restricted expression depending on the tissue[65]. The chains of α collagen IV consist in: 1) An N-terminal domain called 7S 2) **triple-helical collagenous domain** that contains a highly conserved sequence of amino acids Gly-X-Y and 3) a **C-terminal non-collagenous domain called NC1 (Fig 17 A and B)** . These chains α_1 and α_2 are assembled to form heterodimers $\alpha_1\alpha_1\alpha_2$ through areas of specific recognition in the non-collagenous NC1 domains followed by a super rolling of the **triple-helical collagenous domain**. Subsequently, they are secreted to the extracellular matrix and these molecules of collagen type IV are associated to form supramolecular networks that confer **biomechanical stability of the basement membranes**. In addition, this network of collagen IV has an important role **in the interaction of the MB with adjacent cells to promote their migration, proliferation, differentiation and survival**. The accession of these cells to collagen type IV occurs across multiple areas of the two domains, but among them stands out a **CB3 fragment located in the triple helix** containing areas of anchorage for the family of the **integrins** α 1β 1 and α 2β 1.

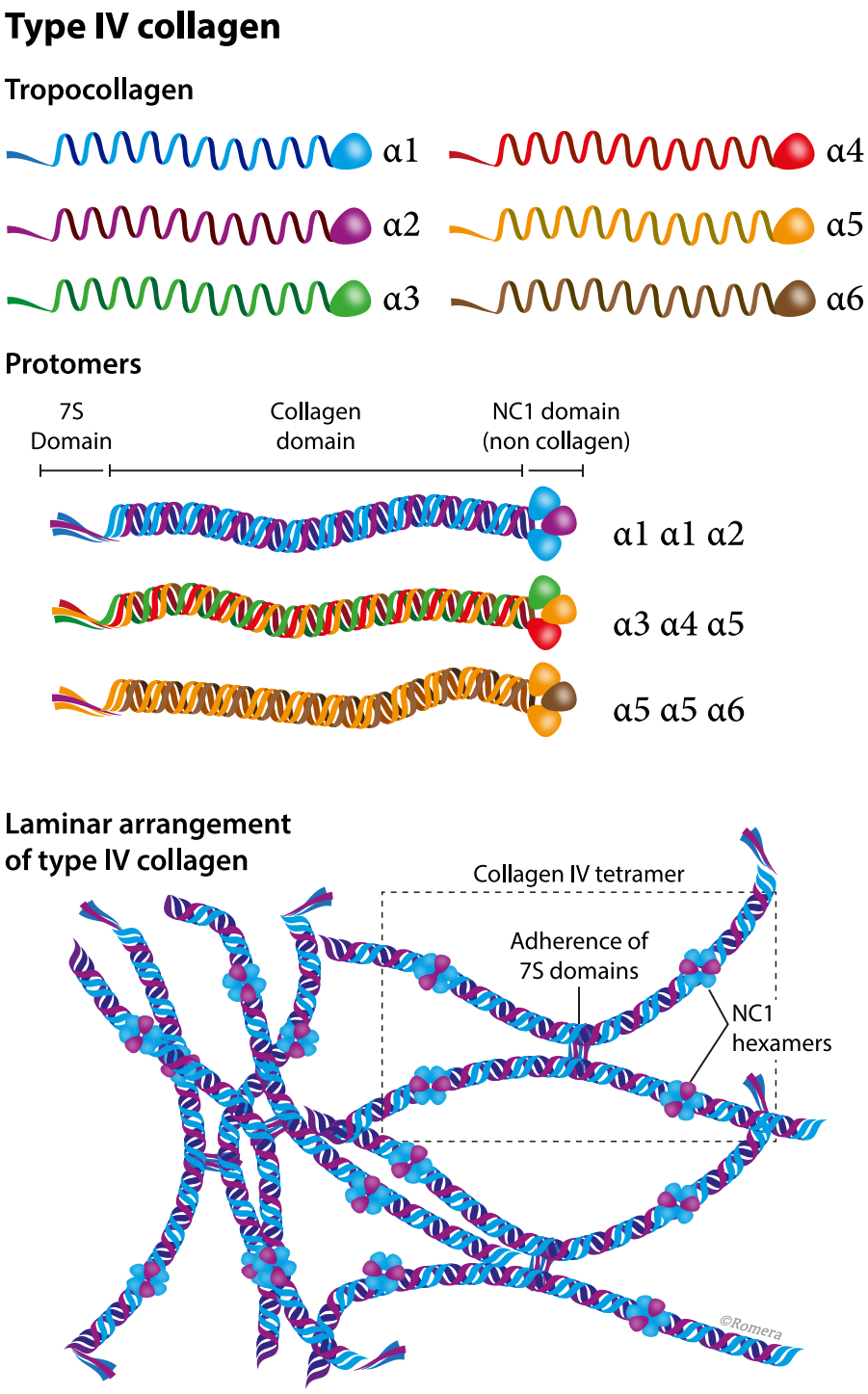

Fig 17 A y B. Molecular Structure of the collagen type IV.

Mutations in the genes that encode these networks of collagen type IV are widely known and it is for years that are described in the literature, in particular **the Alport syndrome**. In summary, these are the most well-known syndromes:

COL4A5 Alport syndrome X-linked (OMIM 301050)

COL4A4/3 →Autosomal recessive Alport Sd (OMIM203780/104200)

COL4A4/3 → Familial benign hematuria

The COL4A1 gene has 52 exons and is located in chromosome 13q34. Mutations in these genes (COL4A1/A2) in both animal models and humans that have led to the recognition of new syndromes linked to functional loss of these genes have recently been described [66]. More than 30 mutations to date linked to this gene have been published and their number is increasing rapidly.[67] We summarize the syndromes linked to mutations in COL4A1 in the following table (**Table 3**):

BSVD	(OMIM 607595)
Porencephaly 1	(OMIM 174780)
Hereditary angiopathy with nephropathy, aneurysms and muscle cramps	(OMIM 611773)
Intracerebral susceptibility to hemorrhage	(OMIM 614519)

Table 3. Syndromes linked to mutations in COL4A1

1-BSVD: this is an AD hereditary disease caused by a heterozygous mutation of the COL4A1 gene, which basically affects the **CNS and the eyeball**. Lanfranconi et al.[68] conducted in 2010 a review of 52 cases with known mutation and found the following presentations: CVA with children hemiparesis, intracranial haemorrhage (ICH), and lacunar stroke. The average age of onset was 36 years (range 14 to 49 years) and **the ICH was recurrent and often caused by a small trauma, activity or anticoagulant therapy.** Other neurological associated findings were intracranial aneurysms and migraine (30% of the cases) [68].

In regard to the ophthalmologic findings described in this syndrome, they are very heterogeneous. When the syndrome was initially described, Vahedi et al. [69] (2003) reported the case of a French family with children hemiparesis in **which all the affected members presented retinal arteriolar tortuosity with hypopigmentation of the fundus of the eye, in addition to the small vessel pathology of CNS (Fig. 18).** A member also presented retinal hemorrhage.

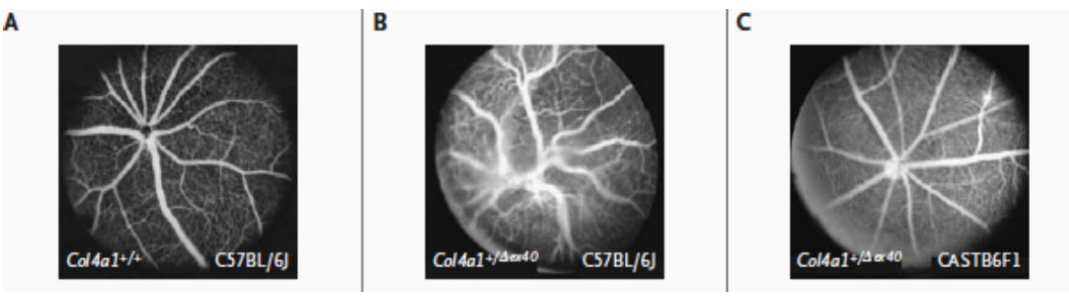

Fig 18. Patient affected with BSVD with arteriolar tortuosity, white matter abnormalities and cerebral hemorrhage (black arrow) published by Vahedi et. Al[69].

Later (2006), Gould DB et al.[56] published more families with this syndrome (including the same family studied by Vahedi) and established that it was due to mutations in the COL4A1 gene. **Curiously, in the other families described subsequently, ophthalmologic findings fundamentally affect the anterior segment and in addition do not usually have retinal arterial tortuosity (Fig 18)** [70]. In summary these anomalies are:

- Congenital cataract, microcornea and corneal opacity.
- Congenital glaucoma, corectopia, polycoria.
- Microphthalmos.
- Axenfeld-Rieger syndrome.

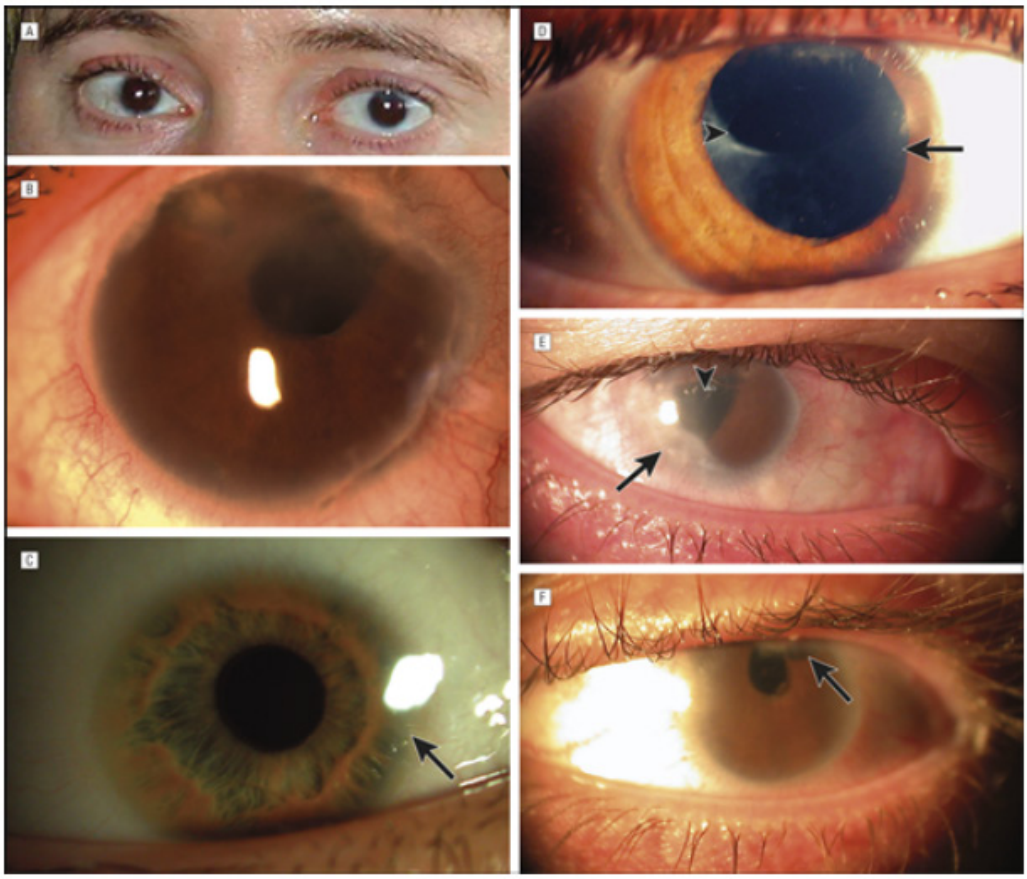

Fig 19. Graphic summary of findings of abnormalities in the anterior segment in family with mutations in the COL4A1 published by Capri I et al. [70]

Van Agtmael et al (2005) [71] and Gould et al (2006) [56] have made animal models of this disease with genetically modified mice with mutations in the col4a1. In them, they also found intracranial hemorrhages (both perinatal as in adults), retinal vascular tortuosity and defects of the ocular anterior segment similar to those described above in humans. Some phenotypes (*Bru mice*) also developed glomerular nephropathy. Curiously, only a line of mice with a different initial genome (C57BL/ 6J) to the other groups, expressed vascular tortuosity of the retina (*Raw mice*) **Fig 20 (B).** This study demonstrates how the ocular phenotype

resulting in mutations of the COL4A1 gene depends on the genetic background in which they occur. The severity of the impairment of the syndrome may also be influenced by this genetic background and environmental factors.

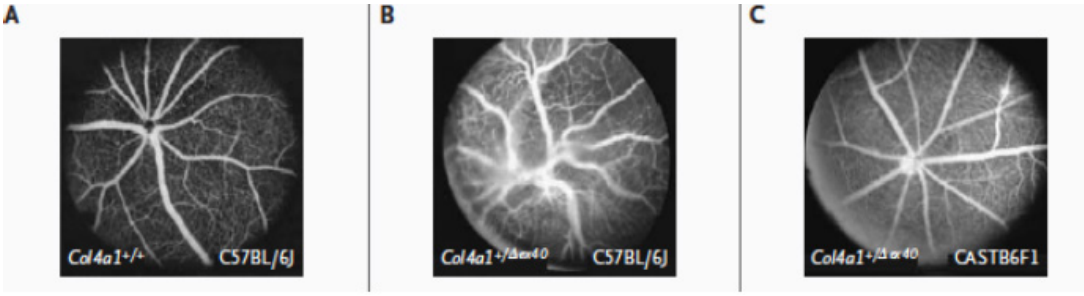

Fig 20. AGF in mouse control (A), in mouse C57BL/ 6J with mutation Col4a1 mutation (B) and in mouse CASTB6F1 with mutation of the col4a1 gene (C). The B pattern shows tortuosity of the retinal vessels in comparison with the other two mice families. Published by Gould et al[56].

2- PORENCEPHALY 1 (POREN1): It is a neurological disease that is characterized by fluid filled cavitations or cysts in the brain. They are believed to be caused by a vascular defect of the arterioles that irrigate the brain parenchyma and that finally involve brain degeneration. The subjects who suffer from this disease typically present hemiplegia, epileptic seizures, and mental retardation, although the degree of affectation is variable [72]. A similar case (**POREN2**) caused by mutations in the COL4A2 gene [73] is also described. Although associations with ocular malformations in some patients are described, these do not usually form part of the typical syndrome's symptoms.

3- SUSCEPTIBILITY TO HEMORRHAGIC STROKE (ICH): Pathological mutations of COL4A1 and COL4A2 (OMIM 614519) in patients of adult age with CVA bleeding without other associated systemic involvement have been identified. None of the mutations affected the highly conserved glycine residue in the evolution of this gene and we know that in the COL4A1 gene give rise to the diseases previously described in early ages of life. In this way, the authors suggest

that these mutations (variants not-glycine) could represent alleles that give rise to mild forms of this disease in adulthood as a result of the interaction with other predisposing factors of ICH [74].

4- HANAC (Hereditary Angiopathy with Nephropathy, Aneurysms and muscle Cramps): It is a disease characterized by involvement of the CNS (aneurysms, leukoencephalopathy), ophthalmological (arteriolar tortuosity and hemorrhages), renal (cysts, alteration of the glomerular filtrate) and muscle (muscle cramps, increased creatinkinase, CK, in serum). Plaisier et al.[75] described in 2005 a French family of 4 generations that presented autosomal dominant familial hematuria associated with extrarenal manifestations: retinal arteriolar tortuosity and muscle cramps. Although a study in two candidate genes was conducted, the genetic cause of this new syndrome was not found. Later in 2007, the same authors published the phenotype of 3 families with this syndrome proposing the acronym of HANAC [75] (summary **Fig. 21**). Renal manifestations in these families included hematuria and bilateral large cysts. A histological study of kidney and skin revealed defects in the basement membrane of both organs. The systemic angiopathy described in these patients affected both small vessels (leukoencephalopathy) and great vessels (aneurysms). The muscle cramps affected 2 of the 3 families described and coincided with high levels of CKs. The retinal arteriolar tortuosity was a frequent finding in all families with HANAC. Occasionally, it was associated with symptomatic haemorrhages that were resolved without sequelae and good visual acuity. Other findings associated with this syndrome were Raynaud's phenomenon and supraventricular arrhythmia.

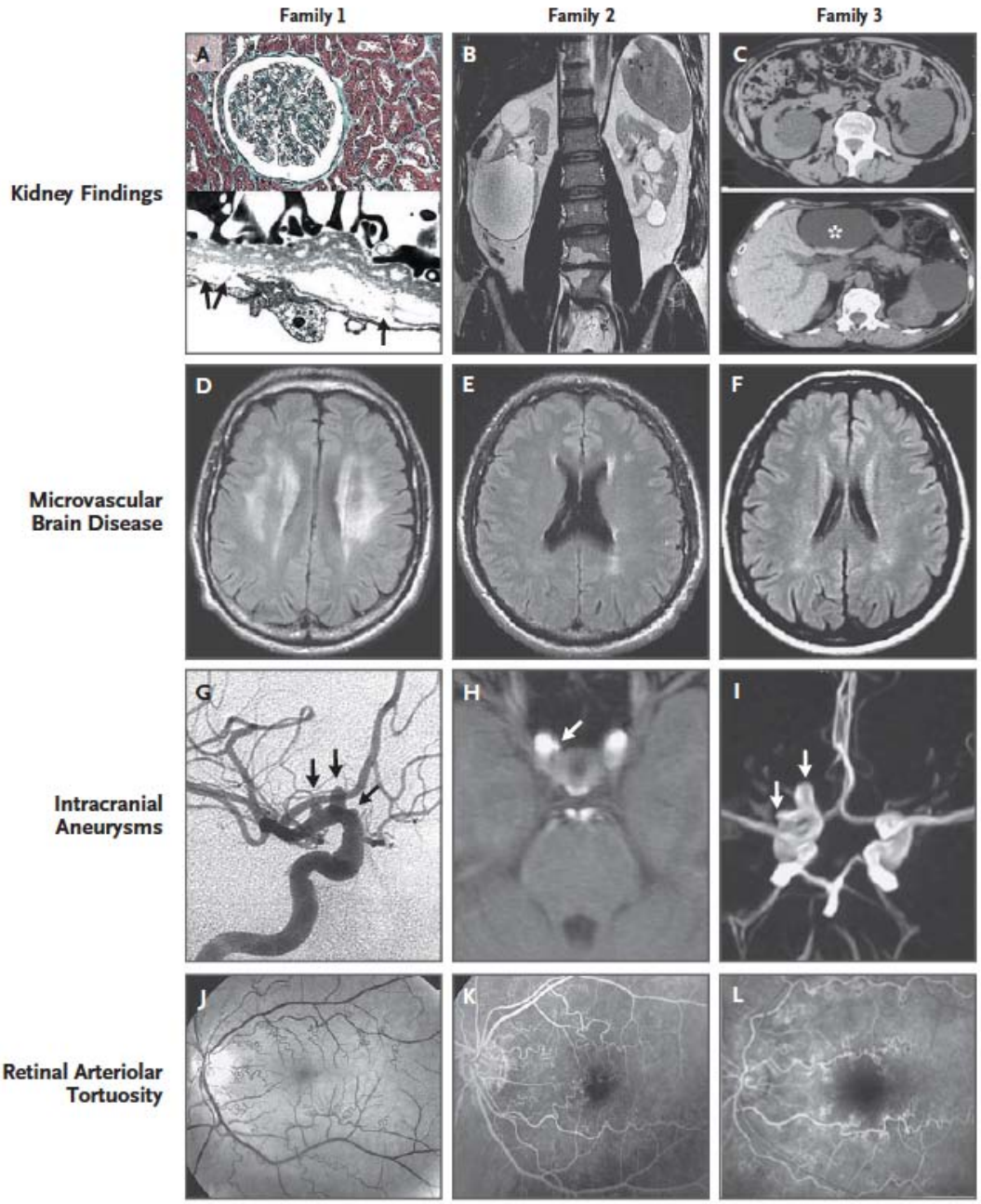

Fig 21. Clinical and histological findings in the HANAC syndrome that include involvement of the CNS, ocular, renal and muscular published by E. Plaisier et al [75].

Genotype in HANAC

All the mutations described so far in the HANAC syndrome affect the glycine residues located in exons 24 and 25 of the COL4A1 gene, suggesting that these exons encode a critical domain for the operation of the triple helix of type IV collagen [76.77] (**Fig. 22**).

TABLE II. *COL4A1* Mutations in Families With HANAC Syndrome and Brain and Eye Restricted Disorders

Families	Nucleotide change	Exon	Amino acid change	Refs.
HANAC syndrome				
Present report				
F1	c.1493G > A	24	p.Gly498Asp	
F2	c.1528G > A	24	p.Gly510Arg	
F3	c.1573_1574GG > TT	25	p.Gly525Leu	
Previous report				
	c.1493G > T	24	p.Gly498Val	Plaisier et al. [2007]
	c.1555G > A	25	p.Gly519Arg	Plaisier et al. [2007]
	c.1583G > A	25	p.Gly528Glu	Plaisier et al. [2007]
Brain and eye restricted disorder	1A > T	1	—	Breedveld et al. [2006]
	c.1769G > A	25	p.Gly562Glu	Gould et al. [2006]
	c.2159G > A	29	p.Gly720Asp	Sibon et al. [2007]
	c.2245G > A	29	p.Gly749Ser	Gould et al. [2005]
	c.2263G > A	30	p.Gly755Arg	Shah et al. [2010]
	c.2413G > A	31	p.Gly805Arg	Vahedi et al. [2007]
	c.3389G > A	39	p.Gly1130Asp	Breedveld et al. [2006]
	c.3706G > A	43	p.Gly1236Arg	Gould et al. [2005]
	c.4267G > C	48	p.Gly1423Arg	Breedveld et al. [2006]
	c.4582-4586dupCCCATG	49	—	Bilguvar et al. [2009]
	c.4738G > C	50	p.Gly1580Arg	De Vries et al. [2009]

Fig. 22. Summary of Plaisier et al.[77] of all mutations in COL4A1 described in HANAC and BSVD.

The evolutionary analysis demonstrates that these glycine residues are highly conserved in all species and that mutations in the same are often pathological (**Fig. 22**). In patients with HANAC, all mutations in the glycine residues are located in a segment of 30 amino acids near the fragment CB3 of the collagenous domain that contains the areas of union specific for integrins. Therefore, it would be possible to establish the following hypothesis: HANAC mutations in the COL4A1 gene produce an alteration in the bending of the collagen type IV that would lead to a faulty interaction between the basement membrane and the adjacent cells, causing the pathology of this syndrome. In addition to the location of these mutations, environmental factors or genetic modifiers can influence the expression of the

phenotype and the degree of severity of the affected organ. Recently, animal models in mice col4a1 $+^{/Lex40}$ have demonstrated the key role of modifier genes in the ocular phenotype [78].

Fig 23. At the start of the figure, the 6 mutations described in HANAC with the substitution of glycine marked in red in the amino acid sequence (489 to 535) that is very similar in all species of evolutionary study.

In 2008, one of the directors of this work (Dr José Antonio Buil) diagnosed a family (father and two daughters) of familial retinal arteriolar tortuosity and proposed this author (J. Crespi) to identify the causative gene. Since the human genome is composed of approximately 20,000 genes, identifying the causal gene for a disease when it is not previously described is an extremely complicated and time consuming task. A paradigmatic example of this complexity is Huntington's disease (HD): in 1983, it was the first hereditary syndrome where a gene was mapped to a chromosome without prior knowledge of its location [79]. Ten years later, in 1993 the gene for HD was identified. The linkage genetic techniques used in this entire process were pioneers and were applied later in the search for many other genetic diseases [80]. **To identify a single gene, Dr Wexler and her team, collected on an island of Venezuela where the prevalence of HD was very high, more than 4000 DNA samples and documented a pedigree with more than 18000 people during this study that lasted more than 20 years [81].**

Currently, with the modernization and new techniques of molecular biology, **the genetic analysis is faster and easier, but it is still essential to know where to look for in the complex human genome.**

For the fRAT disease, we selected COL4A1 as candidate gene using the following assumptions: **the arteriolar tortuosity in the syndromes linked to COL4A1 previously described is very similar (if not identical) to that which is provided in fRAT, and whose funduscopic image is so peculiar, that in fact it is pathognomonic. Therefore, we suggest the hypothesis that fRAT could be caused by mutations in COL4A1 that would result in a "more benign" or restricted only to the retina.** In the second publication in this thesis, we present the results that show for the first time the relationship of COL4A1 with fRAT, thus expanding the number of syndromes linked to this gene (BSVD, POREN1, and HANAC).

PUBLISHED STUDIES

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GENETICS

Next generation sequencing uncovers a missense mutation in *COL4A1* as the cause of familial retinal arteriolar tortuosity

Juan C. Zenteno · Jaume Crespí · Beatriz Buentello-Volante · Jose A. Buil · Francisca Bassaganyas · Jose I. Vela-Segarra · Jesus Diaz-Cascajosa · Maria T. Marieges

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Abstract
Objectives Our aim was to determine the molecular cause of autosomal dominant familial retinal arteriolar tortuosity (FRAT) in a family with three affected subjects.
Material and methods Ophthalmologic evaluation included determination of best-corrected visual acuity (BCVA), slit-lamp and dilated fundus inspection, applanation tonometry, fundus photography, and fluorescein retinal angiography (FA). Molecular methods included whole exome sequencing analysis and Sanger sequencing validation of putative causal mutation in DNA from affected individuals.
Results Typical signs of familial retinal arteriolar tortuosity were observed in all three patients. Exome sequencing identified a heterozygous c.1528G>A (p. Gly510Arg) mutation in *COL4A1*. Sanger sequencing confirmed that all three patients harbored the same pathogenetic mutation in *COL4A1*. The p. Gly510Arg variant in COL4A1 was absent in DNA from an available unaffected daughter, from a set of control alleles, and from publicly available databases.
Conclusions The molecular basis of familial retinal arteriolar tortuosity was identified for the first time, thus expanding the human phenotypes linked to *COL4A1* mutations. Interestingly, the COL4A1 p.Gly510Arg mutation has been previously identified in a family with HANAC (Hereditary Angiopathy with Nephropathy, Aneurysm and Cramps), a multisystemic disease featuring retinal arteriolar tortuosity. No cerebral, neurologic, renal, cardiac or vascular anomalies were recognized in the pedigree described here. These data indicate that identical mutations in COL4A1 can originate both eye-restricted and systemic phenotypes.

Keywords Retinal arteriolar tortuosity · FRAT · COL4A1 · Retinal hemorrhages

Juan C. Zenteno and Jaume Crespí Contributed equally to this work and should be considered equivalent first authors.

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J. C. Zenteno (✉) · B. Buentello-Volante
Genetics Department and Research Unit, Institute of Ophthalmology "Conde de Valenciana" and Biochemistry Department, Faculty of Medicine, National Autonomous University of Mexico (UNAM), Chimalpopoca 14, Col. Obrera, Mexico City, CP 06800, Mexico
e-mail: jczenteno@institutodeoftalmologia.org

J. Crespí · J. A. Buil · F. Bassaganyas · J. I. Vela-Segarra · J. Diaz-Cascajosa · M. T. Marieges
Department of Ophthalmology, University of Barcelona, Hospital de Sant Pau y de la Santa Creu, Barcelona, Spain

Introduction

Increased tortuosity of retinal vessels is a frequent ophthalmoscopic finding and it can occur as a localized or generalized anomaly, affecting one or both eyes, and as a sporadic or inherited trait. Familial retinal arterial tortuosity (FRAT, OMIM %180000), first reported by Beyer in 1958, is an uncommon dominant disorder characterized by marked tortuosity of second-order and third-order retinal arteries with normal first-order arteries and venous system [1]. Typically, vascular tortuosity in FRAT is predominantly located at the macular and peripapillary area and develops during childhood or early adulthood [2–5]. Although the disease may be asymptomatic, most FRAT patients complain of variable degrees of transient vision loss due to retinal hemorrhage following physical exertion or minor trauma (reviewed in [6]). The observation of vertical transmission in affected pedigrees, male to male inheritance, and occurrence in parents and their children in the absence of consanguinity strongly suggests autosomal dominant inheritance [7–9]. To date, approximately 18 familial cases have been reported [6, 8, 9]. In most cases, systemic involvement of non-ocular vascular beds has not

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been demonstrated in FRAT patients, but occasionally other associated vascular abnormalities have been reported, including malformations in the Kieselbach nasal septum, spinal cord vascular mass [3], telangiectasis of bulbar conjunctiva [6], and internal carotid artery aneurysm [10]. While several syndromic genetic entities feature increased retinal arterial tortuosity, isolated FRAT is considered a discrete autosomal dominant entity with an as of yet unknown etiology. In this work, we report the results of exome sequencing analysis in a two-generation family affected with FRAT and provide evidence that a heterozygous missense mutation in *COL4A1* gene is responsible for the retinal phenotype in this pedigree.

Methods

Clinical studies Institutional Review Board (IRB)/Ethics Committee approval was obtained. All patient samples were collected with written informed consent and clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki. A two-generation family from Spain was studied (Fig. 1). In all patients, ophthalmologic examinations included determination of best-corrected visual acuity (BCVA), slit-lamp and dilated fundus inspection, applanation tonometry, fundus photography, and fluorescein retinal angiography (FA). To exclude systemic involvement, hemogram, urianalysis, glomerular filtration rate (GFR) and creatine phosphokinase (CPK) levels measurements, kidney ultrasonography, Doppler echography, magnetic resonance angiography (MRA), and clinical neurological examination were performed in all three patients.

Whole exome sequencing Exome sequencing was performed by Edgebio (Gaithersburg, MD, USA) on a single FRAT patient (father) from this family. Samples were prepared using Illumina's protocol TruSeq DNA Sample Preparation Guide. Briefly, samples were sheared to an average size of 300–400 bp using sonication. DNA fragment ends were repaired and phosphorylated using Klenow, T4 DNA Polymerase and T4 Polynucleotide Kinase. Next, an 'A' base was added to the 3′ end of the blunted fragments, followed by ligation of Illumina Paired-End adapters via T-A mediated ligation. From here, samples were prepared using the NimbleGen protocol outlined in "NimbleGen SeqCap EZ Exome Library SR User's Guide" (Version 3.0). The libraries were amplified using LM-PCR and 1ug of amplified sample libraries were hybridized with Nimblegen's Exome Library baits for 64 h at 47 °C. Captured DNA was then washed and recovered using Streptavidin Dynabeads. The captured DNA was LM-PCR amplified for a total of 17 cycles. The amplified capture DNA library size and concentration were determined using an Agilent Bioanalyzer.

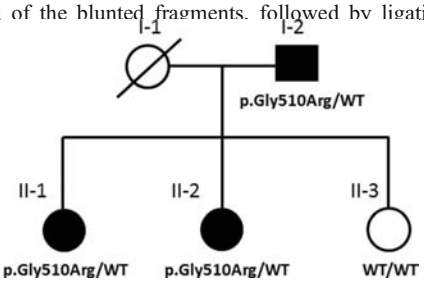

Fig. 1 Pedigree of the family with autosomal dominant FRAT and segregating a heterozygous p.Gly510Arg mutation. *Solid symbols* indicate affected subjects; WT indicates a wild type COL4A1 allele

The captured library was then loaded on a HiSeq 2000 platform for sequencing with a mean exome coverage of 30×. Raw image files were processed by Illumina Pipeline v1.7 for base calling. Single nucleotide polymorphisms (SNPs) and indels were called using an in-house developed software. Identified variants were filtered against the Single Nucleotide Polymorphism database (dbSNP 129, http://www.ncbi.nlm.nih.gov/projects/SNP/snp_summary.cgi), 1,000 genomes project (www.1000genomes.org), and Exome Variant Server (http://evs.gs.washington.edu/EVS/) databases. We excluded the variants that we don't considered pathogenic according to the following criteria: 1. Minor allele frequency (MAF) ≥0.01 from 1000 Human Genome Project database; 2. Located in non-coding regions without affecting splicing site; 3. Synonymous variants without affecting splicing site; 4. Homozygous variations (as we assumed an autosomal dominant transmission in this family). All the other variants were considered pathogenic and summarized for validation. Non-excluded missense mutations were tested for mutational effects by using amino acid substitution prediction tools such as PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), SIFT (http://sift.jcvi.org/), and PANTHER (http://www.pantherdb.org/tools/).

PCR and Sanger sequencing for familial segregation analysis of COL4A1 mutation

Genomic DNA was extracted from peripheral blood leukocytes using an automated system (Qiacube, Qiagene Mexico, Mexico City, Mexico). The exon number 24 of *COL4A1* was amplified by PCR using pairs of primers derived from normal gene sequences (exon 24 Fwd: 5′- CCTTTCTGAGTCCGTC TTGG −3′; Rev: 5′-CACTTACCAGCTCCCACACA −3′ (Ensembl ID ENSG00000187498). Each 25 μl PCR amplification reaction contained 1× buffer, 100 ng of genomic DNA, 0.2 mM of each dNTP, 2 U Taq polymerase, 1 mM of forward and reverse primers, and 1.5 mM MgCl2. PCR products were analyzed in 1.5 % agarose gels, from which the bands with the amplified templates were excised and the DNA was subsequently purified with the help of the MiniElute PCR Purification Kit (Qiagen). Direct automated sequencing of

PCR amplicons was performed with the BigDye Terminator Cycle Sequencing kit (Apllied Biosystems, Foster City, CA, USA). All samples were analyzed on an ABI 3130 Genetic Analyzer (Applied Biosystems). Wild-type and mutated COL4A1 sequences were compared manually. Familial segregation of the mutation was analyzed.

Results

Clinical assessment

Case #1 (father) Case #1 is 53-year old male who was evaluated due to photophobia. He denied any other visual symptom. At examination, best corrected visual acuity was 20/20 in both eyes (OU), and intraocular pressure was 16 mmHg in the right eye (OD) and 17 mmHg in the left eye (OS). Structures of the anterior segment were unremarkable. At funduscopy, marked tortuosity of second-order and third-order arterioles was noted bilaterally. Venous system appeared normal and no evidence of past retinal hemorrhage was noted. In addition, discrete hypopigmentation of the retinal pigment epithelium was observed in both fundi (Fig. 2). Hemogram and urianalysis tests were normal, while glomerular filtration rate (GFR) calculated using the Modification of Diet in Renal Disease (MDRD) Study equation was 91 ml/min/ 1.73 m^2 (normal value: > 60 ml /min/ 1.73 m^2). Kidney ultrasonogram excluded renal anomalies, while Doppler echography did not identify aortic or renal arterial abnormalities. Magnetic resonance angiography (MRA) revealed a small (3 mm in diameter) internal carotid artery aneurysm and no evidence of leukoencephalopathy. No other intracranial vascular lesion was found. No neurologic symptoms were identified and the patient denied having experienced muscle cramps or migraine. CPK levels were 154 u/L (normal values for men: 55–170 U/L).

Case #2 Case #2 is a 21-year old female, the oldest daughter of case #1. At the age of 15 years, she suffered from an episode of exercise-related mild retinal hemorrhage. Her visual acuity was 20/20 (OD) and 20/32 (OS). No anterior segment anomalies were present at biomicroscopic examination. Funduscopic examination revealed increased tortuosity of second-order and third-order arterioles, several round perifoveal intraretinal hemorrhages OD, and a foveal hemorrhage OS (Fig. 3). As observed in her father's fundi, she exhibited a generalized RPE hypopigmentation OU. On follow-up evaluations, hemorrhages resolved spontaneously and visual acuity recovered to normal 20/20. She has experienced several self-resolving events of retinal hemorrhages during the last 5 years. Hemogram and urianalysis tests were normal, while GFR was normal at 98 ml/min/ 1.73 m2. Renal USG results were unremarkable, doppler echography did not identify aortic or renal arterial abnormalities, and MRA did not detect structural anomalies or evidence of leukoencephalopathy. No neurologic symptoms were identified and she denied having experienced muscle cramps or migraine. CPK levels were 115 U/L (normal values for women: 45–135 U/L).

Case #3 Case #3 is an 18-year old female, sister of case #2. She suffered from an episode of exercise-related mild retinal hemorrhage at the age of 13 years. Best corrected visual acuity was 20/20 (OD) and 20/200 (OS). Anterior segment structures were normal OU, IOP was 16 mmHg OU, while funduscopy revealed increased tortuosity or retinal arterioles and bilateral intraretinal hemorrhages (Fig. 3). In OS, a large foveal hemorrhage was observed (Fig. 4). The hemorrhage reabsorbed spontaneously after a 2-month period and no additional episodes of retinal hemorrhages had occurred since then. Results of hemogram and urianalysis tests were normal, while GFR was 104 ml/min/1.73 m2, within normal limits. Renal USG was normal, doppler echography did not identify aortic or renal arterial abnormalities, and MRA did not detect structural anomalies or white matter changes suggestive of leukoencephalopathy. No neurologic symptoms were identified and she denied having experienced muscle cramps or migraine. CPK levels were within normal limits (89 U/L).

Exome sequencing results and mutation validation Whole exome sequencing identified a total of 234 rare non-synonymous heterozygous variants. From this group, 11

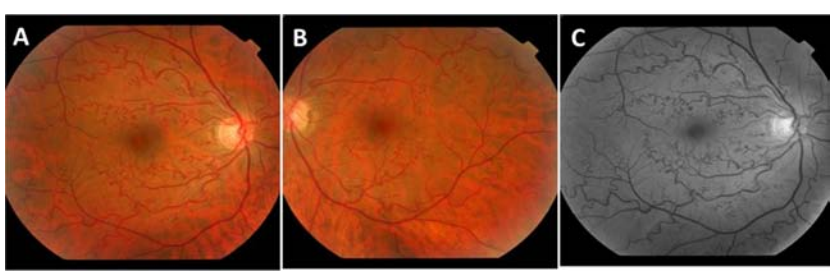

Fig. 2 Fundus photograph of patient #1 showing the pathognomonic pattern of arteriolar tortuosity in right (**a**) and left (**b**) eyes. Red-free retinography (**c**) shows no evidence of retinal hemorrhage and exhibits the striking tortuosity of second and third order arterioles

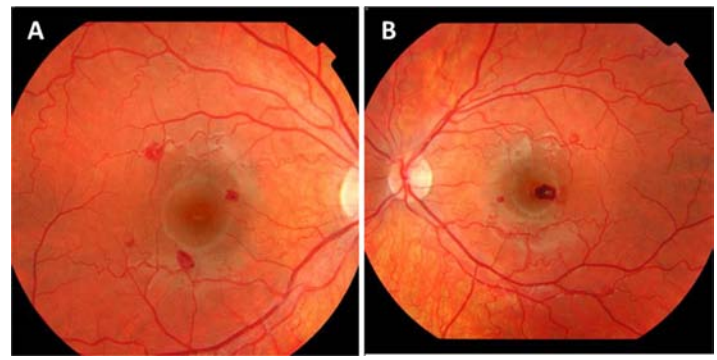

Fig. 3 Fundus image of patient #2 showing marked tortuosity of arterioles and intraretinal hemorrhages at perimacular area in right (**a**) and left (**b**) eye

variants were shown to be deleterious by both Polyphen and SIFT algorithms. Among those, a c.1528G>A (p.Gly510Arg) mutation in COL4A1 was selected to be a strong candidate for the disorder in this family, as this gene has been previously implicated in a broad spectrum of vascular anomalies. Sanger sequencing of *COL4A1* exon 24 demonstrated that the p.Gly510Arg missense mutation co-segregates with disease status in the family (Fig. 5) and was consistently predicted to be damaging by multiple in silico analyses (Polyphen, SIFT, and PANTHER algorithms). In addition, the p.Gly510Arg mutation was absent from DNA of a healthy daughter of case #1, from a set of 200 ethnically matched control alleles, and from the 8,600 exomes in the NHLBI Exome Variant Server. The remaining 10 potentially deleterious missense mutations in other genes (Supplementary table) did not segregate with the phenotype in the family when assessed by Sanger sequencing or were predicted to be benign by Polyphen, SIFT, and PANTHER algorithms.

Discussion

Familial retinal arteriolar tortuosity (FRAT) is an uncommon, autosomal dominant condition characterized by a pathognomonic pattern of progressive and pronounced tortuosity of second-order and third-order arterioles in the macular and peripapillary retinal area. Based on the report of a number of families without detectable extraocular anomalies, FRAT is regarded to be a distinct monogenic disease (OMIM %180000) of unknown etiology. In this work, the exome sequencing-mediated identification of a missense *COL4A1* mutation in affected FRAT subjects from a Spanish FRAT family is reported.

COL4A1 is the most abundant and ubiquitous basement membrane protein. In the eye, COL4A1 is present in the basal lamina of the conjunctiva, corneal epithelium, corneal endothelium, trabecular meshwork, Schlemm's canal, lens, ciliary body, retinal inner limiting membrane, Bruch's membrane and vascular basement membranes [11–14]. The phenotypic spectrum of COL4A1 mutations is wide and includes familial porencephaly [15, 16], cerebral white matter small vessel disease [17], cerebral aneurysms [18], cataract, anterior segment dysgenesis, microcornea [19], nephropathy, muscle cramps [20, 21], and Walker Warburg syndrome [22].

A multisystemic disease featuring retinal arteriolar tortuosity is HANAC (Hereditary Angiopathy with Nephropathy, Aneurysm and Cramps), in which affected patients exhibit retinal arteriolar tortuosity, muscle cramps, and renal disease, whereas the brain phenotype was usually clinically silent [20, 21, 23]. Mutations identified thus far in HANAC patients cluster within a 31 amino acid region (residues 498–528) of

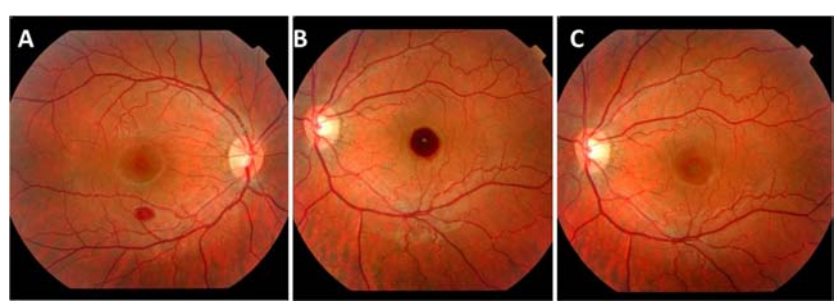

Fig. 4 Fundus photographs of patient #3 demonstrating increased arteriolar tortuosity and intraretinal hemorrhage in right eye (**a**) and arteriolar tortuosity and subretinal hemorrhage involving the fovea in left eye (**b**). Left eye hemorrhage showed spontaneous reabsorption after a period of 2 months (**c**)

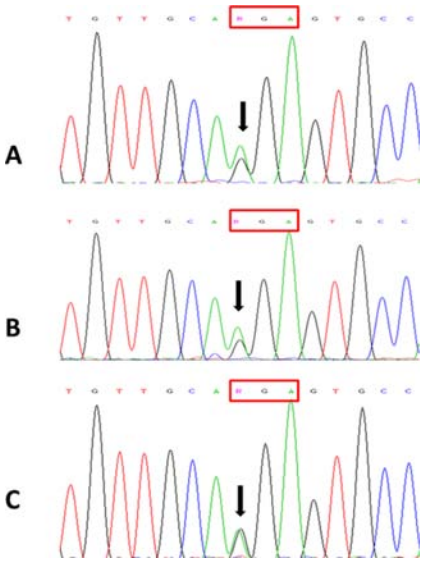

Fig. 5 Partial nucleotide sequence of *COL4A1* exon 24 in DNA from patients #1-#3 (A-C). A heterozygous c.1528G>A mutation (*arrow*), predicting a p.Gly510Arg missense substitution, was identified in affected individuals

Table 1 Comparison of clinical characteristics of the HANAC family reported by Plaisier et al. [23] and the FRAT family in the present study, both carrying an identical p.Gly510Arg mutation in COL4A1

		HANAC			FRAT		
		Plaisier et al. 2010			Present study		
		II-4	III-1	III-2	P1	P2	P3
Eye:	RAT	+	+	+	+	+	+
	Retinal hemorrhages	+	−	−	−	+	+
Kidney:	Bilateral cysts,	−	−	−	−	−	−
	Decreased GFR	+	+	−	−	−	−
Muscle:	Cramps	−	+	+	−	−	−
	elevated CPK	+	+	+	−	−	−
Brain:	Intracranial Aneurysm	−	−	?	+	−	−
	Leukoencephalopathy	−	−	?	−	−	−
	Migraine	−	−	+	−	−	−
Other	Raynaud phenomena	−	+	+	−	−	−
	Arrhythmia	−	+	−	−	−	−

CPK creatine phosphokinase, *GFR* glomerular filtration rate, *RAT* retinal arteriolar tortuosity, ? not tested

the COL4A1 protein that encompasses integrin binding sites [20, 21, 23, 24].

Interestingly, the p.Gly510Arg mutation identified in the FRAT family described in the present work is also located within this critical region, and affects a conserved glycine within the collagenous region of the protein. Pathogenic mutations within the collagenous domain often perturb triple helix assembly and impair secretion of the collagen heterotrimers, and concomitantly, misfolded proteins accumulate within cells [23, 25]. Remarkably, an identical p.Gly510Arg COL4A1 mutation was previously recognized by Plaisier et al. in three subjects from a family suffering from retinal arterial tortuosity (all three patients), Raynaud phenomena (two out of three), migraine (one out of three), and supraventricular arrhythmia (one out of three) and whom were diagnosed as having HANAC syndrome [23]. In the three affected FRAT subjects reported here, no cerebral, neurologic, renal, cardiac or vascular (except for a small carotid aneurysm in one subject) anomalies were recognized, indicating that FRAT could be considered a distinct mild form of COL4A1-related disorder with few (or no) HANAC non-ocular features. Interestingly, HANAC individuals due to the p.Gly510Arg COL4A1 mutation reported by Plaisier et al. [23] and the patients in the present family carrying the same mutation did not exhibit brain anomalies, suggesting an incipient genotype-phenotype correlation (Table 1).

Our data indicates that FRAT can be considered another member of the COL4A1-related group of diseases and that an identical mutation in COL4A1 can result in different clinical spectrum, even within the same family. As previously suggested, besides the location of pathogenic COL4A1 mutations, environmental factors and/or genetic modifiers may influence the phenotypic expression and the severity of the organ involvement in COL4A1-related disease [24]. Further research will be needed to explain why some *COL4A1* mutations originate a pleiotropic effect, while in others they are associated with single organ phenotypes as FRAT. Finally, although our results support that a mutation of *COL4A1* underlies the molecular cause of the disease in the family described here, the involvement of a different gene(s) in other FRAT cases cannot be excluded at this time. Thus, the molecular analysis of additional FRAT sporadic and familial cases would be of great relevance.

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Conflict of interest None.

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ERRATUM

Erratum to: Next generation sequencing uncovers a missense mutation in COL4A1 as the cause of familial retinal arteriolar tortuosity

Juan C. Zenteno[1] · Jaume Crespí[2] · Beatriz Buentello-Volante[1] · Jose A. Buil[2] · Francisca Bassaganyas[2] · Jose I. Vela-Segarra[2] · Jesus Diaz-Cascajosa[2] · Maria T. Marieges[2]

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The original version of this article inadvertently contained mistake. Affiliation number 2 should have been:

Department of Ophthalmology, Autonomous University of Barcelona, Hospital de Sant Pau y de la Santa Creu, Barcelona, Spain

Correct presentation given below.

The online version of the original article can be found at http://dx.doi.org/10.1007/s00417-014-2800-6.

✉ Juan C. Zenteno
jczenteno@institutodeoftalmologia.org

[1] Genetics Department and Research Unit, Institute of Ophthalmology "Conde de Valenciana" and Biochemistry Department, Faculty of Medicine, National Autonomous University of Mexico (UNAM), Chimalpopoca 14, Col. Obrera, Mexico City, CP 06800, Mexico

[2] Department of Ophthalmology, Autonomous University of Barcelona, Hospital de Sant Pau y de la Santa Creu, Barcelona, Spain

3.5 DISCUSSION AND CONCLUSIONS

Since Beyer described this disease in 1958, only 100 cases are described in the literature about fRAT, most of them hereditary. **It is therefore, a rare or minority disease in ophthalmology. However we believe it to be underdiagnosed** either because of unfamiliarity of the Ophthalmologist, either by presenting phenotypes with little symptomatology. In the published article, this disease is for the first time associated with mutations in the COL4A1. We believe that **this article, therefore, will have an important impact on the future management of this disease for several reasons:**

1) COL4A1 is widely expressed in the basement membranes throughout the whole body and is also linked to systemic syndromes, so **patients with fRAT should exclude any systemic pathology previously described, even in the absence of clinical indicators. This is contrary to what it was thought to date about this disease[46], which was considered benign, self-limiting and that it was unnecessary to carry out systemic studies [38].** An example of this is the small carotid artery aneurysm found in the father affected with fRAT in our publication. Although the association of fRAT and carotid aneurysm was already previously described [82], now we know that it is not casual because of the involvement of COL4A1 in the pathogenesis of this disease.

2) The discovery of the causal gene opens options to **perform genetic counselling, modifiable factors counselling and prenatal diagnosis in affected families.** This is especially relevant in the case of COL4A1. Studies in animal models with mutations in COL4A1 suggest that these patients **should take particular**

prevention with the trauma of childbirth (advisable cesarean section for both the carrier mother and the fetus), with traumas during adulthood and with anticoagulant therapy for the high risk of hemorrhagic CVA [56]. This is also valid from now for patients with fRAT with proven mutation in COL4A1. In addition, patients with the same mutation can present different phenotypic symptoms. For example, Takenouchi et al. [83] described the case of a patient diagnosed with HANAC, whose daughter presented PORENCEPHALY and both were heterozygous for the same mutation of COL4A1 (p.G1239R). Another example we have in our article: the Spanish family presents the typical features of fRAT while another French family published by Plaisier et al. presents HANAC and both have the same mutation (p.GLY510Arg). **Environmental factors and genetic modifiers can vary the expression of the gene and change the phenotype.**

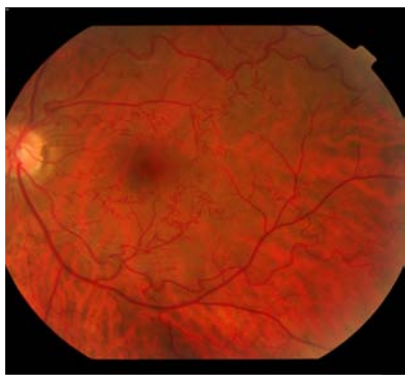
It is important then, that in the genetic counselling we explain all the clinical spectrum of diseases that can occur in case of mutations of COL4A1.

We should mention however, that a single case, does not fully demonstrate yet that fRAT is due to mutations of COL4A1. As we mentioned in the first article published in this work on RP-Nanophthalmos, confirmation is required with more cases to validate this association in a future. **It is also possible, as it happens with many other genetic diseases, that mutations in different genes can give the same symptomology of fRAT.** For example, mutations in genes that encode the laminins, another major protein of the basement membranes, can cause similar phenotypes with ocular involvement, CNS and muscular, both in humans and animal models [84].

For further progress in the study of this disease, we are making a second genetic study in two additional patients diagnosed with fRAT (sporadic cases) that will

hopefully give us more information on the role of COL4A1 and/or other genes involved in this disease (unpublished data).

On the other hand, we are also preparing another article with E. Plaisier to review the ophthalmological features of this disease. We have observed that these patients in addition to the distinctive arterial tortuosity, also presented**: 1) marked hypopigmentation of the EPR 2) cortical cataract in adulthood of rapid evolution (Fig. 24 and 25). They are traits not previously described and that could help define the phenotype of this disease.**



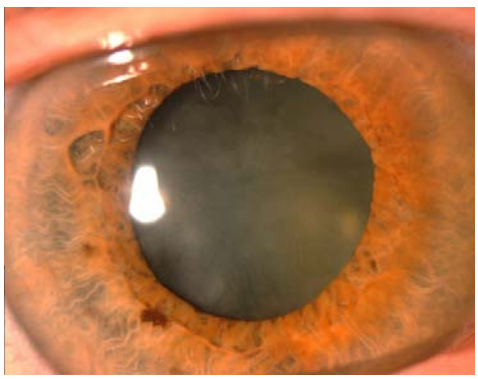


Fig. 24 and 25. Marked hypopigmentation of the EPR and cortical cataract in patients with fRAT.

In the same way as Alport's syndrome (COLA3/5) is associated with anterior lenticonus, it is plausible to think that the cortical cataract in these patients with fRAT (COL4A1) is associated with defects in the folding of type IV collagen, a basic component of the basement membrane of the lens capsule [84]. In regard to the marked hypopigmentation of the EPR the cause is totally unknown for us. In vitro tests, suggest that the hypopigmentation of the EPR is due in part to the direct regulation of genes that encode enzymes related to the synthesis of melanin (PAX6, Mitf, -Catenin)[85]. Therefore, we can speculate that COL4A1 could have a regulatory role in the expression of these genes, influencing the differentiation and pigmentation of the EPR.

In conclusion, we have demonstrated for the first time the association of fRAT with mutations of the COL4A1 gene, which implies a new diagnostic approach in this disease, allows for the possibility of performing genetic counselling and formulate proposals for new treatment and prevention. In addition, we provide ophthalmological clinical features not previously described in fRAT that allow to establish in a future a correlation genotype-phenotype and to differentiate it from other systemic diseases that also present vascular tortuosity.

GENERAL CONCLUSIONS

4. GENERAL CONCLUSIONS

Up to relatively few years ago, the scattered distribution of patients with Rare Diseases has hampered the diagnosis or discovery of new genes involved. Doctors who work with a single isolated patient or a single family may as much make a differential diagnosis and eliminate the best known causes, but they can hardly diagnose the syndrome, and even less likely, they will find the causal gene. The patients and families involved as it is logical, need to know in an appropriate period of time the disease that they suffer and the gene that causes the syndrome. A decade ago, the majority of these patients with Rare Diseases were left in a diagnostic limbo, with little hope for a solution to a rare disease. Both patients and researchers entered a diagnostic odyssey jumping from one gene to another in search of an explanation about the disease.

Two new technological advances have allowed to extremely shorten the time until reaching a diagnosis in Rare Diseases:

1. **"Next-generation DNA sequencing" (NGS)**
2. **The mobilization of patients and affected families in the "social networks (*social media*)".**

Next generation sequencing (NGS), also known as high-throughput sequencing, is the catch-all term used to describe a number of different modern sequencing technologies. These recent technologies allow us to sequence DNA and RNA

much more quickly and cheaply than the previously used Sanger sequencing, and as such have revolutionised the study of Genomics and Molecular Biology.
To illustrate the impact of these changes (technical modernization of Molecular Biology and social networks) serve two examples:

1-) the first one, previously commented, is that of Dr Nancy Wexler, whose team discovered the gene for the disease of Huntington (HD). Nancy Wexler saw her mother and aunt die because of HD. The probability of inheriting the disease was 50 %. She got married and decided not to have children. She spent the following 30 years of her life to search for the gene for the disease that affected her family, having performed more than 4000 DNA analysis and pedigrees with more than 18,000 members. To this end, she founded together with her father organizations of affected patients who carried out events for the spread of the disease, they raised money for its investigation and would contact with first level scientists interested in the elucidation of the disease. Finally, she and her team found the gene. In addition, they created a Prenatal Diagnostic Test for the prevention of the disease. This work is admirable, but the effort and determination to carry out a similar task with all RD is not within reach of everyone. The burnout, in addition, in the person involved is huge. In their own words: "*it has been to live each day playing to the Russian roulette: I did not know if the gun was loaded with the HD gene or not* ".

2-) the second example is recent and it happened in 2010. Matthew Might and Matt Wisley are the parents of a child with a RD not previously described and practically nothing was known about it. Researchers from the University of Duke carried out a first diagnostic approximation by NGS and concluded in a few months that the gene NGLY1 could be a candidate for this disease. Subsequently, they demonstrated in laboratory models that mutations in NGLY1 affect the protein that encodes this gene and alter the path of the deglycosylation 86. Anyway, it was

difficult to prove that this was the causal gene because they had a single affected patient. Finally, they decided to inform the family about their findings, although these were simply a hypothesis. Curiously, one of the parents wrote on the internet (in his blog) the whole story of his son with the intention to give visibility to this RD. This included the Duke's researchers' communication on the heterozygous mutation in NGLY1. It was this blog, discovered by a doctor who treated a patient with a similar clinical, which made it possible to diagnose a second case of this disease 87. Another patient more (in another continent) was discovered when the father of the affected boy sought on the internet the symptoms of his son and found again the blog previously cited. The same parents in this last case were the ones that suggested their doctor sequencing the NGLY1 gene. In this way, a working team was formed consisting of the parents of children affected, doctors and researchers who joined via the internet. 19 months after the first publication, there are already 5 viable proposals for treatment for this disease. The parents have organized themselves to give visibility to the disease in the *social media* and to share all the relevant information on this disease87.

Thus, in less than one decade, the popularization of the social networks and the modernization of genetic techniques (NGS) has permitted a dramatic change in the times of diagnosis and in the coordination of relatives, doctors and researchers implied.

In this research, we have described the genetic cause of two rare or minority diseases in ophthalmology. The impact of these diseases in the patients is significant because in the RP-Nanophthalmos almost all the patients come to legal blindness before the age of 40. In the case of the family with fRAT, the presence of aneurysms of CNS can have important consequences in the morbi-mortality. In both cases therefore, knowing the genetic cause opens the door to develop new therapies.

During the years that the investigation has lasted, it has been crucial to be able to count in an unselfish way with the contribution of leading scientists in different countries that the author of this thesis contacted by internet (especially Juan Carlos Zenteno, of Mexico DF that has carried out all the genetic studies and Beatriz Buentello-Volante, Texas US, that carried out without any price NGS for the father with fRAT). The scientific interest, above any other, moves the research in these minority diseases. Contrary to what happens with the pharmaceutical industry, the hours of research, the genetic findings and the elaboration of possible treatments, often have little or no economic reward for the researchers of RD.

In conclusion, we have described the involvement of MFRP and COL4A1 in two Spanish families diagnosed with rare diseases in ophthalmology. The publication of the results in scientific journals and databases such as OMIM, will help to disseminate these findings favouring the investigation of new therapies against these diseases.

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New genes involved in rare diseases in ophthalmology

Dr. Jaume Crespí received his medical degree from University of Barcelona, Hospital Clínic (1997-2003). He performed his Ophthalmology residency at the Sant Pau´s Hospital, UAB (2004-2008). He received a resident grant from the European Board of Ophthalmology and did a fellowship in Ocular Oncology at Helsinki´s Eye Hospital. He is currently a Senior Retinal Specialist at Sant Pau´s Hospital and at Institut Comtal d´Oftalmologia where he practices surgical and medical retina.

Dr. Crespí is focusing his research interest on macular diseases, anti-VEGF therapy and rare/genetic diseases in Ophthalmology. He is the lead investigator of several clinical studies involving age-related macular degeneration, ocular genetics and diabetic retinopathy. Besides this research interest, he is also active in the education of ophthalmology residents, speaks at national conferences and publishes papers in ophthalmology journals.