

Clinical and genetic investigations of three Moroccan families with retinitis pigmentosa phenotypes

Aymane Bouzidi,^{1,2,3} Majida Charif,^{1,4} Adil Bouzidi,⁵ Ghita Amalou,^{1,2,3} Mostafa Kandil,³ Abdelhamid Barakat,² Guy Lenaers¹

¹MitoLab team, Institut MitoVasc, UMR CNRS 6015, INSERM U1083, SFR ICAT, Angers University, Angers, France;

²Genomics and Human Genetics Laboratory, Institut Pasteur du Maroc, Casablanca, Morocco; ³Team of Anthropogenetics and Biotechnologies, Faculty of Sciences, Chouaib Doukkali University, Eljadida, Morocco; ⁴Genetics, and immuno-cell therapy Team, Mohamed First University, Oujda, Morocco; ⁵Ophthalmology department, Moulay Ismail Military Hospital, Meknes, Morocco

Purpose: Progressive inherited retinal dystrophies, characterized by degeneration of rod photoreceptors and then cone photoreceptors, are known as retinitis pigmentosa (RP), for which 89 genes have been identified. Today, only five Moroccan families with RP with a genetic diagnosis have been reported, justifying our investment in providing further clinical and genetic investigations of families with RP in Morocco.

Methods: The clinical diagnosis based on a combination of a history of night blindness, abnormal rod or rod-cone responses in electroretinography (ERG), and constricted visual field or difficulty perceiving side objects identified three Moroccan families with an RP phenotype. Proband of these families underwent whole exome sequencing (WES), and candidate variants were evaluated for their segregation within family members.

Results: All patients had a history of night blindness and unrecordable rod and cone ERG traces. In addition, one patient had cystoid macular edema, and another had discrete autofluorescence abnormalities, in addition to ellipsoid zone disorganization and narrowed retinal vessels. WES sequencing revealed heterozygous compound mutations in *CRB1*:c.1690G>T/c.1913C>T and in *ABCA4*:c.5908C>T/c.6148G>C and a homozygous *PDE6B* splice mutation c.1920+2T>C.

Conclusions: We provide the first description of Moroccan patients with the RP phenotype harboring pathogenic mutations in the *CRB1* and *ABCA4* genes and the second description of an individual with RP with a *PDE6B* mutation, associated with cystoid macular edema. These data contribute to expand the genetic diagnosis of RP phenotypes in Morocco.

Retinitis pigmentosa (RP) is the most frequent inherited retinal dystrophy (IRD), characterized by degeneration of rod photoreceptors and then cone photoreceptors [1], with an estimated prevalence of 1:4,000 [2]. Night blindness is the earliest symptom, followed by progressive alteration of the peripheral vision, ultimately leading to tunnel vision [3]. Additionally, attenuation of retinal vessels, bone spicule-like pigmentation, and pallor of the optic nerve head are typical hallmarks observed with fundus imaging of the retina [3]. To further confirm photoreceptors' dysfunctions in the progression of the disease, electroretinography (ERG) evaluations help discriminate primary rod photoreceptor degeneration from the following degeneration of cones [1].

Mutations in genes with retina-specific expression are responsible for typical non-syndromic RP, whereas mutations in genes acting in multiple cell types or tissues result more

frequently in systemic manifestations, such as syndromic RP [4,5], which account for more than 30 different clinical presentations [2]. The most frequent syndromic forms are the Usher syndromes, which affect the vision and hearing capacities [6], and the Bardet-Biedl syndromes, which, in addition to the retinal alterations, lead to obesity, post-axial polydactyly, hypogonadism, and renal dysfunction, related to cilia motility dysfunction [7].

Today, 89 genes segregating with all modes of inheritance have been identified as causing non-syndromic RP (from [RetNet](#), accessed on November 2020). Thirty genes can be inherited with autosomal dominant mode, 63 genes with autosomal recessive mode, and three genes with X-linked mode, while mitochondrial inheritance can also occur in the Kearns-Sayre syndrome.

Studies on IRD cohorts originating from North African countries have been mainly performed in Tunisia. Two recent studies provided an overview of the spectrum of genes and mutations in Tunisian families with inherited retinal dystrophies (IRDs), disclosing high genetic heterogeneity. Among 41 families, 18 presented an RP phenotype with variants in 11

Correspondence to: Guy Lenaers, MitoLab Team, Mitochondrial Medicine Research Centre, Institut MitoVasc, Université d'Angers. UMR CNRS 6015, INSERM U1083, CHU Bât IRIS/IBS, Rue des capucins, 49933 Angers cedex 9, France; Phone: 33/0 244 68 84 87; FAX: 33/0 244 68 65 94; email: guy.lenaers@inserm.fr

different genes [8,9]. Conversely, two studies of consanguineous Moroccan families with IRDs reported homozygous mutations in *MERTK* (OMIM: 604705; Gene ID: 10461), associated with a severe form of RP with night blindness starting in early infancy, reduced visual acuity, and macular involvement [10,11]. Two additional families of Moroccan origin with RP were diagnosed among a cohort of 26 families, with homozygous mutations in the *RDH12* (OMIM: 608830; Gene ID: 145226) and *PDE6B* (OMIM: 180072; Gene ID: 5158) genes [12], and another family with RP was identified with a homozygous *ARL2BP* (OMIM: 615407; Gene ID: 23568) splice-site mutation [13].

To expand the genetic diagnosis of RP phenotypes in Morocco, we recruited families with IRDs, selected those with the RP phenotype, and used whole exome sequencing (WES) on probands to infer a molecular diagnosis. This led to the discovery of homozygous and heterozygous composite variants in the *PDE6B*, *CRB1* (OMIM: 604210; Gene ID: 23418), and *ABCA4* (OMIM: 601691; Gene ID: 24) genes.

METHODS

Patients: This study investigated three unrelated Moroccan families, of whom two were consanguineous. After written informed consent was obtained from patients or their parents or both, each patient provided information about geographic

origin, consanguinity, and family and personal medical history. The commitment to ethical principles was approved by the local research ethics committee of the Pasteur Institute in Morocco. This work was performed in accordance with the guidelines of the Declaration of Helsinki and the ARVO statement on human subjects.

The diagnosis of RP was based on the presence of a history of night blindness, eye fundus, autofluorescence imaging, abnormalities in rod or rod-cone-specific ERG responses, peripheral visual field defects or difficulty perceiving side objects (when visual field assessment was not available), and macular optical coherence tomography (OCT) findings (Table 1). Notably, no additional extraocular symptom was encountered in the three probands, indicating the isolated retinal symptoms of the diseases.

Genetic analysis: Genomic DNA was extracted from peripheral blood of probands and their available family members, using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany; Cat No./ID: 51106). DNA from the three probands underwent WES to screen and identify the disease-causing variants.

WES was performed at BGI Tech Solutions (Hong Kong, China), on a BGISEQ-500 sequencer. DNA was randomly fragmented, end-repaired, ligated with adapters and amplified with PCR for several cycles to infer the DNA libraries.

TABLE 1. CLINICAL DATA OF THE THREE MOROCCAN RP PATIENTS.

Patient	RP1.03	RP2.03	RP3.03
Age		17	13
Age at diagnosis		12	9
First symptoms	Night-blindness, difficulty to perceive side objects	Night-blindness, difficulty to perceive side objects	Night-blindness
Consanguinity	No	First cousins	Second cousins
Visual field	Not done	Not done	Severe impairment of the entire visual field with the persistence of tubular vision in both eyes
ERG	Flat traces	Flat traces	Flat traces
Fundus imaging	Not done	Not done	Grayish white aspect in the retinal periphery, slightly pale papilla on the temporal area and decreased retinal vessel size
Auto-fluorescence imaging	Not done	Large hypo-fluorescence region in the central area and hyper-autofluorescence in the inferior area of the optic nerve head	Discrete hyper-autofluorescence ring surrounding the macular area, large hypo-autofluorescence area in the peripheral retina and narrowed retinal vessels.
Macular OCT	Not done	Cystoid macular edema, central thickness of 499µm in right eye and 512µm in left eye	Relative preservation of the foveal structure, disorganization of the ellipsoid zone in the perifovea in both eyes, and epiretinal membrane formation

ERG: Electroretinogram; OCT: Optical coherence tomography.

TABLE 2. GENETIC ANALYSIS AND VARIANT CHARACTERIZATION IN RP PATIENTS.

Patient	Gene	Variant	aa change	gnomAD frequency	Polyphen-2	SIFT	Mutation Taster
RP1.03	CRBI	NM_001257966: c.1690G>T	p.Asp564Tyr	.	damaging	damaging	disease causing
		NM_001257966: c.1913C>T	p.Ser638Leu	1.592e-05	damaging	damaging	disease causing
RP2.03	PDE6B	NM_000283: c.1920+2T>C	p.?	7.974e-06	.	.	disease causing
RP3.03	ABCA4	NM_000350: c.5908C>T	p.Leu1970Phe	0.003	possibly damaging	tolerated	disease causing
		NM_000350: c.6148G>C	p.Val2050Leu	0.00286	possibly damaging	tolerated	disease causing

Then, amplified DNA was captured using the MGIEasy Exome Capture V4 Probe Set (MGI Tech, Hong Kong, China). Captured products were circularized, and rolling circle amplification was performed to produce DNA nanoballs. Each resulting qualified captured library was then loaded on DNBseq sequencing platforms. Reads were aligned to the reference human genome sequence hg19 (GRCh37) using Burrows-Wheeler Aligner, and HaplotypeCaller of GATK (v3.6) was used for variant calling. The mean sequencing depth on target regions was 74-fold, which provided sufficient depth to analyze variants after data quality control. To select the pathogenic candidate variants, we focused on genes known to cause RP ([RetNet](#)). Homozygous and heterozygous compound missense, nonsense, frameshift, and splice-site variants with allele frequencies <0.01 using SNP databases such as the [1000 Genomes](#) Project and [gnomAD](#) exomes were then examined, as the pedigree of the three families suggested an autosomal recessive inheritance pathway.

Selected candidate variants (Table 2), were amplified with PCR using primers designed via [Primer3](#) (Table 3).

Genomic DNA was PCR-amplified with the Hot Goldstar PCR Mix (Eurogentec, Seraing, Belgium), with 94 °C for 5 min, 94 °C for 30 s and Tm °C for 15 s and 72 °C for 30 s (35 cycles), where Tm correspond to annealing temperature for each variant (Table 3), and 72 °C for 7 min. Direct sequencing was performed with the Big-Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Thermo Fisher Scientific, Vilnius, Lithuania) and analyzed on the ABI 3130 Genetic Analyzer (Applied Biosystems). SeqScape v.2.5 software was used to analyze the chromatogram sequences.

Bioinformatics tools: Pathogenicity of missense variants was evaluated using [PolyPhen-2](#), [MutationTaster](#), and [SIFT](#). [Human Splicing Finder](#) v.3.1 was used for splicing mutation analysis.

RESULTS

Clinical description and molecular analysis:

Case 1: RP1.03—The proband is the second child of a non-consanguineous family with two siblings (Figure 1). She

TABLE 3. PRIMER SEQUENCES AND ANNEALING TEMPERATURE FOR SANGER SEQUENCING.

Targeted gene (Family)	Exon	Base sequence	Annealing temperature °C
CRBI (RP1)	6a	F: 5' CAGGTCAATAATCAGTCAAAGG 3'	58
		R: 5' CAAACGAAGGTGTGGATGGC 3'	
PDE6B (RP2)	6b	F: 5' ACCAGTGGGAATGACCAGC 3'	60
		R: 5' CTGTGGCAGTCACACTGG 3'	
ABCA4 (RP3)	15	F: 5' AGAGGCAGACCCTGTCTCAA 3'	58
		R: 5' CAGTGCTCACACCCCAGTG 3'	
ABCA4 (RP3)	43	F: 5' GCTCACACACACTTACCC 3'	58
		R: 5' GACCTGTGAGAGACTCCCTG 3'	
ABCA4 (RP3)	45	F: 5' TTCTGGAGGCGTGAGATCTG 3'	62
		R: 5' TTGGTTTAAGCCCTTGGTGC 3'	

complained of night blindness and difficulty perceiving side objects at 10 years of age. At 12 years, ERG responses of rod and cone photoreceptors were not detectable, with flat traces and no distinguishable a and b waves (Figure 2). The patient had no nystagmus. WES revealed compound heterozygous mutations in *CRB1* exon 6 (OMIM 604210), with one mutation c.1690G>T originating from the father, and the second, c.1913C>T, originating from the mother. The healthy brother of the proband carried only the c.1690G>T mutation (Figure 1). The c.1690G>T mutation causes a missense change of aspartic acid at position 564 to tyrosine, p.Asp564Tyr. The c.1913C>T mutation causes a missense change of serine at position 638 to leucine, p.Ser638Leu, referenced with an allele frequency of 1,592e-05. Both mutations are predicted to have a damaging effect by SIFT and PolyPhen-2, and to be disease causing by MutationTaster (Table 2).

Case 2: RP2.03: The proband was born in a consanguineous family (first cousins) with three siblings (Figure 1). He was diagnosed at 9 years old with RP and complained of night blindness and difficulty perceiving side objects. At the same age, ERG responses showed a major alteration of rod and cone photoreceptors in both eyes; no waves could be recorded. At 11 years, autofluorescence retinal imaging showed a large hypoautofluorescence region in the central area and hyperautofluorescence in the inferior area of the optic nerve head (Figure 3A). Macular OCT revealed intraretinal schisis corresponding to cystoid macular edema (CME) in both eyes and a loss of the ellipsoid zone outside the fovea, reflecting a loss of photoreceptors (Figure 3B). Central macular thickness

was 499 μ m in the right eye and 522 μ m in the left eye. WES results disclosed the homozygous mutation c.1920+2T>C in exon 15 of *PDE6B* (OMIM 180072), a gene known to cause RP40 (OMIM 613801). This mutation was heterozygous in both parents (Figure 1). Its frequency is 7.97e-06 in gnomAD exomes, and it is predicted to be disease causing by MutationTaster. According to Human Splicing Finder v.3.1 software, this mutation causes an alteration of the donor site affecting splicing.

Case 3: RP3.03—The proband was born in a consanguineous family (second cousins) that included five siblings (Figure 1). At 18 years old, he had difficulty with dark adaptation. The visual field revealed a severe impairment of the entire visual field with the persistence of tubular vision in both eyes at 21 years of age (Figure 4B). Scotopic and photopic ERG traces were consistently altered (Figure 4A), indicating rod and cone photoreceptor dysfunctions. Funduscopy examination showed a grayish-white aspect in the retinal periphery, a slightly pale papilla on the temporal side, and decreasing diameter of retinal vessels as we moved away from the papilla (Figure 5A). Autofluorescence funduscopy disclosed a discrete hyper-fluorescence ring surrounding the macula and a large hypoautofluorescence area in the peripheral retina (Figure 5B). Macular OCT showed relative preservation of the foveal structure and the absence of cystic spaces but disorganization of the ellipsoid zone in the perifoveal region of both eyes (Figure 5C). In addition, epiretinal membrane formation was revealed by the macular OCT and seen on the funduscopy image as forming radial

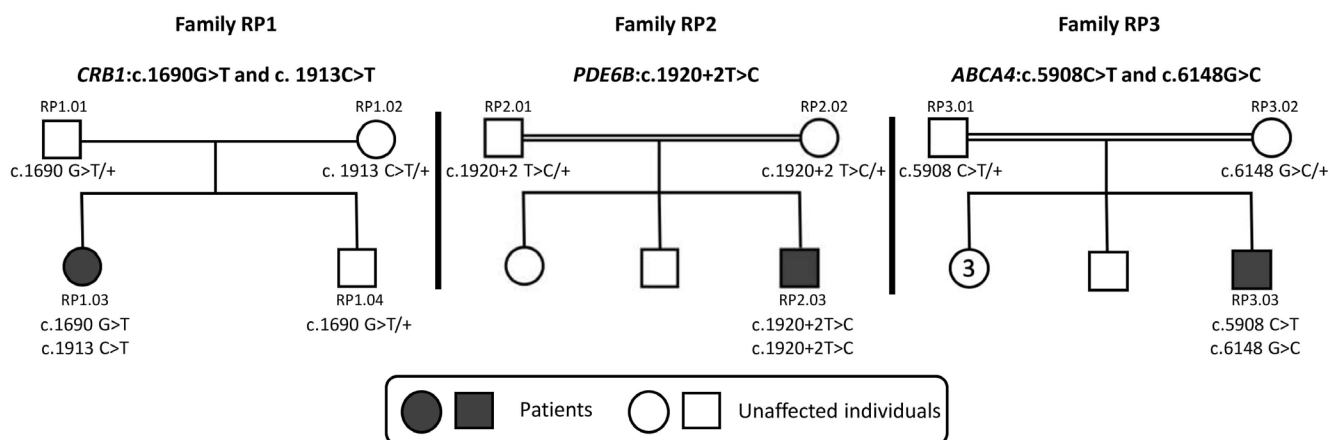


Figure 1. Pedigree of the three unrelated Moroccan families with retinitis pigmentosa with mutation segregation. In family RP1, the proband (RP1.03) carried the compound c.1690G>T and c.1913C>T heterozygous mutations in *CRB1*, transmitted by the father and the mother, respectively. In family RP2, the splice site *PDE6B*: c.1920+2T>C mutation was found homozygous in the proband (RP2.03) and heterozygous in the parents. In family RP3, the proband (RP3.03) carried the heterozygous compound mutations in *ABCA4*, the c.5908C>T and c.6148G>C transmitted by the father and the mother, respectively.

fold. The proband, although born in a consanguineous family, had compound heterozygous mutations in *ABCA4* (OMIM 601691). The first mutation, c.5908C>T, is localized in exon 43 and leads to the missense change p.Leu1970Phe. The second mutation, c.6148G>C, occurs in exon 45 and leads to the missense change p.Val2050Leu. Both mutations are referenced in the gnomAD database with a frequency of 0.00300 and 0.00286, respectively, and are predicted to be possibly damaging and disease causing by PolyPhen-2 and MutationTaster, respectively.

DISCUSSION

In this study, we report three novel Moroccan patients from unrelated families with clinical features suggestive of RP, for which WES revealed pathogenic variants in three different RP genes. The heterozygous *CRB1* mutation c.1690G>T (p.Asp564Tyr) was described in Spain [14-16], in particular in a large consanguineous family with two different subtypes: one related to the RP phenotype with night blindness and diminished visual acuity in early childhood as first symptoms, in addition to non-recordable ERG traces, and one related to the Leber congenital amaurosis (LCA) phenotype, with diminished visual acuity noticed at 1.5 years old,

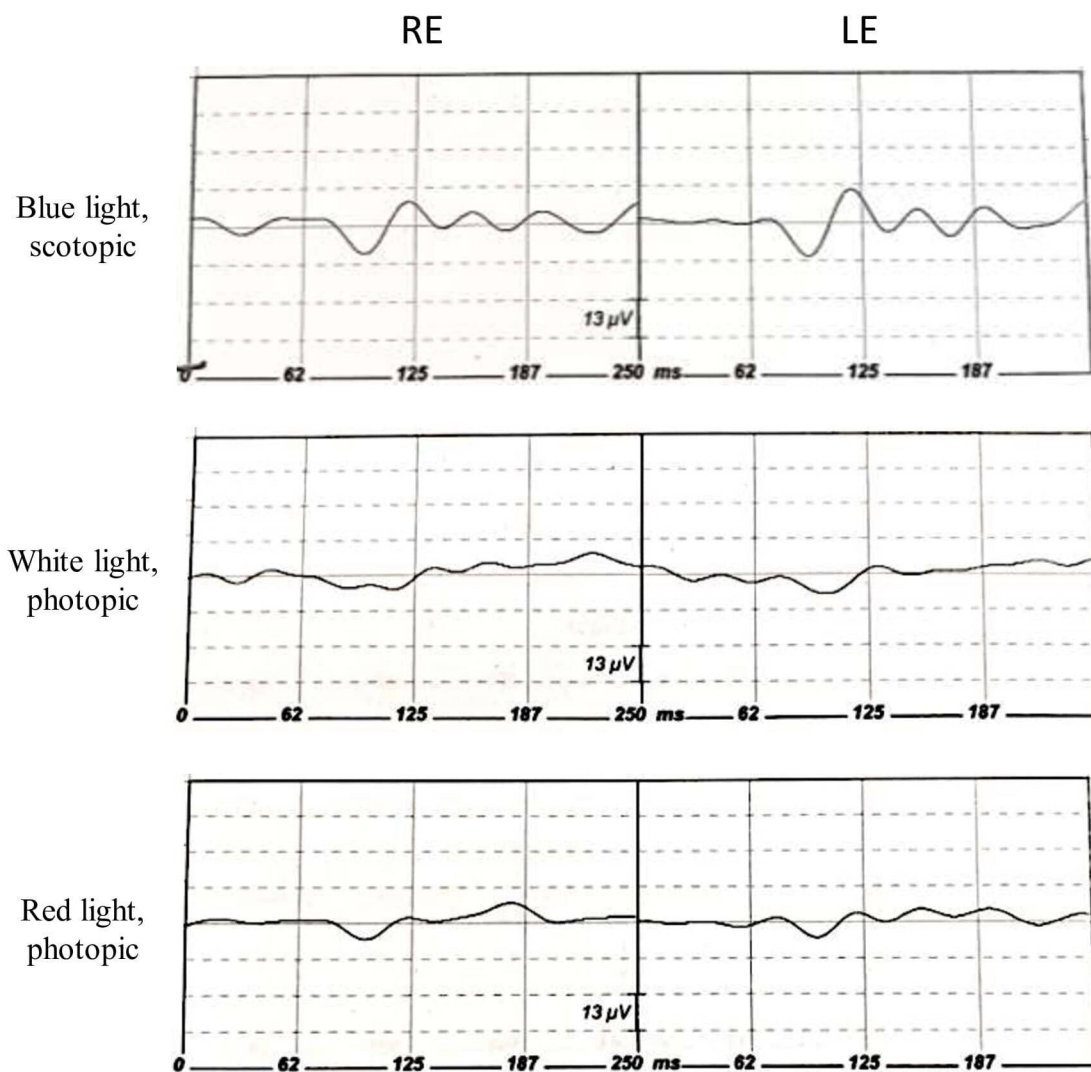


Figure 2. Electroretinogram traces from patient RP1:03. Electroretinography traces of patient RP1:03 performed in scotopic mode with blue light stimulation and in photopic mode with white and red lights do not disclose discernable a and b waves. The 30 Hz flickering light examination was not recorded. RE: right eye; LE: left eye.

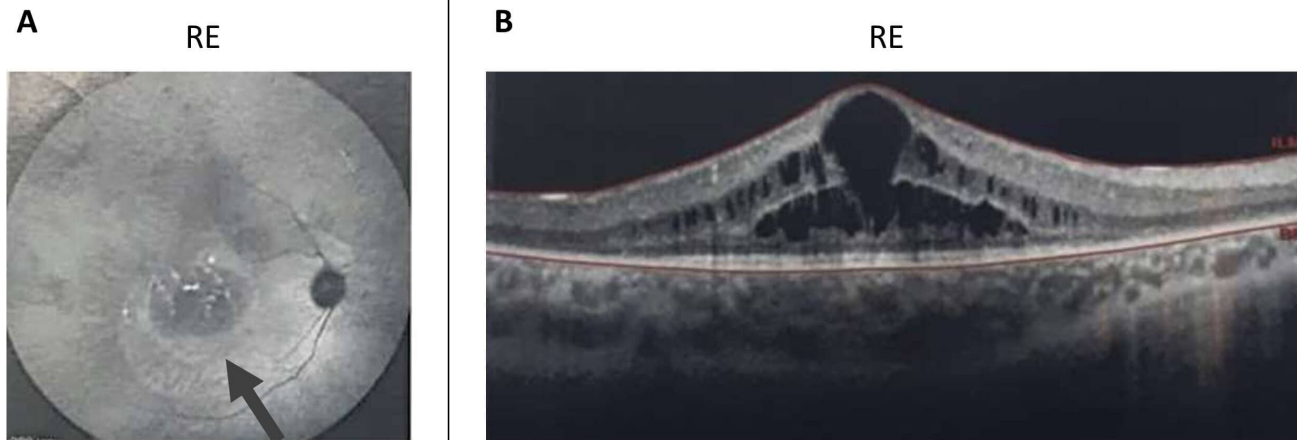


Figure 3. Retinal autofluorescence and OCT imaging in patient RP2:03. **A:** Retinal autofluorescence examination of the right eye (RE) of patient RP2:03, presenting a large hypoautofluorescent central macular area, marked by an arrow, and some hyper-autofluorescence in the inferior area of the optic nerve head. **B:** Macular optical coherence tomography (OCT) of the RE discloses cystoid macular edema and the loss of the ellipsoid zone outside the fovea, reflecting rod photoreceptor loss.

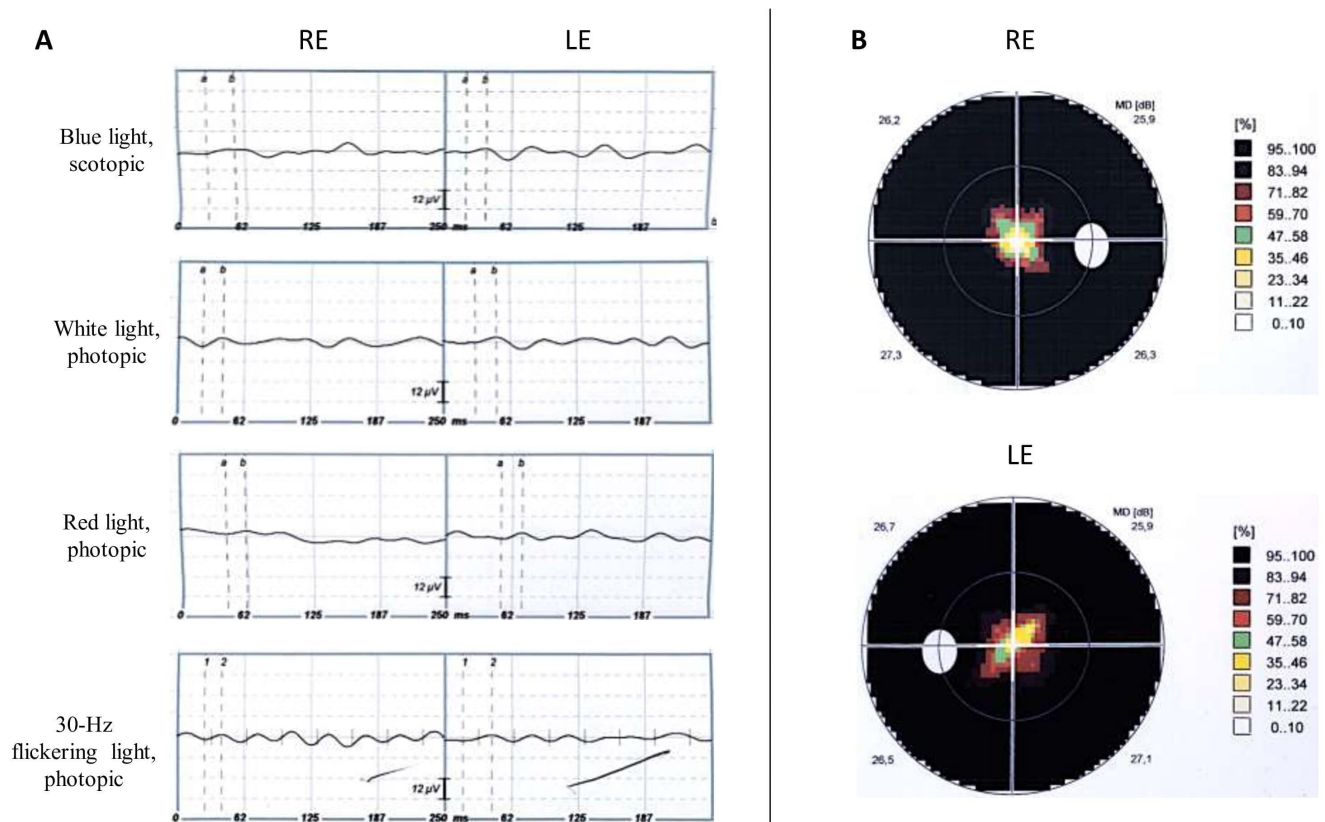


Figure 4. Electrophoretogram traces and visual field of patient RP3:03. **A:** Electrophoretogram traces in the scotopic mode with blue light stimulation and the photopic mode with white and red lights, and 30 Hz flickering light are unrecordable, showing the loss of rod and cone photoreceptors. **B:** The visual field of patient RP3:03 shows a severe impairment of the entire visual field with the preservation of tubular vision in both eyes. RE: right eye; LE: left eye.

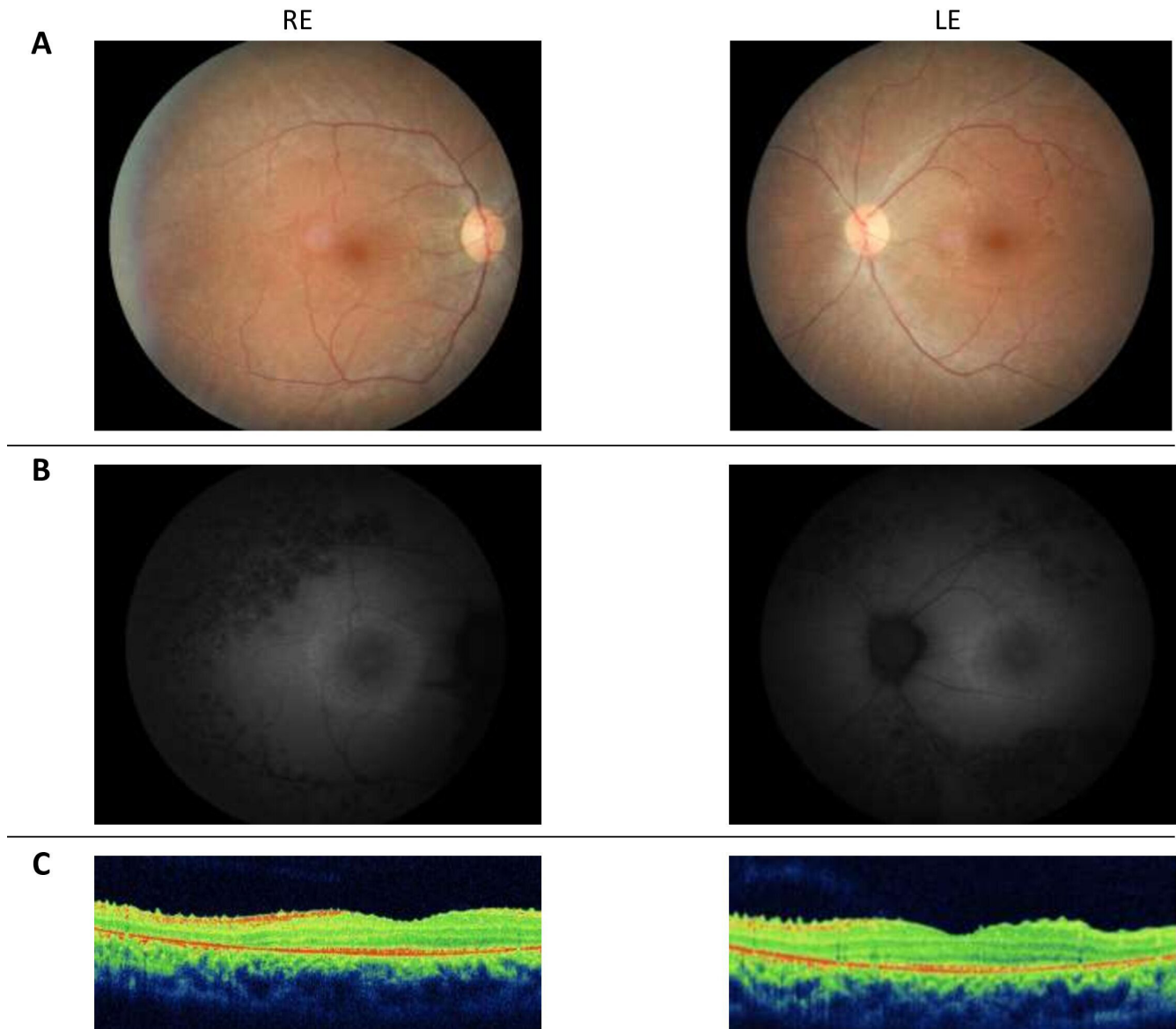


Figure 5. Retinal imaging of patient RP3:03. **A:** Fundus imaging of patient RP3:03 shows a grayish-white region in the retinal periphery, a slightly pale papilla on the temporal side, and progressively decreasing vessel sizes, as we move away from the papilla. **B:** Autofluorescence funduscopy discloses a discrete hyper-fluorescent ring surrounding the macular area and a large hypofluorescent area in the peripheral retina. **C:** Macular optical coherence tomography (OCT) recording shows relative preservation of the foveal structure and the absence of cystic space, but disorganization of the ellipsoid zone in the perifoveolar regions in both eyes, with abnormal epiretinal membrane formation. RE: right eye; LE: left eye.

nystagmus, and non-recordable ERG traces [16]. Individuals with RP were associated with Asp564Tyr/Ile1001Asn and Ile1001Asn/Tyr1161Cys mutations respectively, and individuals with LCA were associated with the Cys896*/Ile1001Asn variants. Recessive mutations in *CRBI* have been described in early-onset RP12 (*RP12*, OMIM 600105) and Leber congenital amaurosis 8 (*LCA8*, OMIM 613835). Because this

patient complained only of visual impairment at 10 years of age, without showing nystagmus, her presentation is likely similar to the early-onset RP12 form. Thus, we report the first occurrence of the *CRBI* p.Asp564Tyr mutation outside Spain, most likely witnessing migration between the two countries, leading to the first description of a Moroccan family with RP with mutations in *CRBI*.

The *PDE6B* c.1920+2T>C mutation that we disclosed in the second patient was reported once in a family with recessive RP with bull's eye maculopathy and significant abnormalities of the retinal epithelium occurring at 20 years old [17]. Interestingly, this latter patient had another mutation in *PRPH2* (OMIM:179605, Gene ID:5961), which was proposed to contribute to the onset of the macular degeneration. Interestingly, the patient also presented with CME, but without an additional mutation that could explain this observation. Macular abnormalities are frequent in patients with RP, and might affect up to 43.8% of eyes with RP, as found in the Italian population, while CME represents another common finding, representing up to 47% of macular abnormalities [18]. CME can occur at any stage of the RP disease [19], but is less likely in the late stages of the degenerative process [19,20]. Thus, the case with the *PDE6B* c.1920+2T>C mutation is the first reported in North Africa, combining RP and CME abnormalities, due to the homozygous mutation in *PDE6B*.

The third patient with RP had two heterozygous *ABCA4* compound mutations c.5908C>T (p.Leu1970Phe) and c.6148G>C (p.Val2050Leu), with 0.003 and 0.00297 allele frequency in gnomAD exomes, or 0.00296 and 0.00198 in gnomAD genomes, respectively. Recessive mutations in *ABCA4* cause various retinopathies, including Stargardt (STGD), cone-rod dystrophies (CRDs), and RP19. Although STGD and CRD are characterized by bilateral progressive defects of the central visual field and of color vision [21,22], the central vision was spared in this patient, a typical finding of RP19 (OMIM 601718). Interestingly, the two mutations in *ABCA4* that we identified were described in STGD [22], CRD [23], and RP [24], and can be found at a homozygous or heterozygous compound status, but they were never reported together in a single individual. In a Danish study that included 677 individuals diagnosed with IRDs [24], two patients with an RP phenotype carried at a heterozygous state the p.Leu1970Phe mutation, which was assigned as a pathogenic variant, according to American College of Medical Genetics and Genomics (ACMG) guidelines. In another study that included 335 patients with STGD, both mutations were considered likely pathogenic, using the ACMG and the Association for Molecular Pathology and Clinical Practice standards and guidelines [22]. Thus, this report combining the two mutations in *ABCA4* in a Moroccan family with RP19 is unique, and suggests that each variant by itself is not pathogenic, as the parents of the proband were unaffected.

Conclusion: This study reports the first implication of mutations in *CRBI* and *ABCA4* in Moroccan individuals with RP and the identification of a second family with mutations in

PDE6B in a patient with an RP combined with CME. All patients had rod and cone abnormalities, suggesting that cone degeneration occurred after that of rods, thus leading to classification of these diseases as true RPs. This data contribute to expanding the genetic diagnosis of RP phenotypes in Moroccan families.

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