

Leila El Matri  
Aude Ambresin  
Daniel F. Schorderet  
Aki Kawasaki  
Mathias W. Seeliger  
Andreas Wenzel  
Yvan Arsenijevic  
François-Xavier Borruat  
Francis L. Munier

## Phenotype of three consanguineous Tunisian families with early-onset retinal degeneration caused by an R91W homozygous mutation in the RPE65 gene

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M. W. Seeliger  
Retinal Electrodiagnostics Research  
Group, Department of Ophthalmology,  
University of Tübingen,  
Tübingen, Germany

A. Wenzel  
Ophthalmology, Retinal Cell Biology,  
University Hospital of Zurich,  
Zurich, Switzerland

L. El Matri and A. Ambresin contributed  
equally to this work.

L. El Matri  
Hedi Rais Institute of Ophthalmology,  
Tunis, Tunisia

A. Ambresin (✉) · D. F. Schorderet ·  
A. Kawasaki · Y. Arsenijevic ·  
F. Borruat · F. L. Munier (✉)  
Department of Ophthalmology,  
Jules Gonin Eye Hospital,  
University of Lausanne,  
1004 Lausanne, Switzerland  
e-mail: aude.ambresin@ophtal.vd.ch  
Tel.: +49-41-216268589  
Fax: +49-41-216268544  
e-mail: francis.munier@ophtal.vd.ch

D. F. Schorderet  
Institut de Recherche  
en Ophthalmologie,  
Sion, Switzerland

**Abstract Purpose:** To identify the genetic defect and to phenotype three consanguineous Tunisian families presenting with early-onset retinal degeneration (EORD).

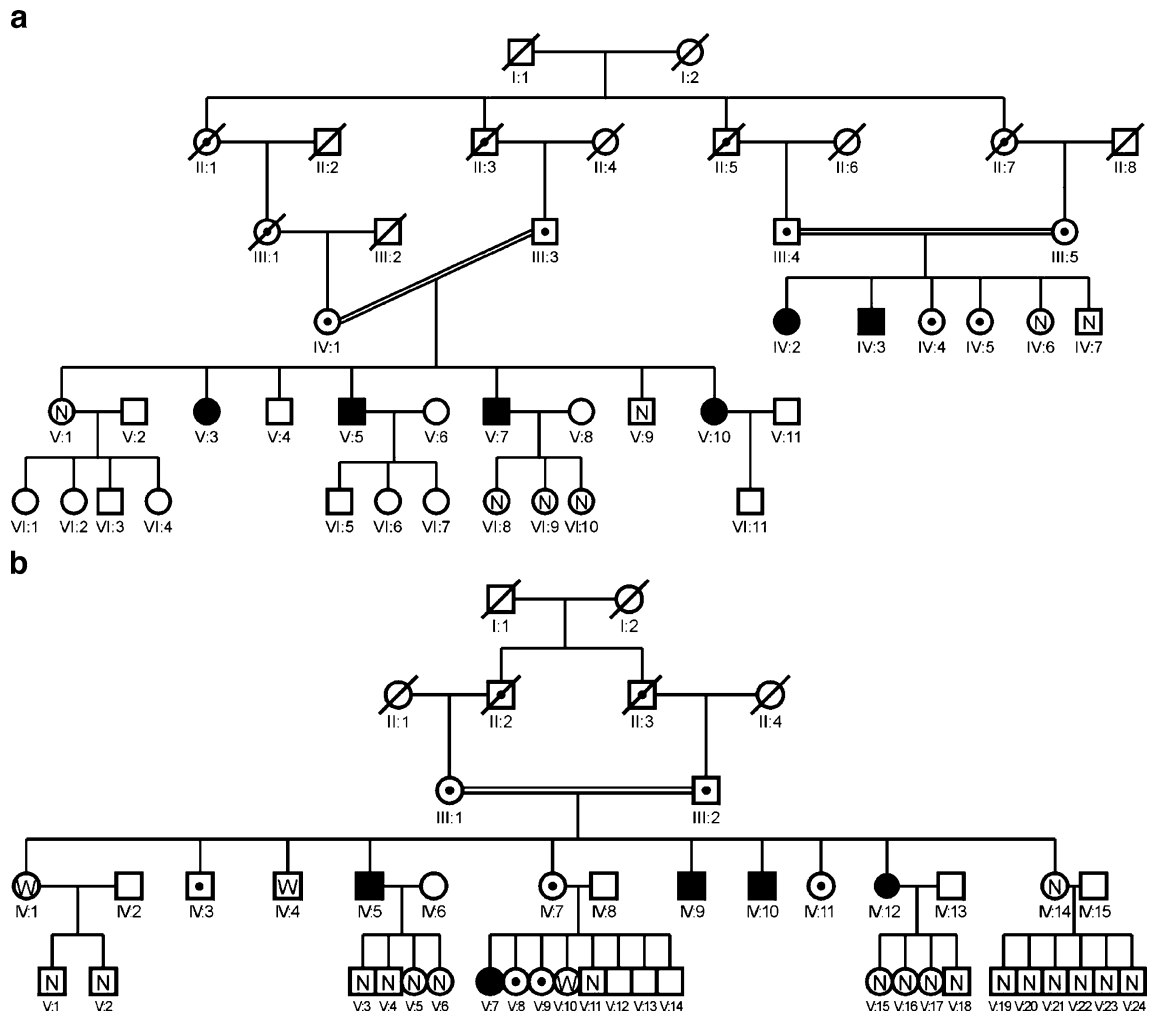
**Methods:** All accessible family members were included. They underwent blood sampling and ophthalmological examination including, when possible, full-field ERG and pupillometry. A genome-wide linkage analysis was initiated. Mutation analysis of the RPE65 gene within the linked interval was performed by bi-directional sequencing. **Results:** Eleven out of 53 examined members were clinically affected with an EORD. Linkage analysis

revealed a maximal lod score of 4.02 ( $\theta=0.1$ ) for the marker D1S207 on 1p31. Mutational screening of the RPE65 gene identified a homozygous R91W mutation co-segregating with the disease in all affected individuals. Eleven homozygotes had nystagmus and acuities ranging from CF to NLP. Two retinal patterns were identified: pattern 1 presented mid-peripheral deep white dot deposits and virtually no clumped pigmentation, whereas pattern 2 showed mid-peripheral pigmented clumps without any white deposits. Homozygotes had no detectable full-field ERG and an abnormal pupillary light reflex. Eleven heterozygotes had normal visual function. **Conclusion:** We identified and characterised an endemic form of early onset rod-cone dystrophy in a consanguineous population from northeastern Tunisia, due to the prevalence of a single RPE65 mutation. Two fundus patterns were identified: white dot deposits in earlier stages and clumped pigment in later stages.

### Introduction

Early-onset retinal dystrophy (EORD) includes heterogeneous hereditary disorders sharing degeneration of both rod and cone photoreceptors [10]. At the most severe end of the spectrum of EORD and accounting for 5% of all retinal dystrophies is Leber congenital amaurosis; a less aggressive form is juvenile retinitis pigmentosa [6, 9, 25].

Among the different genes that have been identified to account for EORD, mutations in the RPE65 gene seem to be the most frequent [3, 12, 25, 30] (approximately 6%) [9]. The RPE65 gene has been assigned to chromosome 1p31 [7] and encodes for a retinal pigment epithelium-specific and highly conserved abundant 65-kDa protein. This protein is a retinol ester-binding protein that plays an important role in the vitamin A cycle, being necessary for



**Fig. 1** **a** Pedigree BH. **b** Pedigree BO. Filled symbols denote individuals  $RPE65^{R91W/R91W}$ ; symbols with dots represent individuals  $RPE65^{WT/R91W}$ ; *W* open symbols show wild-type unaffected

individuals  $RPE65^{WT/WT}$ ; *N* open symbols represent the non-genotyped members with normal phenotype; plain open symbols are the non-examined members

the conversion to 11-cis retinal [7], the chromophore of the visual pigments.<sup>1</sup>

Mice and dogs lacking RPE65 gene (knock-out model) have been developed and studied [22, 28]. Histopathologic examinations of the diseased eyes from these animal models have revealed decreased accumulation of lipofuscin [11] and increased accumulation of retinyl esters in the RPE [16]. Partial functional and structural recovery of the retina after gene therapy in the RPE65 null mutation dog was recently reported [19].

The human molecular pathology of the RPE65 gene is not fully described and little is known regarding genotype/

phenotype correlations and natural history of specific disease-causing mutations [3, 5, 8, 20, 21, 25].

In this study three families harbouring a single RPE65 mutation were fully characterised, as precise phenotyping is likely to represent an important prerequisite for future trial of gene therapy.

## Patients and methods

Fifty-three members of three consanguineous nuclear families (Fig. 1) with EORD who originated from the same village in Cap Bon, northeastern Tunisia were included in the study. They underwent complete ophthalmological examination and, when possible, fluorescein angiography, colour vision test, computerised visual field, full-field ERG and pupillometry.

<sup>1</sup>Moiseyev et al identified the RPE65 protein as the isomerohydrolase transforming all-trans retinyl-ester into 11-cis retinol. Moiseyev G, Chen Y, Takahashi Y, Wu BX, Ma JX. RPE65 is the isomerohydrolase in the retinoid visual cycle. Proc Natl Acad Sci U S A. 2005 Aug 30;102(35):12413–12418

With their consent, blood samples were obtained from 26 family members.

### Clinical and electrophysiological examinations

Visual acuities were tested with Snellen chart and illiterate E chart. Computerised visual fields were measured with Humphrey Systems (Program 30–3, Humphrey Field Analyzer II, San Leandro, CA, USA). Colour vision was tested using the Farnsworth D-15 saturated dichotomous test. Presence of nystagmus was evaluated. The anterior segment was examined with a slit lamp (Haag–Streit), and funduscopy was performed by binocular indirect ophthalmoscopy. Fundus photographs and fluorescein angiography were performed with a fundus camera (Topcon). The electroretinography was performed under topical anaesthetic (tetracaine monodoses) using an ERG unit (Veritas; LKC, Gaithersburg, USA) according to the ISCEV standards (International Society for Clinical Electrophysiology of Vision). Pupillometry was carried out using a prototype portable pupillometry device (Neuroptics, Irvine, CA, USA). The device gave a 1-s diffuse light stimulus to one eye ( $6.2 \text{ mW/cm}^2$ ) while recording the iris and pupillary margins continuously for 1 s after the light stimulus. Internal software analysis automatically calculated the maximum area, minimum area, percentage of contraction amplitude, latency and velocity. Normative data based on age and baseline pupil size were provided by the manufacturer. Five measurements of the pupil light reflex per eye were obtained and averaged to minimise confounding effects from short-term variability.

### Molecular analysis

Genomic DNA was isolated from peripheral blood leucocytes as described elsewhere [15]. Genotyping was performed using 396 fluorescent dye-labeled dinucleotide repeat markers –FAM, \_HEX or NED (Prism Linkage

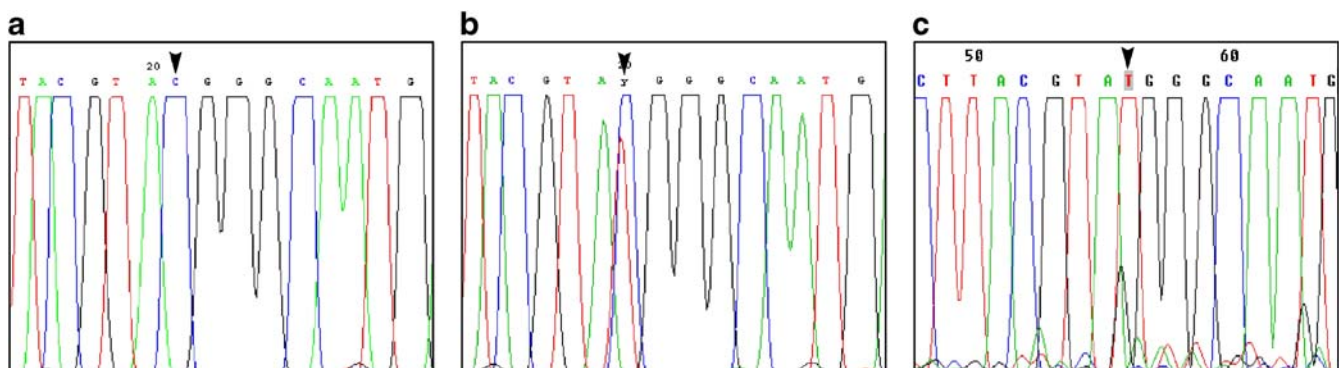
Mapping Set, Version 2; Applied Biosystems) following instructions from the manufacturer. PCR products were pooled and denatured at  $90^\circ\text{C}$  for 2 min. Markers were electrophoresed on denaturing polyacrylamide Performance Optimized Polymer 4 gels (POP-4; Applied Biosystems) on an automatic DNA sequencer (ABI-Prism 310 Genetic Analyser). Data were collected and analysed using Genescan and Genotyper software packages. Linkage analysis was performed with MLINK using equal allele frequency and a new mutation rate of  $10^{-4}$ .

Once linkage had been established, each of the exons of the best candidate gene was amplified by PCR as previously described. The PCR products were then screened for mutations by single-strand conformational polymorphism (SSCP) analysis. The abnormal conformers were sequenced bidirectionally.

### Results

The three nuclear families belonging to two distinct pedigrees (BH and BO) are shown in Fig. 1. Eleven of the 53 examined family members were clinically affected with EORD. Blood samples were obtained from 10 of them and from 16 unaffected family members. Linkage analysis revealed a maximal lod score of 4.02 ( $\theta = 0.1$ ) for marker D1S207 on chromosome 1p31, mapping close to the RPE65 gene locus. Mutational analysis of the RPE65 gene identified a homozygous R91W missense mutation (CGG to TGG) in exon 4 (Fig. 2c), co-segregating with the disease in all affected individuals. Analysis of the blood samples from the 16 clinically unaffected members revealed 12 heterozygotes  $\text{RPE65}^{\text{WT}/\text{R91W}}$  and 4 wild-type  $\text{RPE65}^{\text{WT}/\text{WT}}$  (Fig. 2a,b).

Details of the ten examined homozygotes are summarised in Table 1. None had signs of syndrome-associated features. They all had congenital night blindness and no photophobia. They had useful vision in the first decade of life, allowing reading acquisition, but had to learn Braille at the end of the first decade. They progressively lost vision in the third decade of life to the point of disability. They were aged 19–55 years



**Fig. 2** Genomic sequences: missense mutation R91W (CGG to TGG) in exon 4 of the RPE65 gene. **a** Wild-type missense mutation  $\text{RPE65}^{\text{WT}/\text{WT}}$ . **b** Heterozygotes mutation  $\text{RPE65}^{\text{WT}/\text{R91W}}$ . **c** Homozygotes  $\text{RPE65}^{\text{R91W}/\text{R91W}}$

**Table 1** Details of homozygous individuals

Patient/ age (years)/ sex	Acuity (ETDRS)	Ambulatory vision, up to	Refraction	Lens	Macula	Disk	Peripheral pigment clumps	Retinal vessels	Fluorescein angiography	Pattern
Pedigree BH										
Patient IV: 2/55/F	BE:LP	No, 50 years	NA	APoNSPCOcat	Atrophic	Slight pallor	++	Attenuated	NA	2
Patient IV: 3/49/M	BE:LP	No, 21 years	RE: S +3.00, C-3.75 LE:S-1.5, C-3.00	NSPCOcat	Atrophic, RPE changes	Slight pallor	++	Attenuated	Confluent lobular macular choriretinal filling defect hypofluorescence, central and peripheral pigment clumps blockage hypofluorescence	2
Patient V: 3/54/F	BE:LP	No, 47 years	NA	NSPCOcat	Atrophic, RPE changes	Pale	+	Attenuated	NA	2
Patient V: 7/43/M	BE:NLP	No, 30 years	RE: S+ 2.25, C-1.5 LE:S+5.5, C-2.00	PCOcat	Atrophic	Pale	(+)	Attenuated	NA	2
Patient V: 10/38/ F	RE: CF LE: HM	No, 30 years	NA	PCOcat	Atrophic, sparse RPE changes	Slight pallor	(+), mid-pe- ripheral white dot deposits	Attenuated	NA	1
Pedigree BO										
Patient IV: 5/43/M	BE: LP	No, 22 years	RE:S-8.75, C-2.5 LE: S-7.00, C-0.75	APoPCOcat	Atrophic	Slight pallor	++	Attenuated	Lobular partial confluent hypofluorescence	2
Patient IV: 9/41/M	RE: LP LE:NLP	No, 32 years	RE: S-1.25, C-0.5 LE:S+0.75, C-2.5	PCOcat	Atrophic	Slight pallor	+++	Attenuated	NA	2
Patient IV: 10/36/ M	BE: LP	Yes	RE:S-7.5, C-1.00 LE:S-7.5, C-3.25	PCOcat	Atrophic	Slight pallor	++	Attenuated	Mid-peripheral confluent lobular hypofluorescence	2
Patient IV: 12/32/ F	RE: CF LE: LP	Yes	NA	None	Atrophic	Slight pallor	Absent	Attenuated	NA	1
Patient V: 7/19/F	BE: CF	Yes	RE:S-6.5, C-3.5 LE:S-10.5, C-2.0	PCOcat	RPE mottling	Healthy	Absent, mid- peripheral white dots	Normal	Early macular hypofluorescence due to CMO with late pooling, early peripheral hypo- fluorescence, late mottled hyper- fluorescence	1

Po, polar; PCO, posterior capsular opacity; A, anterior; P, posterior; NS, nuclear sclerosis; cat, cataract

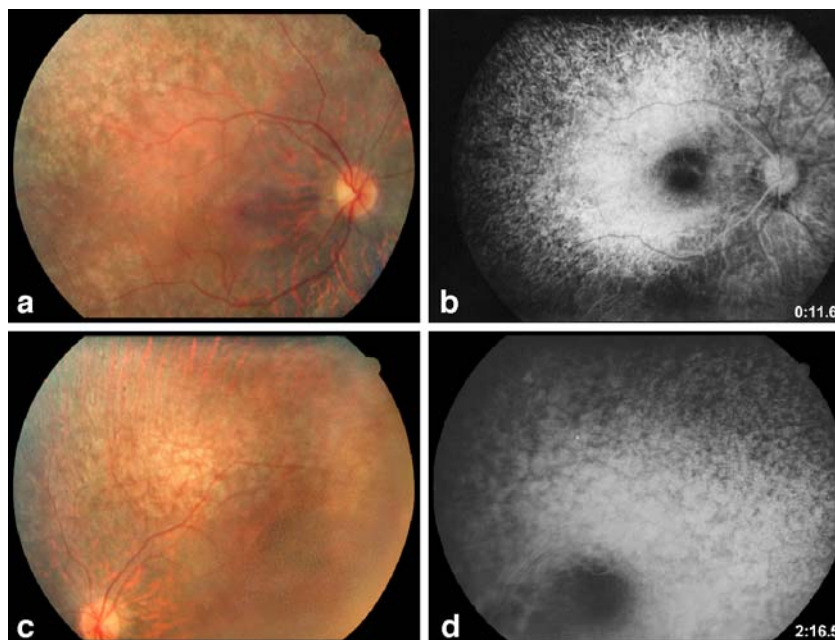
**Table 2** Funduscopy and fluorescein angiography findings

Pattern	Fundus colour photography	Fluorescein angiography
Pattern 1 ( <i>n</i> =3)	<ol style="list-style-type: none"> <li>1. Healthy disk</li> <li>2. Macular RPE changes or cystoid macular oedema</li> <li>3. Normal or slightly attenuated retinal vessels</li> <li>4. Peripheral grey discoloration of the retina and white dot deposits with virtually no clumped pigmentations</li> </ol>	<ol style="list-style-type: none"> <li>1. Macula: early hypofluorescence probably due to foveal cysts</li> <li>2. Periphery: early hypofluorescence due to late filling of the inner choroid with late irregular fluorescence due irregular dying away RPE.</li> </ol>
Pattern 2 ( <i>n</i> =7)	<ol style="list-style-type: none"> <li>1. Pale disc</li> <li>2. Macular atrophy</li> <li>3. Attenuated vessels</li> <li>4. Clumped pigmentation</li> </ol>	<ol style="list-style-type: none"> <li>1. Macula: Large partially confluent lobular area of hypofluorescence in early and late frames due respectively to absence of the inner choroid and cystoid macular oedema</li> <li>2. Periphery: annular patches masking the normal background fluorescence corresponding to clumped pigmentations</li> </ol>

with visual acuities ranging from CF to NLP. All had congenital nystagmus decreasing with age. There was no trend towards specific refraction default. All had brown irides and all but one had posterior capsular cataract. Bilateral anterior polar cataract was found in two patients.

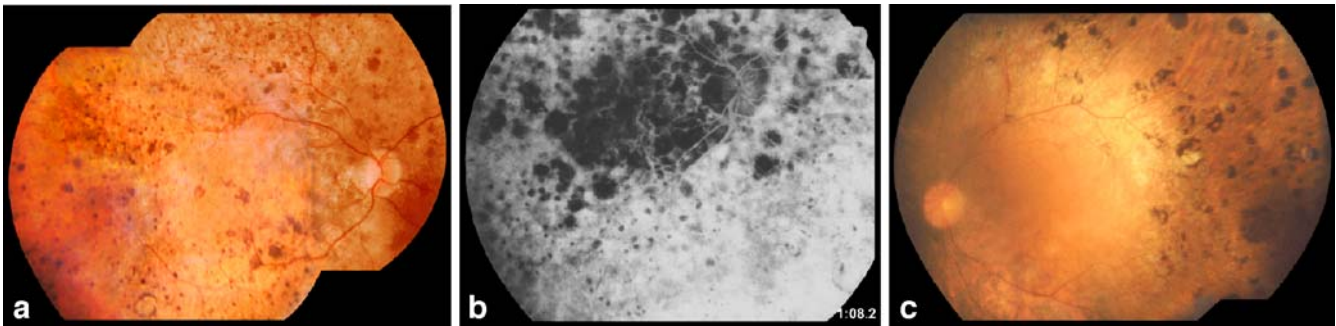
Patient V:14, pedigree BO, a 19-year-old myopic female with uncorrected distance visual acuity of CF, had a blue–

yellow axis on the Farnsworth dichotomous D-15 test and the Humphrey visual field showed a partial island of central vision in the left eye. Patient V:10/38/F, pedigree BH, and patient IV:12/32/F, pedigree BO, were not able to perform either the Farnsworth dichotomous D-15 or the Humphrey visual field tests but could distinguish saturated red, blue, and green colours and showed very restricted confrontational



**Fig. 3** Pattern 1: fundus colour photography and fluorescein angiography. **a.** Fundus colour photograph, right eye. Patient V:7/19/F, pedigree BO. Confluent white dot deposits and grey discoloration of the RPE localised outside the temporal vascular arcades. Peripapillary atrophy. **b.** Fluorescein angiogram, early frame, right eye. Patient V:7/19/F, pedigree BO. Mid-peripheral hypofluorescence caused by diffuse alteration of the RPE and lack of the choriocapillaris. Cystoid hypofluorescence in the macular area due to cystoid macular oedema. **c.** Fundus colour photograph, left eye. Patient

V:7/19/F, pedigree BO. As in **(a)**. **d.** Fluorescein angiogram, late frame, left eye. Patient V:7/19/F, pedigree BO. Persistence of mid-peripheral diffuse hypofluorescence and well-delimited islands of hyperfluorescence in the posterior pole and outside the temporal vascular arcades corresponding to the area of white dot deposits and grey discoloration of the RPE demonstrated in **(a)** and **(c)**. Petaloid hypofluorescence in the macular area due to cystoid macular oedema



**Fig. 4** Pattern 2: fundus colour photography and fluorescein angiography. **a** Composed fundus colour photograph, right eye. Patient IV:3/49/M, pedigree BH. Typical mid-peripheral pigmented clumps of different sizes either entirely pigmented or with central depigmentation. **b** Fluorescein angiogram, late frame, right eye. Patient IV:3/49/M, pedigree BH. Posterior pole confluent hypofluorescence due to alteration of the RPE and lack of the

choriocapillaris in the macular area. Masking effect at the posterior pole and mid-periphery due to clumped pigmentation. **c** Composed fundus colour photograph, left eye. Patient IV:2/55/F, pedigree BH. Typical mid-peripheral pigmented clumps of different sizes either entirely pigmented or with central depigmentation. Blurred view of the posterior pole due to lens opacities

visual fields. For the others, both colour vision and visual field were not testable.

Fundus and fluorescein angiography findings were subdivided into two patterns (Table 2). Pattern 1 included relative vascular and disc preservation, mid-peripheral deep white dot deposits, grey coloration of the peripheral retina and virtually no clumped pigmentations. Angiography ( $n=1$ ) showed peripheral hypofluorescence in early and late frames due to lack of choriocapillaris, mid-peripheral hyperfluorescence increasing during the transit, reflecting an unhealthy EP, and partial preservation of the choriocapillaris. Hypofluorescence of the macular area may have been due to cystoid macular oedema (Fig. 3). Pattern 2 included attenuated vessels and optic disc pallor, mid-peripheral clumped pigmentation and absence of white dots. Angiography ( $n=3$ ) showed a lack of choriocapillaris in the periphery as well as in the posterior pole. Peripheral and central masking in the zone corresponding to clumped pigment (Fig. 4). The mean age in pattern 1 was

$29\pm 12.02$  years and in pattern 2,  $47\pm 5.45$  years ( $p=0.03$ ). Patients in pattern 1 had better vision than pattern 2 (NS) (Table 1).

Eleven of the 13 diagnosed heterozygotes were assessed. They were aged 34–86 years. All had normal vision, ranging from 20/50 to 20/20, except one (patient III: 1/65/F, pedigree BO) who was diabetic and had high myopia and age-related cataract (visual acuity OU 20/200). All had normal confrontational visual fields and none had nystagmus. Funduscopy was normal.

Both scotopic and photopic ERG responses were not detectable in the 8 homozygotes tested. ERG responses were normal in the two heterozygotes tested.

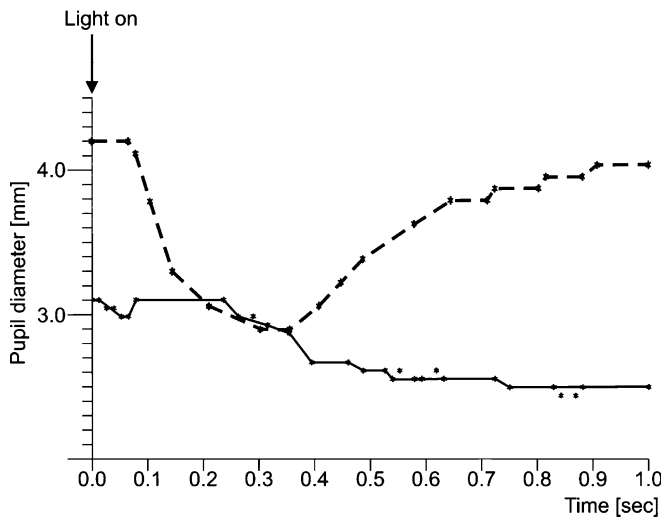
Pupillometry was performed in nine homozygotes, three heterozygotes and two wild-type controls. The controls showed normal contraction amplitude, velocity and latency. The pupil data of three members of the pedigree BH (patients V:7, V:3, IV:2) were excluded because inadequate numbers of reliable samples (fewer than five) were

**Table 3** Pupillometry results in pedigrees BH and BO

Patient/ age (years)/sex	Contraction amplitude (CA)		Latency (LAT)		Contraction velocity (V)		RPE65: R91W mutation, WT wild type
	CA RE	CA LE	LAT RE	LAT LE	V RE	V LE	
Pedigree BH							
Patient V:3/54/F	0.01*	NR	NR	NR	NR	NR	R91W/R91W
Patient V:10/38/F	0.49*	0.70*	0.47*	0.31*	0.59*	0.65*	R91W/R91W
Patient IV:4/40/F	1.12	1.24	0.23	0.23	1.68	2.11	WT/R91W
Pedigree BO							
Patient III:1/65/F	1.13	0.99	0.26*	0.26*	1.64	1.79	WT/R91W
Patient IV:5/43/M	0.63*	0.72*	NR	0.49*	NR	1.34*	R91W/R91W
Patient IV:11/34/F	1.18	1.19	0.24	0.23	1.65	1.83	WT/R91W
Patient IV:12/32/F	1.13	0.89	0.28*	0.28*	1.56	1.47	R91W/R91W
Patient V:7/19/F	0.59*	0.54*	0.31*	0.42*	0.73*	0.60*	R91W/R91W

\*Beyond one standard deviation

\*Normal ranges: contraction amplitude 0.81–1.97; latency  $\leq 0.25$ ; contraction velocity  $\geq 1.44$



**Fig. 5** Single monocular recordings to light flash in two patients (solid curve homozygote; dashed curve heterozygote)

obtained due to nystagmus. Pupillary responses of the 8 other family members (5 homozygotes, 3 heterozygotes) are reported in Table 3. Waveforms of an heterozygote (pedigree BO: patient IV.11) and an homozygote (pedigree BH: patient IV:3) are shown in Fig. 5. Patient V:3/54/F, pedigree BH, showed recordable responses only in the contraction amplitude in the right eye, allowing no further conclusions. Evaluation of the pupillary light reflex revealed diminished contraction amplitude, prolonged latency time and decreased contraction velocity in three homozygotes with poor vision (two of pattern 1, one of pattern 2). One additional homozygote with visual acuity of CF RE and LP LE (patient IV:12/32/F, pedigree BO, pattern 1) had a subnormal contraction amplitude with only minimal prolongation of the pupillary latency time. Pupillometry was normal in two heterozygotes, and a third heterozygote (patient III:1/65/F, pedigree BO) who was also diabetic showed a mildly prolonged latency time.

## Discussion

RPE65 mutations are a common cause of EORD [14] and may be responsible in 6.1–11.4% of patients with mutations in homozygous or compound heterozygous form [9, 26]. The disease-causing mutation mechanism of the missense mutation R91W is not yet known but has already been reported as a recurrent mutation arising on different ethnic backgrounds [26]. This change together with the R91Q mutation was described as causing both Leber congenital amaurosis (LCA) and autosomal recessive retinitis pigmentosa in compound heterozygotes and LCA in homozygotes [14, 18]. The R91W mutation may exert its pathogenic effect by changing the tertiary structure of the protein after the replacement of arginine, a positively charged, linear strong proton donor, by

tryptophan, an uncharged aromatic proton donor [3]. Specifically, Thompson et al. predicted that the substitution at arginine 91 may perturb the local beta-pleated sheet structure into an alpha helix, possibly inactivating a functional domain necessary for the synthesis of 11 cis-retinal [26].

In our study of three nuclear families, we examined 53 members of whom 11 were affected, the larger series described up to date with a single homozygous RPE65 mutation. Childhood visual function in homozygotes is good enough to acquire reading in the first decade of life but deteriorates to LP by the third decade of life. They all had congenital nystagmus, night blindness and no photophobia. Restricted visual field was common to all of them and becomes non-testable by the third decade of life. Colour vision could be evaluated by the Farnsworth dichotomous D-15 test for the only patient in the second decade, indicating an initial partial preservation of the cone system. Only saturated red, green and blue colours could be recognised by the third decade and none could be distinguished by the fourth decade. All this findings suggests that R91W phenotype is initially a rod–cone dystrophy. Although visual function was reduced since early childhood, the overall performance was better than usually seen in LCA [4, 10]. Our findings are in keeping with the previous phenotypical descriptions of RPE65 mutations in humans and dogs [2, 5, 19] but inconsistent with findings reported in RPE65 knock-out mice [23]. One possible explanation is that the survival time of cones relates to the size of the organism, so that mice may lose their cone functionality very early, whereas it is preserved longer in dogs and humans.

Two retinal patterns in homozygotes emerged based on fundus and fluorescein angiogram. Based on the work of Moiseyev et al. [16], we postulate that the peripheral white dot deposits seen on funduscopy of pattern 1 patients may represent accumulation of retinyl esters in the RPE. Their presence should be further assessed in other RPE65 gene mutations. White dots and clumped pigment have been described in other disease-causing mutations (mutations in NR2E3, RLBP1, RDH5 genes) [17, 24, 27, 29]. The significant difference in age between the two patterns suggested that they may represent a continuum in the evolution of the disease. All homozygous patients showed a complete loss of ERG responses and an abnormal pupillary reflex. However, a recordable reflex could be obtained in some of the homozygotes, suggesting that pupillometry may be one objective means of assessing and monitoring visual function in these patients as well as in patients who may benefit from future gene therapy [1]. In contrast, heterozygous patients showed normal visual function, ERG and pupillometry. Younger siblings should be further identified and tested.

Early-onset rod–cone dystrophy along with white dot deposits and typical absent or minimal autofluorescence, as described by Lorenz et al. [13], may become extremely

helpful criteria in the clinical diagnosis of RPE65 mutations.

In conclusion, we identified and characterised an endemic form of early onset retinal dystrophy. It presents as an early-onset rod-cone dystrophy in a highly inbred population of Cap Bon in northeastern Tunisia, due to the prevalence of a single homozygous missense R91W mutation in exon 4 of the RPE65 gene, most likely caused by a founder effect. Two patterns were identified: the white

dot deposits in earlier stages and the clumped pigment in later stages may represent a continuum. Thorough phenotyping is likely to be of great assistance in identifying these patients and will give guidance for future gene therapy.

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