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CHARACTERIZATION OF A PORTUGUESE LCA FAMILY SECONDARY TO HOMOZYGOUS *RPE65* MUTATION

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TRABALHO REALIZADO SOB A ORIENTAÇÃO DE

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Abbreviations:

A – Adenosin

AAV – Adeno-associated virus

AAV2 – Adeno-associated virus type 2

Arg - Arginine

BCVA – Best Corrected Visual Acuity

c. – codon

cDNA – complementary DNA

Dcc/Ncc - Ocular alignment at Distance with correction/Near with correction

dHPLC – Denaturing High Pressure Liquid Chromatography

DNA – Deoxyribonucleic acid

DTL fiber – Dawson Trick Litzkow fiber

EORD - Early-onset Retinal Disease

EPP - Estimate of Pathogenic Probability

ERG – Electroretinogram

FAF – Fundus autofluorescence

FST –Full-field Sensitivity Test

G- Guanine

Gln – Glutamine

IOL – Intraocular Lens

LCA – Leber Congenital Amaurosis

LCA2 – Leber Congenital Amaurosis type 2

LRAT - Lecithin Retinol Acyltransferase

mfERG – Multifocal Electroretinogram

OCT - Optical Coherence Tomography

ONL – Outer Nuclear Layer

PCR – Polymerase Chain Reaction

PRC – Photoreceptor cell

RP – Retinitis Pigmentosa

RPE – Retinal Pigment Epithelium

SD-OCT - Spectral-Domain OCT

TD-OCT – Time Domain OCT

TPLR - Transient Pupillary Light Reflexes

Abstract

Introduction: Leber congetinal amaurosis type 2 is an autosomal recessive degenerative retinal disease presenting with severe vision loss, wandering nystagmus and reduced cone and rod ERG signal from an early age. It results from a two-allele mutation in the *RPE65* gene, disrupting the expression of a key-enzyme for the visual cycle pathway. Recently, gene therapy and pharmacological clinical trials have been gaining recognition due to successful efficacy and relative safety results. This study reports the first Portuguese family diagnosed with LCA2 and discusses their eligibility for available clinical trials.

Methods: Four individuals affected with LCA2, two males and two females whose diagnosis was based on molecular genetic analysis and visual and retinal function studies, were included in this study. Patients underwent complete ophthalmological examination and molecular testing. Phenotypical characterization included panretinal fundus photos, electroretinogram, optical coherence tomography and fundus autofluorescence.

Results: Patients revealed significant phenotypic heterogeneity, though the LCA2 hallmark features were present: reduced ERG signal, no evidence of outer nuclear layer in OCT, decreased best corrected visual acuity in all patients (less than 20/40) and early onset nystagmus, in most patients.

Discussion: Although LCA2 was genetically confirmed in all patients, in two, central massive photoreceptor loss evidenced on OCT and extremely low BCVA results exclude them as potential candidates for pharmacological or gene therapy clinical trials. Significant phenotypical heterogeneity is present in this family. We suggest the implementation of early genetic based LCA2 diagnosis, supported by further investigation on genotype-phenotype correlation as well as better diagnostic strategies.

Resumo

Introdução: A Amaurose Congénita de Leber tipo 2 é uma doença autossómica recessiva degenerativa da retina que progride com nistagmo, perda de visão acentuada e resposta elétrica retiniana reduzida desde uma idade muito precoce. Resulta de uma mutação homozigótica do gene *RPE65*, bloqueando a expressão de uma enzima chave para o ciclo visual. Recentemente, ensaios clínicos de terapia génica e novas estratégias farmacológicas têm ganho amplo reconhecimento dentro da comunidade científica devido ao sucesso dos seus resultados sobre eficácia e segurança. Este trabalho analisa a primeira família portuguesa diagnosticada com LCA2 e discute a possibilidade da sua inclusão nos ensaios clínicos disponíveis.

Métodos: Dois doentes do sexo masculino e duas do sexo feminino, cujo diagnóstico foi baseado em análise molecular e estudos de função visual e retiniana, foram incluídos neste estudo. Os doentes foram submetidos a um exame oftalmológico completo. A caracterização fenotípica incluiu retinografia pan-retiniana, electroretinograma, tomografia de coerência ópitca e autofluorescência da retina.

Resultados: Embora a comparação dos resultados revele uma heterogeneidade fenotípica, as características paradigmáticas de LCA2 estão presentes: forte diminuição na amplitude no ERG, ausência da camada nuclear externa no OCT, diminuição da melhor acuidade visual corrigida em todos os doentes juntamente com nistagmo de aparecimento precoce na maioria deles.

Discussão: Apesar da confirmação do diagnóstico de LCA2, em dois dos doentes a perda maciça de fotorreceptores exclui estes candidatos de ensaios clínicos. Sugere-se a implementação de diagnóstico genético precoce de LCA2, prosseguindo a investigação em melhores estratégias diagnósticas e de seguimento, assim como a procura por um conhecimento mais exato sobre a correlação genótipo-fenótipo.

Keywords

Leber Congenital Amaurosis, *RPE65* gene, gene therapy, clinical trials, SD OCT, visible photoreceptor (outer nuclear) layer

Introduction

LCA is a rare hereditary retinal degeneration that results in severe vision loss at an early age [1]. Clinical descriptions of the disease were first published in 1869 by Theodor Leber, who reported the symptoms of a young adult with severe blindness from birth, wandering nystagmus, pigmented retinopathy and amaurotic pupils [2]. Later in 1954, Franceschetti and Diertle defined the severe reduction of measured ERG as the cornerstone of LCA [3]. Numerous pre-clinical studies researching LCA's responsible genetic mutations led to the discovery of more than a dozen genes that play different roles in the phototransduction pathway [4]. One of these genes is *RPE65* and its mutations are responsible for autosomal recessive LCA type 2 (LCA2), representing 6% of all LCA cases [5].

The *RPE65* gene is highly expressed in the retinal pigment epithelium where it encodes the retinoid-isomerase enzyme, responsible for converting all-trans retinyl ester to 11-cis-retinol. This enzyme is essential to the canonical pathway of the visual cycle [6]. A block in this step of the visual cycle causes a lack of visual chromophore 11-cis-retinal, increases thermal activation of the phototransduction pathway as well as substract accumulation of lipid droplets containing all-trans-retinyl esters, leading to later retinal degeneration and photoreceptor loss [7].

Clinically, LCA2 patients are visually less responsive shortly after birth or within the first years of life [4]. Best corrected visual acuity (BCVA) may vary from 20/32 to 20/200 in the first thirty years, but afterwards BCVA less than 20/200 is

common [8]. There is abnormal oculomotor behavior, typically wandering eye movements and faster oscillations, pointing to a sensory nystagmus [9]. Additionally, oculodigital sign (poking or eye rubbing), keratoconus/keratoglobus, cataracts and strabismus may be present. Fundus appearance often show signs of intraretinal pigment migration with bone-spicule pigmentation, attenuated retinal blood vessels, macular atrophy and optic nerve pallor [1]. Abnormal ERG even at youngest ages, undetectable rod ERGs and only reduced cone ERG [10,11] is observed with, little measurable kinetic field by the end of the third decade of life [12]. Finally, mid-peripheral dysfunction as a later feature where only central and peripheral islands remain [13], belong to the typical findings. OCT, along with ERG, is critical for evaluation of LCA2; retinal thickness and outer nuclear layer signal are measured, the latter being proportional to photoreceptor density. Both measures appear to be reduced in LCA2 [14,15].

LCA, along with autosomal recessive early-onset retinitis pigmentosa, early-onset retinal dystrophy and severe early childhood-onset retinal dystrophy, comprise a heterogeneous group of disorders affecting rod and cone simultaneously [11]. Because this group represents a *continuum*, there is some diagnosis ambiguity [4]. As a critical differential diagnosis, non-LCA forms of inherited Retinitis Pigmentosa, contrasting with LCA, present with rod mediated changes first, including nyctalopia and peripheral vision loss, before slowly progressive reduction in ERG cone responses and central vision. Classically, intra-retinal pigment epithelial migration results in the classic fundoscopic appearance of bone spicule pigmentation, indicative but not universal for RP [1].

Though a quantitative relationship between disease severity (phenotype) and genotype has not been definitely established [4,16], there is some evidence of a

correlation between specific mutation in a single gene and phenotype. Regarding mutations affecting the *RPE65* gene, Philip *et al.* developed an objective algorithm to calculate an "estimate of pathogenic probability" (EPP) based on the prevalence of a specific variation, its segregation within families, and its predicted effects on protein structure [17].

In contrast to the majority of the retinal degenerative disorders, in LCA2 there is a disproportional loss of photoreceptor function [18] compared to retinal degeneration, where patients usually show greater photoreceptor preservation for their severe visual loss [14]. This feature is a prerequisite for gene therapy and pharmacological options, explaining a crucial eligibility criterion – the evidence of an intact ONL confirmed using OCT, suggesting intact photoreceptor machinery [4].

Therapeutic options for LCA2 have been mainly supportive [1], albeit gene and pharmacological clinical trials are starting to gather strong scientific evidence. Pharmacological research has pointed out that Vitamin A metabolite 11-cis-retinal serves as the visual chromophore and shows promise in the treatment of LCA2 [19,20]. Similarly, gene therapy has recently gathered great enthusiasm among scientists [4]. Since 2007, gene therapy trials in human LCA2 patients, using recombinant AAV vectors and based on a multitude of basic, pre-clinical and clinical research, have been reported by three groups and proof-of-concept of gene replacement efficacy has been demonstrated [21-27]. Despite methodological differences between the three groups, all have shown that gene therapy is effective and relatively safe.

Here, we present the first portuguese family diagnosed with LCA2, with focus on their genotype and phenotypical features, compare with the clinical descriptions available in the literature and discuss their eligibility for pharmacological or gene therapy clinical trials.

Materials and Methods

Patients and control population

Four individuals affected by LCA2, whose diagnosis was based on visual and retinal function studies, are all members of the same family. Two females and two males, ages between 31 and 58, were included in this study. All affected individuals are followed at the Centre for Hereditary Eye Diseases of the Department of Ophthalmology, Centro Hospitalar Universitário de Coimbra. Probands and affected family members presented at our clinic mostly due to visual impairment (loss of central vision) or funduscopic changes that fit the clinical diagnosis of LCA.

All individuals included in the study were informed about its objectives and volunteered to participate. Informed consent was obtained from all subjects according to the tenets of the declaration of Helsinki. The study was approved by the Ethics Committee of the Centro Hospitalar Universitário de Coimbra.

Clinical Examination

Ophthalmological examination included assessment of BCVA after manifest or cycloplegic refraction, pupillary reflexes, ocular motility, slit-lamp examination and fundus examination using a non-contact 78-diopter lens. Fundus photography was performed with a TOPCON TRC 50X (Topcon Optical, Tokyo, Japan).

Electroretinogram

Multifocal ERGs were recorded using DTL fiber electrodes, after a light adaptation period of 10 minutes and pupil dilation with tropicamide, before fundus photography, with a commercial system (RETIscan System; Roland Consult) (Kutschbach, 1997). Refractive errors were corrected in relation to the viewing distance.

The stimulus used in the mfERG consisted of 61 hexagons covering a visual field of up to 30° and presented on a 20-inch monitor at a viewing distance of 33 cm. Luminance was 120 cd/m2 for white hexagons and approximately 1 cd/m2 for black hexagons, resulting in a Michelson contrast of 99%. The hexagonal areas increased with eccentricity to compensate for local differences in signal amplitude because of differences in cone density across the retina (leading to a fourfold change in hexagon area size). Each hexagon was temporally modulated between light and dark according to a binary m-sequence (frame rate, 60 Hz). Observers were instructed to fixate a small black cross in the center of the stimulus. Fixation was continuously checked by means of online video-monitoring during the approximately 8-minute recording sessions. To improve fixation stability, sessions were broken into 47-second segments; eight trials were recorded in total. Signals were amplified with a gain of 100,000 and were band-pass filtered (5–300 Hz).

Reference and ground electrodes were attached to the ipsilateral outer canthus and forehead, respectively. The surface electrode impedance was less than 10 k_. Analyses were performed with the system software (RETIscan; Roland Consult) and standard statistical packages. First-order kernels were used for mfERG analysis because of their close correlation with the function of the outer retina [28]. The obtained local ERGs responses were normalized by the area of stimulus delivery to obtain a density response (nV/deg^2). For each hexagon, the peak amplitude of P1—defined as the difference between N1 and P1 amplitudes—the N1 peak, and the implicit time of P1 component were computed. To easily evaluate spatial differences of the local ERG responses, responses from the 61 elements were divided into averages of five concentric rings around the fovea.

Optical Coherence Tomography

OCT was performed with commercially available equipment in three LCA2 patients. We used an OCT device (Spectralis Spectral-Domain OCT; Heidelberg Engineering, GmbH, Dossenheim, Germany) to obtain cross-sectional images centered in the macula, The newer Spectral (or Fourier) Domain OCT (SD-OCT) uses a significantly faster, non-mechanical technology. The SPECTRALIS® SD-OCT simultaneously measures multiple wavelengths of reflected light across a spectrum, hence the name spectral domain. The SPECTRALIS system is 100 times faster than TD-OCT and acquires 40,000 A-scans per second. The increased speed and number of scans translates into higher resolution and a better chance of observing disease. Scan of high axial resolution of 10 _m or less, transversal resolution of 10 _m, and longitudinal scan range of 2 mm, were obatained to allow evaluation of retinal thickness and layer integrity in the macula. Special attention was payed to the RPE/photoreceptor interface.

Fundus autofluorescence

Both infrared and fundus autofluorescence imaging were performed using the HRAII (Heidelberg Engineering, Dossenheim, Germany) according to the manufacturer's recommendations.

Molecular genetic analysis

Genomic DNA was extracted using an automated DNA extractor (BioRobot EZ1, Qiagen, Hilden, Germany). The *RPE65* gene was e PCR-amplified using previously described primers and conditions [29]. To detect sequence changes, *RPE65* gene were screened by dHPLC using a WAVE ™ DNA Fragment Analysis System (Transgenomic). The PCR amplicons from control DNA and test DNA were combined

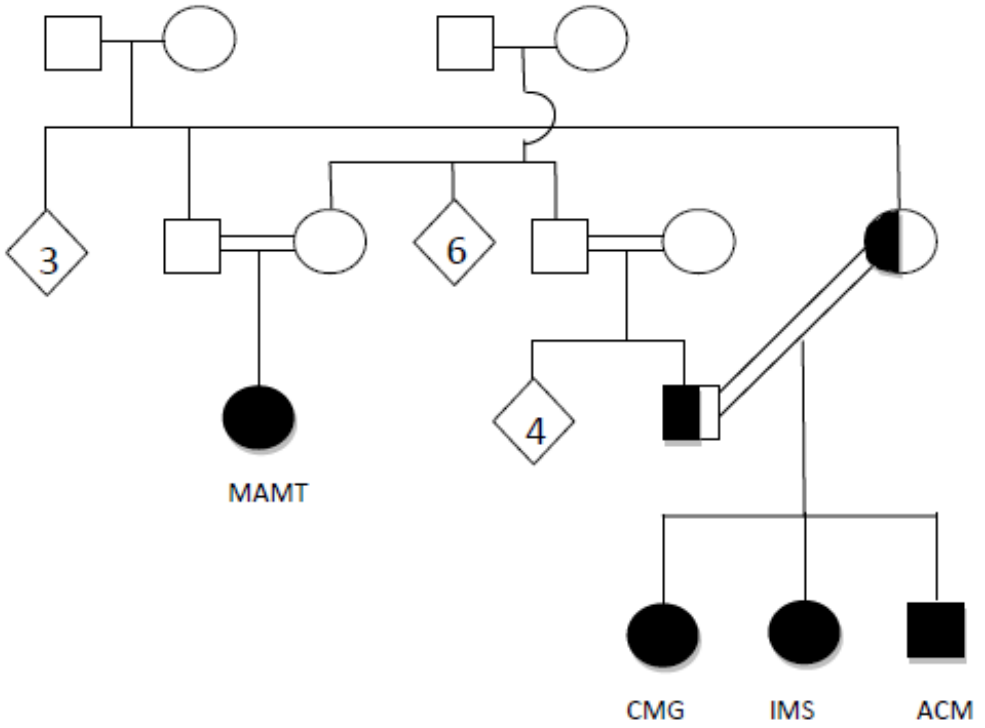
in 1:1 ratio and were loaded (5µl) on a C_{18} reserved-phase column (DNA Sep™ column; Transgenomic). The column mobile phase consisted of an acetonitrile gradient formed by mixing buffers A and B (WAVE Optimized™; Transgenomic). The flow rate was set at 0.9 ml/min and DNA was detected at 260 nm. For each amplicon, three optimum temperatures for hetero- and homodimer detection were determined empirically. The chromatograms obtained with the control and test samples were compared for the peak number and shape, for each temperature. All abnormal heteroduplexes obtained were, then, sequenced. Amplification products were purified with QIA-quick Gel Extraction Kit (Qiagen). Sequencing reactions were performed using the 4-dye terminator cycle sequencing ready reaction kit (BigDye DNA Sequencing Kit, Applied Biosystems, Foster City, California). Sequence products were purified through fine columns (Sephadex G-501, Princetown Separations, Adelphia, New Jersey) and resolved in an ABI Prism 3130 (Applied Biosystems). In those cases, in which no mutation was detected using dHPLC screening, the *RPE65* gene was directly sequenced to guarantee that all sequence changes were identified.

Results

We screened the *RPE65* gene for underlying LCA2/early-onset rod-cone dystrophy by direct sequencing of the coding exons and flanking intronic regions in each proband. A homozygous *RPE65* missense mutation was identified, representing a c.272 G>A transition in exon 4 leading to a Arg 91 Gln mutation.

All probands with homozygous *RPE65* mutations were available for clinical examination (Table 1). A family's genogram is shown (Fig. 1) and the OCT, FAF, fundus and ERG results are summarized in the Tables (Table 2 and 3).

Fig.1 – Genogram



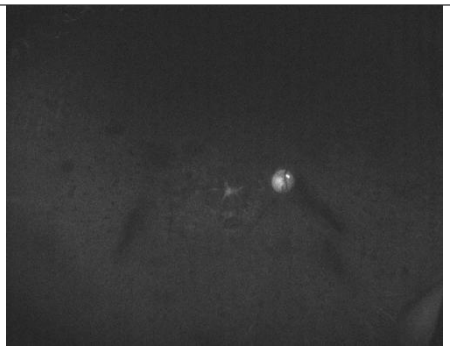
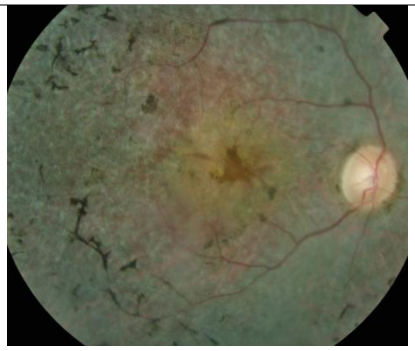
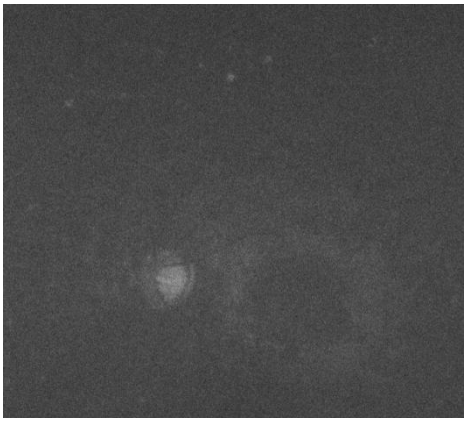
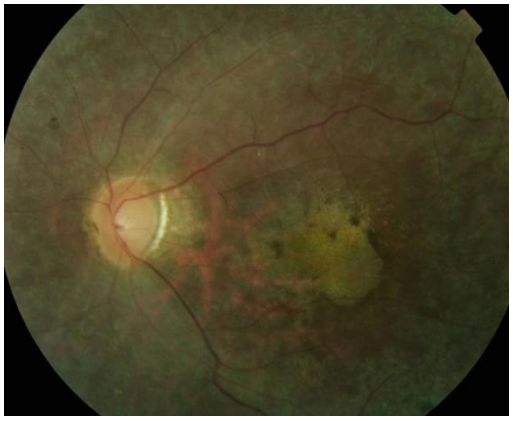
Male is represented by a square and female is represented by a circle. Filled square/circle represents an affected individual carrying homozygous *RPE65* mutation and half filled square/circle represents an individual carrying heterozygous *RPE65* mutation.

Table 1. Summarized clinical examination results

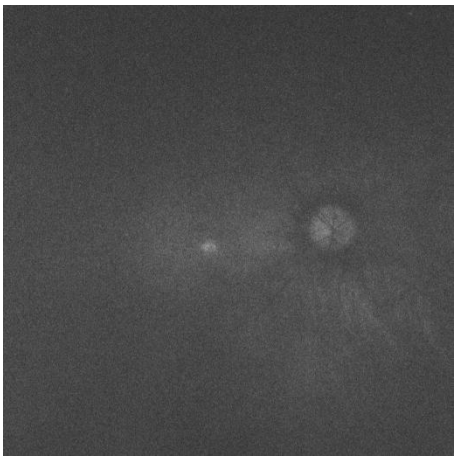
	MAMT	ACM	CMG	IMS
AGE	58	31	36	35
BCVA	<20/1000 bilateral	<20/1000 bilateral	OD 3/10, OS 6/100	OD 3/10 OS 2/10
CORRECTION	---	OD +5,00 OS +3,00	---	OD -2,00 OS -1,50
STEREOPSIS	Absent	Absent	Absent	Absent
PUPILLARY REFLEXES	Sluggish	Sluggish	Sluggish	Sluggish
EYE MOVEMENTS	Horizontal nystagmus	Horizontal nystagmus	No nystagmus	Vertical and torsional nystagmus
DCC/NCC	Exotropia	Exotropia	Orthotropia	Exotropia
SLIT-LAMP EXAM	Pseudophakia	Within normal limits for age group. No cataract.	Within normal limits. No cataract.	within normal limits for age group. No cataract.
FUNDUS	Severe central and peripheral RPE atrophy, white deposits and rare bone spicules in the periphery, vessels of reduced caliber and optic pallor	Severe central and peripheral RPE atrophy, white deposits in the retinal periphery, vessels of reduced caliber and optic pallor	Relative preservation of central retina. Whitish deep deposits in the mid and far periphery. Reduced vessel caliber. Rare bone spicules in the mid periphery	Relative preservation of central retina. Whitish deep deposits in the mid and far periphery. Reduced vessel caliber. Rare bone spicules in the mid periphery
IMMUNESUPPRESSION	No	No	No	No
PREGNANCY/ BREASFEEDING	---	---	No	No

Abbreviations used: BCVA (best corrected visual acuity), Dcc/Ncc (Ocular alignment at Distance with correction/Near with correction), OD (right eye), OS (left eye).

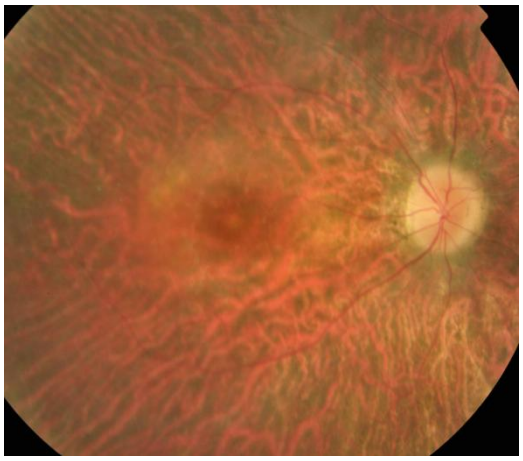
Table 2. FAF and Fundus results

	FAF	FUNDUS
MAMT		
	Left eye FAF displaying almost complete absence of autofluorescence except for a faint star temporal to the disc in the central macula related with the beaten bronze lesion	Right eye fundus image displaying optic pallor, vessels of reduced caliber, central macular beaten bronze pigmentation, whitish deep deposits spread throughout the posterior pole and periphery, peripheral bone spicules and patchy REP atrophy.
ACM		
	Left eye FAF displaying almost complete absence of autofluorescence except for a very faint ring temporal to the disc surrounding a central area of complete atrophy.	Left eye fundus. Optic pallor, thin vessels, severe central macular atrophy and hyperpigmented ring, patchy peripheral RPE atrophy with rare bone spicules.

CMG

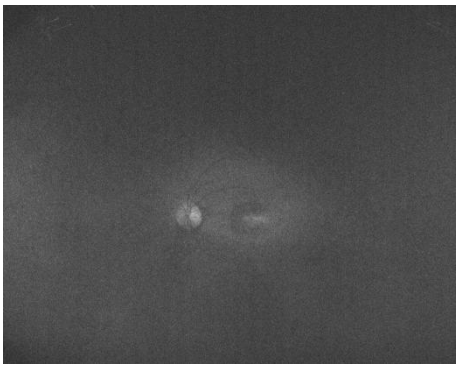


Right eye FAF displaying almost complete absence of autofluorescence except for a spot of hyperautofluorescence in the central macula.

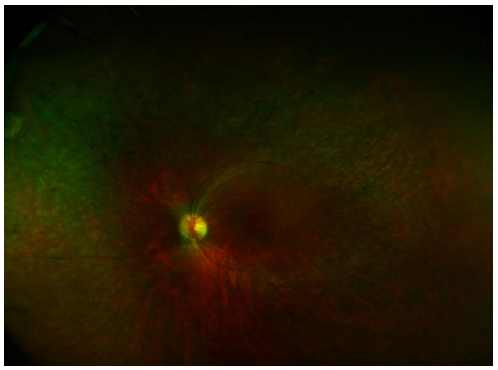


Right eye fundus image displaying optic pallor, vessels of reduced caliber, central macular beaten bronze pigmentation, rare whitish deep deposits spread throughout the posterior pole and periphery and patchy RPE atrophy.

IMS

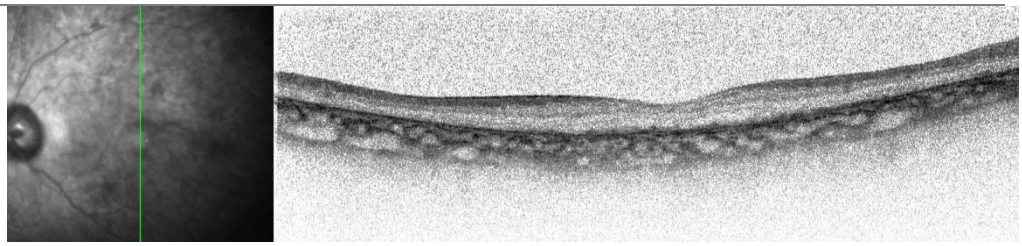
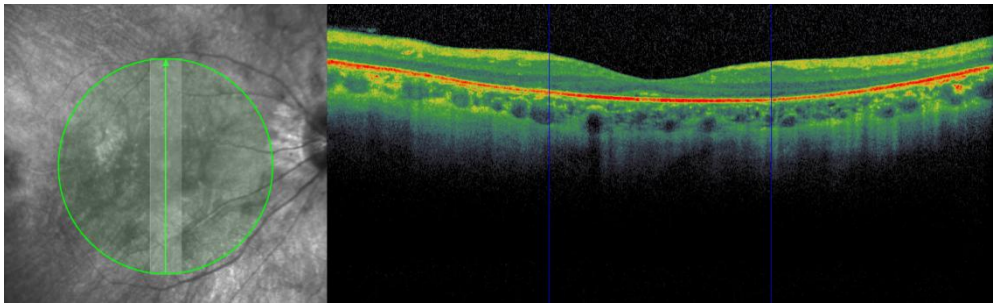
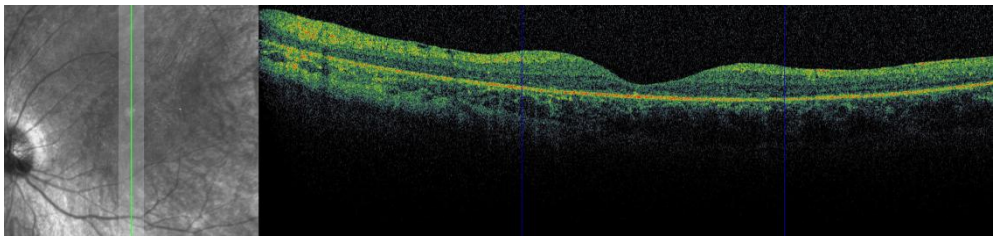


Right eye FAF displaying almost complete absence of autofluorescence except for a streak of hyperautofluorescence in the central macula surrounded by a fainter halo.



Left eye panretinal fundus photo displaying central and peripheral RPE atrophy, whitish deposits, patchy areas of RPE atrophy and rare bone spicules in the far periphery.

Table 3. OCT Results

	OCT
MAMT	 Left eye SD-OCT vertical scan displaying a very thin retina completely disorganized and almost absent RPE/PRC interface, and significantly disrupted inner retinal layers.
ACM	Not available due to severe nystagmus and insufficient technical quality.
CMG	 Left eye SD-OCT vertical scan displaying a thin retina with a central islet of RPE/PRC interface, and relatively preserved inner retinal layers.
IMS	 Left eye SD-OCT vertical scan displaying a thin retina with a central spot of RPE/PRC interface, and relatively preserved inner retinal layers.

MAMT is a 58 year-old female, born to consanguineous parents with no past family history of retinopathies. Her first disease symptoms started shortly after birth with manifest horizontal nystagmus. She complained of nyctalopia, impaired peripheral vision and severe visual acuity loss since her early childhood. The patient describes worsening of her symptoms especially after her first year in College. She stopped

reading at the age of 20 and later in 1997 she underwent bilateral cataract surgery (phacoemulsification with IOL implantation). Upon ophthalmological examination, BCVA was below 20/1000 in both eyes, pupillary reflexes were absent bilaterally as well as stereopsis. There was abnormal ocular motility, where manifest horizontal nystagmus, and variable angle exotropia, for distance and at near, were evident. There was no apparent fixation preference. Slit-lamp examination revealed pseudophakia bilaterally. Fundus examination disclosed severe central and peripheral RPE atrophy, white deposits and rare bone spicules in the periphery, vessels of reduced caliber and significant optic pallor. On OCT there was almost absent RPE/PRC interface and relatively preserved inner retinal layers. She is not taking any immunosuppressive drugs nor is she suffering from any immunosuppressive condition.

ACM is a 31 year-old male, born to consanguineous parents. Like MAMT, ACM complained of nyctalopia since birth and only started using large print books after age 13. At that time, his visual acuity was approximately 20/400. On ophthalmic examination, BCVA was again below 20/1000 bilaterally, there were bilateral reduced pupillary reflexes and absent stereopsis. His ocular motility revealed horizontal nystagmus of variable characteristics. Bilateral significant ptosis was also identified. Additionally, the patient revealed a variable angle exotropia with no fixation preference. Slit-lamp examination disclosed anterior segments within normal limits. Fundus examination revealed optic pallor, severe central and peripheral RPE atrophy, white deposits and rare bone spicules in the periphery and vessels of reduced caliber. OCT results are not available due to severe nystagmus and insufficient technical quality. He is not immunodeficient.

CMG is a 36 year-old female, born to consanguineous parents. First symptoms included nyctalopia since early childhood. Her visual acuity progressively worsened,

especially in the last 8 years, with BCVA of 3/10 in the right eye and 0,6/10 in the left eye. Ocular motility assessment failed to evidence manifest nystagmus. Under slit-lamp examination, anterior segments were within normal limits. Fundus examination revealed relative preservation of central retina, whitish deep deposits in the mid and far periphery, reduced vessel caliber and rare bone spicules in the mid periphery. These findings are compatible with diagnosis of early onset rod-cone-dystrophy. Her OCT results showed a thin retina with a central islet of RPE/PRC interface, and relatively preserved inner retinal layers. She is not pregnant nor breastfeeding and she is immunocompetent.

IMS is a 35 year-old female and born to consanguineous parents. CMG and ACM are siblings. She suffered from early onset nyctalopia, complaining about severe difficulty with transitions from light to dark and vice-versa. Her BCVA is 3/10 in her right eye and 2/10 in her left eye. Ocular motility assessment revealed torsional/vertical nystagmus with exotropia of variable angle for distance and near, without fixation preference. Slit-lamp examination confirmed anterior segments within normal limits for the age group. Fundus examination again showed relative preservation of central retina, whitish deep deposits in the mid and far periphery, reduced vessel caliber and rare bone spicules in the mid periphery. OCT results were similar to her sister's, displaying a thin retina with a central islet of RPE/PRC interface, and relatively preserved inner retinal layers. She is not pregnant nor breastfeeding and she is immunocompetent.

It is important to underscore that ERG was performed in all individuals, and was completely flat both in photopic and scotopic conditions (ISCEV guidelines) (data not shown).

Discussion

Notwithstanding that all patients involved in this study present the same homozygous *RPE65* missense mutations, namely a c.0272 G>A transition in exon 4, it is interesting to observe a peculiar intrafamilial phenotypic heterogeneity. Visual acuity results in this family are diverse, varying from less than 20/1000 to 3/10. These results are consistent with many studies showing that BCVA may in fact substantially vary in the first three decades of life [12], mainly representing milder losses, although severe losses, as exemplified by ACM, were reported as well [30]. After the third decade of life, most patients have acuities of 20/200 or worse [8]. Another diverging phenotypic feature is the different refraction correction in emmetropic patients – ACM hyperopic eyes contrasting to IMS myopic eyes. Furthermore, MAMT is the only family member who underwent cataract surgery by the age of 41. Albeit it is not well known where there is genetic influence of *RPE65* mutations in cataract and refraction diseases, there is some evidence of a correlation between LCA2, cataracts and myopic eyes [31,32]. Equally interesting is the lack of nystagmus in CMG, when confronted with horizontal nystagmus in MAMT and ACM and vertical and torsional nystagmus in IMS. This may be partially explained by a better fixation capacity provided by a higher BCVA in CMG, although other underlying factors must exist, because IMS's BCVA does not significantly differ from CMG's. Moreover, fundus examination results enhance intra-family phenotypic heterogeneity by confronting two diverging findings: MAMT's and ACM's retinas show severe central and peripheral RPE atrophy, white deposits and rare bone spicules in the periphery whereas the retinal images from IMS and CMG depict relative preservation of central retina and whitish deep deposits in the mid and far periphery. These findings raise the crucial question of which and how determinant are the underlying environmental and genetic modifying factors. Thus it would be

interesting to explore other retinal expressed genes (associated with both LCA and/or Retinitis pigmentosa) to identify other potential mutations that segregate appropriately with the observed phenotype. This would not be a case of trigenic inheritance, rather a case o genetic modification/worsening of the phenotype.

Today, two major therapy possibilities define the track of the most recent clinical trials, with the latter showing the most promising results: pharmacological and gene therapy. Within the pharmacological options, synthetic oral cis-retinoid, QTL Inc.'s QTL 091001 yielded visual improvements in patients with RP or LCA due to either of two genetic mutations – RPE65 and LRAT mutations, according to two clinical trials [33,34]. Regarding gene therapy, three independent clinical trials of *RPE65* gene therapy intervention by subretinal administration of deficient recombinant AAV2 vectors carrying the wildtype human *RPE65* cDNA were initiated in 2007 and results in 18 patients covering maximum follow up periods ranging from 90 days to 1.5 years have been published to date [21-26]. Additionally, a Phase 3 open-label randomized controlled trial of gene therapy started in October 2012. Despite the methodical differences, all groups have shown that gene therapy is effective and relatively safe. Some of the groups reported transient macular blebs, retinal detachment, self-limited intraocular inflammation and antibody to the AAV2 capside production [21-27]. Regarding efficacy results, Maguire *et al.* observed overall improvements in visual field assessment, pupillometry, ocular motility and functional tests [24,27]; on the other hand, Hauswirth *et al.* used other testing strategies and demonstrated improved dark adapted FST results [22,23,25,26]. For the majority of the patients, ERG results did not improve [21-27].

In furtherance of the patient's eligibility, the following criteria must be met: first, the genetic diagnose of a *RPE65*- associated retinal disease; second, clinical

diagnosis of LCA2 or EORD, and best corrected visual acuity of 20/40 or worse in the study eye, but not worse than hand motion in both the treated eye and the fellow eye; and third, a visible photoreceptor (outer nuclear) layer on an OCT scan [35]. Most important exclusion criteria are immunosuppressive states and pregnancy/breastfeeding. These excluding criteria regard the possibility of subclinical systemic effects, as suggested in canine studies [36,37], where virus can spread inadvertently to the other eye, optic nerves and to the brain. None of the patients use any immunosuppressive drugs, suffer from any immunosuppressive disease nor is pregnant/breasfeading.

Subjacent to the first and second proposed criteria are some uncertainties. Firstly, a predictive relation between a pair of mutant *RPE65* alleles and resulting disease severity is currently unknown. Although a correlation between missense mutations and remaining wild-type enzyme function was established [17], in which extent it is proportional to the patient's phenotypic presentation is still controversial [16], making an inclusion criterion according to a certain *RPE65*-mutation less reliable. Second, the clinical diagnosis encloses some ambiguity depending on the age of diagnosis, clinical impression, timing and tests performed and variability of disease expression [4]. Third, a visible photoreceptor (outer nuclear) layer on a SD OCT scan is a *sine qua non* proposition, meaning that patients in advanced stages of LCA, where no intact or at least existing but malfunctioning cellular machinery is available, is no candidate for gene or pharmacological therapy. This inclusion criterion is associated with the concept of a time window opportunity, justified by the disproportion between visual function and photoreceptor loss in *RPE65*-associated retinal dystrophies, contrasting with other retinal dystrophies where loss of light sensitivity (in linear units) is proportional to the square of ONL thining [14,18]. This opportunity window is defined by a visual loss caused only by a diminished concentration of visual

chromophore 11-cis-retinal, increased thermal activation of the phototransduction pathway and intact visual pathway anatomy, the latter already suggested by high-resolution MRI image studies [38]. With further photoreceptor destruction, this window opportunity is closed.

Unfortunately, only two of our patients meet all criteria. In the other two relatives, ACM and MAMT, there is neither evidence of the photoreceptor layer (as depicted in MAMT's OCT) nor sufficient BCVA (in both ACM and MAMT), meaning that the window of opportunity for this therapeutic approach was shut. Although this conclusion raises the hypothesis of a correlation between age, quantity and quality of remaining photoreceptors and therefore eligibility for gene therapy, it raises as well a common misconception about *LCA2*. This proposition is not supported in any given cross-sectional sample of *RPE65*-LCA patients in the first three decades of life since substantial inter-patient variability of the human disease allows no reliable predictions of disease severity with age. Nonetheless, there are some dose escalation clinical trials [39] and murine model studies [40,41] that consistently showed overall improvement in younger patients. Consequently, a certain younger age is not synonym for an intact photoreceptor layer; although on average, according to the disease's pathophysiology, with an earlier accurate diagnosis, there is greater chance for the patient to be within the window opportunity.

Therefore, in the future, an increased diagnose objectivity combined with an extensive knowledge on the genotype-phenotype correlation would be critical for a disease diagnosis within the mentioned window opportunity, making possible the inclusion of a greater number of patients. As gene therapy evolves, genetic diagnostic testing will be a paramount in identifying patients with pre-phenotypic variants of the disease. Custom microarrays could be used to detect a battery of specific known

mutations without having to screen genes individually [1]. The method of measuring retinal function activity with dark adapted FST, combined with microperimetry and TPLR help to monitor quantitatively rod- and cone-based photoreceptor function and could refine diagnosis and follow-up strategies [1,4,13].

In conclusion, because two of the patients – MAMT and ACM - already are in an advanced stage of the disease, where the criteria of an evidence of the photoreceptor layer and sufficient BVCA are not met, their inclusion in a gene therapy or pharmacological clinical trial in no longer an option. This study suggests the need of an early genetic based LCA2 diagnosis, since younger patients with greater populations of viable photoreceptors stand to gain the most from early intervention with gene and pharmacological therapy.

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