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Multimodal Imaging in Autosomal Dominant Cone-Rod Dystrophy Caused by Novel *CRX* Variant

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Keywords
Cone-rod dystrophy · *CRX* · Multimodal imaging · Inherited retinal dystrophies · Optical coherence tomography angiography

Abstract
Aim: To characterize by multimodal approach the phenotype of patients from a 3 generations pedigree, affected by autosomal dominant cone-rod dystrophy (CRD), found to carry a novel pathogenic variant in the cone-rod homeobox-containing (*CRX*) gene. ***Methods:*** Examination of the adult patients included the following tests: visual acuity, multicolour imaging, spectral domain optical coherence tomography (SD-OCT), fundus autofluorescence (FAF) and OCT angiography (OCT-A) recordings. In a 2.5-year-old child, cycloplegic refraction, fundoscopy, ocular motility evaluation and electrophysiological exams were performed. Next Generation Sequencing of patients' DNA has been carried out. ***Results:*** A novel *CRX* pathogenic variant has been identified in our patients. The 2.5-year-old child in the third generation was found to have inherited the variant, with no clinical signs of the condition, but electroretinographic abnormalities in the scotopic component. In the adult patients, diffuse atrophy of the retinal pigment epithelium/photoreceptor complex in the macular region was evident at the OCT and FAF, while OCT-A showed choriocapillaris density reduction. ***Conclusions:*** Multimodal study allowed the characterization of a peculiar form of CRD. The novel pathogenic variant seems to have a different effect on the phenotype if compared with a previously described similar one, giving an insight into the pathogenic mechanism of *CRX*-related retinal dystrophies and offering valuable information that could lead to the development of possible future therapies.

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Introduction

Inherited Retinal Dystrophies (IRDs) include a vast number of pathologic traits, characterised by high genetic and phenotypic heterogeneity. Part of this spectrum is represented by cone-rod dystrophies (CORDs), progressive inherited retinal disorders predominantly characterized by cone dysfunction in the early stages and subsequent rod degeneration [1]. The estimated prevalence is 1/40,000, and so far 33 implicated genes and at least 5 additional loci have been identified [2]. Transmission patterns in familial cases can be autosomal dominant, autosomal recessive and X-linked.

Patients mapping to the CORD2 locus have been found to carry pathogenic variations in the cone-rod homeobox containing gene (*CRX*, located at 19q13, MIM 602225), also involved in an autosomal dominant form of Leber Congenital Amaurosis (LCA) [3, 4].

CRX is a photoreceptor-specific homeodomain transcription factor gene. In adults, it is expressed predominantly in photoreceptors and pinealocytes, playing a significant role in the differentiation and maintenance of photoreceptor cells by synergistic interaction with other transcription factors such as neural retina-specific leucine zipper protein (NRL) and retinal homeobox protein (RAX) [5–7]. Variations in the *CRX* gene cause the autosomal dominant form of CORD mapped at 19q13, either by haploinsufficiency or by a dominant negative effect [5].

Pathogenic variations in the *CRX* gene have been reported in 2.35% of LCA, in 4.76% of CORD and in 0.80% of Retinitis Pigmentosa (RP) cases [8]. *CRX* variation types as well as their localization within the gene are not associated with phenotypic differences (CORD vs. LCA vs. RP), indicating a lack of genotype – phenotype correlation [8].

At present, no treatment is available for *CRX*-related IRDs, but great efforts of the scientific community are being directed towards the feasibility of molecular therapies. In this perspective, great importance is given to both the genetic and clinical characterization of affected patients.

In this study, we described 3 members of a 3-generation pedigree affected by an autosomal dominant form of CORD with the aim of describing genotype-phenotype correlations by carrying out a comprehensive clinical characterization by multimodal approach. Patients underwent molecular genetic characterization revealing a novel *CRX* and an additional *CNGA3* variation.

Methods

Three members of a 3-generation family affected by autosomal dominant cone-rod dystrophy (CRD) have been studied. Patients' characteristics are described in Table 1. Patient 1 (Pt1) is our proband, patient 2 (Pt2) is her father and patient 3 (Pt3) is her son.

Ophthalmological characterization of Pt1 and Pt2 included the measurement of best corrected visual acuity (BCVA; expressed by Snellen equivalent fraction), dilated fundoscopy, multicolour imaging, Spectral domain-optical coherence tomography (SD-OCT), blue autofluorescence (FAF; Spectralis HRA+, Heidelberg Engineering, Heidelberg, Germany), OCT angiography (OCT-A; OptovueAngioVue System, Optovue Inc, Fremont, CA, USA). In Pt3 (aged 2.5 years at the time of evaluation) cycloplegic refraction, dilated fundoscopy, ocular motility evaluation and full-field electrophysiology including visual evoked potentials and photopic and scotopic electroretinogram (ERG) recordings performed with skin electrodes (Retimax, CSO, Florence, Italy). By using skin electrodes and ISCEV standards recording settings, full-field flash scotopic ERG after 20 min of dark adaptation was performed. Right after the young patient was exposed to 20 min of light adaptation to record the full-field flash photopic ERG. We also recorded flash visual evoked potentials showing normal responses (data not shown).

Following phenotyping, patients underwent an interview during which time information was collected about symptoms, age of onset and family history. At that stage, the genetic nature of the disease was explained and the molecular genetic testing has been prospected.

Blood Samples were Collected and Sent to the MAGI's Laboratories, were 200 μL were used for DNA extraction using a commercial kit (Blood DNA kit E.N.Z.A., Omega bio-tek; Norcross, GA, USA).

Proband's DNA was sequenced using a custom-made oligonucleotide probe library. Briefly, exons and intron-exon junctions of a panel of CORD genes (*CNGA3*, *GUCY2D*, *C8orf37*, *PROM1*, *GUCA1A*, *CERKL*, *SEMA4A*, *CRX*, *AIPL1*, *RPGRIP1*, *ABCA4*, *PITPNM3*, *PRPH2*, *ADAM9*, *RPGR*, *CDHR1*, *RIMS1*, *RAX2*) were enriched through liquid phase hybridization technology and analysed by massive parallel sequencing (Illumina MiSeq, PE 2 × 150 bp Protocol). Obtained sequences were mapped to the human reference sequence GRCh38. The mean coverage resulted in 310.79× with a coverage of at least 25× for 97.95% of the target region. Sequence variant calling was performed using 3 calling tools: GATK Unified Genotyper, Varscan (version v2.3) and Bcftools of SAMTools (version 0.1.19-44428cd); the filter-based annotation was performed using *Annovar* software and public databases such as 1000 Genomes (http://www.1000genomes.org/), dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP) and Exome Variant Server (evs.gs.washington.edu/EVS) databases; variant-disease association databases: Human Gene Mutation Database (HGMD), HumsVar (http://omictools.com/humsavar-tool) and LOVD (Leiden Open Variation Database). The pathogenicity of variants was predicted *in silico* by using 3 different software: Mutation Taster (http://www.mutationtaster. org/), SIFT (Sorting Intolerant From Tolerant, http://sift.jcvi.org/www/SIFT_enst_submit.html) and PolyPhen-2 (Polymorphism Phenotyping v2, http://genetics.bwh.harvard.edu/pph2/index.shtml).

The reported nucleotide variants were confirmed by Sanger sequencing(CEQ8800 Sequencer, Beckman Coulter).

Putative pathogenic variants were screened in proband's affected father to study the genotype-phenotype segregation and in her son to evaluate the disease transmission.

This study is a retrospective case series description that does not require the approval of the Ethics Committee. Written, informed consent was obtained prior to their inclusion in this study. The informed consent forms include consent for the use of anonymised genetic results for scientific publications. The research adhered to the tenets of the Declaration of Helsinki.

Results

Our proband (Pt1), aged 31 at the time of our observation, was referring to progressive visual acuity decay since she was 17. The diagnosis of retinal dystrophy had been already made elsewhere and previous documentation was produced including a report of electrophysiological exams performed when she was aged 25, revealing a dysfunction of both cones and rods photoreceptors. Additional reported symptoms were photophobia and impaired dark adaptation. Patient reported her father to be affected by the same disease and therefore we asked him to go through an examination (Pt2) before proceeding with molecular genetic investigations.

Pt2 was aged 62 at the time of our first observation. He referred decay in visual acuity since his early twenties. He produced an ophthalmologist's certificate reporting his BCVA being 0.1 (Snellen equivalent) in both eyes when aged 32. The patient underwent the same set of investigations as his daughter. His right eye examination and imaging were performed with difficulty due to an asteroid hyalosis and unsteady fixation. Clinical features of our patients are reported in Table 1.

The collected data about the family history excluded the presence of other affected family members, although Pt2's father died aged 35 and it has been highlighted by the patient that in the old days, the presence of debilitating conditions was easily misdiagnosed or even hidden.

The proband's male child (Pt3), aged 2.5 years, did not show any sign suggesting low vision such as nystagmus, misalignment or poor fixation, but since in the verbal age, he complained undefined discomfort when in dim light conditions. Electrophysiological testing showed reduced b-wave amplitude in the scotopic ERG and normal a- and b- wave implicit times as compared to age-matched controls. The photopic responses revealed amplitude and implicit time values within the normal limits. The traces were clearly detectable with a high level of reproducibility (Fig. 1). Visual evoked potentials revealed normal P100 latency and N75-P100 amplitude (data not shown).

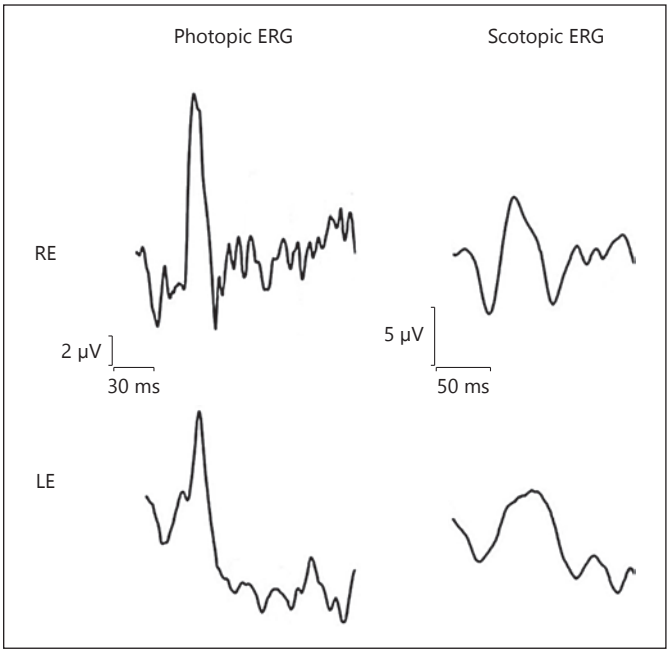

Fig. 1. Patient number 3 full-field flash ERG layouts. The scotopic electroretinogram shows reduced b-wave amplitude and normal a- and b- wave implicit times as compared to age-matched controls. The photopic responses revealed amplitude and implicit time values within the normal limits. ERG, electroretinogram; RE, right eye; LE, left eye.

Table 1. Clinical features of CORD patients

Patients	BCVA RE-LE (Snellen Equivalent)	Age at onset (referred)	Symptoms
1	0.1–0.08	17	LVA, PH, NYC
2	0.2–0.08	21	LVA, PH, NYC
3	ND	No clinical symptoms	NYC (probable)

CORD, cone-rod dystrophies; BCVA, best corrected visual acuity; RE, right eye; LE, left eye; LVA, low vision acuity; PH, photophobia; NYC, nyctalopia; ND, not detectable.

In the multimodal imaging context, in Pt1 and Pt2, multicolour images showed extensive macular atrophy. Blue FAF revealed an absence of autofluorescence corresponding to the ophthalmoscopically evident atrophy areas. SD-OCT showed the absence of photoreceptoral layer and IS-OS junction in the macular area. Finally, OCT-A images, despite the presence of motion artefacts related to fixation instability, revealed a significant reduction in the choriocapillaris

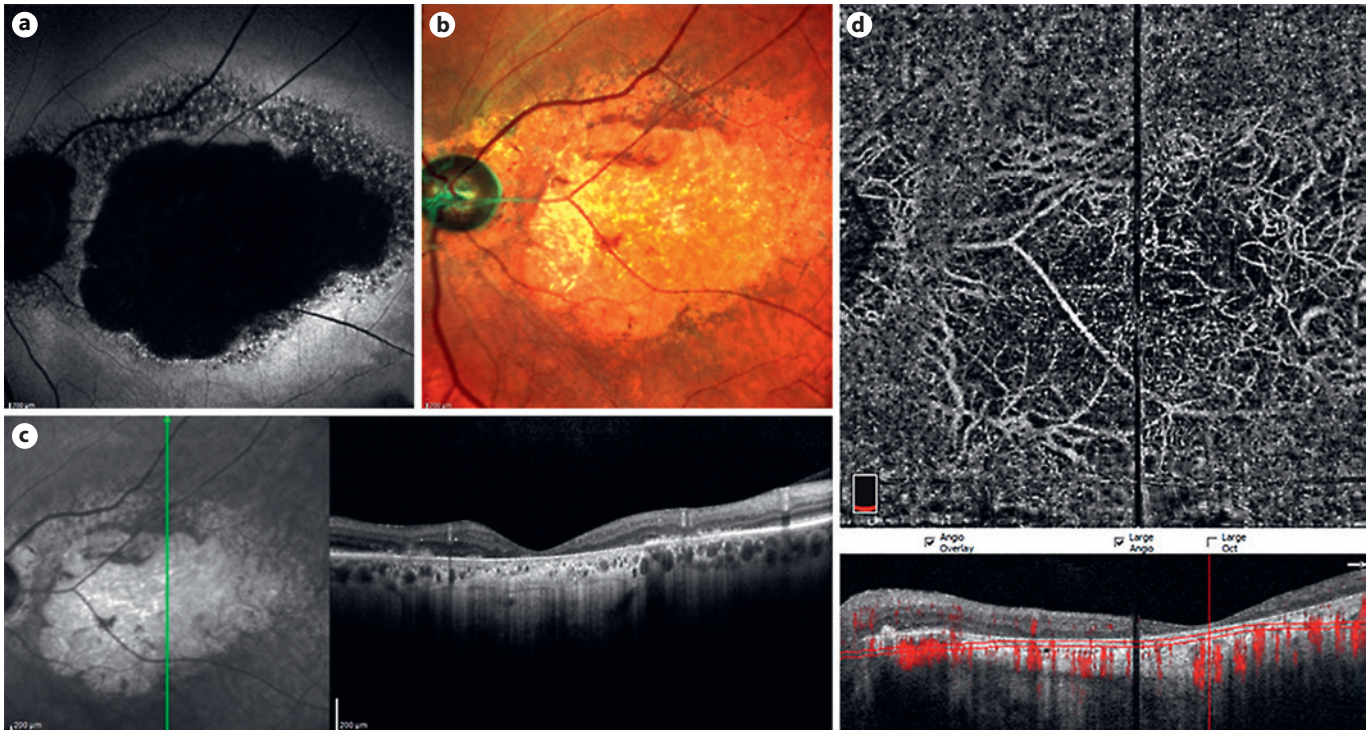

Fig. 2. Patient number 2 Multimodal Imaging. **a** Blue autofluorescence revealing decreased or absent autofluorescence corresponding to the atrophic areas. **b** Multicolour images showing macular atrophy. **c** SD-OCT scan through the fovea showing the absence of photoreceptoral layer and IS-OS junction in the macular area. **d** OCT-A showing a significant reduction in choriocapillaris density (central dark line is an artefact due to poor fixation).

density in an area corresponding to the atrophic one detected both with the multicolour and the autofluorescence imaging (Fig. 2).

The phenotype did not show any relevant differences between father and daughter in regard to their age.

Patients' phenotype characterization and pedigree evaluation allowed the possibility to define the model of inheritance of the CRD in this family as autosomal dominant.

Molecular testing in the proband revealed the presence of a novel *CRX* gene variant c.429del: p.(Pro145Leufs*42; NM_000554) and of a second variant in the *CNGA3*gene: c.1618G > A: p.(Val540Ile; NM_001298). The *CRX* variant has never been previously described but has been considered pathogenic, since it is a frameshift variant that leads to a premature stop codon thus shortening the protein. *In silico* analysis also suggested a causative role. The *CNGA3* variant has been previously described as pathogenic in recessive cone dystrophies; however, being in an heterozygous state in all family members, it cannot be considered pathogenic, although a modifier effect cannot be excluded.

The *CRX* variant was also confirmed to be present in the proband's son and in her affected father through targeted sequencing.

Sequence chromatogram of the *CRX* variation and family pedigree are shown in Figure 3.

Discussion

In this family, phenotypes were not different between the 2 adult patients in terms of age of onset, imaging characteristics and severity.

The evaluation of exhibited past years documentation of clinical features (BCVA and visual field) together with the referred age of onset of photophobia and nyctalopia, allow the possibility to define both severity and progression as matching in the 2 adult family members. Interestingly, CORD2 patients traditionally reported that the primary defect is in the night vision and this seems to be the case of the younger Pt 3 in consideration of behavioural and electrophysiological data. Conversely, adult Pts 1 and 2, despite reporting nyctalopia, mainly complain about

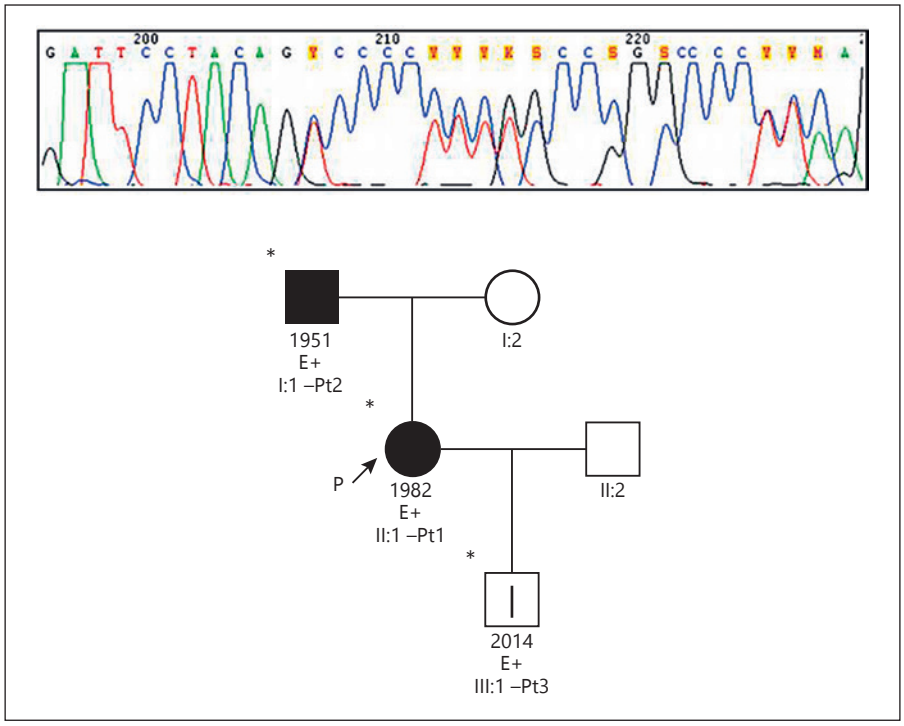

Fig. 3. Electropherogram and segregation of the CRX variant in the CORD family. Sanger sequence chromatogram and family pedigree illustrating cosegregation of the p.(Ser143Argfs*44) *CRX* variation identified in CORD affected patients. * Documented clinical evaluation. Year of birth is indicated below each symbol. E+, positive to genetic test; |, asymptomatic/presymptomatic carrier; P, proband.

photophobia coupled with vision decay starting around the beginning of the second decade of life. This could be interpreted as an initial rods dysfunction with potential subsequent and progressive cones involvement.

The p.(Pro145Leufs*42) variant is novel and lies in a codon that is highly conserved among species. Only one study can be found in literature reporting a similar heterozygous variation c.429_430delTCinsA or p.(Ser143Argfs*44) associated with an LCA phenotype, far more severe than the one our patient had [9]. It is intriguing to note that when comparing the 2 variations, the only differences concern the first 2 amino acids (namely the 144 and the 145) involved in the frameshift p.(Ser143Argfs*44), while the remaining 42 amino acids are identically shifted until the premature stop codon, at position 186, thus loosing 114 amino acids in respect of the wild-type sequence (Fig. 4). Both variants conserved the homeodomain, thus retaining the DNA-binding capability, and both result in a C-terminus truncated CRX protein that fails to activate transcription, being the essential portion for the CRX-mediated transcriptional in the C-terminal region, between amino acids 200 and 284, that is completely lost.

The reason why these 2 variations resulted in phenotypes of such different severity could be difficult to understand. The marked phenotypic heterogeneity among patients bearing *CRX* variations is known [10] and has been described even in patients carrying the same variation

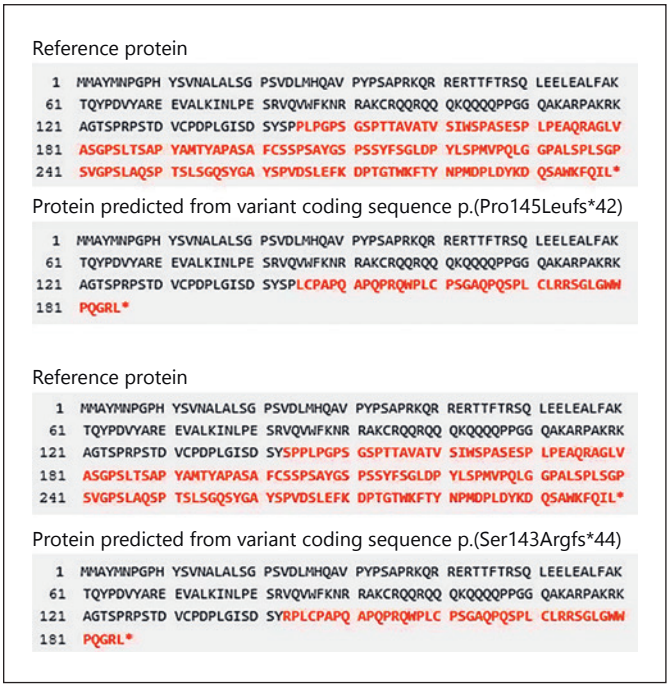

Fig. 4. Aminoacid sequence resulting from the 2 similar CRX variations. Comparison between p. (Pro145Leufs*42) and p.(Ser143Argfs*44) *CRX* frameshift variants, respectively, associated with CORD and LCA.

[11]. Furthermore, phenotypic variability was demonstrated in *crx*-mutant mouse models as a consequence of graded changes in photoreceptor gene expression. Since CRX acts as a transcriptional factor in photoreceptor transcription, mainly as activators but also as a repressor, *CRX* variations can have a deep impact on the delicate balance of cellular pathways critical for photoreceptor function and maintenance [12].

Both variations fall in the class III (antimorphic frameshift/nonsense variations with intact DNA-binding) of a 4-degree system of classification, as reviewed by Tran and Chen [13]. Therefore, patients' phenotypic differences led by these 2 proteins, which are predicted to be prematurely stopped with the same length (AA 186) and with the only difference of 2 amino acids, could be explained by the mechanism of the allele-specific overexpression of the mutant CRX protein; that is, a different level of expression of the mutant CRX protein that interferes with the function of wild type CRX, impacts the disease severity, as shown in mouse models with at least 2 different class III variations [14]. It is not clear though if this phenomenon is conserved for all Class III variations and which are the underlying cellular mechanisms involved [13]. Alternatively, we can speculate that these frameshift variations are likely to stop protein translation due to nonsense-mediated mRNA decay [15] and, in this case, other genetic factors are therefore probably involved to explain the profound effect on the resultant phenotype. For a better comprehension, further investigations are then required.

In conclusion, genetic characterization of IRDs is a crucial point for the identification of molecular mechanisms underlying pathological phenotypes.

In the era of next generation sequencing (NGS) technology, very often multiple gene variants that are potentially causative are identified and the role of each one needs to be assessed.

Our findings confirm the phenotypic heterogeneity of *CRX*-related IRDs. Moreover, the p.(Pro145Leufs*42) variation is novel, thus broadening the spectrum of *CRX* variations described so far with our report.

While the novel *CRX* identified variant is clearly pathogenic, the role of the second *CNBA3* variant is inconclusive, as it is present in a heterozygous state in all the patients. The recessive character and the role of CNGA3 protein in the retinal function (very different from the CRX one) induce us to exclude an additive pathogenic effect.

Advanced imaging is acquiring great value in the characterization of the different phenotypic expressions of genetic retinal dystrophies. Definition of the retinal structure alterations can give new information about underlying pathological mechanisms and the correlation with the different genes can give insights in the gene function itself. To our knowledge, there are no previous studies specifically correlating *CRX*-related IRDs and OCT-A features.

The interpretation of our finding of choriocapillaris loss in our patients is controversial regarding its nature. It could be a direct consequence of retinal pigment epithelium (RPE) atrophy suggesting a role of the RPE in the modulation of choriocapillaris structure and function. RPE, in fact, is known to produce vascular endothelial growth factor (VEGF) and VEGF receptors are expressed on the choroidal endothelium facing the RPE [16]. Conversely, choroidal vessels providing the vascular support to outer retinal layers could be primarily responsible, suggesting a possible pathogenetic role of choriocapillaris atrophy in photoreceptors degeneration [17]. CRX is known to have a role in retinal development and maintenance and expression studies reveal its presence in both cone and rod photoreceptors, possibly in the bipolar cells [18] but not in the RPE neither in any level of the choroid [19]. This data, coupled with the observation that areas of impairment appear to be overlapping at OCT-A and FAF, lead us to conclude that choriocapillaris' reduced density is more likely to be a consequence of the Photoreceptor/RPE complex dysfunction rather than a primarily localized defect.

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Disclosure Statement

The authors report no conflicts of interest.

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