DEEP PHENOTYPING OF PROM1-ASSOCIATED RETINAL DEGENERATION

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SYNOPSIS/ PRECIS

This is the first in-depth analysis including AOSLO in patients with PROM1-RD. The evidence of residual cones in both autosomal dominant and recessive disease presents an opportunity for potential therapeutic intervention.

ABSTRACT

Background/Aims: The purpose of this study was to investigate retinal structure in detail of subjects with autosomal-dominant (AD) and autosomal-recessive (AR) *PROM1*-associated retinal degeneration (*PROM1*-RD), study design: institutional, cross-sectional study

Methods: Four eyes from four subjects (three with ad and one with ar) *PROM1*-RD were investigated by ophthalmic examination including best-corrected visual acuity (BCVA) and multimodal retinal imaging: fundus autofluorescence (FAF), spectral-domain optical coherence tomography (SD-OCT) and adaptive optics scanning light ophthalmoscopy (AOSLO). Quantitative assessment of atrophic lesions determined by FAF, thickness of individual retinal layers and cone photoreceptor quantification was performed.

Results: BCVA ranged from 20/16 to 20/200. Initial pathologic changes included the presence of hyperautofluorescent spots on FAF imaging, while later stages demonstrated discrete areas of atrophy. In all patients, thinning of the outer retinal layers on SD-OCT with varying degrees of atrophy could be detected depending on disease-causing variants and age. Cone density was quantified both in central and/or at different eccentricities from the fovea. Longitudinal assessments were possible in two patients.

Conclusions: *PROM1*-RD comprises a wide range of clinical phenotypes. Depending on the stage of disease, the cone mosaic in *PROM1*-RD is relatively preserved and can potentially be targetted by cone-directed interventions.

SUMMARY BOX

- **What is already known on this topic:** *PROM1*-associated retinal degenerations can be caused autosomal dominant and recessive leading to different phenotypes.

- **What this study adds**: Both types of *PROM1*-associated retinal degenerations are characterized by multi-modal imaging down to a celullar level including Adaptive-optics scanning light ophthalmoscopy

- **How this study might affect research, practice or policy**: The evidence of residual cones in both autosomal dominant and recessive disease presents an opportunity for potential therapeutic intervention, for which the proof of principle has been shown in the knockout mouse model.

INTRODUCTION

The *PROM1* gene codes a protein called Prominin 1 (PROM1; also known as CD133 and AC133), which contains two large, highly glycosylated extracellular loops and a cytoplasmic tail.[1-3] Originally used as a human stem cell-specific marker[4], its role both during the formation and organization of disks within the photoreceptor outer segment (OS) [2], as well as in cytoplasmic function by regulating autophagosome maturation and trafficking of the retinal pigment epithelium (RPE) has been elucidated;[3 5] also extraocular manifestations have been described.[3 6]

Retinal phenotypes ranging from milder forms such as bull's-eye maculopathy (BEM) and isolated macular dystrophy to panretinal cone, cone-rod and rod-cone dystrophy, have been reported;[7-9] There are both autosomal dominant [7] and autosomal-recessive forms.[9]

Basic research has paved the way for potential pharmacotherapy in *PROM1*-related retinal degeneration (RD),[10] and recent advances in adeno-associated viral vector-based gene therapy and optogenetics could become further successful treatment options.[9 11]

In order to prepare future therapeutic approaches and to design appropriate clinical trials, the "Natural History of the Progression of Atrophy Secondary to Stargardt Disease type 4 (STGD4)" (ProgStar- 4 Study) was launched.[3] It is known that the onset and progression of the diseases varies considerably in patients, even within a single family,[2 7 10 12] and molecular mechanisms underlying the variation in progression of *PROM*1-RD may reflect variation in environmental factors or modifier genes.[10]

However, little is known about how specifically cone photoreceptors are affected by *PROM1* sequence variants. Adaptive optics (AO) enables advanced retinal imaging for in-depth phenotyping with cellular resolution of the photoreceptor mosaic by correcting for the monochromatic aberrations of the eye. Using a confocal detector, cones can be visualised based on their waveguidung ability which is thought to be based on intact photoreceptor outer segments (OS).[13-15] The split detection technique exploits the non-confocal multiple-scattered light to resolve photoreceptor inner segments; it applies the subtraction of images divided by their sum by capturing the light to the left of the confocal aperture with one detector and the light to the right of it with another.[16 17]

The purpose of this study was to investigate the retinal structure on a cellular scale in subjects with different forms of *PROM1*-RD.

METHODS

Twelve patients from four pedigrees with likely disease-causing variants in *PROM1* were identified at Moorfields Eye Hospital (MEH), London, UK. Six patients were recruited to the ProgStar-4 study[3] and these were also invited to participate in the deep phenotyping study at MEH (i.e. additional imaging by AO Scanning Light Ophthalmoscopy; AOSLO), which was approved by the Ethics Committee of MEH, and separate written informed consent was obtained from all subjects prior to enrollment. The research followed the tenets of the Declaration of Helsinki. AOSLO imaging was possible in four patients from three pedigrees.

Ocular examination

Ocular examination included best-corrected visual acuity (BCVA) according to the “Early Tretament of Diabetic retinopathy study“ (ETDRS) protocol and biomicroscopy of the anterior segments. The right eye of each patient was chosen for AOSLO imaging.

Fundus autofluorescence

Fundus autofluorescence (FAF) images were obtained after pupil dilation using a custom FAF acquisition software that was formalized and deployed for exclusive use [18], which implements the concept of short-wavelength reduced-illuminance autofluorescence imaging described by Cideciyan et al.[19 20] Atrophic lesions were analyzed by two independent graders (GS and RWS) according to previously established grading protocols using the Heidelberg Eye Explorer version 6.3.4.0.[18 20 21] The optic nerve head served as a reference point for “100% level of darkness“, while the background AF evident in the periphery of the image served as the reference for normal AF. Areas with level of darkness ≥ 90% in reference to the optic nerve head or blood vessels were defined as “definitely decreased autofluorescence” (DDAF), whereas darkness levels ranging between 50 and 89% darkness were defined as “questionably decreased autofluorescence” (QDAF).[3 20 21]

Spectral-domain optical coherence tomography (SD-OCT)

Using a Heidelberg Spectralis device, an infrared reflectance image and a SD-OCT 20° x 20° volume scan consisting of 49 B-scans centered on the anatomical fovea (Automatic Real Time; ART mean of ≥9 frames) were acquired. Images were independently graded by two unmasked graders (GS, MG) with adjudication by a senior investigator (RWS) in cases of disagreement between the initial graders. Images with insufficient quality (ungradable images) were excluded from analysis. A SD-OCT grading application, OCTOR 3.0 developed by Doheny Image Analysis Laboratory (DIAL) and validated at Doheny Imaging Readling Center, was used for viewing, annotating and quantifying OCT scans. A DIAL algorithm was used to generate automated segmentations which served as a starting point for manual readjustments, to support quantitative assessment using OCTOR 3.0 as previously described.[22] Results for thickness of the individual layers were derived from grading for the inner subfield, inner ring and outer ring of the ETDRS subfield grid.[22]

At least 25 of 49 B-scans per volume scan were graded per eye/visit using the adopted grading method.[23] In each of the selected B-scans, the following boundaries, presented in innermost (anterior) to outermost (posterior) order were segmented (see Supplementary Figure 1):

- Inner Limiting Membrane (ILM)
- (Dendritic) Outer Plexiform Layer (OPL)
- External Limiting Membrane (ELM)
- Inner Segment-Outer Segment (IS-OS) Junction or ellipsoid zone (EZ)
- Retinal Pigment Epithelium (RPE)
- Inner choroid boundary (ICB)

In B-scans where a given layer had been completely absent, the immediately adjacent posterior boundaries were snapped together resulting in a thickness value of 0. By applying these boundaries, the following layers were outlined and segmented:

- Mean inner retinal thickness (IR): generated from the two boundaries: ILM and inner boundary of OPL
- Mean ONL thickness (ONL): OPL and ELM
- Mean IS thickness (IS): ELM and IS-OS junction

- Mean OS thickness (OS): IS-OS Junction and inner boundary of RPE cell layer
- Mean RPE thickness and intact area: RPE Cell Layer inner boundary and inner choroid boundary
- Mean total retinal thickness (TR) and intact area: ILM and inner choroid boundary

The data were compared to a normative database from 20 healthy, age-matched individuals either within or greater than the standard deviations (SD) from the normal mean.

AOSLO Imaging of the Photoreceptor Mosaic

AOSLO was attempted in all six subjects participating in the ProgStar-4 study using a previously described custom-built AOSLO at Moorfields Eye Hospital (MEH)/UCL Institute of Ophthalmology, London, UK.[24] The procedure included simultaneous confocal and split detection (non-confocal). Image sequences of at least 150 frames each were obtained over the foveal centre (exploiting the foveal reflex of the vitreous/ILM interface) or the preferred retinal locus (PRL) and strips extending from that landmark to 5 degrees in the temporal, superior, nasal and inferior directions. Each image sequence was processed by a desinusoiding algorithm before individual frames were selected, registered and averaged to increase the signal-to-noise ratio for subsequent analysis.[25] The resulting images were aggregated into a single montage (Adobe Photoshop; Adobe Systems, Inc., San Jose, CA, USA) in layers for the different detection schemes by using a custom-built automated software.[26] The scale was determined as previously described[24]: first in degrees per pixel in an image of a Ronchi ruling of known spacing, after each imaging session, followed by the linearly scaling of the value by using each subject's axial length. Cone density was used for quantitative assessment of the photoreceptor mosaic as previously described:[24 27] cone coordinates from every given foveal image were extracted following manual annotation by a single, experienced grader (MK). The peak (foveal) cone density (PCD, cones/mm^2) was calculated by using the coordinate arrays where possible. The average (± SD) of two 55µm square sampling windows was calculated to determine the mean cone density at eccentric regions of interest (ROIs) in the superior, inferior, temporal and nasal directions away from either the PCD or the PRL.

The ROIs were cropped and imported into a custom software (MOSAIC, Translational Imaging Innovations, Rayleigh, NC) for cone counting using either confocal or split-detection imaging by two experienced graders (MK and NS). Bound cone density was then calculated by dividing the total number of bound Voronoi cells in an ROI by the total bound Voronoi area within the ROI, as previously described.[15 28] The mosaics were also qualitatively characterised.[24] The eligibility criteria for AOSLO analysis were strict; low quality images (due to involuntary eye motion or low signal-to-noise ratio) and non-contiguous montages were excluded from further analysis.

RESULTS

Analyzable montages with sufficient image quality could be acquired in four patients at least once and in two, also longitudinal data could be obtained. The limiting and confounding factors for successful image acquisiton in *PROM1*-RD included unstable fixation and lack of structure. Despite the high theoretical transverse resolution of the AOSLO (2 microns) some foveal cones could not be resolved due to the phenotype of the condition (clusters of non-waveguiding cones). All patients showed unremarkable anterior segments.

Patient P1

This 45-year old male (patient ID2 of the ProgStar-4 study[3]), harbouring the *PROM1*(NM_006017.3):c.1117C>T (p.Arg373Cys) variant as determined by bidirectional Sanger sequencing, presented with BCVA of 20/16 (ETDRS score 88 letters) in the right eye. In FAF imaging, the foveal centre appeared to be regular; however, it was surrounded by multiple tiny spots of both DDAF and hyperautofluorescence (Figure 1a). This area of heterogeneity measured 20.83±0.16 mm^2. SD-OCT analysis showed preservation of the foveal center and thinning of the RPE, OS, ONL and total retina in the inner and outer ETDRS rings (Table 1a,b; Figure 2a).

In AOSLO imaging, cones within the foveal centre could not be fully resolved in order to be reliably quantified, with the location of the foveal centre defined as the centre of the non-wave-guiding area (crossing point of maximum height and width). The highest cone density directly adjacent to the foveal centre (hence not PCD)

measured 72315 cones/mm^2. The rest of the eccentric ROIs are shown in table 2 and the nasal strip in Figure 3. In the confocal AOSLO images, a bull's-eye maculopathy (BEM) pattern could be observed due to the wave-guiding cones in a shape of a ring at about 2 degrees away from the fovea (Figure 3 and supplementary Figure 2).

Table 1a: Mean retinal thicknesses [µm] for individual layers of patients are provided for the outer ring, inner ring and central subfield of the ETDRS-ring after manual correction and centration onto the anatomical fovea									
	Retinal pigment epithelium			Outer segments			Inner segments		
	Outer ring	Inner ring	Central subfield	Outer ring	Inner ring	Central subfield	Outer ring	Inner ring	Central subfield
P1	20,00	16,10	23,40	6,00	1,90	4,00	32,70	23,10	39,40
P2	19,50	19,80	21,10	0,30	0,50	5,40	30,60	5,00	23,60
P3: first visit	17,10	16,80	18,90	7,60	0,0	0,0	31,60	6,60	0,0
P3: 24 months later	17,80	13,40	11,50	8,50	0,2	0,5	24,90	0,70	0,0
P4: first visit	29,50	28,10	32,80	7,80	4,20	3,20	36,20	24,60	28,90
P4: 24 months later	17,20	17,80	19,90	7,80	4,00	3,00	36,70	22,30	29,60
Normative Data (mean standard deviation)[Y]	31,4±3,3	30,4±3,1	32,8±4,8	20,0±4,3	18,5±4,7	24,5±4,4	29,0±1,9	32,9±2,1	34,4±2,3

Table 1b: Mean retinal thicknesses [µm] for individual layers of patients are provided for the outer ring, inner ring and central subfield of the ETDRS-ring after manual correction and centration onto the anatomical fovea, as well as for the total scanned area

	Outer nuclear layer			Inner retina			Total retina		
	Outer ring	Inner ring	Central subfield	Outer ring	Inner ring	Central subfield	Outer ring	Inner ring	Central subfield
P1	68,30	64,00	96,80	140,90	162,60	81,80	268,50	275,10	248,70
P2	36,20	54,70	75,70	132,50	128,90	62,8	195,30	215,30	192,90
P3: first visit	58,40	28,80	48,20	106,80	140,60	86,20	220,30	213,40	182,00
P3: 24 months later	56,20	35,70	38,50	110,30	149,10	100,60	222,00	214,20	183,10
P4: first visit	69,10	54,80	83,80	122,90	133,90	69,20	264,40	242,10	217,90
P4: 24 months later	63,50	48,50	62,80	121,00	132,20	68,50	250,00	228,10	198,80
Normative Data[Y]	88,7±5,4	103,2±6,3	111,4 ±11,5	141,9±10,4	159,6±8,6	67,0±12,1	310,9±12,8	344,6±13,5	270,0±19,3

Y derived from 20 healthy subjects without any retinal pathology

Table 2: Mean (± SD) cone densities [cones/mm^2] for distinct regions of interest (ROIs) away from the foveal center [µm] are provided derived from adaptive optics scanning laser ophthalmoscope imaging for patients P1, P2 and P4)

Patient ID		PCD	Inferior			Temporal			Superior			Nasal		
			1°	2°	5°	1°	2°	5°	1°	2°	5°	1°	2°	5°
P1	Cone density	n/a	34774 (±197)	14656 (±2189)	5674 (±1651)	32159 (±69)	14105 (±973)	n/a	n/a	11515 (±2157)	7879 (±1393)	37629 (±339)	14711 (±856)	9696 (±615)
	ROI	0	287,5	575	1437,5	287,5	575	n/a	287,5	575	1437,5	287,5	575	1437,5
P2	Cone density	32505 (±517)	14270 (±1095)	9862 (±325)	n/a	14050 (±953)	9862 (±737)	n/a	10849 (±487)	n/a	n/a	13775 (±1283)	10413 (±1696)	n/a
	ROI	0	314,9	629,8	n/a	314,9	629,8	n/a	314,9	n/a	n/a	314,9	629,8	n/a
P3 First Visit	Cone Density	34135 (±755)	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
P3 Two years later	Cone Density	32409 (±275)	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	ROI	0	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
P4 First visit	Cone density	n/a	23030 (±1877)	n/a	4683 (±249)	n/a	n/a	4628 (±467)	21872 (±1032)	n/a	5950 (±467)	18457 (±1816)	13939 (±569)	5839 (±827)
P4 Two years later		n/a	19091 (±1249)	n/a	4353 (±487)	n/a	n/a	n/a	16088 (±1177)	n/a	5509 (±271)	17356 (±1383)	12287 (±569)	5344 (±605)

	ROI	0	281,8	n/a	1409	n/a	n/a	n/a	281,8	n/a	1409	281,8	563,6	1409

PCD= peak cone desnity; n/a = not applicable; SD = standard deviation

Patient P2

Direct testing for mutations in a Stargardt/Macular dystrophy panel by next generation sequencing (NGS) and consecutive Sanger sequencing detected mutations in this 31-year old male (ID 5 of the ProgStar-4 study[3]): a reported recessive mutation, *PROM1*(NM_006017.3):c.1354dup (p.Tyr452LeufsTer13) and a novel insertion/deletion in the *PROM1* gene, *PROM1*(NM_006017.3):c.630_c.630+8delGGTAAAAACinsAACTTGAATGAAA, which is classified as pathogenic according to the American College of Medical Genetics guidelines [29]. Both variants are predicted *in silico* to likely undergo nonsense-mediated mRNA decay and are loss-of-function variants [30]. BCVA was 20/80 (ETDRS score 55 letters) in the right eye. FAF imaging showed a central area of DDAF (measuring 0.49±0.01 mm^2) with an area of surrounding QDAF and additional QDAF spots with a summed area of 0.21±0.02 mm^2 (Figure 1b), although the posterior pole revealed a mostly preserved and homogeneous autofluorescence pattern. SD-OCT revealed a preserved but thinned foveal architecture, with atrophy of the outer retinal layers (Figure 2b, Table 1) at the peripheral macula.

Peak cone density was 32505±517 cells/mm^2 and cone counts were also possible in all meridians but not beyond two degrees of eccentricity due to lack of photoreceptor structure (Table 2).

Patient P3

This 54-year old female patient (ID 6 of the ProgStar-4 study[3]) from a different pedigree than P1 harbouring the same *PROM1*(NM_006017.3):c.1117C>T (p.Arg373Cys) variant, presented with a BCVA of 20/200 (ETDRS score 35 letters) in the right eye. FAF revealed a central DDAF lesion measuring 1.45±0.02 mm^2 and total lesion size of DDAF and QDAF comprised 8.82±0.22 mm^2 (Figure 1C). On SD-OCT imaging, the corresponding atrophy was reflected by reduced mean thickness of retinal sublayers (Table 1, Figure 2C).

AOSLO imaging was possible at the foveal centre and revealed a PCD of 34135±755 cells/mm^2. There was no remnant photoreceptor structure outside the foveal avascular zone within 5 degrees in all meridians, in keeping with SD-OCT.

Re-evaluation after 24 months showed a BCVA of 20/800 (ETDRS score 5 letters) in either eye. Lesion size of DDAF enlarged to 1.79±0.91 mm^2 and total lesion size (DDAF+QDAF) to 9.42±0.27 mm^2 (Figure 1D). SD-OCT showed further

reduction of mean thicknesses of retinal sublayers (Table1). AOSLO imaging showed a decline of PCD to 32409±275 cells/mm^2.

Patient P4

This patient (age at initial visit 31 years, daughter of P3 and ID 7 of the ProgStar-4 study[3]), and also carrying the *PROM1*(NM_006017.3):c.1117C>T (p.Arg373Cys) variant) was imaged longitudinally. At baseline, BCVA was 20/25 (ETDRS score 80 letters) in the right eye. FAF imaging did not show any areas of DAF, but faint dot-like discrete hyperautofluorescence was observed, in keeping with that seen in P1; these were however too faint for a reliable delineation (Figure 1E, 1F). SD-OCT showed a preserved RPE (Figure 2D), however with thinning of the outer segments and ONL (Table 1a). PCD could not be reliably determined, however peripheral cone counts could be performed (Table 2). After 24 months, BCVA was 20/40 (ETDRS score 70 letters) in the right eye. FAF imaging remained unchanged, however, in AOSLO imaging the number of cones decreased at all analysed areas (Table 2).

DISCUSSION

High-resolution retinal imaging provides potential surrogate outcome measures for degenerative diseases of the retina and may be accepted by the regulating authorities for therapeutic trials.[31] However, the ideal modalities for consecutive imaging and tracking of disease progression are still to be elucidated: FAF was chosen as the primary endpoint in ProgStar-4 in keeping with other studies investigating retinal degenerations.[20 21 32] The earliest manifestations seen in our patients, especially cases with the autosomal-dominant c.1117C>T variant, are hyperautofluorescent patterns at the macula, with consecutive atrophy development; a previous report speculated that this hyperautofluorescence might be caused by a window defect representing the AF of the RPE through an atrophic outer retina.[33] However, previous studies from *PROM1*-transgenic mice demonstrated abnormal lipofuscin-like deposits in the RPE, indicating that this cell type is also compromised,[2] and the role of *PROM1* in the regulation of photoreceptor autophagy in RPE cells was also described.[5]

Recently, quantitative AF (qAF) measurements demonstrated levels within normal limits in a patient with dominant *PROM1*-RD, whereas a patient with more severe autosomal recessive *PROM1*-RD (of similar age) showed substantially increased qAF levels.[34] It has been shown that *PROM1* is required for the maintenance of the expression levels of *ABCA4* and *RDH12*, which is consistent with the idea that *PROM1* is also involved in the regulation of the visual cycle, especially at the reducing step of all-trans-retinal to all-trans- retinol,[10] or a possible role of *PROM1* for an indirect effect on lipofuscin accumulation, through *ABCA4* dysfunction, resulting from a disrupted outer segment structure.[34] In advanced stages (like P3), the basic concept of FAF imaging to measure atrophy development may serve as a potential outcome measure.[31] However, the absence of any atrophic lesion (even QDAF as indicated in Figure 2E and 2F) as in patient P4, may restrict the application of this imaging modality (even of qAF), especially in early stages of diseases in which photoreceptors (and RPE) are amenable to rescue via pharmaco-, gene augmentation, or gene editing therapy.[11] Nevertheless, early intervention is

extremely important because significant photoreceptor loss occurs before the development of visual symptoms.[33]
Patients with such early stages appear to be ideal candidates for AOSLO imaging as this study show cases, which is – to the best of our knowledge - the first published series of AOSLO imaging in patients with *PROM1*-RD, both for autosomal dominant and autosomal recessive traits. Three of these patients carried the previously described AD *PROM1*(NM_006017.3):c.1117C>T (p.Arg373Cys) variant [7 9]; in one patient (P2), *PROM1*-RD was inherited in an AR fashion. The phenotypes of these patients – with P2 being the youngest of these cases but showing the largest amount of retinal atrophy as indicated by retinal thinning on SD-OCT - confirm previous reports that recessive disease is associated with early-onset severe panretinal degeneration.[2 12] This is supported by our findings, when AOSLO imaging of cones at several locations was feasible, although SD-OCT already showed significant thinning of the OS band. In contrast, the dominant *PROM1*(NM_006017.3):c.1117C>T (p.Arg373Cys) variant causes predominantly macular degeneration that mainly affects cone photoreceptors with later-onset dystrophy predominantly involving the macula.[2 9]

In two of the AD patients, longitudinal imaging and analysis was possible, and progressive cone loss, albeit mild, but importantly larger than the standard deviation, could be documented. In the aforementioned patient P4 with no areas of decreased FAF, AOSLO using the split-detector technique provided a robust method to visualize cone inner segment structure in a manner that appears to be independent of the integrity of the outer segment.[17] Individuals affected by *PROM1*-RD therefore are candidates for AOSLO imaging, in keeping with other retinal degenerations, where the outer segments are lost before the remainder of the photoreceptor cell.[16 17]
The pentaspan transmembrane domain glycoprotein encoded by *PROM1* is specifically localised to membrane protrusions at the base of rod and cone outer segments, where its key roles include disc morphogenesis and subsequent photopigment sorting [2], with wild-type PROM1 preferentially localising to basal disc membranes in rod OS and throughout outer rims of disc lamellae of cone OS.[9] Our quantitative SD-OCT analysis supports the paradigm of the outer segments' degenerative process, although a thinning of the ONL in all cases throughout the macula could also be observed, which is in alignment with a previously reported

case.[33] However, while Pajewala et al. did not describe a change in OCT imaging over a period of three years, we were able to demonstrate a decline in mean retinal thicknesses over two years in patient P4 by semi-automated segmentation of the features and affected layers in SD-OCT images which is an extremely laborious process. Furthermore, due to the disorganisation of the outer retina, it is very challenging to accurately segment the relevant boundaries and categorise tissues into the appropriate layers.[22]

This is the first in-depth analysis including AOSLO in patients with *PROM1*-RD. The evidence of residual cones in both autosomal dominant and recessive disease presents an opportunity for potential therapeutic intervention, for which the proof of principle has been shown in the knockout mouse model.

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Gernot Schliessleder: analysis and interpretation of data for the work, drafting the work, final approval of the version to be published and agreeing to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Angelos Kalitzeos: acquisition, analysis and interpretation of data for the work, revising the work critically for important intellectual content, final approval of the version to be published and agreeing to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Navjit Singh: acquisition, analysis and interpretation of data for the work, revising the work critically for important intellectual content, final approval of the version to be

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FIGURE LEGENDS

Figure 1: Examples from fundus autofluorescence (FAF) imaging of the right eye of the four patients. **Panel A (P1)**: The fovea of patient 1 exhibits the normal reduced FAF centrally due to photopigments of the photoreceptor outer segments and macular pigment. There are dots of definitely decreased autofluorescence (DDAF; examples highlighted by yellow arrows) and surrounding hyperautofluorescence (green arrow). Complete lesion of AF heterogeneity is encircled by the blue line measuring 20,83 mm^2. The white dashed line indicates the location of the spectral-domain optical coherence tomography B-scan in Figure 2, panel P1). The white rectangle outlines the region in which cone densities in Figure 3 were analysed. **Panel B (P2)**: Patient 2 shows a central lesion of DDAF (yellow line) which was surrounded by an area of questionably decreased autofluorescence (QDAF; green line) and additional small areas of DAF (blue arrows). The white dashed line indicates the location of the spectral-domain optical coherence tomography B-scan in Figure 2, panel P2) **Panels C+D (P3)**: Lesion of DDAF (yellow line) and QDAF (blue line) at first visit (panel C) and after 24 months (panel D). The white dashed line in panel C indicates the location of the spectral-domain optical coherence tomography B-scan in Figure 2, panel P3). **Panels E+F (P4):** FAF images from patient 4 at first visit and after 24 months. There are no areas of reduced AF, but tiny and faint hyperautofluorescent spots (green arrows). The white dashed line in panel E indicates the location of the spectral-domain optical coherence tomography B-scan in Figure 2, panel P4).

Figure 2: SD-OCT B-scans through the fovea of all four patients (P1 to P4) are shown. Patients with the autosomal dominant *PROM1*(NM_006017.3):c.1117C>T (p.Arg373Cys) variant (P1, P3, P4) all show reduced thicknesses of the outer retinal layers in the parafoveal region (orange arrows). In patient P2 with autosomal recessive disease (*PROM1*(NM_006017.3):c.1354dup (p.Tyr452LeufsTer13) and *PROM1*(NM_006017.3):c.630_c.630+8delGGTAAAAACinsAACTTGAATGAAA), the retinal layers at the foveal center were thinned, but present (between blue arrows), and there was atrophy of the outer retinal layers at the peripheral macula.

Figure 3: Confocal adaptive optics scanning laser ophthalmoscopy (AOSLO) (top row) and non-confocal split-detection AOSLO (middle row) derived photoreceptor mosaics of the right eye of patient P1, showing outer and inner segments, respectively. White squares (55 microns square) labelled A in the confocal image and B and C in the split-detection image (1, 2 and 5 degrees away from the foveal center, respectively) are magnified in the bottom row and were used for cell annotations. Dashed white lines indicate the location of the transfoveal SD-OCT B-scan shown in Figures 1 and 2.

Supplementary Figure 1: In each of the selected B-scans, the following boundaries, presented in innermost (anterior) to outermost (posterior) order were segmented: inner limiting membrane (ILM) – red line; inner boundary of outer plexiform layer (OPL) – blue line; external limiting membrane (ELM) – yellow line; inner segment-outer segment (IS-OS) junction or ellipsoid zone (EZ) – orange line; photoreceptor outer segment layer (OS) and retinal pigment epithelium (RPE) inner boundary – green line; inner choroid boundary (ICB) – purple line. In B-scans where a given layer had been completely absent, the immediately adjacent posterior boundaries were snapped together resulting in a thickness value of zero.

Supplementary Figure 2: AOSLO confocal images overlayed on the FAF image of the right eye of P1 indicating the extent of photoreceptor imaging performed across the four directions away from the fovea. The nasal strip is zoomed in Figure 3.