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Progressive Loss of Cones in Achromatopsia.

An Imaging Study using Spectral-Domain Optical Coherence Tomography

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Key words: Achromatopsia, gene therapy, OCT, cone photoreceptor, cone cell degeneration.

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Abstract

Purpose: Achromatopsia (ACHM) is a congenital autosomal recessive cone disorder with a presumed stationary nature and only a few causative genes. Animal studies suggest that ACHM may be a good candidate for corrective gene therapy. Future implementation of this therapy in humans requires the presence of viable cone cells in the retina. We investigated the presence of cone cells in ACHM as a function of age.

Methods: We evaluated the appearance and thickness of all retinal layers in 40 ACHM patients (age range 4-70 years) with known mutations in the *CNGB3*, *CNGA3* and *PDE6C* genes using spectralis domain optical coherence tomogram (SD-OCT; Heidelberg Spectralis). A comparison was made with 55 healthy age-matched controls.

Results: The initial feature of cone cell decay was loss of inner- and outer segments with disruption of the ciliary layer on OCT, which was observed as early as age 8 years. Cone cell loss further progressed with age, and occurred in 8/19 (42%) patients below 30 years, and in 20/21 (95%) of those aged 30+ years. Retinal thickness was significantly thinner in the fovea of all patients (126 µm in ACHM vs 225 µm in controls, $P<0.001$); this correlated with age (beta=0.065; $P=0.011$). Fovea hypoplasia was present in 24/30 (80%) of patients, and in 1/55 controls.

Conclusions: ACHM is not a stationary disease. The first signs of cone cell loss already occur in early childhood. If intervention becomes available in the future, our results imply that this should be applied in the first decades.

Achromatopsia (ACHM) is a congenital cone photoreceptor disorder with a presumed stationary course. The estimated prevalence is 1:30.000. ACHM is characterized by low visual acuity, photophobia, nystagmus, severe color vision defects, and a presumably normal macular appearance.[1] The inheritance is autosomal recessive, and the known responsible genes are *CNGA3*, *CNGB3*, *GNAT2,* and *PDE6C.*[2-5] Together, these genes explain the majority (>90%) of all ACHM cases. Although it is known that these genes code for essential proteins in the cone phototransduction cascade, repair of the gene defects is not feasible as yet.

A promising therapy which is currently under investigation is cone-targeted gene therapy. The first results of animal studies showed that *CNGB3, CNGA3* or *GNAT2* knockout mice and dogs responded well to adeno-associated virus gene therapy. In these rescued animals, cone ERG amplitudes recovered to nearly normal levels.[6,7] The next step in this development will be gene therapy in humans with cone dysfunction. For that purpose, it is crucial to know whether the non-functional cones are present in the macula and still viable.[8]

The aim of this study was to investigate the presence of cone cells as a function of age in ACHM. We compared foveal morphology in 40 ACHM patients of various ages with 55 healthy age-matched controls using a new spectralis domain optical coherence tomogram (SD-OCT; Heidelberg Spectralis). This device has a high reproducibility[9], and a better resolution than the conventional OCT, and its images correlate well with histopathology in vivo.[10]

Methods

Study population

ACHM patients (N=40; N=77 eyes) were ascertained from the Dutch achromatopsia patient organization (AchroNed) as well as from various ophthalmogenetic centers in the Netherlands (Erasmus Medical Center Rotterdam, The Rotterdam Eye Hospital, Radboud Nijmegen Medical Center, Sensis Institute Grave). Diagnostic criteria were: poor visual acuity since birth, congenital nystagmus, photophobia, color vision disturbances in three axes, and absent or residual cone responses with normal rod responses on full-field electroretinogram (ERG). All patients were screened for mutations in the *CNGA3*, *CNGB3, GNAT2* or *PDE6C* gene. Controls (N=55; N=110 eyes) were unrelated persons accompanying patients, or health workers derived from the Erasmus Medical Center, who had best-corrected visual acuity (BCVA) of 0.8 (20/25) or higher and absence of eye diseases. Controls were age-matched with patients per decade. The study was approved by the Medical Ethics Committee of Erasmus Medical Center and adhered to the tenets of the Declaration of Helsinki. All patients provided signed, informed consent for participation in the study, retrieval of medical records, and use of blood and DNA for research.

Clinical examination and OCT

All ACHM patients underwent a complete ophthalmologic examination, including best-corrected Snellen visual acuity, refractive error, color vision testing (HRR, Ishihara), ERG, and 35º fundus photography centered on the macula (Topcon TRC 50IX). We performed SD-OCT on all eyes using Heidelberg Spectralis® HRA+OCT version 4.0 (Heidelberg Engineering with TruTrack™ eye tracking and Heidelberg Noise Reduction™) according to the principles described elsewhere.[11]

Heidelberg Eye Explorer version 1.61 software was used for all measurements. We performed a single section action (one B-scan, 30 degrees, 768 pixels) to obtain a longitudinal section across the center of the macula, and we performed a volume scan (19 B-scans, 20x15 degrees, 512 pixels, 12 frames per B-scan) to ensure capturing the center of

the fovea. When the nystagmus in ACHM patients was so severe that it impaired the tracking system of the OCT, settings for resolution, speed, the number of B-scans and the number of frames per B-scan were adjusted.

Retinal thickness measurements in the fovea included the following structures: outer nuclear layer (ONL), inner and outer segments of the cone cells, and retinal pigment epithelium (RPE). Measurements were determined per ETDRS area[12] using the automated measurements from the software. We calculated retinal thickness in the fovea manually by searching the thinnest point in the fovea with the Heidelberg Eye Explorers tool. We placed points to outline the boundaries of the foveal pit, and then used the system's measurement tool to calculate depth and width of the fovea. In subjects with foveal hypoplasia or a hypodense area ('bubble'), measurements were performed manually as well. In these cases, we placed points to outline the boundaries of the extra retinal layers or the 'bubble', measured these with the system's measurement tool, and then subtracted these values from the total retinal thickness given by the automated measurements.

Statistical analysis

Frequency differences between ACHM patients and controls were compared using Student's *t*-test for continuous variables, and one-way ANOVA for categorical variables. First, we calculated between-eye correlations for the OCT measurements. Correlations between continuous variables, e.g. foveal thickness and disease status (ACHM, control) were calculated with a bivariate Pearson's correlation test; correlations between categorical variables, e.g. macular appearance, were analyzed with Spearman correlation analysis. Differences in presence of cone and RPE cell disruption were analyzed using the Chi-square test. Within the cases, we further examined whether age, visual acuity and/or macular appearance influenced foveal thickness with linear regression analysis.

Results

Clinical features

Baseline characteristics of the study population are presented in Table 1.

Table 1: Clinical characteristics of patients with achromatopsia and age-matched controls.

Variable	Achromatopsia N total = 40	Controls N total = 55
Mean age (SD‡)	34 (19)	32 (18)
0-9 years	6	6
10-19 years	6	7
20-29 years	4	13
30-39 years	8	9
40-49 years	8	9
50-59 years	5	7
60-70 years	3	4
Male	21	23
Female	19	32
Nystagmus	40	0*
BCVA <0.10 †	4	0*
BCVA >= 0.10	36	55*
Emmetropia	16	37*
Myopia	11	14*
Hypermetropia	13	4*
Molecular defect		
CNGB3 mutations	33	
CNGA3 mutations	2	
PDE6C mutations	5	
No mutations	0	55*

*P<0.05 for the difference between achromatopsia patients and controls

‡ SD: Standard Deviation

† BCVA: Best Corrected Visual Acuity

We examined 77 eyes of 40 patients with ACHM, and all 110 eyes of 55 control persons. All patients showed a pendular nystagmus, were photophobic, and had BCVA 0.05-0.20. Refractive errors (<2D or >2D) were significantly more present among patients (24/16 vs 18/37, P=0.004). Gene defects in patients were mostly present in the *CNGB3* gene

(83%;33/40). We did not find a genotype-phenotype correlation, i.e., the genes were equally distributed among those with and without OCT abnormalities.

OCT findings

All OCT parameters were highly correlated between right and left eyes ($R^2 \geq 0.90$). To ensure the best images for analysis, we used the eye which was least affected by the congenital nystagmus for all subsequent measurements. Observations on OCT and macular appearance are summarized in Table 2.

Table 2: Measurements of the macular appearance on fundus photographs and on OCT in achromatopsia patients and controls

	Achromatopsia N total=40	**Controls** N total=55	***P***
Macular appearance on fundus photographs, n			
No aberrations	15	38	
Foveal reflex absent	6	17	
(Subtle) RPE alterations	13	0	<0.001
Bull's eye with RPE degeneration	3	0	
Area of RPE atrophy	3	0	
Macular appearance on OCT*, N			
Retinal thickness of ETDRS† area 1 (fovea), mean (µm)			
Age stratum	n total		
0-9 yrs	6	163 (SD‡ 22)	215 (SD 17)	
10-19 yrs	6	176 (SD 37)	217 (SD 6)	
20-29 yrs	5	113 (SD 16)	222 (SD 13)	
30-39 yrs	7	116 (SD 55)	229 (SD 12)	<0.001
40-49 yrs	8	91 (SD 82)	230 (SD 22)	
50-59 yrs	5	103 (SD 53)	223 (SD 19)	
60-70 yrs	3	126 (SD 28)	218 (SD 7)	
Total	40	127 (SD 59)	222 (SD 14)	
Retinal thickness of ETDRS area 2-9 (parafovea), mean (µm)			
0-9 yrs	6	301 (SD 8)	354 (SD 7)	
10-19 yrs	6	305 (SD 14)	352 (SD 13)	
20-29 yrs	5	290 (SD 22)	354 (SD 15)	
30-39 yrs	7	289 (SD 25)	363 (SD 27)	<0.001
40-49 yrs	8	301 (SD 17)	367 (SD 17)	
50-59 yrs	5	287 (SD 23)	368 (SD 6)	
60-70 yrs	3	308 (SD 8)	356 (SD 7)	

Total	40	297 (SD 19)	359 (SD 16)	
Foveal hypoplasia , N				
0-9 yrs	6	2	0	
10-19 yrs	6	3	0	
20-29 yrs	5	2	0	
30-39 yrs	7	5	0	<0.001
40-49 yrs	8	5	1	
50-59 yrs	5	4	0	
60-70 yrs	3	3	0	
Total	40	24	1	
Loss of cone inner- and outer segments, N				
0-9 yrs	6	1	0	
10-19 yrs	6	1	0	
20-29 yrs	5	4	0	
30-39 yrs	7	8	0	<0.001
40-49 yrs	8	6	0	
50-59 yrs	5	5	0	
60-70 yrs	3	3	0	
Total	40	28	0	
Intraretinal bubble, N				
0-9 yrs	6	1	0	
10-19 yrs	6	1	0	
20-29 yrs	5	3	0	
30-39 yrs	7	7	0	<0.001
40-49 yrs	8	4	0	
50-59 yrs	5	5	0	
60-70 yrs	3	3	0	
Total	40	24	0	
RPE atrophy, N				
0-9 yrs	6	0	0	
10-19 yrs	6	0	0	
20-29 yrs	5	0	0	
30-39 yrs	7	0	0	<0.001
40-49 yrs	8	2	0	
50-59 yrs	5	2	0	
60-70 yrs	3	3	0	
Total	40	7	0	

*OCT: Optical Coherence Tomography

†ETDRS: Early Treatment Diabetic Retinopathy Study

‡SD: Standard Deviation

Loss of cone inner- and outer segments (IS and OS) with interruption of the ciliary layer (the connecting cilium of the photoreceptors), was the most frequent retinal abnormality among patients (Figure 1A/B). This feature was present in 28/40 (70%) of all ACHM, and showed a strong association with age. In the age group 0-10, only one (1/7; 14%) child had this feature; while in the oldest age-group all patients (7/7, 100%) showed this characteristic (Figure 2). Fundus photographs of patients with only loss of IS and OS (n=2) showed no abnormalities.

A 'bubble', an optical empty cavity, was visible in the cone cell layer in 24/40 (60%) ACHM patients (Figure 1C/D). Smaller bubbles coincided with loss of outer segments while bigger bubbles also involved the inner segments of cone photoreceptor cells. Fundus photographs of patients with a bubble and intact RPE layer on OCT (n=19) showed RPE mottling (n=11), no foveal reflexes (n=2), or no abnormalities (n=6). The presence of a bubble on OCT was not significantly related to BCVA.

Disruption of the RPE cell layer was visible in 7/40 (18%) of all patients (Figure 1E/F). This was only present in those beyond 40 years of age. Fundus photographs evidenced the RPE disruption in the majority of cases (6/7; 86%). BCVA was not significantly reduced.

We determined the total thickness of the ONL, IS and OS of the cone photoreceptors, and RPE layer in the fovea and of all retinal layers in the nine ETDRS areas. These layers were significantly thinner in patients (126 µm in fovea of ACHM; 225 µm in fovea of controls, $P<0.001$), and this reduction was significantly correlated with age (1.2 µm (standard error 0.33) decrease per year; beta=0.065; $P=0.011$). Patients without any signs of retinal degeneration (all layers intact on OCT; n=12) also had thinner layers (mean thickness 179 µm in fovea of ACHM; 225 µm in fovea of controls, $P=0.05$). Controls all had a foveal thickness of at least 215 µm and showed no reduction with age. Refractive error did not correlate with foveal thickness ($P=0.822$).

Foveal hypoplasia

In the fovea of 80% (24/30) of ACHM patients, we noticed multiple ganglion cell layers. These extra layers indicate a lack of formation of the foveal pit, i.e., foveal hypoplasia. This

phenomenon was always present bilaterally. It occurred in all age-groups of patients, and in one 51-year old control person with a Snellen visual acuity of 1.25 in both eyes, no color vision defects, and a normal macular appearance. Those with foveal hypoplasia had a less steep foveal slope than those with a normal fovea (92 µm versus 133 µm, *P*=0.02), but had no differences in foveal width (2004 µm versus 2126 µm, *P*=0.20) (Figure 3). Fundus photographs showed absent foveal reflexes only in 50% of patients with hypoplasia, and revealed normal macular appearance in the remaining half.

Discussion

Our study provides evidence that cone cells die progressively in ACHM. The first signs of decay were loss of IS and OS with a disruption of the ciliary layer on OCT, followed by appearance of an evolving bubble with cell loss in the cone photoreceptor layer. The end-stage was characterized by atrophy of the RPE. This cascade of events had its onset predominantly in the second decade, and showed a strong association with age thereafter. Before any signs of decay were visible, the foveal and parafoveal regions were already significantly thinner in ACHM patients than in age-matched controls. With the appearance of cone cell degeneration, thinning of the retina became more pronounced.

A strength of this study was the use of the recently developed SD-OCT. The resolution of this device is 50 times higher than the conventional Stratus OCT, making it possible to distinguish the different retinal layers, and to analyze changes on a cellular level.[13] Moreover, the SD-OCT has settings which are adjustable for the individual patient, facilitating measurements in patients with congenital nystagmus and in young children.[9] A limitation of the study was the relatively small sample size and the cross-sectional character of the study. Only larger studies with longer follow-up can overcome this problem. Nevertheless, to our knowledge, the current investigation is the largest imaging study in ACHM to date. The wide age-range in subjects who were otherwise homogeneous in genotype and disease-onset enables interpretation of the clinical course over time.

Studies investigating retinal morphology in ACHM have been scarce. Former histopathology studies of individual patients contradict each other on the presence and number of cones in the fovea.[14,15] Falls et al. described a normal number of foveal cones with aberrant morphology in one ACHM patient.[14] Contrasting, Glickstein et al. reported a patient who had no detectable foveal cones.[15] Two in vivo imaging studies using the Stratus OCT opposed each other likewise; one reported a normal macular thickness while the other showed a significant decrease.[16,17] We believe that these contradictory findings may result from differences in age of the patients. In favor of this view are the results from a study on knockout mice which had absent CNG3 channels. Cones were present at the age of two months, but no cones could be detected at eight months.[18,19]

Foveal hypoplasia was a frequent phenomenon in the ACHM patients of our study (24/30;80%). This has been reported in histology studies, but not yet confirmed in in vivo imaging studies.[15,20] Foveal hypoplasia is not specific for ACHM, but is also present in other congenital eye disorders such as ocular albinism and aniridia.[21,22] In the healthy eye, foveal development takes place at 24-36 weeks of gestation by thinning of the ganglion cell layers and thickening of the ONL. The final relocation of the inner nuclear and ganglion cell layers to the periphery takes places at four months after birth, resulting in uncovered foveal cone nuclei thereafter.[23] Our ACHM patients with foveal hypoplasia appeared to have missed these early foveal developments; they showed no thickening of the ONL, persistent ganglion cell layers, and a significantly less steep foveal slope (Figure 3). Remarkably, one control person also had foveal hypoplasia with extra ganglion cell layers. The difference with the ACHM patients was that this person had a normal thickness of the ONL. We do not know the exact mechanism behind this abberant retinal development. However, the range in the measurements of the width (range 1665-2629 µm; SD: 257) and the slope of the fovea (range 91-173 µm; SD: 20) was large in our healthy controls, indicating that normal foveal development varies considerably among subjects with normal visual acuity. The diagnosis of foveal hypoplasia was by far more sensitive on OCT than on fundus photographs, suggesting that abberant foveal development may be more common than presumed. Half of the ACHM

patients with foveal hypoplasia on OCT showed a normal macular appearance. In these patients, a minor slope was often present giving the impression of a normal foveal reflex (Figure 3).

What do our results indicate for the application of future therapies? They imply that the foveal cones of ACHM patients, although reduced in number, are morphologically intact at birth. However, after the first decade, cone cell loss occurs progressively in a relatively short time period. Therefore, our results suggest that if gene therapy becomes available in the future, earlier application may be preferable to later application.

In conclusion, our study provides profound evidence that ACHM is not a stationary disease, but a disorder which shows progressive loss of cone photoreceptors. SD-OCT is a valuable, non-invasive tool to visualize the severity of the cone decay, and a helpful means to give insight in the cellular changes present in ACHM.

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Legends to Figures

Figure 1: Fundus photographs and Optical Coherence Tomography (OCT) images showing progressive loss of cones in patients with achromatopsia.

A. Normal OCT in a 9-years-old patient with mutations in the *CNGA3* gene (c.847C>T / c.1709G>T).

Description of all visible retinal layers: NFL: nerve fiber layer; GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer; IS / OS: inner segments and outer segments of the cones; CC: ciliary layer (connecting cilium); RPE: retinal pigment epithelium.

B. Loss of cone photoreceptor inner- and outer segments with disruption of the ciliary layer on OCT and normal macular appearance on fundus photograph in an 8-years-old patient. Mutations were detected in the *CNGB3* gene (c.1148delC / c.991-3T>G).

C. Small 'bubble' with absent cone photoreceptors in the fovea on OCT, and normal macular appearance on fundus photograph in a 15-years-old patient with mutations in the *CNGB3* gene (c.1148delC / c.1148delC).

D. Large 'bubble' with absent cone photoreceptors in the fovea on OCT, and normal macular appearance on fundus photograph in a 21-years-old patient with mutations in the *CNGB3* gene (c.1148delC / c.1148delC).

E. Foveal bubble and moderate RPE cell layer disruption on OCT, and macular RPE atrophy on fundus photograph in a 49-years-old patient with mutations in the *CNGA3* gene (p.D260N / p.D162V).

F. Complete cone and RPE cell layer disruption in the fovea on OCT, and macular RPE atrophy on fundus photograph in a 56-years-old patient with mutations in the *CNGB3* gene (c.1148delC / c.886-896del11insT).

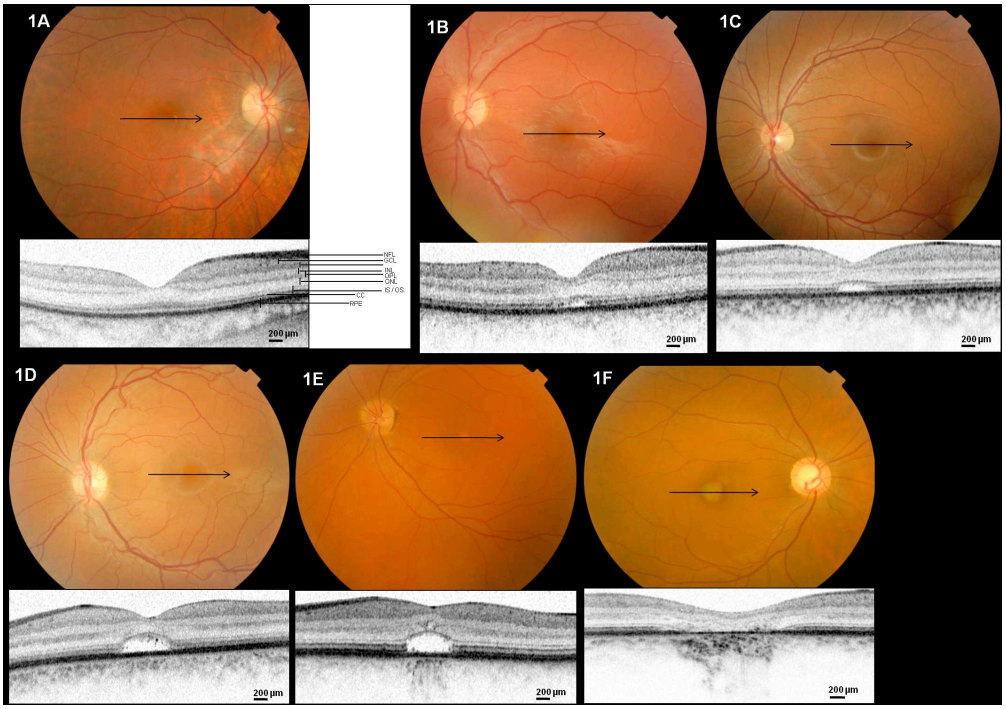
Figure 2: The proportion of achromatopsia patients with cone cell degeneration (grey) and RPE atrophy (black) per age-group in percentages. The total number of patients per stratum is given below the age-range. The youngest patient with signs of cone cell decay was 8 years old.

Figure 3: Fundus photographs and Optical Coherence Tomography (OCT) images showing foveal hypoplasia.

A. Normal OCT and macula appearance in a 26-years-old control person, outer nuclear layer (ONL) thickness: 100 µm.

B. Foveal dip hypoplasia on OCT and no abnormalities on fundus photograph in a 51-years-old control person with normal ONL thickness: 103 µm.

C. Foveal hypoplasia on OCT and no abnormalities on fundus photograph in a 27-years-old patient with achromatopsia and mutations in the *CNGB3* gene (c.1148delC / c.1148delC). Note the decreased thickness of the ONL: 70 µm.



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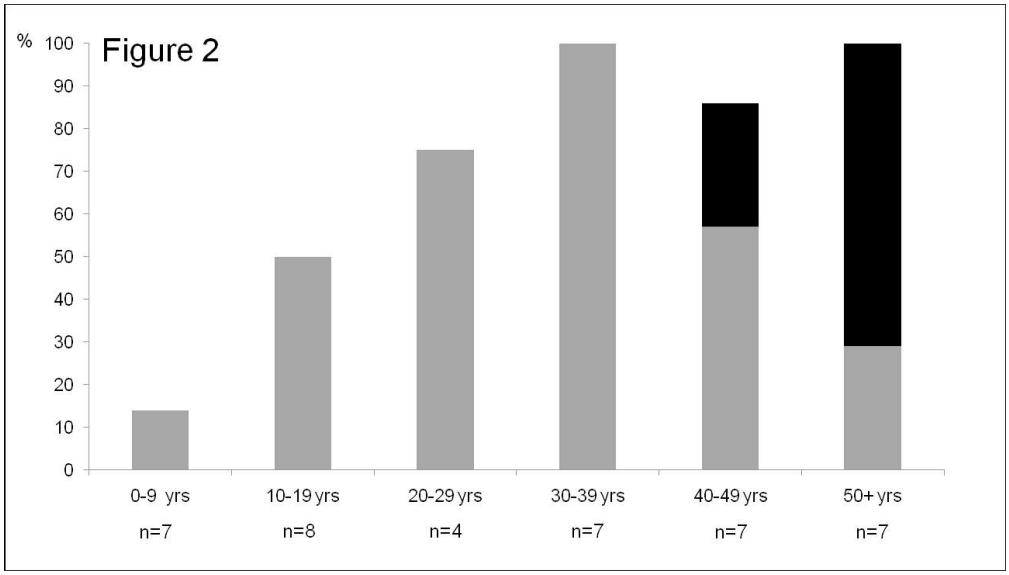

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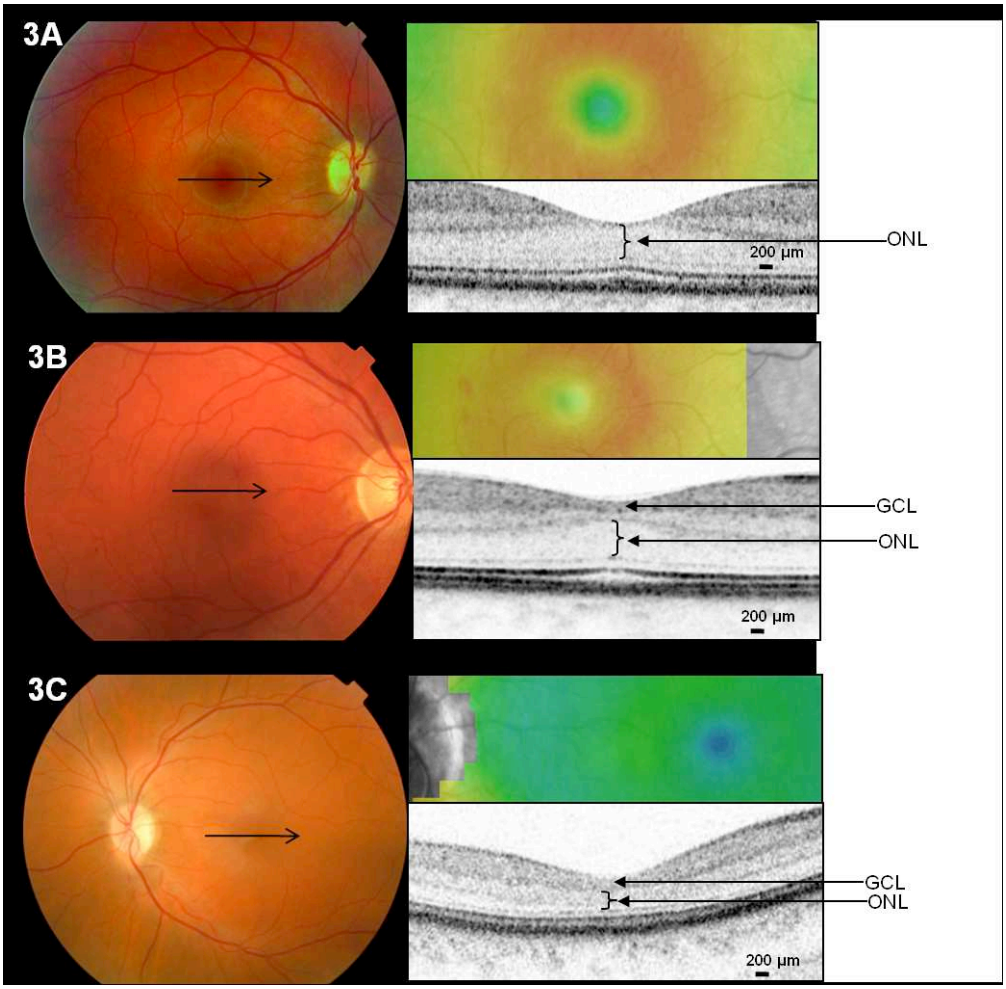

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