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Title: A prospective longitudinal study of retinal structure and function in achromatopsia

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Abstract

Purpose: To longitudinally characterize retinal structure and function in achromatopsia (ACHM) in preparation for clinical gene therapy trials.

Methods: 38 molecularly confirmed ACHM subjects underwent serial assessments, including spectral domain optical coherence tomography (SD-OCT), microperimetry, and fundus autofluorescence (FAF). Foveal structure on SD-OCT was graded and compared for evidence of progression, along with serial measurements of foveal total retinal thickness (FTRT) and outer nuclear layer (ONL) thickness. FAF patterns were characterized and compared over time.

Results: Mean follow-up was 19.5 months (age range at baseline: 6-52 years). Only 2 of 37 subjects (5%) demonstrated change in serial foveal SD-OCT scans. There was no statistically significant change over time in FTRT (p=0.83), ONL thickness (p=0.27), hyporeflective zone (HRZ) diameter (p=0.42), visual acuity (p=0.89), contrast sensitivity (p=0.22), mean retinal sensitivity (p=0.84), and fixation stability (p=0.58). Three distinct FAF patterns were observed (n=30): central increased FAF (n=4), normal FAF (n=11), and well-demarcated reduced FAF (n=15); with the latter group displaying a slow increase in the area of reduced FAF of 0.03 mm^2 over 19.3 months (p=0.002).

Conclusions: Previously published cross-sectional studies have described conflicting findings with respect to the age-dependency of progression. This study, which constitutes the largest and longest prospective longitudinal study of ACHM to date, suggests that although ACHM may be progressive, any such progression is slow and subtle in most patients, and does not correlate with age or genotype. We also describe the first serial assessment of FAF, which is highly variable between individuals, even of similar age and genotype.

Abstract word count: 246

INTRODUCTION

Achromatopsia (ACHM) is an autosomal recessive cone dysfunction syndrome affecting about 1 in 30,000 people, and characterized by the presentation in infancy of pendular nystagmus, poor visual acuity, and photophobia.[1] Electroretinography (ERG) demonstrates absent cone responses and normal or near normal rod responses,[2, 3] with psychophysical testing revealing normal rod function but absent or severely reduced cone function.[4] To date, five genes have been found to be associated with ACHM, all encoding components of the cone-specific phototransduction cascade. The two most common of these are *CNGA3*[5] and *CNGB3*,[6] encoding the α- and β-subunits of the cGMP-gated cation channel respectively, and together account for approximately 70% of cases.[7] Disease-causing sequence variants have also been identified in *GNAT2*,[8] *PDE6C*,[9] and *PDE6H*,[10]; each responsible for < 2% of cases.[8-10]

Several studies have recently demonstrated the effectiveness of using a gene-replacement approach to restore cone function in dog and mouse models of ACHM,[11-14] and the neuroprotective protein ciliary neurotrophic factor has also been shown to induce a transient restoration of cone function and visually directed behavior in the *cngb3* dog model.[15] Given these promising results, there are plans to begin human clinical trials in the near future. This makes the accurate measurement and stratification of retinal structure and function in ACHM critical, both in terms of patient selection and subsequent assessment of treatment response.

ACHM has been classically described as stationary,[1, 2, 7] however, several recent studies have suggested it is a progressive condition. Thiadens et al[16] examined 40 ACHM patients using spectral-domain optical coherence tomography (SD-OCT), and reported that increased cone cell 'decay' and retinal thinning was correlated with age, and began in early childhood. They reported that cone loss, as assessed on SD-OCT, occurred in 42% (8 of 19) of the achromats aged less than 30 years old, whereas this finding was observed in 95% (20 of 21) of patients aged older than 30 years. Thomas et al[17] also noted in their study (n=13) that the existence of a hyporeflective zone (HRZ), and outer nuclear layer (ONL) thinning were both age dependent. In contrast to these studies, Genead et al[18]

carried out a variety of investigations to assess macular structure, including SD-OCT (n=12), and found that there was significant structural variability even within individuals of the same genotype and age. They also found adaptive optics scanning light ophthalmoscopy (AOSLO) evidence of residual cone inner segment structure in all but one of the nine patients assessed, including the oldest patient (55 years), suggesting that progressive and complete loss of cones in ACHM may not be inevitable, and was not obviously age-dependent. Sundaram et al[19] examined 40 ACHM patients aged 6 to 52 years old, and found no correlation between age and visual acuity, total foveal thickness, foveal ONL thickness, or inner segment ellipsoid (ISe) intensity and cone loss on SD-OCT.

All of these studies are inherently limited by their cross-sectional nature, and the debate surrounding whether there is, or is not, age-dependent cone loss highlights the need for prospective longitudinal studies of large cohorts of molecularly-proven patients. Thomas et al[20] followed-up eight patients longitudinally, over a mean period of 16 months. They reported that the five younger patients (aged <10 years) demonstrated progressive disturbance at the foveal photoreceptor inner segment/outer segment (IS/OS) junction on OCT; but the three older patients (aged >40 years) did not. Due to the small number of patients they were not able to perform statistical comparisons between the two visits.

A further means of assessing macular integrity is through the use of fundus autofluorescence (FAF),[21] which can act as a surrogate measure of photoreceptor health.[22] There are limited data concerning FAF patterns in ACHM. Michaelides et al[23] reported that the FAF appearance was normal in five affected members of a *GNAT2* family. In a recent study of 10 achromats, Fahim et al[24] suggested an age-dependent change in FAF, with younger patients exhibiting increased foveal autofluorescence (AF), whilst older patients had reduced AF with discrete borders corresponding to outer retinal defects on SD-OCT. Greenberg et al[25] also observed FAF abnormalities including increased and/or decreased AF (n=17). However, again these were cross-sectional studies of relatively small size, with no longitudinal FAF data in ACHM published to date. There is a need to assess whether FAF signals in

ACHM change over time, which might aid assessment of any progression, genotype characterization, and potentially serve as a marker for response to future interventions.

Here we performed serial assessments of visual acuity, contrast sensitivity, microperimetry (MP), SD-OCT, and FAF, in the largest longitudinal study of molecularly proven patients to date, with the goal of providing statistically supported conclusions regarding the relative frequency and rate of any observed progression.

METHODS

Subjects

Forty subjects that had been characterized both phenotypically (including electrophysiologically) and genotypically in a previously published cross-sectional study[19] were prospectively asked to return for follow-up assessment at an interval of between 12 and 24 months after baseline assessment. The study protocol adhered to the Tenets of the Declaration of Helsinki, and was approved by the Moorfields Eye Hospital Ethics Committee. Informed consent was obtained from all subjects before entering the study.

Clinical assessments

A detailed follow-up assessment was undertaken which included best-corrected visual acuity (BCVA) using an Early Treatment Diabetic Retinopathy Study chart, contrast sensitivity assessment using the Pelli-Robson chart at 1 m, MP, SD-OCT, and FAF. All assessments were prospectively standardized to be undertaken in the same conditions in the follow-up assessment as at baseline assessment.

SD-OCT

After pupillary dilation with tropicamide 1% and phenylephrine 2.5% eye drops, line and volume scans were obtained using a Spectralis SD-OCT on both eyes (Heidelberg Engineering, Heidelberg, Germany) using the same protocol as used in the baseline cross-sectional study.[19] Briefly, the volume acquisition protocol consisted of 49 B-scans (124 µm between scans; 20°x20°), with Automatic Real Time eye tracking used whenever possible. During follow-up assessment, the

Spectralis SD-OCT was engaged in its follow-up mode, in order to ensure that the same scanning location was identified at both time points. This is achieved by setting the baseline scan as the reference scan, and the inbuilt software then ensures that the SD-OCT laser is directed to the same retinal location during subsequent image acquisition.[26] The lateral scale of each image was estimated using the axial length of the corresponding eye, obtained from the Zeiss IOL Master (Carl Zeiss Meditec, Jena, Germany).

Qualitative assessment of foveal morphology was undertaken by grading foveal structure on SD-OCT images into 1 of 5 categories: (1) continuous inner segment ellipsoid (ISe), (2) ISe disruption, (3) ISe absence, (4) presence of an HRZ, or (5) outer retinal atrophy, including loss of RPE (Figure 1). The presence or absence of foveal hypoplasia was also noted, and was defined as the persistence of ≥1 inner retinal layer (outer plexiform layer, inner nuclear layer, inner plexiform layer, or ganglion cell layer) through the fovea. Consensus grading was established by 3 independent examiners (J.A., Joe.C., and M.M.). Measurements of foveal total retinal thickness (FTRT) (internal limiting membrane to RPE distance), foveal outer nuclear layer (ONL) thickness and, where relevant, HRZ diameter, were made by a single observer (J.A.) at baseline and follow-up scans, using the digital calipers built-in to the software (Heidelberg Eye Explorer, Heidelberg Engineering, Heidelberg, Germany), and a 1 pixel : 1 µm display with maximal magnification. In cases of foveal hypoplasia, the distance between the posterior outer plexiform layer and the external limiting membrane (ELM) was taken as the ONL thickness. The mean of three measurements was used. Due to the optimum image resolution, imaging speed and follow-up acquisition mode and eye-tracking used in the Spectralis SD-OCT, retinal thickness measurements using this device have been shown to be highly reproducible,[26] and this method of assessing foveal thickness in the assessment of macular pathology has been used elsewhere.[27, 28]

Fundus autofluorescence

Fundus autofluorescence (FAF) was performed at baseline and follow-up assessments using the AF mode built in to the Spectralis confocal scanning laser ophthalmoscope (Heidelberg Engineering,

Heidelberg, Germany). All images were acquired after mydriasis as described above, and after the SD-OCT image acquisition protocol was complete; due to the relatively intense lights used during FAF acquisition and the photophobic nature of achromats. Images were acquired using a 30° field, with an optically pumped solid-state laser producing an excitation wavelength of 488 nm. The induced AF was detected through a barrier filter of 500 nm, and acquired after focusing the retinal image using the 820 nm infrared mode and sensitivity adjustment at 488 nm.

In the case of patients who had areas of abnormally reduced AF signal, measurements of this area were performed by describing this region with a mouse-driven cursor, and recording the area given by the in-built image analysis software (Heidelberg Eye Explorer, Heidelberg Engineering, Heidelberg, Germany); this method has been used in the assessment of progressive macular disease elsewhere.[29, 30] All FAF measurements were made by a single observer (J.A.), and for each FAF image the mean of three area measurements was calculated and used for further analysis.

Microperimetry

Microperimetry (MP) was performed in all subjects on both eyes, and after pupillary dilation, in a darkened room using the MP1 microperimeter (Nidek Technologies, Padova, Italy) in follow-up mode, and otherwise using the same testing conditions as at baseline assessment.[19] Two tests were undertaken on each eye, using a customized test grid of 44 retinal locations situated within an 8° radius in order to cover the macular and para-macular region, and over which the mean retinal sensitivity was recorded. During each test the non-tested contralateral eye was occluded.

Background illuminance was set within the mesopic range (1.27cd/m^2) and patients maintained fixation by looking at a 2° target. A variable intensity Goldmann size III (4mm^2) stimulus of 200ms duration was used as the testing stimulus. A 4-2 testing strategy was employed, with the intensity of the stimulus reduced in 4dB steps until the patient no longer detected it. The stimulus intensity then increased in 2dB steps until detected once again. Projection of the stimulus into the blind spot at 30 second intervals tested for false positive errors. Fixation stability was assessed using the bivariate contour ellipse area (BCEA) analysis, which represents an area in degrees where 68% (i.e. one

129 standard deviation) of fixation points are located[31]; this value is reported by the Nidek software. An
130 active eye tracking system ensured accurate stimulus projection in relation to retinal landmarks in
131 order to correct for fixation errors.

132

133 ***Data analysis methods***

134 Histogram plots were used to verify the normality of data before the use of any parametric tests.
135 Statistical analyses were performed using GraphPad Prism, version 5 (GraphPad Software Inc., La
136 Jolla, CA). As the left eye had been selected for further analysis in the original cross-sectional study
137 (which had found no significant difference in measured parameters between eyes),[19] this eye also
138 underwent detailed further analysis in the follow-up study. Patient identification numbers were kept
139 the same as in the cross-sectional study for ease of reference. Variability indicators (±) represent one
140 standard deviation in normally distributed metrics.

141 **RESULTS**

142 Thirty-eight of the 40 patients (95%) who were assessed at baseline were able to attend for follow-
143 up assessment within the 12-24 month study recall window. Two female patients were unable to
144 return; one due to the onset of unrelated long-term illness (patient #25) and the other due to
145 pregnancy (patient #32). Of the 38 patients that were followed up, 20 were male (53%) and 18 were
146 female (47%). The mean age of the follow-up cohort was 25 years old at baseline (± 12.5; range 6 –
147 52 years), with a mean follow-up interval of 19.5 months (± 2.8 months; range 13 – 24 months).
148 Table 1 summarizes the clinical findings, and includes age at baseline, interval of follow-up period,
149 genotype, and functional and structural parameters over time.

150 ***Best corrected visual acuity and contrast sensitivity***

151 The mean BCVA at baseline was 0.92 logarithm of the minimum angle of resolution (logMAR) (±
152 0.13; range 0.74 – 1.32 logMAR), and was not significantly different to the mean BCVA at follow-up,
153 which also measured 0.92 logMAR (± 0.11; range 0.74 – 1.28 logMAR) (paired t-test; p=0.89). The
154 mean of the logarithm of the contrast sensitivity (logCS) at baseline of 1.16 logCS (± 0.23; range 0.5 –

1.55 logCS) was also not significantly different to that measured at follow-up of 1.21 logCS (± 0.32; range 0.25 – 1.65 logCS) (paired t-test; p=0.22); where higher logCS values indicate better contrast sensitivity.

SD-OCT

In 37 of the 38 patients that underwent serial SD-OCT, foveal scans were acquired at baseline and follow-up that were comparable across time points. In one patient (patient #1) foveal follow-up SD-OCT images could not be obtained due to poor fixation, and this patient was excluded from further SD-OCT analysis. Two of these 37 patients (5%) demonstrated progression on SD-OCT between time points; patient #2 (aged 10 years old at first visit) progressed from category 1 (continuous ISe layer) at baseline to category 2 (ISe disruption) over a 20 month period, and patient #31 (aged 33 years old at first visit) progressed from category 2 (ISe disruption) to category 4 (HRZ) over the same number of months (Figure 2). However, these observed changes were subtle in patient #2, and the SD-OCT findings of patient #31 at baseline, with the high reflectivity foveal lesion, are not typically seen in ACHM. No other patients showed evidence of progression, either in terms of transition to another SD-OCT category or progression within a category (Figure 1).

The right eye SD-OCT images of all patients were also graded at baseline and follow-up assessment, given that in the cross-sectional study no parameter measured demonstrated any significant difference between eyes,[19] and so presumably any change over time observed in both eyes would more likely represent real disease progression. Both patients with SD-OCT evidence of progression in the left eye showed similar deterioration in the right eye (Figure 2); whilst the remaining patients had the same SD-OCT appearance in the right eye at baseline and follow-up, as was the case for their left eyes. The changes in patient #2 were again subtle in the right eye, but that the SD-OCT changes were mirrored in both eyes in both patients #2 and #31 would lend support to the view that the morphological change detected in these two patients was real.

The remaining 35 patients (95%) showed no evidence of deterioration in SD-OCT appearance on qualitative assessment (Figure 1). No patients had a change in the presence or absence of foveal hypoplasia between baseline and follow-up assessment.

Foveal Total Retinal Thickness, ONL Thickness, and HRZ Diameter

Foveal total retinal thickness (FTRT) was measured in all 37 patients who had serial foveal SD-OCT assessments. There was no statistically significant change between visits (i.e. follow-up thickness minus baseline thickness; negative numbers indicating thinning over time) in FTRT in the cohort, with mean change of -0.1 µm (95% confidence interval (CI) -1.3 to +1.1 µm; range -9 to +4 µm) (paired t-test; p=0.83) (Table 1).

Three of the 37 patients (8%) that underwent serial foveal SD-OCT scanning (patients #10, #28 and #30) did not have a sufficiently structurally distinct ONL layer which could be accurately measured, and so were excluded from the ONL thickness analysis. The mean ONL thickness change in the remaining 34 patients of -0.6 µm (95% CI -1.7 to +0.5 µm; range -10 to +3 µm) was not statistically significant between assessments (paired t-test; p=0.27) (Table 1). Only one patient had ONL thinning of ≥10 µm (patient #2 with 10 µm); which is in keeping with the qualitative deterioration of outer retinal structure on SD-OCT observed in this patient. In the 9 patients who had a HRZ at both time point assessments, there was no statistically significant change in the diameter of the HRZ over a mean follow-up time of 19.6 months in this group (paired t-test; p=0.42).

Microperimetry

All 38 patients underwent follow-up MP testing on ≥2 occasions, and the mean of these tests was used for subsequent analysis. The mean retinal sensitivity of this cohort at baseline was 16.6 dB (± 3.4; range 3.1 – 19.9 dB), and was not significantly different to that measured at follow-up (16.5 dB ± 3.07; range 5.8 – 16.5 dB) (paired t-test; p=0.84) (Table 1). The fixation stability measured at baseline

(median = 9.1°; range 1.7 – 65°) was also not significantly different from that at follow-up (median = 8.2°; range 1.7 – 61.8°) (Wilcoxon matched-pairs signed rank test; p = 0.58) (Table 1). At baseline assessment six subjects had a scotoma (defined as 0 dB sensitivity in ≥ 1 retinal location). Follow-up MP assessment also showed that the same 6 patients had scotomas. In addition, one further patient (patient # 5, aged 17 years old at baseline assessment) developed a scotoma that was not apparent on initial testing, and which was evident in both follow-up MP tests 16 months later (Figure 3). However, the change in point sensitivity is small and would fall within inter-test variability limits, as can be seen in the variation in sensitivities in the points around those that developed an absolute scotoma.

Fundus autofluorescence

In 30 of the 38 patients (84%), baseline and follow-up FAF images were of sufficient quality to enable accurate classification and, where necessary, measurement of the area of reduced FAF signal. Several patients experienced discomfort with the bright light needed to acquire FAF images, and given their extreme photophobia, this contributed to relatively lower patient concordance and successful acquisition compared to other serial assessment modalities. We observed three FAF patterns at baseline (n=30): 1) a normal FAF pattern - seen in 11 patients (37%); 2) an abnormal central increase in FAF – seen in 4 patients (13%); and 3) a discretely bordered abnormal central reduction in FAF – seen in 15 patients (50%) (Figure 4). No patients changed the type of FAF pattern they displayed between baseline and follow-up assessments, and no patients had different FAF patterns between eyes.

Given that the reduced FAF pattern had well-demarcated borders amenable to quantification, the area of reduced FAF was measured at baseline and follow-up in the 15 patients with this FAF phenotype. There was a small but statistically significant increase in median area of reduced FAF between assessments (Wilcoxon matched-pairs test; p=0.002), with the median change being +0.03

229 mm^2 (IQR +0.01 to +0.13 mm^2; range 0 to +0.28 mm^2), where positive numbers indicate an increase
230 in the area of reduced FAF signal over the mean follow-up time in this sub-group of 19.3 months.

231 The mean age of patients in the group with a normal FAF appearance was 27.6 years (range 10 - 52
232 years); 16.0 years (range 11 - 29 years) in the group with an increased FAF pattern and 29.3 years
233 (range 11 - 49 years) in the group with a reduced FAF pattern. There was no statistically significant
234 difference in the ages between the three FAF pattern groups (Kruskal Wallis test; $p<0.05$). In one of
235 the two patients in whom we detected evidence of progressive change on SD-OCT (patient #31),
236 there was only a minimal increase in the area of reduced FAF area of +0.01 mm^2 over a follow-up
237 time of 20 months; the other patient who progressed on SD-OCT (patient #2) had a normal FAF
238 pattern at both time points.

239 We observed a statistically significant correlation between more disordered SD-OCT structure
240 (category 1 to 5) and a more abnormal FAF pattern (graded from normal through central increased
241 FAF to central decreased FAF) (Spearman r = 0.55; p=0.002).

242

243 ***Genotype-phenotype correlation***

244 Of the two patients that progressed on qualitative SD-OCT assessment, one had disease-causing
245 variants in *CNGA3*, and the other in *CNGB3*, suggesting no significant association of progression in
246 *CNGA3* vs. *CNGB3*. This would also be in agreement with our previous findings that there was no
247 association between severity of SD-OCT phenotype or presence of foveal hypoplasia, and
248 genotype.[19] However, with only two patients appearing to show progression on SD-OCT, the
249 numbers of progressors by genotype are too small to be anything other than suggestive.

250 When we analyzed the *CNGA3* and *CNGB3* groups separately, we again did not find any statistically
251 significant difference between the baseline and follow-up measures in BCVA (Wilcoxon matched-
252 pairs test; *CNGA3* p=0.75; *CNGB3* p=0.72), contrast sensitivity (*CNGA3* p=0.06; *CNGB3* p=0.11), MP
253 mean sensitivity (*CNGA3* p=0.33; *CNGB3* p=1.0) or MP mean BCEA (*CNGA3* p=1.0; *CNGB3* p=0.41).

There was also no statistically significant difference between the *CNGA3* and *CNGB3* groups in the change in FTRT or ONL thickness (FTRT: Mann Whitney U test; p=0.33; ONL thickness: unpaired t-test; p=0.58).

In the case of the *CNGA3* and *CNGB3* patients, there was no significant association between the numbers of patients exhibiting each of the three observed FAF patterns and their genotype (Chi^2 test; p=0.40).

DISCUSSION

This study is, to the best of our knowledge (PubMed search 06/02/2014; keywords: achromatopsia, rod monochromatism), the largest prospective longitudinal study of ACHM with the longest mean follow-up time of 19.5 months, and sufficient patient numbers for statistical analysis looking for change over time.

Our cohort did not show any evidence of a statistically significant change over time in BCVA, contrast sensitivity, or MP measures of retinal sensitivity and fixation stability, either as whole, or as separate *CNGA3* and *CNGB3* genotype sub-groups. Only two of 37 patients (5%) followed up longitudinally demonstrated evidence of progressive outer retinal changes according to independently graded qualitative SD-OCT analysis over the follow-up period. Thirty-five patients (95%) demonstrated no qualitative changes on their SD-OCT images between baseline and follow-up assessment. This agrees with our longitudinal quantitative assessment of both TRFT and ONL thickness, both of which showed no statistically significant change in the cohort over the time course of this study. Neither of the two patients (#2 and #31) that had a qualitative change in SD-OCT had any significant deterioration over time (defined as worsening of more than two standard deviations from baseline measurements) in their BCVA, contrast sensitivity, MP mean sensitivity or MP mean BCEA. It has been shown in other inherited retinal conditions that SD-OCT changes may precede deterioration of clinical assessment parameters such as BCVA[32] and FAF[33], and so despite a failure to demonstrate

deterioration in other clinical measures used in this study, the SD-OCT changes in these two patients likely represent real change. Serial assessment using other sensitive retinal imaging techniques such as AOSLO, may shed further light on the earliest indicators of any progression in ACHM.

We saw no patients with an ONL or central macular thickness (CMT) thinning of >10 μm, which contrasts with Thomas et al[20], who report that the five pediatric patients in their study (aged <10 years at first visit) showed SD-OCT evidence of thinning of both CMT and ONL over a mean follow-up time of 13 months. Three of these 5 children appear to have had an ONL thinning of >10 μm, although the numerical values are not stated. The three adults in their study (aged >40 years) showed only minimal variation in these parameters over a mean follow-up of 20 months, in keeping with our study. However, in at least two of the children, the evidence for progression was not unequivocal. Thomas et al[20] described how patients #1 and #2 had developed an outer retinal hyper-reflective zone, which they interpreted as an indication of progression. It should be noted however, that the same two patients had a generalized increase in reflectivity of several other layers in their respective follow-up OCT images, including the nerve fiber layer and ganglion cell layer, and so the possibility that this apparent increase in outer retinal reflectivity over time may be artifactual remains. Furthermore, they were unable to perform any statistical analyses due to their small sample size. The reason for the difference in our respective findings regarding the evidence for progressive thinning of the ONL and/or CMT may be related to the fact that we had only 3 children aged ≤10 years old at first visit. However, our cohort did contain a total of eight children aged ≤12 years old at first visit, and the much larger number of patients in our cohort and the consequent ability to carry out a statistical analysis would lend further support to our findings. We also observed no statistically significant change in the diameter of the HRZ in the 9 patients in our study who had this finding at both time point assessments.

Fundus Autofluorescence

Our findings of three distinct FAF patterns is in broad agreement with those of Fahim et al[24]. We also found a very small but statistically significant increase in the area of reduced FAF over time amongst patients that displayed that pattern; indicating that serial FAF may play a role in assessing change in ACHM. There was no statistically significant difference between ages in the three FAF patterns. However, no patients changed their FAF pattern during the time-course of this study, and the rate of increase in the size of reduced FAF was very slow (median 0.02 mm^2/year); by comparison, FAF atrophy rates in age-related macular degeneration (AMD) are several orders of magnitude greater, in the realm of several mm^2 / year.[29] In addition, there were three patients aged ≤ 13 years in the group that had a normal FAF pattern, and one young patent (aged 11 years old) that had a reduced FAF pattern. This again is in keeping with a highly variable disease, where age and genotype are insufficient predictors of phenotype (Figure 4). However, caution needs to be exercised in over-interpreting this data and no definitive conclusions can be reached without larger cohorts.

We also observed a moderately strong correlation between degree of foveal SD-OCT structural disorganization and degree of FAF abnormality. FAF intensity may plausibly progress in sequence, from normal signal through increased FAF signal to subsequent FAF signal reduction. It is also possible that the five SD-OCT categories represent a sequence in the severity of foveal outer retinal structure disorganization (from a continuous ISe to outer retinal atrophy); thereby suggesting that the observed correlation of SD-OCT outer retinal architecture to FAF pattern may be of prognostic value, in addition to potentially acting as a further possible metric in treatment trials. Longer follow-up with larger molecularly confirmed cohorts, combined with more quantitative assessment of FAF, are required to establish whether SD-OCT structural abnormality precedes or follows FAF abnormalities in ACHM.

The diagnostic and prognostic relevance of FAF in ACHM has yet to be established. Robson et al[34] demonstrated that increased FAF in cone or cone-rod dystrophy is associated with reduced rod and cone sensitivity, with scotopic sensitivity reductions being milder than photopic losses. In contrast, in

AMD increased FAF may be more indicative of rod as opposed to cone dysfunction.[35] We found that the MP mean retinal sensitivity (averaged between the two assessments at each time point) was significantly higher in the increased FAF group (median 18.71 dB) compared to the decreased FAF group (median 17.33 dB) (Mann Whitney U test; p=0.008), but did not find any statistically significant difference in retinal sensitivity between normal FAF and increased FAF groups (Mann Whitney U test; p=0.19). In terms of monitoring for progressive change, it would seem that this measure, like the others we have investigated, would need to be assessed over a greater time span, given the likely slow rate of change – and again given the variability, this highlights the need to consider patients on an individual basis in terms of potential suitability for intervention. The use of further quantitative FAF analyses[24, 35, 36] may increase the sensitivity of FAF assessment in ACHM, but it is still likely that any progression by this measure will occur at a very slow rate in a subset of patients.

Age-dependency of progression

In their retrospective study, Thiadens et al[37] reported that, over a mean follow-up of 15 years, 12% of the ACHM patients they reviewed had a deterioration in BCVA; in contrast with other cross-sectional studies which have not found an age-dependent deterioration in BCVA.[16, 17, 19] In their longitudinal study of 8 patients, Thomas et al[20] also showed no evidence of a decrease in BCVA over the mean follow-up of 18 months. Khan et al[3] also reported that 6 to 12 years had elapsed between their detecting and subsequently failing to detect photopic ERG response in two affected adult *CNGB3* individuals. Given the aforementioned timespans, it is likely that any progression in this condition is very slow and possibly subtle; it may be that to see clear evidence of progression in more patients, longitudinal studies of a much longer time course that may extend into many years or even decades would be required.

There is also significant phenotypic variation between individuals in terms of the existence and time course of any such progression. This phenotypic variability is illustrated in the findings of OCT studies[16, 19, 38-40] and AOSLO studies.[18, 41, 42] Such variation may confound attempts to look for progression en bloc in what is likely to be a more heterogeneous condition than previously thought. Our data suggests that each individual patient's phenotype, and the significance and time course of any progression, does not correlate with genotype and is highly variable between individuals. Probing this more deeply may require more detailed longitudinal cellular phenotyping with ultrastructural assessments such as AOSLO, potentially in concert with AO-guided psychophysical assessments. The need for such deep-phenotyping has clear implications for the selection and monitoring of patients for anticipated therapeutic interventions. For most of the retinal parameters measured, our initial cross-sectional study did not find any association with *CNGA3* or *CNGB3* genotype.[19] However, we did observe that retinal sensitivity was significantly higher in the *CNGB3* group. This longitudinal study did not identify any measured parameters that worsened significantly between time points when each genotype group was analyzed alone.

The longitudinal findings herein broadly agree with our initial cross-sectional study that age and genotype are not necessarily the critical factors in selecting patients for gene therapy. The vast majority of parameters assessed in this study did not change over time, and those that did, either did so very slowly, or in a very small proportion of patients. This supports the view that the potential treatment window for gene therapy may not only be wider in terms of age than has been previously suggested, but that it may remain open for a longer period. Our data also lend support to the view that ACHM is heterogeneous, and that factors other than age and genotype are likely to influence the phenotype. Such factors may have an as of yet unknown influence on any planned therapeutic interventions.

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Figure Captions

FIGURE 1 Longitudinal spectral-domain optical coherence tomography (SD-OCT) scans in achromatopsia. Left panel column: Baseline scans indicating genotype, patient number and age at initial scan. Right panel column: Follow-up scans indicating patient number and interval between baseline and follow-up scans in months. Central panel column shows foveal magnification (x3) of corresponding scan (grey arrows). Shown are the left eyes of a typical subject from each of the five

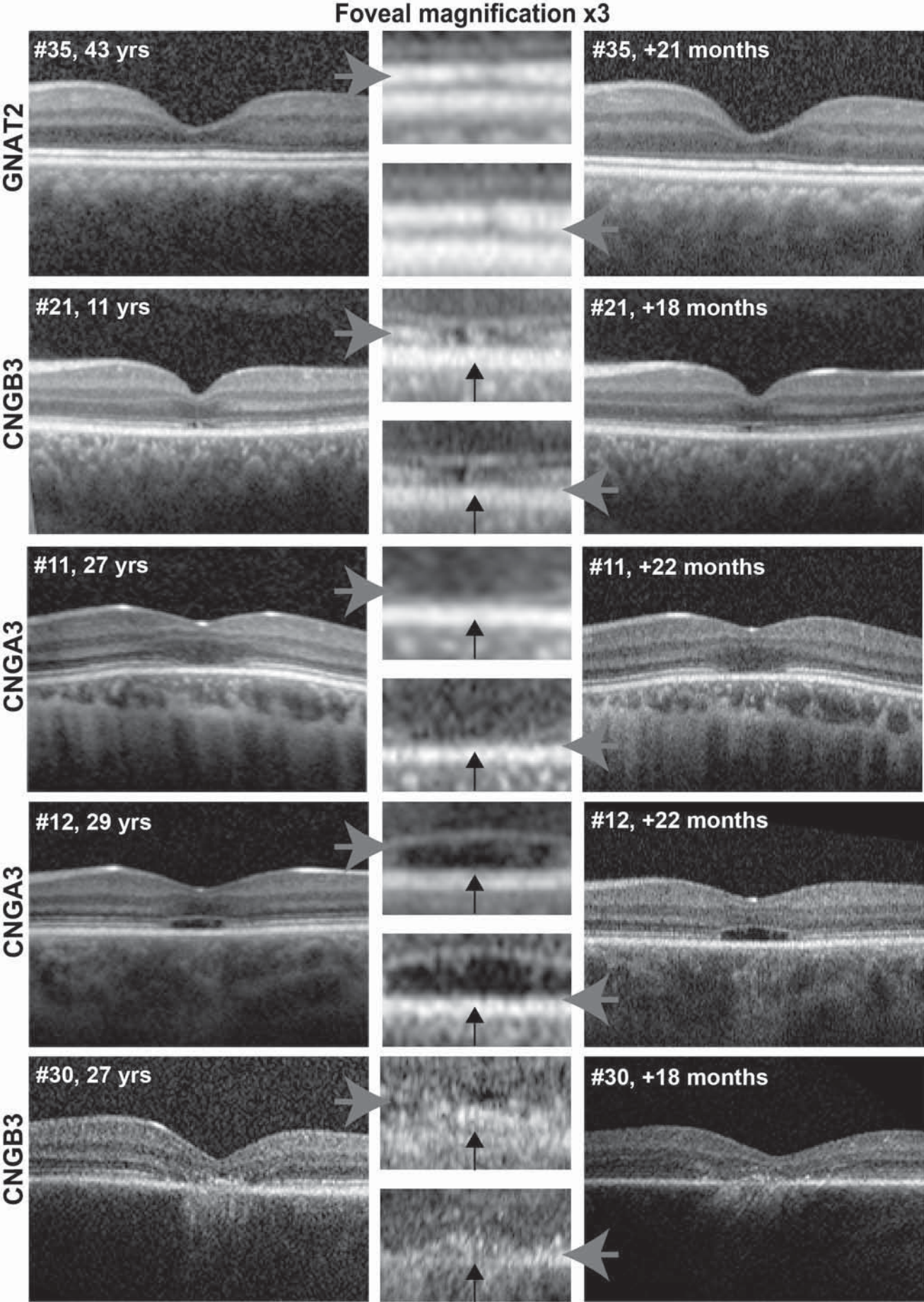
SD-OCT categories, all of whom demonstrated no change in SD-OCT structure over the study period (from the top row down: patient #35 with a continuous inner segment ellipsoid (ISe) band at both time points; patient #21 with a disrupted ISe layer at both time points; patient #11 with an absent ISe layer at both time points; patient #12 with a hyporeflective zone (HRZ) at both time points; and patient #30 with outer retinal atrophy at both time points). Non-magnified images represent 4500µm horizontally and 1000µm vertically; image scaling has been corrected for axial length. Scale bar, 200µm.

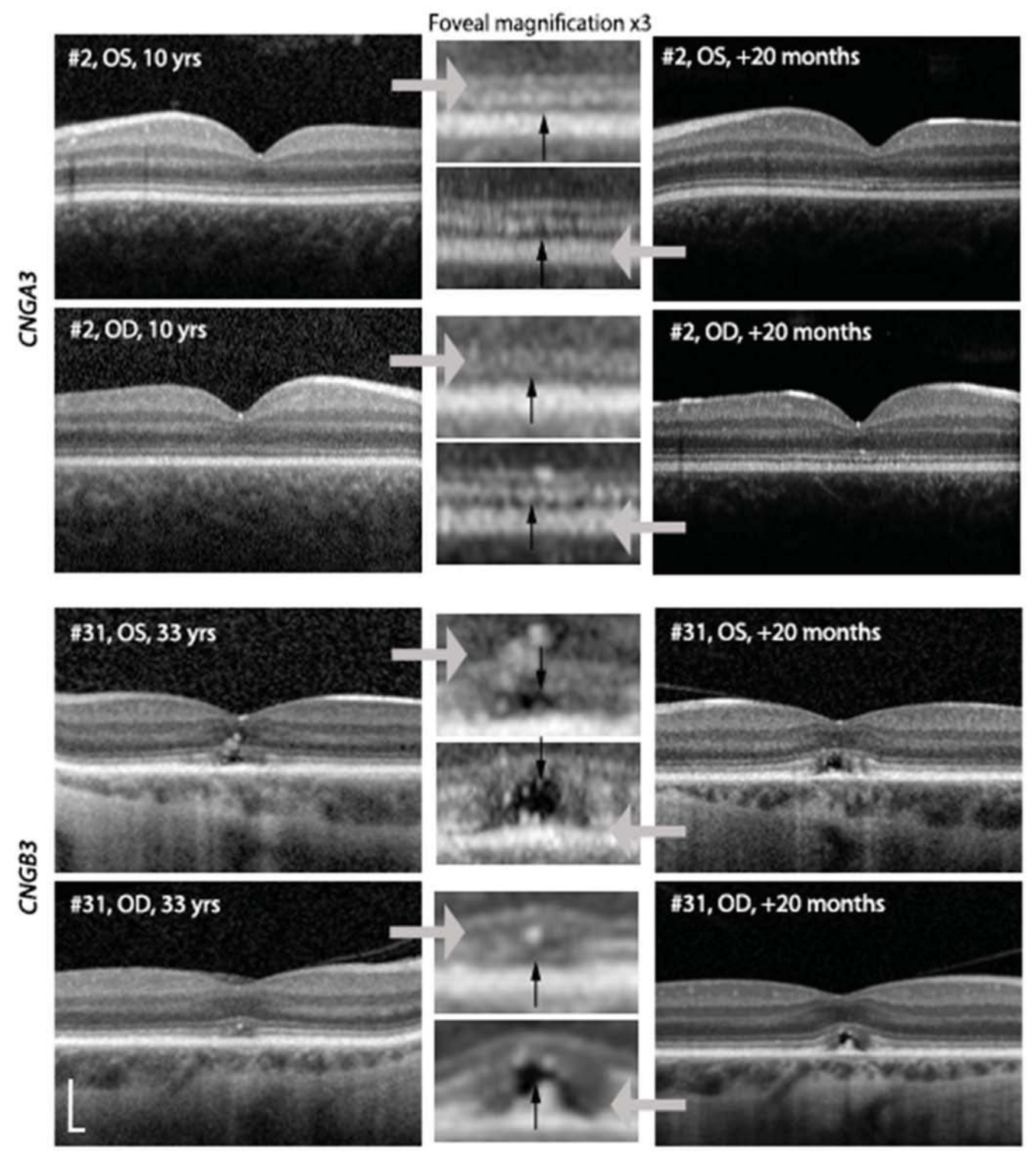
FIGURE 2 The left and right eye scans of the two achromatopsia subjects who demonstrated a change in SD-OCT appearance over the time course of the study. Layout is as in Figure 1. Top two panel rows: Patient #2 showed a continuous inner segment ellipsoid layer (ISe) at the fovea at initial scan in both the left (OS) and right (OD) eyes, and subsequently a disrupted ISe layer in both eyes at follow-up scan 20 months later (black arrows). Bottom two panel rows: Patient #31 showed a disrupted ISe layer at initial scan in both eyes and a hyporeflective zone (HRZ) 20 months later (black arrows). Non-magnified images represent 4500µm horizontally and 1000µm vertically; image scaling has been corrected for axial length. Scale bar, 200µm.

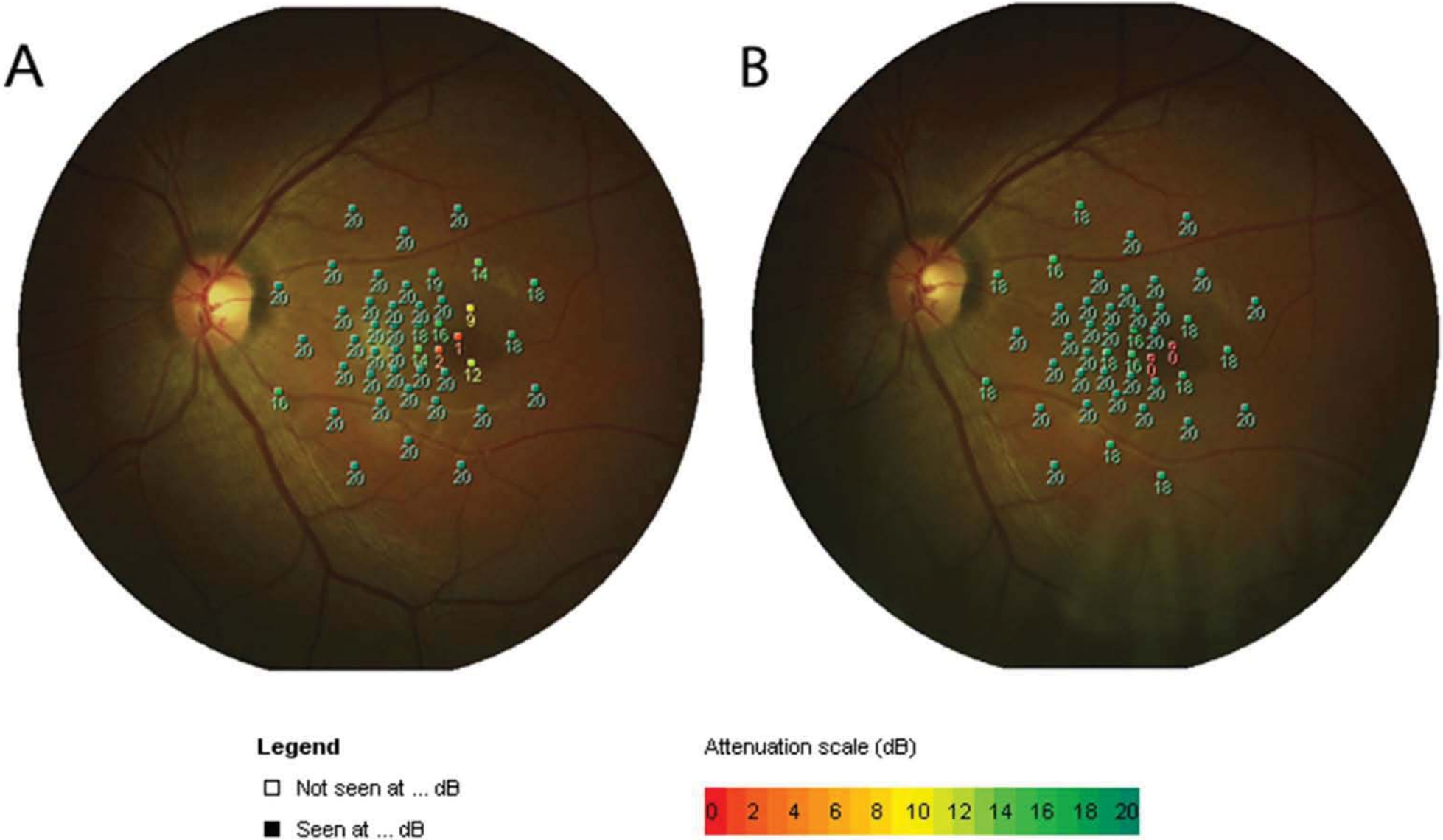
FIGURE 3 The microperimetry findings in patient #5 who developed a scotoma during this study. Left panel a): MP findings at baseline. Numbers indicate retinal sensitivity (dB) at that location. Right panel b): MP findings 16 months later indicate an absolute scotoma at two locations.

FIGURE 4 Longitudinal FAF in achromatopsia, demonstrating the three patterns observed (patient number, genotype and age at baseline acquisition indicated in left panels; patient number and follow-up interval in months indicated in right panels). Top panel row: Pattern 1: Reduced FAF signal centrally with a well-demarcated border. Middle panel row: Pattern 2: Normal FAF appearance.

Bottom panel row: Pattern 3: A central increase in FAF. These patterns were not statistically significantly age dependent. All of the three above patients harbor *CNGA3* disease-causing alleles and are of a similar age, which serves to highlight both the wide phenotypic variation seen in achromatopsia even within the same genotype, and also the lack of strict age-dependency.


Foveal magnification x3
GNAT2
#35, 43 yrs
#35, +21 months
CNGB3
#21, 11 yrs
#21, +18 months
CNGA3
#11, 27 yrs
#11, +22 months
CNGA3
#12, 29 yrs
#12, +22 months
CNGB3
#30, 27 yrs
#30, +18 months


Foveal magnification x3
#2, OS, 10 yrs
#2, OS, +20 months
CNGA3
#2, OD, 10 yrs
#2, OD, +20 months
#31, OS, 33 yrs
#31, OS, +20 months
CNGB3
#31, OD, 33 yrs
#31, OD, +20 months


A
B
Legend
Not seen at ... dB
Seen at ... dB
Not projected
Attenuation scale (dB)
0 2 4 6 8 10 12 14 16 18 20

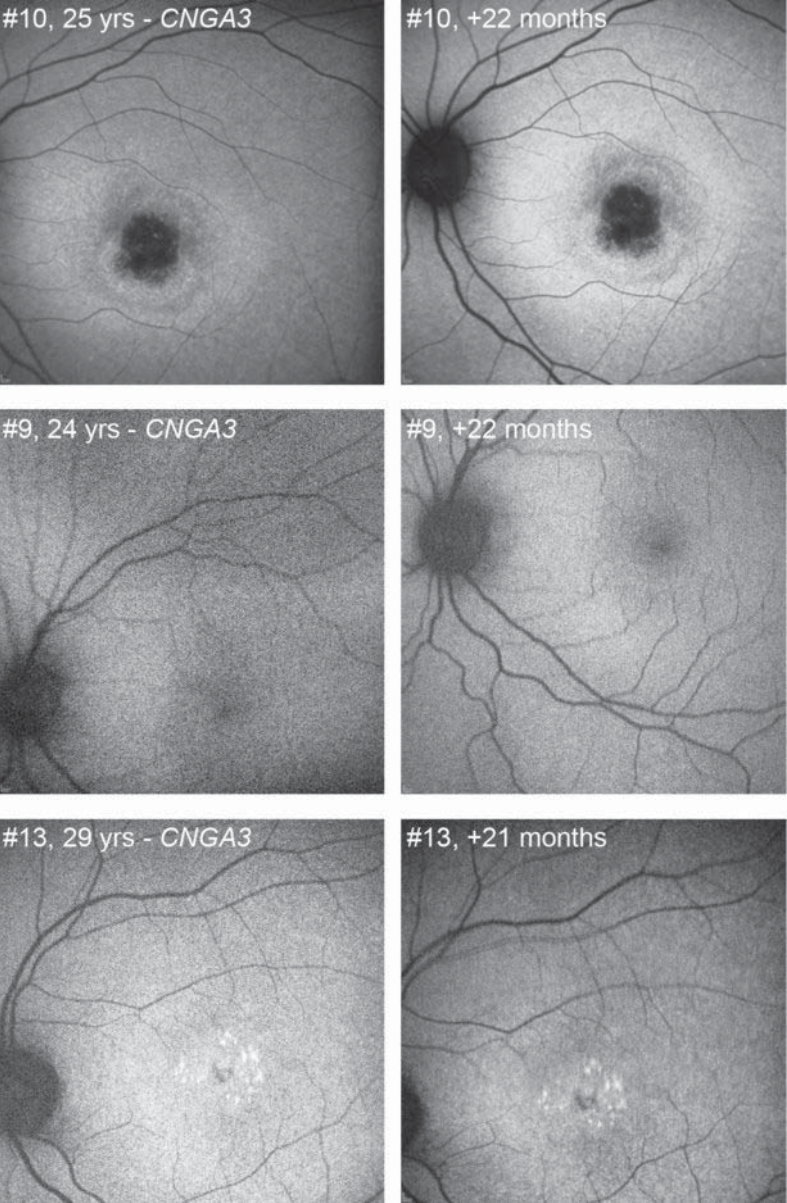

#10, 25 yrs - *CNGA3*
#10, +22 months
#9, 24 yrs - *CNGA3*
#9, +22 months
#13, 29 yrs - *CNGA3*
#13, +21 months

TABLE 1. Summary of Clinical Findings

Patient #	Age at baseline (yrs)	F/u interval (mnths)	Genotype	BCVA (logMAR)		Contrast sensitivty (logCS)		MP mean sensitvity (dB)		MP mean BCEA (°)		SD-OCT catergory*		Change in FTRT between assessments (μm)	Change in ONL thickness between assessments (μm)	Central FAF pattern at both assessments
				b	f/u	b	f/u	b	f/u	b	f/u	b	f/u			
1	7	21	CNGA3	0.94	0.86	1.30	1.40	14.90	16.20	44.04	36.70	3	N/A	N/A	N/A	N/A
2	10	20	CNGA3	0.80	0.82	1.25	1.55	19.67	19.75	15.82	8.05	1	2	+3	-10	Normal
3	11	16	CNGA3	1.08	1.04	0.90	1.10	14.90	14.50	40.70	61.80	1	1	+2	+2	N/A
4	11	17	CNGA3	0.94	1.00	1.05	1.30	17.60	19.50	14.75	17.90	2	2	+3	-1	Increased
5	17	16	CNGA3	0.80	0.86	1.20	1.35	18.35	18.55	6.00	7.35	3	3	-9	-1	Reduced
6	19	21	CNGA3	0.80	0.92	0.65	0.50	17.57	14.85	3.51	3.45	2	2	+2	0	Reduced
7	22	16	CNGA3	1.16	1.06	1.05	1.00	15.55	15.35	3.91	5.90	2	2	+3	+3	Normal
8	22	22	CNGA3	0.92	0.92	1.35	1.50	17.60	19.15	5.48	4.45	4	4	-3	-7	Normal
9	24	22	CNGA3	0.90	0.86	1.20	1.35	19.30	18.55	16.80	8.35	2	2	+2	+2	Normal
10	25	22	CNGA3	0.84	0.84	1.35	1.45	3.10	5.80	3.61	24.40	5	5	+4	N/A	Reduced
11	27	22	CNGA3	0.84	0.76	1.05	1.55	17.67	17.80	7.92	4.25	3	3	-7	-6	Reduced
12	29	22	CNGA3	0.74	0.90	1.20	1.25	13.55	15.20	37.43	7.60	4	4	+3	+2	Reduced
13	29	21	CNGA3	0.90	0.92	1.55	1.55	19.20	18.25	7.74	5.00	2	2	-8	-3	Increased
14	31	22	CNGA3	0.90	0.92	1.45	1.25	17.95	17.00	2.00	8.90	4	4	-2	+2	Normal
15	31	21	CNGA3	0.84	0.84	1.25	1.50	17.63	15.90	17.88	36.10	2	2	0	-4	N/A
16	34	21	CNGA3	0.90	0.82	1.20	1.05	13.80	14.90	10.20	14.40	2	2	-1	+3	Normal
17	35	21	CNGA3	0.96	0.96	0.90	1.00	14.17	15.90	13.15	7.60	2	2	-4	+2	Normal
18	49	17	CNGA3	0.80	0.74	1.30	1.30	17.15	18.25	2.23	2.95	4	4	+1	+2	Reduced
19	6	17	CNGB3	1.08	1.08	1.20	1.10	14.75	16.50	65.00	28.30	1	1	+2	-2	N/A
20	11	15	CNGB3	0.82	0.86	1.05	1.15	19.65	19.20	19.13	28.20	4	4	0	-1	Reduced
21	11	18	CNGB3	0.90	0.88	1.35	1.20	18.35	19.00	5.23	3.30	2	2	0	+3	Increased
22	13	21	CNGB3	0.90	1.00	1.00	1.10	18.93	19.25	8.62	8.80	3	3	-3	+3	Normal
23	12	16	CNGB3	0.76	0.80	1.30	1.35	19.80	19.05	5.97	3.95	2	2	+3	-4	Normal
24	13	18	CNGB3	0.90	0.82	1.30	1.50	19.27	19.95	6.54	6.00	1	1	-8	+1	Increased
26	18	24	CNGB3	0.74	0.76	1.25	1.40	18.40	17.80	3.70	4.10	4	4	+3	+2	Reduced
27	19	17	CNGB3	0.86	0.88	1.35	1.35	18.50	17.10	3.75	2.60	4	4	+3	-3	N/A
28	23	13	CNGB3	0.76	0.80	1.25	1.55	18.35	17.75	6.32	6.40	5	5	+4	N/A	Reduced
29	24	20	CNGB3	0.90	0.92	1.45	1.65	15.00	14.80	3.27	3.60	1	1	-2	-2	N/A
30	27	18	CNGB3	1.20	1.04	1.15	1.00	14.63	15.75	38.02	44.95	5	5	+2	N/A	Reduced
31	33	20	CNGB3	0.96	0.84	0.90	1.20	16.65	18.00	15.12	11.90	2	4	+3	+3	Reduced
33	47	13	CNGB3	0.96	1.00	1.10	1.15	19.35	17.00	7.30	5.00	4	4	+3	-1	Reduced
34	29	24	GNAT2	0.88	0.94	1.20	0.25	14.30	14.80	2.09	1.70	4	4	-2	0	Reduced
35	43	21	GNAT2	1.10	1.10	0.70	0.75	14.83	12.30	16.56	15.70	1	1	-4	-1	Reduced
36	49	21	GNAT2	0.94	1.04	1.05	0.75	13.43	13.20	10.31	11.70	1	1	-1	-1	Normal
37	52	21	GNAT2	1.00	1.00	0.50	0.65	14.80	14.30	20.90	18.95	1	1	+1	0	Normal
38	43	21	PDE6C	1.32	1.28	1.05	0.80	7.77	7.70	17.15	11.85	3	3	+2	+1	Reduced
39	19	21	?	0.96	0.92	1.45	1.55	19.57	18.80	9.54	20.10	3	3	+2	-5	N/A
40	23	21	?	0.86	0.74	1.20	1.35	19.83	19.75	16.14	10.85	3	3	0	0	N/A

#, patient number (as in Sundaram et al[19]); b, baseline; f/u, follow-up; BCVA, best corrected visual acuity; OD, right eye; OS, left eye; LogMAR, logarithm of the minimum angle of resolution; LogCS, logarithm of contrast sensitivity; MP, microperimetry; dB, decibels; BCEA, bivariate contour ellipse area; SD-OCT, spectral domain optical coherence tomography; ISe, inner segment ellipsoid; RPE, retinal pigment epithelium; N/A, not assessable. FTRT, foveal total retinal thickness. ONL, outer nuclear layer. '+' indicates an increase in metric over time between assessments; '-' indicates a reduction over time; FAF, fundus autofluorescence; ?, no disease causing variant identified in *CNGA3*, *CNGB3*, *GNAT2*, *PDE6C* or *PDE6H*. *SD-OCT category: 1 = continuous ISe; 2 = ISe disruption; 3 = ISe absence; 4 = hyporeflective zone present; 5 = outer retinal atrophy.