A Cross-Sectional and Longitudinal Study of Retinal Sensitivity in *RPE65*-Associated Leber Congenital Amaurosis

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Purpose. *RPE65*-associated Leber congenital amaurosis (*RPE65*-LCA) is an early-onset severe retinal dystrophy associated with progressive visual field loss. Phase I/II and III gene therapy trials have identified improved retinal sensitivity but little is known about the natural history of retinal sensitivity in *RPE65*-LCA.

Methods. A total of 19 subjects (aged 9 to 23 years) undertook monocular full-field static perimetry of which 13 subjects were monitored longitudinally. Retinal sensitivity was measured as mean sensitivity (MS) and volumetrically quantified (in decibel-steradian) using visual field modeling and analysis software for the total (V_{TOT}), central 30° (V_{30}) and central 15° (V_{15}) visual field. Correlation was evaluated between retinal sensitivity and age, best-corrected visual acuity (BCVA), contrast sensitivity, vision-related quality of life, and genotype. Test-retest reliability was also investigated.

Results. V_{30} was identified to have a strong, weak, and moderate correlation with age, BCVA and contrast sensitivity respectively. Furthermore, V_{30} was identified as having a weak linear relationship with the mobility and independence domains of the vision-related quality of life questionnaire. Longitudinal analysis demonstrated a slow loss of retinal sensitivity in this cohort. Subjects with at least one *RPE65* nonsense variant appeared to show greater progressive loss of retinal sensitivity in the second decade of life than those without.

Conclusions. Volumetric assessment of central 30° visual field sensitivity, $V_{30,}$ is a useful independent measure of retinal function and, in our data, represented the best metric to monitor deterioration of retinal sensitivity in *RPE65*-LCA. Furthermore, functional correlation with genotype may enable more informed prognostic counseling.

Keywords: retinal sensitivity, perimetry, visual field, retina, Leber congenital amaurosis, LCA, LCA2, clinical trials, endpoints

Leber Congenital Amaurosis (LCA) was first described in 1869 by Theodore Leber and is now used to describe a group of severe recessively inherited, early infantile-onset rod-cone dystrophies.[1] It is thought to affect between 1 in 33,000[2] to 1 in 81,000,[3] and is believed to account for ≥5% of all inherited retinal diseases.[2] Presentation is from birth with severe visual impairment, nystagmus, and poor pupillary responses. To date, 25 genes have been identified to account for approximately 70% to 80% of cases, with *RPE65*-associated LCA accounting for approximately 5% to 10%.[1] *RPE65*-associated LCA, similar to most forms of LCA, is associated with progressive retinal degeneration and loss of visual function. Progressive peripheral visual field loss is one of the hallmarks of rod-cone dystrophies and has been described, most commonly, with kinetic perimetry, in LCA.[4–6]

Following successful gene replacement in canine[7] and mouse models[8,9] a total of four phase I/II trials[10–13] and one phase III trial[14] have shown proof of principle that subretinal injection of a recombinant adeno-associated virus (AAV) vector containing the *RPE65* cDNA can improve retinal function and vision. Interestingly, all trials identify improvements in retinal sensitivity or functional vision, but using varying assessments, such as Goldmann kinetic perimetry, static Humphrey visual field testing, full-field light sensitivity threshold testing (FST), Nidek microperimetry, dark-adapted static perimetry, and performance score using the multiple luminance mobility test (MLMT).[13–17] Furthermore, following such successes the FDA has recently approved an ocular gene therapy for *RPE65*-associated LCA.

Goldmann kinetic perimetry has historically been utilized to test the entire visual field and locating borders between seeing/nonseeing areas. The operator-dependent nature of Goldmann kinetic perimetry has made the assessment difficult to standardize, poorly reproducible and is furthermore difficult to quantify.[18,19]

In comparison automated static perimeters, such as the Octopus (Haag Streit, Köniz, Switzerland) and Humphrey Field Analyzer (Carl Zeiss, Oberkochen, Germany) have been found to be superior at detecting visual field loss within the central 30° earlier, with greater standardization, and without the need for skilled perimetrists.[20,21] Furthermore, static perimetry has been more sensitive in identifying subtle sensitivity gradients. The development of a new fast full-thresholding strategy, German Adaptive Threshold Estimation (GATE), now allows relatively quick full-field static perimetry.[22,23]

Additionally, FST has been used to assess global light sensitivity in patients with severe retinal degenerative diseases with low vision and has been used to demonstrate improvement in a phase III interventional trial.[14,24] Advantages have included the potential use in patients with very low vision; however, it should be noted that this is a global assessment of light sensitivity, rather than an assessment of retinal sensitivity at multiple discrete locations.

The conversion of static visual field sensitivity into a hill of vision (HOV) allows for volumetric assessment of visual field sensitivity as a physical unit (decibel-steradian) rather than as an arithmetic mean sensitivity.[25] Volumetric assessments of retinal sensitivity are particularly useful in subjects with poor fixation and varying degrees of nystagmus, especially in comparison with pointwise indices of sensitivity such as mean sensitivity and mean deviation. A further advantage of a volumetric measure over pointwise analysis, is that following entire visual field testing and modeling, specific regions of interest can be defined and measured. This can be based specifically on defined geographic regions (e.g., a central circle of a selected radius, such as the central 30°), or by isosensitivity topography lines defining regions of greater remaining sensitivity, or by regions targeted by delivery of gene replacement therapy. These advantages are particularly noteworthy in inherited retinal disease where poor fixation, variable nystagmus, and nonuniform loss of retinal sensitivity is common.

Here we present cross-sectional and longitudinal full-field static perimetry data using a customized test grid on 37 eyes of 19 molecularly proven *RPE65*-LCA patients. We also explore correlation of retinal sensitivity, using the mean sensitivity (MS) as well as volumetric measures of the HOV with age, visual acuity, contrast sensitivity, vision-related quality of life questionnaires, and *RPE65*-LCA sequence variants.

Methods

Subjects

A total of 19 subjects (aged 9 to 23 years) with molecularly confirmed *RPE65*-associated LCA undertook monocular full-field static perimetry, assessment of visual acuity and contrast sensitivity. A subset of 10 adults also undertook a vision-related quality of life questionnaire. Furthermore, a subset of 13 patients were monitored for at least 10 months of which seven subjects undertook full-field static perimetry three times in each eye, within 3 months. Of the 13 subjects monitored longitudinally, results from a group of eyes were excluded as these eyes were recruited into an interventional study. 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 prior to entering the study.

Static Full-Field Perimetry

Static full-field perimetry was performed using the Octopus 900 (Haag Streit AG) controlled by the vendor-supplied software (EyeSuite; Haag Streit AG). The test was conducted monocularly, in a dark room, with the fellow eye patched. Patients were read a standardized set of neutral instructions on how to respond to the stimulus during testing.[26] Where appropriate, the distance refraction was used, with additional plus correction for near provided for testing of the central 30° field.

Patients were instructed to fixate on a cross with a background illuminance of 10 cd/m^2 (31.4 apostilbs). Static perimetry used the GATE strategy (Haag Streit AG, Köniz, Switzerland), stimulus size V, a 200-ms presentation and a radially designed, centrally condensed grid of 164 test locations that extended radially to 80° temporally, 67° inferiorly, and 55.5° nasally and superiorly. Data sets of x, y, and z, where x and y are the Cartesian coordinates of the test location and z is the differential luminous sensitivity values were exported from the perimeter and imported into the visual field modeling and analysis (VFMA) software application for further analysis, (Office of Technology Transfer and Business Development [OHSU], Portland, OR, USA).[25] Supplementary Figure S1 demonstrates the 164 point grid used and an example of the static perimetry results from a subject with *RPE65*-associated LCA.

The vendor software (Haag-Streit AG) generated mean sensitivity (MS), mean deviation and the reliability factor, which is calculated as a percentage of both false-positive and false-negative responses divided by the sum of positive and negative catch trials presented. Testing was repeated if the reliability factor was higher than 20.

Once imported into VFMA, the sensitivity data were fit in non-Euclidian space with a thin-plate, radial-based spline, producing a 3-dimensional model of the HOV with sensitivity on the z axis and the boundary (in unit steradian) of the test grid as the x, y base of the hill of vision.[25] The volume (in unit decibel-steradian, dB-sr) beneath the surface of the thin-plate spline representation of the HOV and within the external boundary of the grid was quantified. The total volume of the entire HOV (V_{TOT}) was calculated using a selection process that conformed to the external boundary (in unit steradian) of the entire test grid. Additional regions of interest (ROI), for example, the central 30° field of vision, V_{30}, and the 15° HOV portion, V_{15}, were measured using circle selections of 30° and 15° diameters, respectively, as separate metrics of the central portion of the visual field. The details of such analysis have been previously described.[26]

Visual Acuity

LogMAR best corrected visual acuity (BCVA) was assessed, monocularly, with an Early Treatment Diabetic Retinopathy Study chart. Precision Vision lightboxes were used (Precision Vision, Woodstock, IL, USA) and were illuminated with two cool daylight 20-watt fluorescent tubes, with the overhead lights turned off, so that no more than 161.4 lux should fall at the center of the chart.

Contrast Sensitivity

Contrast sensitivity was assessed, monocularly, using the Pelli-Robson chart at 1 meter, with room lighting allowing a chart luminance of 100 cd/m^2.

RPE65 Variant Detection, Pathogenicity Prediction, and Genotype Group Classification

All probands, and parents where possible, provided a fresh sample of peripheral blood. The probands' sample was sent to the Manchester Centre for Genomic Medicine (Manchester, UK) to extract genomic DNA from leukocytes for targeted next-generation sequencing (NGS) of the coding regions of 176 retina-

associated genes. Parental blood was used to confirm the variants to be either in cis or in trans by cosegregation analysis with Sanger direct sequencing. A report was then provided identifying the sequence variants and subsequent protein sequence changes, in the tested genes, including the *RPE65* gene. Where possible, results of cosegregation were also provided.

Each report was reviewed and further in silico molecular genetic analysis was performed to confirm the pathogenicity and predictive effects of the detected variants. Pathogenicity of all the detected variants was analyzed with three software prediction programs (accessed date: December 1, 2016): Mutalyzer (https://www.mutalyzer.nl, in the public domain), mutation taster on-line tool (http://www.mutationtaster.org, in the public domain), and human splicing finder program version 3.0 (http://www.umd.be/HSF3/HSF.html, in the public domain). First, Mutalyzer, was used to confirm the amino acid change caused by the nucleotide change of each variant (transcript: NM_000329.2). Furthermore, this provided information on the effects of any frameshift and subsequent stop. Second, to investigate any predicted effects on splicing of all missense variants, we used the mutation taster online tool and the human splicing finder program version 3.0. Third, we investigated the predicted effects of intronic variants on splicing, using the human splicing finder program version 3.0.

All variants were classified as either non-null or null, according to preset definitions. Null mutations were defined as variants with definitely or likely deleterious protein damage, such as stop-gained variants, intronic variants with significant splice site alteration, missense variants with significant splice site alteration, and deletion/insertion variants causing frameshift. Non-null mutations were defined as missense (without significant splice site alteration) or in-frame insertion/deletion variants.

Patients were classified into three genotype groups based on the severity of the predicted mutational damage: (1) mild, where a patient had two non-null variants; (2) intermediate, where a patient had one non-null variant and one null variant; and (3) severe, two null variants.

Vision-Related Quality of Life Questionnaires (VRQoL)

The 28-item impact of vision impairment (IVI) questionnaire was used as a measure of patients' perception of vision related restriction on their activity and quality of life in a subset of 10 *RPE65*-LCA adults.[27] Patients rated their perceived impact of vision limitation from "not at all," "a little," "a fair amount," to "a lot," which are allocated number scores from 1 to 4 for analytical purposes. The final response of "can't do this for other reasons" was excluded from analysis. The raw data was then converted using Rasch analysis into three IVI scales: (1) reading and accessing information (9 items), (2) mobility and independence (11 items), and (3) emotional wellbeing (8 items), using previously described methods.[27] The resulting scores are expressed in logits (log of the odd units) and higher scores signify better VRQoL.

Longitudinal Analysis of Retinal Sensitivity

A subset of 19 eyes in 13 patients were monitored with full-field static perimetry for a range of 10 to 22 months.

Statistical Analysis

Test-retest reliability was investigated by calculating the intraclass correlation coefficient (ICC), based on a single rater, absolute-agreement, 2-way mixed-effects model, calculated using statistical software (StataCorp, College Station, TX, USA). To minimize the clustering effect of using data from both eyes, only results from the left eye of all subjects were analyzed.

We examined V_{30}, age, visual acuity, contrast sensitivity, and VRQoL metrics for normality using the Shapiro-Wilk test. This confirmed that the data did not deviate from a normal distribution ($P > 0.05$). A Pearson's product-moment correlation was run to assess the relationship between V_{30} and age, visual acuity, contrast sensitivity and VRQoL, to generate a correlation coefficient (r). Statistical analyses were performed using a statistical software package (JMP; SAS Institute, Cary, NC, USA). To minimize the clustering effect of using data from both eyes, only the results from left eyes were used when investigating the correlation between V_{30} and age, visual acuity, and contrast sensitivity. In contrast, when investigating the correlation between V_{30} and VRQoL, the eye with the better BCVA was used, to account for the effect of ocular dominance in activities of daily living. Correlations between V_{TOT}, V_{30}, V_{15}, and age were then examined using Steiger's test.[28]

Furthermore, we fitted a multilevel mixed effects linear regression model to V_{30} and examined the effects of the three genotype groups on V_{30}, adjusting for age. The gradients of the three linear regression lines were subsequently examined to investigate any change in V_{30} with age. The three gradients of the three genotype groups were then compared with each other, using a post-hoc contrast comparison. The statistical analysis was performed using the Stata statistical software (StataCorp).

Longitudinal data was also investigated to identify any change in V_{30} during follow-up. A significant change was defined as a change greater than the estimated coefficient of repeatability. The estimated coefficient of repeatability was calculated as:

$$\pm 1.96 \times \sqrt{2} \times \text{the within subject standard deviation}$$

To estimate the within subject standard deviation, a 1-way ANOVA model was fit to the data incorporating data from baseline data from 37 eyes from 19 subjects. The statistical analysis was performed using statistical software (StataCorp) Furthermore, the confidence intervals for the coefficient of repeatability were also calculated.

Results

Patient Demographics

A total of 19 patients completed full-field static perimetry, assessment of contrast sensitivity and best corrected visual acuity. Of these, 10 were male (53%) and 9 female (47%). Table 1 shows patient demographics and genotypes. The ages ranged from 9 to 23 years, with a mean age of 17 years (SD: ±3.8 years).

Of note, subjects MM_0304 (aged 19 years old) and MM_0392 (aged 11 years old) were unable to complete static perimetry reliably in their left and right eyes respectively at their baseline visits. However, they were both able to complete the test reliably with both eyes at the next follow-up visit. MM_0292 (aged 10 years old) was unable to complete static perimetry reliably in the left eye at her first follow-up visit, but was able to at her next visit. Subject MM_0234 (aged 12 years old) could only complete static perimetry reliably in the right eye despite two attempts with either eye. These four instances were the only times assessments had to be repeated.

Comparison of Conventional and Volumetric Metrics of Retinal Sensitivity

The correlation between V_{TOT} and MS was investigated (Supplementary Fig. S2). As expected, the correlation

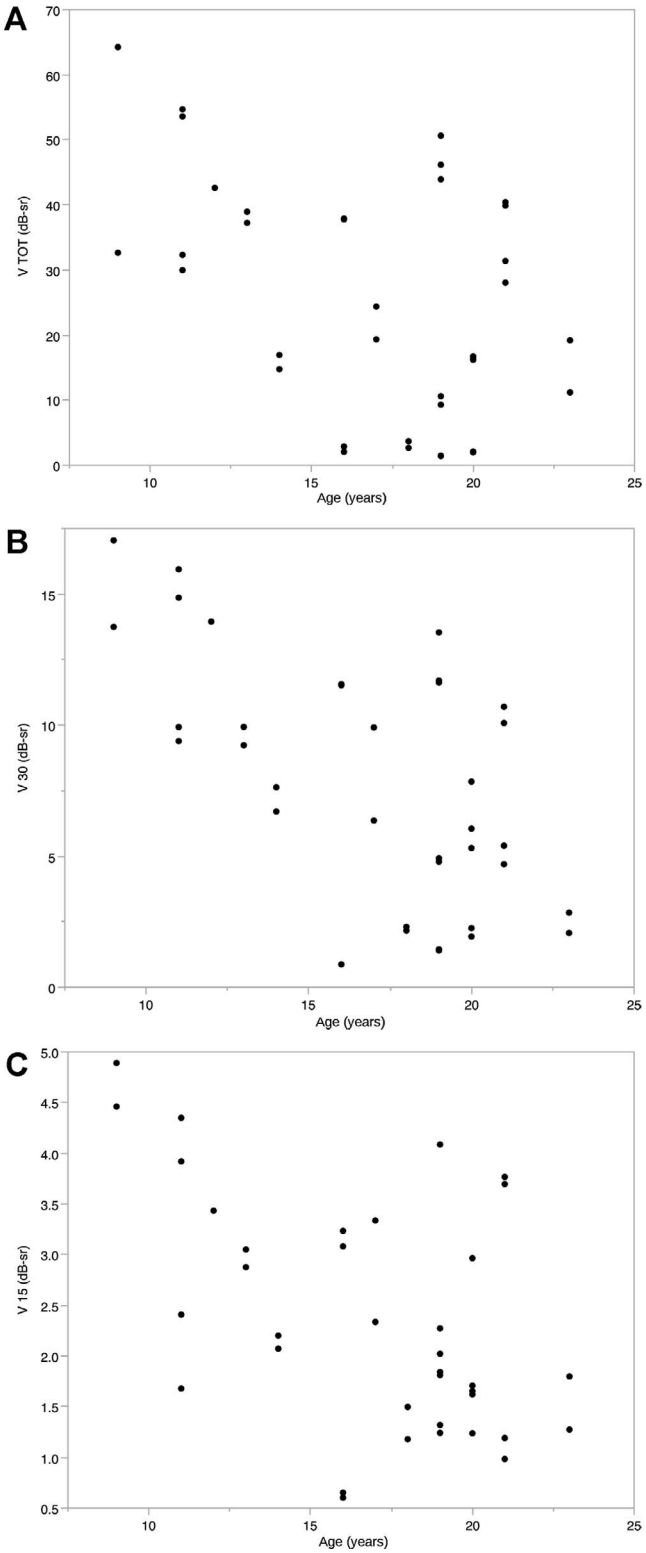

Figure 1. Visual field sensitivity against age. Shown are *scatterplots* (including both eyes of all patients, $n = 37$) of (**A**) V_{TOT}, (**B**) V_{30}, and (**C**) V_{15} against age, demonstrating a decline in retinal sensitivity with increasing age across all three metrics.

coefficient shows a strong, positive linear correlation between both metrics ($r = +0.98$). However, it should be remembered that the utility of MS would be limited in detecting subtle, localized changes in retinal sensitivity (as may be seen in progression or following intervention) in comparison to volumetric measures.

Test-Retest Reliability

Test-retest reliability was investigated in seven subjects undertaking static perimetry three times. ICC results are shown in Table 2 and as values for V_{TOT}, V_{30}, and V_{15} are all greater than 0.90 we conclude that static perimetry, assessed using these three metrics, all demonstrate strong test-retest reliability.[29]

Retinal Sensitivity Correlation With Age

Figures 1A, 1B, and 1C show scatterplots of the total HOV (V_{TOT}), central 30° HOV (V_{30}), and central 15° HOV (V_{15}), respectively, against age, demonstrating the decline in retinal sensitivity with increasing age. To investigate the relationship further, Figure 2A, Supplementary Figure S3, and Supplementary Figure S4 show scatterplots of V_{30}, V_{TOT}, V_{15} in the left eyes only of subjects with a linear regression line provided. Interestingly, the correlation coefficient between V_{30} ($r = -0.64$) and age suggests a moderate to strong linear relationship. In comparison, the correlation coefficient between V_{TOT} ($r = -0.58$) and age and V_{15} ($r = -0.55$) and age suggest a moderate linear relationship. Steiger's test revealed no statistically significant differences ($P = 0.37$) between the correlation of the three different retinal sensitivity metrics and age.

Retinal Sensitivity Correlation With Visual Acuity and Contrast Sensitivity

Supplementary Figure S5 shows a linear regression line between V_{30} and BCVA. This shows a weak linear relationship ($r = -0.30$). Supplementary Figure S6 shows a linear regression line between V_{30} and contrast sensitivity which identifies a moderate linear relationship ($r = +0.46$).

Retinal Sensitivity Correlation With VRQoL

A total of 10 adults undertook the Impact of Vision Impairment (IVI) questionnaire. Figures 3A through 3C demonstrate the correlation between the three domains of reading and accessing information ($r = -0.14$), mobility and independence ($r = -0.34$), and emotional wellbeing ($r = -0.16$). Notably, this suggests a weak linear relationship between the central 30° HOV and mobility and independence. Hypothesizing that mobility and independence may be better correlated with more central measures of retinal sensitivity, the correlation between the central 15° HOV with mobility and independence was investigated. This showed a moderate ($r = -0.46$) negative linear relationship.

RPE65 Genotype-Pathogenicity Correlation

We investigated the effect of the 3 genotype groups on V_{30} adjusting for age (Fig. 2B). We identified that there was no variation in V_{30} with age in the "mild" genotype group ($n = 5$ between the ages of 11 to 21 years ($P > 0.05$). In contrast, we identified a worsening of V_{30} with age in the "intermediate" ($n = 7$) and "severe" ($n = 6$) genotypes between the ages of 10 to 23 years ($P < 0.05$) and 11 to 20 years ($P < 0.05$), respectively. Furthermore, pairwise comparison of the rate of change of V_{30} over time, identified a significant difference between the "mild" and "intermediate" groups ($P < 0.05$). However, no significant difference was found in the rate of change of V_{30} over time between the "mild" and "severe" groups ($P > 0.05$).

Table 1. Cohort Demographics*

Patient Identifier	Sex	Age	Genotype Group	*RPE65* Variant 1 Nucleotide Change	*RPE65* Amino Acid Change, Effect 1	*RPE65* Variant 2 Nucleotide Change	*RPE65* Amino Acid Change, Effect 2	VA - OD	VA - OS
MM_0220	F	18	Mild	c.1078C>A	p.Pro363Thr, missense	c.1078C>A	p.Pro363Thr, missense	0.74	0.78
MM_0229	M	18	Intermediate	c.11+5G>A	Splice region, splice site alteration	c.1102T>C	p.Tyr368His, missense	0.32	0.12
MM_0231	M	14	Severe	c.1451G>A	p.Gly484Asp, splice site alteration	c.1451G>A	p.Gly484Asp, splice site alteration	0.9	1.1
MM_0234	M	12	Mild	c.271C>T	p.Arg91Trp, missense	c.1102T>C	p.Tyr368His, missense	0.6	0.7
MM_0252	M	16	Severe	c.11 + 5G>A	Splice region, splice site alteration	c.245G>A	p.Arg82Lys, splice site alteration	0.66	0.64
MM_0255	M	19	Severe	c.1451G>A	p.Gly484Asp, splice site alteration	c.1451G>A	p.Gly484Asp, splice site alteration	0.64	0.74
MM_0262	F	21	Mild	c.118G>A	p.Gly40Ser, missense	c.955G>A	p.Glu319Lys, missense	0.20	0.30
MM_0264	F	16	Mild	c.272G>A	p.Arg91Gln, missense	c.1306G>A	p.Gly436Arg, missense	0.76	0.94
MM_0277	F	13	Mild	c.271C>T	p.Arg91Trp, missense	c.271C>T	p.Arg91Trp, missense	0.72	1.66
MM_0283	F	11	Severe	c.353G>A	p.Arg118Lys, splice site alteration	c.353G>A	p.Arg118Lys, splice site alteration	0.6	0.64
MM_0292	F	9	Intermediate	c.74C>T	p.Pro25Leu, missense	c.11+5G>A	Splice region, splice site alteration	0.48	0.62
MM_0289	F	20	Intermediate	c.989G>A	p.Cys330Tyr, missense	c.1443_1445delAGA	p.Glu481del, in-frame deletion	0.72	0.82
MM_0304	F	19	Severe	c.11+5G>A	Splice region, splice site alteration	c1341_1342dupCT	p.Cys448SerfsTer4, frameshift	0.78	0.8
MM_0309	M	18	Severe	c.859G>T	p.Val287Phe, splice site alteration	c.859 G>T	p.Val287Phe, splice site alteration	1.3	1.48
MM_0313	M	20	Intermediate	c.1398C>G	p.Tyr466Ter, stop-gained	c.1464T>A	p.Ser488Arg, missense	0.7	0.4
MM_0340	M	17	Intermediate	c.370C>T	p.Arg124Ter, stop-gained	c.952T>A	p.Tyr318Asn, missense	0.8	0.7
MM_0350	M	21	Intermediate	c. 130C>T	p.Arg44Ter, stop-gained	c.1543C>T	p.Arg515Trp, missense	1.3	1.4
MM_0349	M	23	Intermediate	c. 130C>T	p.Arg44Ter, stop-gained	c.1543C>T	p.Arg515Trp, missense	1.1	1.4
MM_0392	F	11	Mild	c.47T>C	p.Phe16Ser, missense	c.1292A>G	p.Tyr431Cys, missense	0.6	0.8

VA, visual acuity; OD, right eye; OS, left eye.

* Shows sex, age at baseline assessment, genotype group, *RPE65* variants, corresponding *RPE65* amino acid changes, and its effect and baseline visual acuities.

Longitudinal Analysis of Retinal Sensitivity

We monitored 19 eyes in 13 patients with full-field static perimetry for a minimum of 10 months. The mean follow-up was 16.5 months (range, 10–22 months). Figure 4, and Supplementary Figures S7 and S8, respectively show the change in V_{30}, V_{TOT}, and V_{15} in these subjects. In addition, rate of change (dB-sr/year) was calculated for each patient. In this cohort, the average V_{TOT}, V_{30}, and V_{15} rate of change was found to be –2.17 dB-sr/year, –0.60 dB-sr/year, and –0.16 dB-sr/year, respectively (Table 3).

The coefficient of repeatability and its confidence interval were calculated and are presented in Supplementary Table S1. Of all the subjects tested, subject MM_0304 showed a loss of retinal sensitivity greater than test-retest variation (TRTV) in all three metrics and subject MM_0292 showed a loss greater than TRTV in V_{TOT} alone.

Discussion

We have investigated and volumetrically quantified retinal sensitivity as assessed by automated static perimetry, using the VFMA software, in a cohort of 19 subjects with *RPE65*-associated LCA, aged 9 to 23 years old.

In this cohort, we identify that mean sensitivity and volumetrically quantified retinal sensitivity show a strong correlation. However, given the benefits of volumetric indices with regard to subjects with poor fixation, nystagmus, and nonuniform loss of retinal sensitivity, we feel they are inherently better placed to identify change either during the natural history or following intervention.

We investigated how V_{TOT}, V_{30}, and V_{15} varied with age. V_{30} ($r = –0.64$) exhibited a stronger correlation with age compared to V_{TOT} ($r = –0.58$) and V_{15} ($r = –0.55$). While no statistically significant difference was noticed between the three metrics, the results suggest V_{30} as the best metric to monitor deterioration of visual function in cohorts of children

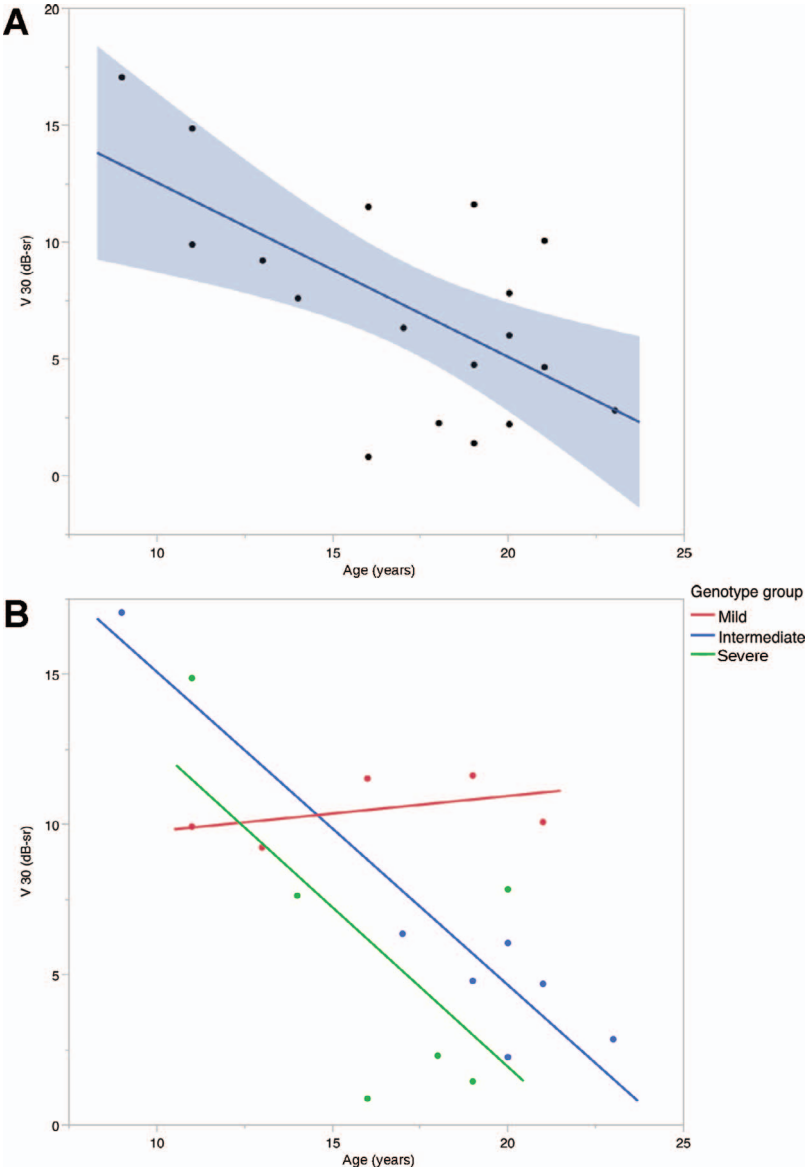

Figure 2. **(A)** V_{30} compared to age. Shown is a *scatterplot* of V_{30} of the left eyes ($n = 18$) of all patients against their age, with the corresponding linear regression line. *Shaded area* denotes confidence interval for regression line. This demonstrates a strong, negative linear correlation ($r = -0.67$) between central 30° visual field sensitivity and age. **(B)** V_{30} compared to age, in the three genotype groups. Shown is a *scatterplot* of V_{30} of the left eyes of all patients against age, with linear regression lines for those in the "mild" ($n = 5$, $r = +0.46$, *red*), "intermediate" ($n = 7$, $r = -0.95$, *blue*), and "severe" ($n = 6$, $r = -0.67$, *green*) genotype groups. This demonstrates the relationship between the central 30° visual field sensitivity and age after separating out the three genotype groups.

and young adults. Furthermore, we identified a weak and moderate relationship between V_{30} and BCVA and contrast sensitivity, respectively; suggesting that V_{30} is a useful independent measure of visual function in *RPE65*-associated LCA.

We investigated the correlation between V_{30} and VRQoL quantified using Rasch-transformed data from the IVI questionnaire. Interestingly, a weak linear relationship between V_{30} and mobility and independence was identified, compared to a moderate ($r = -0.45$) linear relationship between V_{15} and

Table 2. Test-Retest Reliability

		95% Confidence Interval		F Test With True Value 0			
	ICC	Lower Bound	Upper Bound	Value	*df*1	*df*2	Sig
V_{Tot}	0.986	0.951	0.997	204.28	6	12	< 0.001
V_{30}	0.961	0.868	0.993	67.24	6	12	< 0.001
V_{15}	0.963	0.874	0.993	70.65	6	12	< 0.001

Results of ICC calculation in statistical software (Stata; StataCorp) using a single-rater, absolute-agreement, 2-way random effects model.

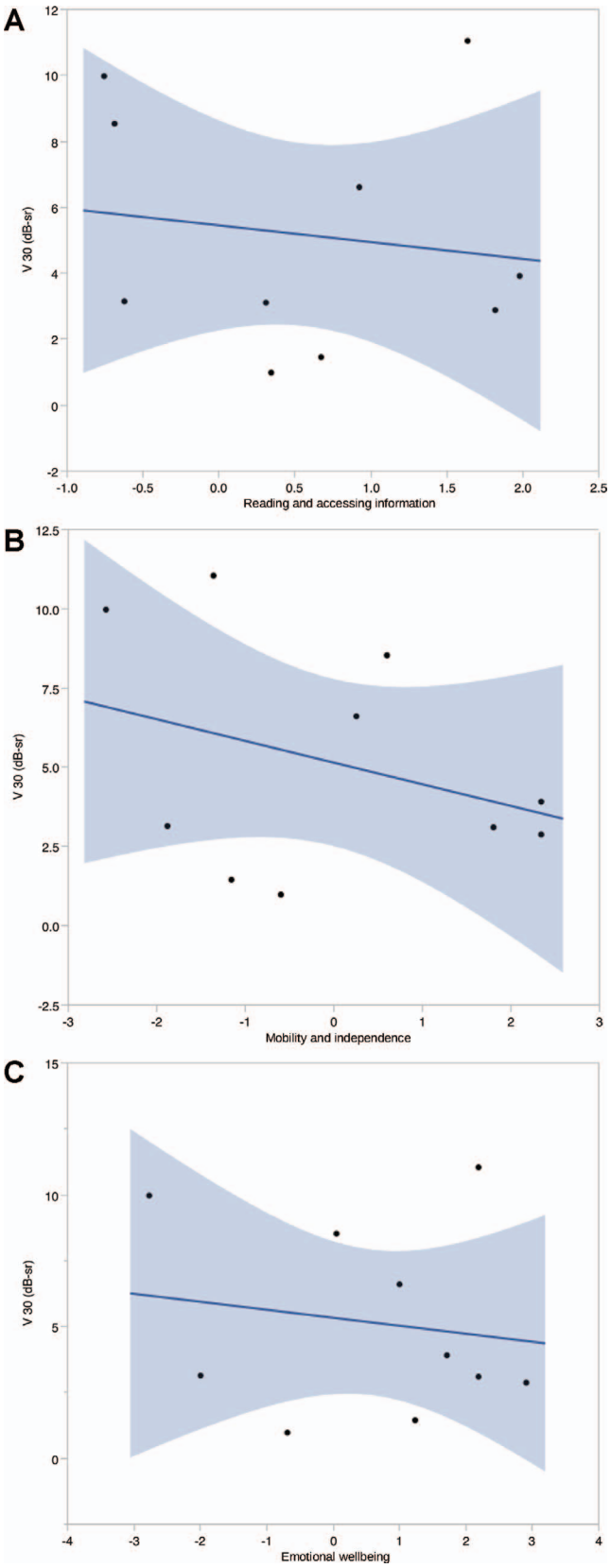

Figure 3. V_{30} compared to vision-related quality of life ($n = 10$). Scatterplot and linear regression line of V_{30} against (**A**) reading and accessing information, $r = -0.14$, (**B**) mobility and independence, $r = -0.34$, and (**C**) emotional well-being, $r = -0.16$. This demonstrates a weak linear relationship between central 30° visual field sensitivity and the three domains of vision related quality of life. *Shaded area* denotes confidence interval for regression line.

mobility and independence. It therefore appears that patients do not feel more limited by their mobility and independence as more central visual field is affected.

Using in silico molecular genetic analysis patients were stratified into three genotype groups based on severity (mild, moderate, and severe). We identified no variation in V_{30} with age in the "mild" genotype group, in contrast to worsening of V_{30} with age noted in the "intermediate" and "severe" genotypes. This suggests that patients with a mild genotype may have a milder and more slowly progressive phenotype between the ages examined.

In the following 13 subjects for an average of 16.5 months, we observed an average V_{TOT}, V_{30}, and V_{15} rate of change of –2.17 dB-sr/year, –0.60 dB-sr/year, and –0.16 dB-sr/year, respectively (Table 3). This suggests a slow progressive loss of retinal sensitivity. The magnitude of progression seems to vary among subjects with some (e.g., MM_0304), showing a greater progression. As shown in Figure 4 and Supplementary Figures S7 and S8, some patients demonstrate an increase in their retinal sensitivity from baseline. However, interestingly, when comparing the change in V_{30} in all patients over the length of the longitudinal study, only 6 out of 19 eyes (MM_0262, right eye; MM_0277, both eyes; MM_0292, right eye; MM_340, left eye; and MM_0349, right eye) show an increase in retinal sensitivity over time, of which two subjects (MM_0262, right eye and MM_0277, right eye) increase very minimally by 0.1 dB-sr/year (Table 3). We feel such variability can be explained by the slow nature of loss of retinal sensitivity and the learning effect of performing static perimetry.

Interestingly, only one subject (MM_0304) showed a loss of retinal sensitivity greater than TRTV in V_{TOT}, V_{30}, and V_{15}, with a further subject (MM_0292, right eye) demonstrating a loss in retinal sensitivity greater than TRTV in V_{TOT} alone. This is possibly in part due to the phenotypic variability seen in *RPE65*-associated LCA and the different ages of the subjects assessed, resulting in considerable difference in retinal sensitivity between patients. We therefore suggest longitudinal progression is best measured individually rather than as a cohort, and loss of retinal sensitivity may require more than 12 months' follow-up.

RPE65-associated LCA has been investigated extensively with kinetic perimetry demonstrating narrower isopters in older, compared to younger, cohorts.[4,5,30,31] This is exemplified well in a study by Jacobson et al.[30] who identified that 29 subjects had kinetic visual fields measurable by a large, bright target (V4e) which was used to map the varying patterns of visual field and subsequently quantified as a percentage of normal. Advantages of kinetic perimetry lie in detecting sharp boundaries (of particular value in neuro-ophthalmic conditions), the ability to investigate the entire field of vision and those with complex field loss. As such, it is regularly used in the assessment of both driving eligibility and disability. However, key disadvantages of kinetic perimetry include the difficulty in defining shallow scotomas, the ceiling and floor effects of large and small targets respectively, the high test-retest variability, difficulty in quantifying both results and patient performance and the operator dependent nature of the assessment.[5,6] In comparison, static perimetry is better placed to identify and define shallow scotomas, has quantify reliability parameters and is less operator dependent (with a comparatively shorter learning curve for the perimetrist). Importantly, it is also better placed to quantify both global and local pointwise parameters (including, mean sensitivity, mean deviation, and volumetric measures), which allow the investigation of regions of interest by topographic isosensitivity or by geographic selections.[7] Of note, the resolution of static perimetry is limited by the grid spacing

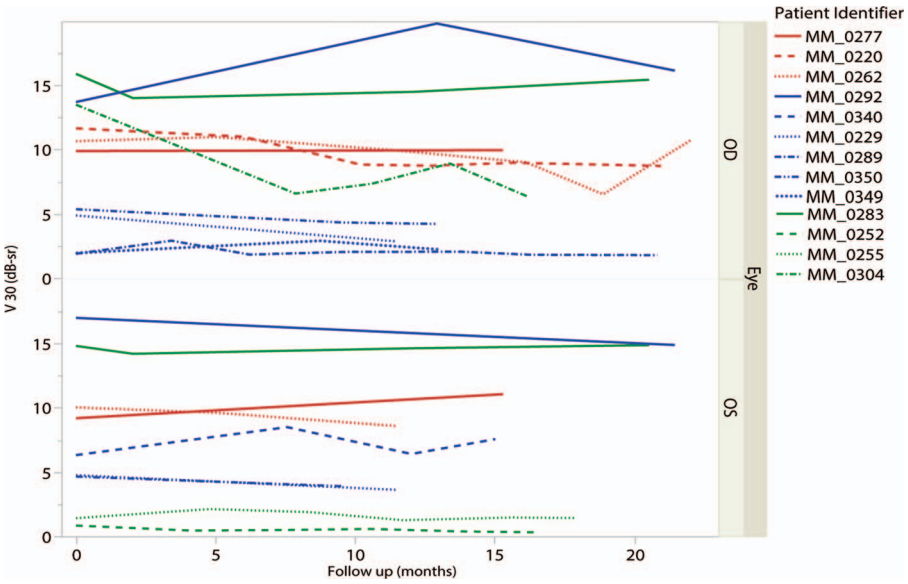

Figure 4. Change in central 30° visual field sensitivity, V_{30}, over time in a subset of 13 patients. Shown is a line graph of V_{30} measurements in subjects over time. Subjects have also been color-coded by genotype group (*red*: mild, *blue*: intermediate, and *green*: severe). This demonstrates the slow loss of V_{30} over time.

and is poor at defining sharp borders. As such, we feel static perimetry is better placed than kinetic perimetry to reliably assess and quantify retinal sensitivity in *RPE65*-associated LCA.

There are, however, some limitations to our study. Acknowledging the between subject difference in retinal sensitivity, future studies would benefit from multiple baseline assessments per subject to ideally quantify test-retest variability per subject and ideally per eye. Additionally, ideally a greater number of subjects could be included, however given the rarity of the condition this may prove difficult. Furthermore, longer follow up would also be of value.

In conclusion, this study identifies the central 30° visual field sensitivity (V_{30}), measured using static perimetry and analyzed with VFMA, as an important metric to monitor for clinically significant deterioration of visual function in cohorts of children and adults with *RPE65*-associated LCA. Furthermore, this study demonstrates that *RPE65*-associated LCA patients, over a broad age group (aged 9 to 23 years) and a broad level of visual acuity (0.20 logMAR to 1.66 logMAR), can reliably undertake such challenging and clinically validated static perimetry assessments. Such assessments of static perimetry can further be analyzed using volumetric indices and a radially designed, centrally condensed grid that can aid accurate assessment of retinal sensitivity in the presence of poor fixation and varying degrees of nystagmus. This highlights the importance of such assessments as appropriate measures of retinal sensitivity rather than as exploratory in nature, as previously

Table 3. Visual Field Sensitivity Rate of Change

Patient Identifier	Age	Mutation	Sex	Follow up	Eye	V_{TOT}	V_{30}	V_{15}
MM_0220	18	Mild	F	21	OD	−4.1	−1.7	−0.4
MM_0229	19	Intermediate	M	11	OD	−4.4	−2.1	−0.8
MM_0229	19	Intermediate	M	11	OS	−3.6	−1.2	−0.3
MM_0252	16	Severe	M	17	OS	−1.2	−0.4	−0.3
MM_0255	19	Severe	M	18	OS	0.0	0.0	0.1
MM_0262	21	Mild	F	11	OS	−2.6	−1.5	−0.3
MM_0262	21	Mild	F	22	OD	0.0	0.1	0.0
MM_0277	13	Mild	F	15	OD	−1.9	0.1	0.1
MM_0277	13	Mild	F	15	OS	−1.9	1.5	−0.2
MM_0283	11	Severe	F	21	OD	−1.3	−0.3	0.0
MM_0283	11	Severe	F	21	OS	0.3	0.0	0.0
MM_0292	9	Intermediate	F	21	OD	10.1	1.4	−0.2
MM_0292	9	Intermediate	F	21	OS	−15.1	−1.2	−0.3
MM_0289	20	Intermediate	F	21	OD	−0.1	−0.1	0.1
MM_0304	19	Severe	F	16	OD	−20.7	−5.3	−1.0
MM_0340	17	Intermediate	M	15	OS	1.7	1.0	0.4
MM_0349	23	Intermediate	M	13	OD	9.3	0.3	0.3
MM_0350	21	Intermediate	M	10	OS	−5.5	−1.0	−0.2
MM_0350	21	Intermediate	M	13	OD	−0.3	−1.1	0.0
Average	**16.9**	**N/A**	**N/A**	**16.5**	**N/A**	**−2.17**	**−0.60**	**−0.16**

Shown are the number of months follow-up and the rate of change (dB-sr/year) of three metrics of visual field sensitivity.

suggested.[14] This portion of the visual field loss appears to progress at a slow but definite rate over many years. Additionally, we suggest that the rate of progression can be correlated, to an extent, with the predicted mutational damage of *RPE65* variants using in silico analysis. This is supported by knowledge that in vitro assessments have shown varying isomerohydrolase activity with different amino acid substitutions in *RPE65*.[32] Gene therapy for *RPE65*-associated LCA has been shown to be safe with varying levels of efficacy and durability noted in both phase I/II and III studies.[10,13,14,16,17,33] We suggest that full-field static perimetry and quantification of the central 30° visual field sensitivity is an appropriate, robust, and accurate measure of visual function in these patients, before and after intervention.

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