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) underwent 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. (ClinicalTrials.gov number, NCT02714816.)

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 33,0002 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 canine7 and mouse models8,9 a total of four phase I/II trials10–13 and one phase III trial14 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 perimeter, and performance score using the multiple luminance mobility test (MLMT).15–17 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 perimetry.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 point-wise 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 form 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-associated 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, vision-related quality of life questionnaires, and RPE65-LCA sequence variants.

**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/m² (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° 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 (VTOT) 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, vs, and the 15° HOV portion, v15, 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/m².

**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/HSFHtml, 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. Subjects 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. 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 V₃₀, 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 V₃₀ 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 V₃₀ and age, visual acuity, and contrast sensitivity. In contrast, when investigating the correlation between V₃₀ 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 V₃₀, V₅₀, V₁₅, and age were then examined using Steiger’s test.

Furthermore, we fitted a multilevel mixed effects linear regression model to V₃₀ and examined the effects of the three genotype groups on V₃₀, adjusting for age. The gradients of the three linear regression lines were subsequently examined to investigate any change in V₃₀ 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 V₃₀ 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:

\[
\pm 1.96 \times \sqrt{2} \times \text{within subject standard deviation}
\]

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 V₅₀ and V₁₅ was investigated (Supplementary Fig. S2). As expected, the correlation...
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 \( V_{\text{TOT}}, V_{30}, \) 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.\(^2\)

**Retinal Sensitivity Correlation With Age**

Figures 1A, 1B, and 1C show scatterplots of the total HOV (\( V_{\text{TOT}} \)), 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 \( V_{30}, V_{\text{TOT}}, V_{15} \) 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 \( V_{\text{TOT}} \) \( r = -0.58 \) 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 \( P > 0.05 \).
Retinal Sensitivity in RPE65-LCA

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>G                       | p.Pro365Thr, missense             | c.1078C>G                       | p.Pro365Thr, 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.Gly848Asp, splice site alteration | c.1451G>A                   | p.Gly848Asp, 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.Arg821ys, splice site alteration | 0.66   | 0.64   |
| MM_0255 M          | 19  | Severe| c.1451G>A                                               | p.Gly848Asp, splice site alteration | c.245G>A                   | p.Arg821ys, splice site alteration | 0.64   | 0.74   |
| MM_0262 F          | 21  | Mild| c.118G>A                                                   | p.Gly480ser, 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.980G>A                                                  | p.Cys330Tyr, missense              | c.1443_1445delAGA           | p.Glu81del, in-frame deletion       | 0.72   | 0.82   |
| MM_0304 F          | 19  | Severe| c.11+5G>A                                                   | Splice region, splice site alteration | c.1341_1342dupCT         | p.Cys448SerfsTer4, frameshift    | 0.78   | 0.8    |
| MM_0309 M          | 18  | Severe| c.859G>T                                                  | p.Val287Phe, splice site alteration | c.859G>T                   | p.Val287Phe, splice site alteration | 1.3    | 1.48   |
| MM_0513 M          | 20  | Intermediate| c.1598C>G                                              | p.Tyr166Ter, stop-gained            | c.1464T>A                   | p.Tyr166Asn, missense             | 0.7    | 0.4    |
| MM_0340 M          | 17  | Intermediate| c.370C>T                                                  | p.Arg124Ter, stop-gained            | c.952T>A                   | p.Arg124Ter, stop-gained           | 0.8    | 0.7    |
| MM_0350 M          | 21  | Intermediate| c.130C>T                                                  | p.Arg447Ter, stop-gained            | c.1543C>T                   | p.Arg447Ter, stop-gained           | 1.3    | 1.4    |
| MM_0349 M          | 23  | Intermediate| c.130C>T                                                  | p.Arg447Ter, stop-gained            | c.1543C>T                   | p.Arg447Ter, stop-gained           | 1.1    | 0.8    |
| MM_0392 F          | 11  | Mild| c.4T>C                                                     | p.Phe16Ser, missense                 | c.1292A>G                   | p.Tyr341Cys, 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 V30, VTOT, and V15 rate of change in these subjects. In addition, rate of change (dB-sr/year) was calculated for each patient. In this cohort, the average VTOT, V30, and V15 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 VTOT 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 VTOT, V30, and V15 varied with age. In this cohort, we identify that mean sensitivity and change in VTOT, V30, and V15 rate of change was found to be −16.5 months (range, 10–22 months). Figure 4, and Supplementary Figures S7 and S8, respectively show the change in V30, VTOT, and V15 rate of change in these subjects. In addition, rate of change (dB-sr/year) was calculated for each patient. In this cohort, the average VTOT, V30, and V15 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 VTOT alone.

While no statistically significant difference was noticed between the three metrics, the results suggest V30 as the best metric to monitor deterioration of visual function in cohorts of children
and young adults. Furthermore, we identified a weak and moderate relationship between V30 and BCVA and contrast sensitivity, respectively; suggesting that V30 is a useful independent measure of visual function in RPE65-associated LCA.

We investigated the correlation between V30 and VRQoL quantified using Rasch-transformed data from the IVI questionnaire. Interestingly, a weak linear relationship between V30 and mobility and independence was identified, compared to a moderate ($r = -0.45$) linear relationship between V15 and age after separating out the three genotype groups.

![Figure 2](https://example.com/fig2.png)

**Figure 2.** (A) V30 compared to age. Shown is a scatterplot of V30 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) V30 compared to age, in the three genotype groups. Shown is a scatterplot of V30 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.

| Test-Retest Reliability | 95% Confidence Interval | F Test With True Value 0 |
|-------------------------|-------------------------|-------------------------|
|                          | ICC                     | Lower Bound | Upper Bound | Value | df/1 | df/2 | Sig  |
| $V_{30}$                | 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.
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 with age in the “mild”
genotype group, in contrast to worsening of V30 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 VTOT, V30, and V15 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 V30 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 VTOT, V30, and V15, with
a further subject (MM_0292, right eye) demonstrating a loss in retinal sensitivity greater than TRTV in VTOT 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. This is exemplified
well in a study by Jacobson et al. 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 respective-
ly, the high test-retest variability, difficulty in quantifying both
results and patient performance and the operator dependent
nature of the assessment. 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 peri-
meter). 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
insensitivity or by geographic selections. Of note, the
resolution of static perimetry is limited by the grid spacing

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**Figure 3.** V30 compared to vision-related quality of life (n = 10). Scatterplot and linear regression line of V30 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.
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 (V30), 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 shown.

### Table 3. Visual Field Sensitivity Rate of Change

| Patient Identifier | Age | Mutation | Sex | Follow up | Eye | VTOT | V30  | V15  |
|--------------------|-----|----------|-----|-----------|-----|------|------|------|
| 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. 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. 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. 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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