Outcome Measures for Clinical Trials of Leber Congenital Amaurosis Caused by the Intrinsic Mutation in the CEP290 Gene

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Submitted: January 26, 2017
Accepted: April 12, 2017

PURPOSE. To determine efficacy outcome measures for clinical trials of Leber congenital amaurosis (LCA) associated with a common intrinsic mutation in the CEP290 gene.

METHODS. CEP290-LCA patients (ages 5–48) with the intrinsic mutation (c.2991+1655A>G) were studied as a retrospective observational case series using clinical methods and with full-field sensitivity testing (FST), optical coherence tomography (OCT), autofluorescence imaging (NIR-RAFI), transient pupillary light reflex (TPLR), oculomotor control and instability (OCI), a mobility course, and a questionnaire (NEI-VFQ). Patients were investigated cross-sectionally but a subset was able to be followed longitudinally.

RESULTS. With FST, there was no rod function; cone sensitivities had a wide range from not detectable to near normal. OCT analyses indicated retained central photoreceptors with normal distal laminae. Based on OCT and FST, most patients had dissociation of structure and function. TPLR was nondetectable in the majority of patients, with responders demonstrating severe losses in light sensitivity. OCI was abnormal in most patients. NEI-VFQ scores had a similar range to those of other severe retinopathies. Mobility scores were consistent with FST sensitivities. In patients examined with FST, OCT, and NIR-RAFI over long-term intervals (7–10 years), there was limited but detectable disease progression.

CONCLUSIONS. Efficacy would be a quantitative change in foveal cone function and possibly distal laminar structure. FST provides a subjective photoreceptor-based outcome; OCT and NIR-RAFI can assess photoreceptor and RPE structure. TPLR and OCI can provide objective measures of postretinal transmission. Minimal change over a decade indicates that there is no practical value in natural history studies.

Keywords: cones, rods, optical coherence tomography, autofluorescence imaging, visual acuity, LCA10, NPHP6, pupillometry, oculomotor control

Leber congenital amaurosis (LCA) is a group of severe early-onset inherited retinal degenerations (IRDs) that are now better understood because gene identification helped reveal the different disease mechanisms underlying this clinical presentation.1,2 Small and large animals with similar genetic defects to the human LCA diseases were also recognized and the opportunity was created to test novel therapies. Now, we have entered an era when gene-specific therapies for LCA are being considered or are already in clinical trials (for example, LCA2 or RPE65-LCA5).

One of the more common molecular subtypes of LCA is caused by mutation in the gene encoding CEP290 (Centrosomal protein 290), which has been localized in the outer retina to the photoreceptor cilium.4 CEP290 mutations are also a cause of nonoculocutaneous phenotypes.5 A frequently occurring CEP290 mutation is an intronic mutation (c.2991+1655A>G) that creates a splice donor site that permits a 128-bp cryptic exon insertion and leads to premature termination (p.C998X) of protein synthesis.5–8

Pivotal question is whether there are ways to alter the genetic consequences and possibly ameliorate the severe visual disease resulting from mutation in CEP290. Gene augmentation therapy is theoretically possible but is constrained by the packaging capacity of the viral vector, which in the case of adeno-associated virus (AAV) is not sufficient to deliver large cDNAs such as CEP290. There are, however, variations on the conventional theme of AAV and full-length CEP290, such as fragmented AAV, carrying truncation mutants and the use of lentiviral platforms.9 Other concepts, such as antisense oligonucleotides7 or gene editing,10 have also been proposed. When such gene-based proposals are made, however, it is assumed that there are residual retinal photoreceptor cells that can be targeted. Otherwise, the disease becomes a candidate for other therapies that do not require retained photoreceptors,
such as regenerative medicine, optogenetics or a retinal prosthesis.\textsuperscript{11–14}

In LCA patients, how are we able to determine if there are retained photoreceptors despite severe visual disturbances? Optical coherence tomography (OCT) has allowed retinal structural details to be imaged in patients. \textit{CEP290}-LCA is one of the forms of LCA with a retained photoreceptor cell layer despite severe visual loss.\textsuperscript{14} The residual ONL (outer nuclear layer) is mainly central-foveal; and evidence has been provided that there is early loss of more peripheral ONL.\textsuperscript{15,16}

Given the possibility of therapy, which outcome measures would be valuable and feasible to monitor in this visually severe retinopathy with relatively preserved central outer retinal structure? Conventional outcomes, such as visual acuity,\textsuperscript{17} need to be supplemented. Here we study with cross-sectional and longitudinal measurements from \textit{CEP290}-LCA patients, specifically with the intronic mutation (c.2991+1655A>G), to decide on clinically feasible efficacy outcomes for this disease. With these data, we are now positioned to develop a protocol for a future clinical trial of therapy for this previously untreatable and incurable disorder.

\section*{Methods}

\subsection*{Human Subjects}

The present studies were approved by the institutional review board at the University of Pennsylvania. Informed consent was obtained and all procedures adhered to the tenets of the Declaration of Helsinki. There were 22 patients, representing 20 families, with LCA caused by \textit{CEP290} mutations, diagnosed clinically and by molecular genetics (Supplementary Table S1). All subjects underwent a complete eye examination. The study was a retrospective observational case series (between 2000 and 2016); not every study method was able to be performed on every patient. Electroretinograms (ERGs) were not performed as a part of this study, but results were available from medical records in 19 of the patients.

\subsection*{Full-Field Stimulus Testing (FST)}

FST was performed in 20 patients. Visual sensitivities were measured with red (peak 637 nm) and blue (peak 465 nm) stimuli in the dark-adapted state using published methods.\textsuperscript{18–20} The difference between blue and red sensitivities determined whether there was cone- or rod-mediated perception. Sensitivity to the red stimulus was used as a metric for cone function.

\subsection*{Optical Coherence Tomography}

OCT was able to be performed in 21 patients. Cross-sectional imaging with line scans along the horizontal meridian crossing the fovea was mainly performed with a spectral-domain OCT unit (RTVue-100; Optovue, Fremont, CA, USA); images from a subset of patients were acquired using a time-domain OCT unit (OCT3; Carl Zeiss Meditec, Dublin, CA, USA). Data were resampled to equal density (512 samples per 30°) and processed with custom programs (MATLAB 7.5; MathWorks, Natick, MA, USA) using reported methods.\textsuperscript{21} Two parameters of central retinal structure were measured: ONL thickness of the foveal peak and extent of the residual central ONL island. The ONL thickness was the average of three samples, one obtained at the foveal pit and one on each side of the fovea along the horizontal meridian at 0.5° eccentricity. Extent of the central ONL island was defined as the retinal distance between the foveal pit and an eccentric point where the ONL reached a criterion thickness. The criterion thickness was defined with consideration of the resolution of the instruments used and the asymptote of ONL thickness outside the central peak when detectable. Among the patients studied, 10 showed an asymptotic ONL thickness ranging from 6.0 to 12.5 μm beyond the central peak, yet within the recorded scans. Axial resolution in retinal tissue of OCT3 and spectral-domain instruments are better than 12.5 μm.\textsuperscript{22} Thus, the ONL criterion thickness was taken as 12.5 μm. In one patient (P15) ONL in the nasal direction at all visits never reached 12.5 μm, but instead had an asymptote of 20 μm. In this case, the nasal extent was defined using a criterion thickness of 20 μm.

Longitudinal data permitted comparison of foveal ONL thickness and central ONL island extent for visits that were several years apart. Measured longitudinal changes were compared with test-retest variability estimates derived from pairs of independent scans obtained on different days within the same visit (n = 7; five patients with Optovue recordings and two with OCT3 recordings). In each scan, ONL was segmented and foveal ONL thickness and ONL temporal and nasal extents were measured. Test-retest variability was defined as the 95% repeatability coefficient of these parameters calculated as $1.96 \times \sqrt{2sw}$, where $sw$ is the within-person SD obtained by ANOVA.\textsuperscript{23}

\subsection*{Near-Infrared Excited Reduced-Illuminance Autofluorescence Imaging (NIR-RAFI)}

NIR-RAFI was able to be performed in 20 patients. A confocal scanning laser ophthalmoscope (HRA2 or Spectralis HRA without OCT; Heidelberg Engineering, Heidelberg, Germany) was used to record NIR-RAFI as previously described.\textsuperscript{15,16,24} In brief, images were obtained with 790-nm NIR excitation light (100% indocyanine green laser power setting; and 95% or 105% detector sensitivity for HRA2 or Spectralis HRA, respectively; automatic normalization off), and emission signals were collected between 810 and 900 nm. High-speed mode was used for acquisition, and multiple images were registered and averaged to increase the signal-to-noise ratio. In most cases, a 30° lens was used. Depending on the equipment used and oculomotor characteristics of individual patients, averaging was performed either during acquisition with the manufacturer’s automatic real-time method or postacquisition image processing was used with custom programs to align and average individual images visually judged to be lacking shearing artifacts resulting from eye movements. Boundaries of the centrally retained islands were drawn manually at the transition from higher to lower signal; location of the anatomical fovea was transferred from OCT scans; and the extent of the central island from the fovea was quantified.

\subsection*{Transient Pupillary Light Reflex (TPLR)}

TPLR was performed in 21 patients. The direct TPLR was recorded using previously published methods.\textsuperscript{20,25–29} In brief, TPLR luminance-response functions were derived from full-field stimuli of short duration (100 ms) with increasing intensities of green (−6.6 to +2.3 log scot-cd.m²), orange (−4.5 to +1.3 log scot-cd.m²), and white (+2.5 log scot-cd.m²) lights, presented monocularly in the dark-adapted (>1 hour) state. The pupil was imaged in the dark under infrared illumination. For each stimulus presented, a video clip (with a >1-second prestimulus baseline) was digitized and analyzed frame-by-frame. A 6-mm-diameter calibration target was recorded for all subjects to allow determination of absolute pupil size. TPLR amplitude was defined as the signed difference between the pupil diameter at a fixed time (0.9 second) after the onset of the stimulus and the prestimulus baseline. Luminance-response functions were fit with a hyperbolic function and the
threshold scotopic luminance evoking a criterion response (0.3 mm, limit of spontaneous oscillations in pupil diameter$^{35}$) was determined for green and orange stimulus series. The difference between the thresholds estimated from two colors was used to determine photoreceptor mediation.

Data obtained on two consecutive days were used to estimate the test-retest variability for baseline pupil diameter and TPLR amplitude. For this purpose, 95% limits of agreement were calculated considering repeated measures$^{30–32}$ with the R statistical software (https://cran.r-project.org/web/packages/MethComp/index.html)$^{33}$

Oculomotor Control and Instability (OCI)

OCI was able to be performed in 16 patients. Video imaging of the eye was accomplished with a confocal scanning laser ophthalmoscope (Spectralis HRA; Heidelberg Engineering) using near-infrared (815 nm) reflectance mode (30° lens; 25% laser power setting; 55% detector sensitivity; automatic normalization off; high-speed recording mode). The focus was held constant at +20 diopters (D) for all subjects. The patient was instructed to “look straight ahead” in primary gaze. The camera head was moved along the z-axis to bring the iris into focus (without changing the focus knob), and then the camera was moved laterally such that both eyelids were visible and centered in the frame, and the medial (nasal) canthus was just visible at the edge of the frame. In a dark room (light from the computer monitor was blocked from reaching the subject’s eye) two sets of 30-second-long video recordings were performed per eye. One set was with the internal fixation turned off to record oculomotor characteristics under “open-loop” conditions without visual feedback (independent of the level of remnant vision in each patient). The second set was recorded with the internal fixation target turned on. Some patients and all normal subjects were able to see the target and fixate and thus the recording represented “closed-loop” conditions with visual feedback. For patients who were not able to see the fixation target, the second set was effectively a duplicate of the first set.

The videos were imported into an image processing software (Image) software; http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). Frame-by-frame analysis was performed to remove blinks and to quantify the location of the center of pupil. The center was estimated from the visible edges of the pupil with respect to a reference placed at the medial canthus. Image pixels were converted to millimeters with the use of a calibrated target imaged similarly. The coordinates of the mean normal primary gaze were defined with respect to the medial canthus reference. Mean gaze offset was quantified as the radial distance of the center of pupil from the mean normal primary gaze locus averaged over the recording epoch. Oculomotor instability was quantified by the variability (2 SD) of the radial distance. An overall OCI measure was defined as the sum of the mean gaze offset and oculomotor instability parameters.

Obstacle Course and Scoring

Obstacle course mobility was determined in 16 patients. This indoor obstacle course was built in a room (5 m long × 4 m wide × 2.75 m high) normally used for the clinic. Lateral walls made of Styrofoam delimited the course, leaving a central area of 4.6 × 2.7 m (Supplementary Fig. S1). Eye-level obstacles (Styrofoam squares and circles) were suspended from wire tracks below ceiling level that crossed the room every 1.4 m. Each of these obstacles could be located at any of four positions along the track. Floor-level obstacles were also part of the course and could be positioned at various locations along the course. Obstacles were designed to fall away from the patient if touched and thus were not hazardous. The patient was guided by an investigator from an anteroom into the mobility course area and instructed to walk through the course and reach a door at the far end of the room while trying to avoid any obstacles encountered en route. The far-end door consisted of a Styrofoam panel covered with white fabric over a darker background wall. Ambient room illumination was 100 lux. The course obstacle set was rearranged between each of the trials using a randomized table of positions.

The patients received a training session for the task and the test session occurred on the following day. Three trials (full-course traversals) were performed for the right eye, left eye, and both eyes. The number of patient incidents (bumps to obstacles and walls; need for reorientations due to changes of direction inconsistent with the instructions$^{34}$) per trial was noted. The mobility scores for each eye and both eyes were obtained by adding the number of incidents noted for each of the three trials.

Visual Function Questionnaire

The National Eye Institute Visual Function Questionnaire-25 (NEI-VFQ-25) was administered by telephone to 19 patients (3 patients could not be reached) and scored$^{35}$; scores range from 0 to 100 and higher scores indicate better vision. The three items in the “Driving” subscale of VFQ-25 were not included.

Results

Twenty-two patients with CEP290-LCA were studied with clinical examinations (Supplementary Table S1): 20 were able to be examined with FST (except P2 and P10, both unable to cooperate); 21 were able to be examined by OCI and NIR-RAFI (only P10, with keratoconus, could not be imaged; P9 could be imaged but had an unusual structural phenotype; Supplementary Fig. S2); TPLR could be performed in 21 patients (P10 could not cooperate for the testing); OCI and mobility performance were performed in 16 patients (the methods reported here were designed specifically after 2009 to address a need for such assessments; 6 patients, first seen before 2009, were not available to return for these studies); and NEI-VFQ was performed by telephone interview in 19 patients (3 patients could not be reached after multiple attempts for this interview). Historical records of rod and cone ERGs were reviewed and were nondetectable in all 19 patients who had this testing.

Visual Function in CEP290-LCA

Patients with the intronic mutation in CEP290-LCA had best-corrected visual acuities (VAs) that ranged from 20/50 to no light perception (NLP). There were anterior segment abnormalities (cataract and keratoconus; details in Supplementary Table S1) but data could be collected from the testing eyes with two exceptions. P4 OS had such density of cataract that imaging in that eye was not possible, and the keratoconus in P10 made imaging not feasible. Ranking of the patient VAs in the better-seeing eye (Fig. 1A, left column) and the fellow eye (right column) indicates that there was no clear relation of VA to age in this cohort. (Spearman’s $\rho = -0.28$ and $-0.30$, with $P = 0.25$ and $P = 0.19$ for OD and OS, respectively; $n = 20$ patients). Adjacent to the VAs are cone sensitivities by dark-adapted FST. Of note, four patients homogeneous for the intronic mutation (P6, P8, P15, and P18) were among those with the best retained vision. Chromatic FST showed that all patients with recordable sensitivities had
no measurable rod function and the results were co-
mediated. Visual function data are not shown for P2 and P10, who were unable to perform the test. Cone FST results were linearly related to logMAR measures of VA (n = 14 eyes of seven patients excluding light perception [LP] and NLP; r² = 0.72, P < 0.0001; hand motion [HM] was assigned a logMAR value of 2.28 ± 0.7 log units). The long-term change between FST in -LCA patients of -LCA patients with measurable sensitivities on two visits separated by 8 to 10 years. Gray dashed lines show short-term (1 day) test-retest variability (±2 SD).

**Is CEP290-LCA Stationary or Progressive, Once Peripheral Structure Is Lost in Early Life?**

Our previous cross-sectional studies of CEP290-LCA patients showed that almost all patients, independent of severity of visual dysfunction, retained a central island of photoreceptor nuclear layer.9,15,16 A clue that there could be a natural history of slow degenerative change in the typically retained central islands was in the analysis of the width of the islands along the horizontal meridian of OCTs in CEP290-LCA patients of different ages.9 In the current cohort of CEP290-LCA patients with the intronic mutation, we tested the hypothesis that this is a progressive disease by studying not only cross-sectional data but also long-term longitudinal data with OCT and a complementary data set with en face imaging.

First, OCTs and en face central fundus NIR-RAFI images from a cross-sectional data set were quantified (Fig. 2). An OCT section in a normal individual across 30° of retina centered on the fovea shows multiple laminae representing the nuclear, synaptic, and fiber layers (Fig. 2A, upper left). There is a peak of ONL thickness at the foveal depression and thinning with increasing eccentricity, but a maintained level of ONL to the edges of the scan. P13 at age 15 has normal peak foveal ONL thickness, but ONL is reduced to a barely discernible level by approximately 7 to 8° eccentricity. Lamination distal to the ONL is present in the normal scan but it appears indistinct in the patient's scan, as reported previously15,16 (Fig. 2A, lower left).

ONL was able to be quantified in the horizontal scans across the central retina of 20 CEP290-LCA patients representing a large age spectrum (Fig. 2B). Foveal ONL thickness was within or above normal limits in all but one patient (P20, at age 32). As a function of age, foveal ONL thickness tended to decrease (rate, 0.95 μm/°; Fig. 2B, lower left panel); but, this was not statistically significant (P = 0.06; r² = 0.18). Temporal (T) and nasal (N) extents of ONL, on average, did not differ significantly (P = 0.9, t-test). We asked whether T and N extents tended to change with age. Linear regression fit to the data showed slopes of -0.065 and -0.058 degrees per year for T and N extents, respectively (Fig. 2B, right panels); these rates were not statistically significant (P = 0.21, r² = 0.08, for both T and N).

An NIR-RAFI image from the normal individual has a high signal near the fovea (Fig. 2A, upper right) considered to be due to the increased melanin optical density of the RPE cells at that location.24,38 P13 has a discrete slightly elliptical central area of high signal (Fig. 2A, lower right), indicating a region of...
FIGURE 2. Retinal imaging in CEP290-LCA: cross-sectional data. (A) Upper left: OCT scan in a normal subject (age 23) across the horizontal meridian through the fovea. Lower left: OCT in patient P13 (age 15) with CEP290-LCA. Retinal layers are labeled: ONL, inner nuclear layer (INL), ganglion cell layer (GCL), and nerve fiber layer (NFL). Upper right: NIR-RAFI of the macula in the normal subject. Lower right: NIR-RAFI in the patient with CEP290-LCA. (B) OCT data. Upper left: Horizontal ONL profile of P13 shown in the OCT scan in (A) compared with normal limits (gray band represents normal mean ONL ± 2 SD). The dotted line represents the criterion ONL thickness of 12.5 μm. Two vertical lines denote temporal (T) and nasal (N) extents of ONL in this patient. ONL extent was defined as the eccentricity of the intersection of the horizontal ONL profiles with the criterion value (dotted line). Lower left: ONL peak at the fovea as a function of age in patients (black circles; n = 20 patients) and normal individuals (n = 27, ages 4–58, gray circles). Light gray dashed lines indicate the 95% prediction interval of linear regression fit to the normal data. The dark gray line is a linear regression fit to the patient data. Upper right: Nasal retinal ONL extent from fovea as a function of age.
healthy RPE with retained melanization transitioning to a surrounding area of demelanization.\textsuperscript{15,16}

En face NIR-RAFI signals were able to be characterized in the 20 CEP290-LCA patients (Fig. 2C). All showed a central elliptical region of higher signal as in the representative image of a patient (P18) shown (Fig. 2C, inset); for P20, with the unusual foveal loss of NIR-RAFI signal (Supplementary Fig. S2), the perifoveal boundary was measured. The extents of the detectable boundaries along the four cardinal meridians are shown (Fig. 2C). Linear regression slopes were not significantly different from zero: $-0.039, -0.02, -0.036$, and $-0.044$ degrees per year ($P = 0.18, 0.27, 0.33, 0.23$; $r^2 = 0.10, 0.07, 0.05, 0.08$, respectively) along the superior (S), inferior (I), T, and N meridians, respectively. Thus, OCT and NIR-RAFI results taken together suggest a retained central elliptical region of approximately 13-degree width and 10-degree height in the great majority of the patients. There were, however, two notable exceptions to this pattern. One 13-year-old patient (P9) had foveal schisis with extensive retinal thinning from the foveal area to the optic nerve; and a 40-year-old patient (P20) had foveal loss of ONL surrounded by more preserved outer retinal structure (Supplementary Fig. S2).

Longitudinal imaging data for CEP290-LCA have not been reported to date. Within our cohort, we have measurements from both imaging modalities in a subset of these patients (Fig. 3). P11 at ages 19 and 29 provides an example; we compared the horizontal OCT scans and NIR-RAFI en face images at these ages and quantified the images. The ONL in the two OCTs separated by a decade appears to show limited change; measurements indicate no change in the foveal ONL peak but $4.5$ degrees of reduced ONL extent into the T retina, whereas no change is detectable in the N extent (Fig. 3A). N and T extents apparent on NIR-RAFI also indicate reduction only in the T meridian of the ellipse of high signal (Fig. 3B). P15 at ages 20 and 30 provides a further example of longitudinal imaging data. The OCT scans appear similar but quantitation of ONL indicates a reduction of 28 $\mu$m at the foveal peak at age 30; both measures fall within the normal range. The N extent of ONL is unchanged but the T extent is reduced by 3 degrees (Fig. 3C). The high signals in the NIR-RAFI images in P15 show a generalized constriction of the boundaries (Fig. 3D).

All longitudinal OCT and NIR-RAFI data for the subset of patients are plotted (Figs. 3E, 3F). OCT data in eight eyes of eight patients obtained serially over an average of 8.75 years (range, 7–10 years) indicate that peak ONL at the fovea is not changed more than intervisit variability in six of eight eyes, and all foveal thickness measurements except those of P20 were above the lower limit of the normal range (76 $\mu$m). Nasal extent of ONL changed more than expected from intervisit variability (1.4 degrees) in two of eight eyes; the T extent was reduced at the later age in five of six eyes. In a subset of seven eyes of seven patients, NIR-RAFI was obtained serially over an average of 9 years (range, 4 to 10 years). The average rate of constriction in the seven eyes with serial follow-up was 0.35 degree per year along the T meridian but only 0.12 to 0.14 degree per year along the other three meridians (S and I not shown). In summary, there was neither a significant reduction in peak foveal ONL ($P = 0.07$; paired $t$-test) nor ONL nasal retinal extent ($P = 0.08$); but there was a significant reduction of ONL extent into the T retina ($P < 0.001$) and significant changes in NIR-RAFI data ($P < 0.05$). Based on these imaging data, it can be concluded that the disease is not stable but shows a slow progression.

**Pupillometry as an Outcome Measure in CEP290-LCA**

A bright short-duration achromatic stimulus presented to a normal dark-adapted eye evokes a transient fast pupil constriction followed by a slower redilation (Fig. 4A). Fewer than half ($9/21 = 43\%$) of the current cohort of CEP290-LCA patients also responded to a bright short-duration achromatic stimulus with a detectable and fast TPLR (Fig. 4B), whereas most ($12/21 = 57\%$) showed no detectable change in pupil diameter (Fig. 4C). There were no statistically significant differences ($P > 0.1$; $t$-test) between the baseline dark-adapted pupil sizes of normal subjects (mean $= 6.5 \pm 0.4$ mm), TPLR-responder CEP290-LCA patients (5.9 $\pm 1.1$ mm), and nonresponders (6.2 $\pm 1.4$ mm).

The amplitude of pupillary constriction in the TPLR-responder CEP290-LCA patients was significantly smaller than normal when measured at a fixed time of 0.9 second ($0.75 \pm 0.38$ mm versus $2.16 \pm 0.20$ mm; $P < 0.0001$) or at peak constriction ($0.87 \pm 0.45$ mm versus $2.41 \pm 0.36$ mm; $P < 0.0001$) (Figs. 4A, 4B). In all pupillary responders, chromatic luminance-response functions were used to determine the sensitivity of the TPLR to light and estimate the underlying photoreceptor mediation at response threshold. In a representative normal subject, the threshold for TPLR responses was near $-5$ log scot-cd.m$^{-2}$ (Fig. 4D). With increasing stimulus luminance, TPLR grew in amplitude and accelerated in timing; scotopically matched green and orange stimuli produced similar TPLRs throughout the measurement range, implying a dominant role for the rod photoreceptor system driving the pupils. On the other hand, the representative CEP290-LCA patient P17 demonstrated reduced light sensitivity with barely detectable responses near $-1.5$ log scot-cd.m$^{-2}$ (Fig. 4E). As compared with scotopically matched green stimuli, orange stimuli produced larger and faster TPLR amplitudes (Fig. 4E). Luminance-response results could be fit with similar saturating functions for green and orange stimuli for normal subjects (Fig. 4F) and for the CEP290-LCA patients who were TPLR responders (Fig. 4G).

Pupillary light sensitivity was defined based on the threshold stimulus intensity expected to evoke a criterion (0.5 mm) pupillary constriction amplitude. In all responding CEP290-LCA patients, thresholds were significantly and substantially elevated between 4.2 and 5.6 log units from normal (Fig. 4H, upper). Chromatic threshold differences suggested that in eight of nine pupillary responders, the cone system was likely dominating the TPLR; in one of nine patients, there was evidence of the rod system contributing at a minimum to the TPLR evoked by the green stimulus (Fig. 4H, lower). The extent of the pupillary sensitivity loss was similar to the perceptual sensitivity loss (Fig. 4I).
amplitude; however, there was general consistency of TPLR dynamics. Baseline dark-adapted pupil diameters tended to be 0.40 mm larger on day 1 and 95% limits of agreement were 0.74 to +1.54 mm (Supplementary Fig. S3B). TPLR amplitude did not have a bias between the days (mean difference +0.04 mm) and 95% limits of agreement were +0.47 to +0.38 mm (Supplementary Fig. S3C).

Oculomotor Control: Eye Position and Stability

Video images of eyes illuminated with near-infrared light in the dark were used to quantify the gaze position and stability over time. A normal eye in primary (straight-ahead) gaze corresponds to a pupil center position that is on average 13.7 mm temporal and 3.3 mm superior with respect to the medial canthus (Fig. 5A). Excursions of 30° visual angle from primary...
Figure 4. Pupillometry in CEP290-LCA. (A–C) Dynamics of pupil constriction to a 100-ms duration achromatic bright stimulus in a representative normal (A), CEP290-LCA patients grouped into those with detectable responses (n = 9 patients) (B), and those without (n = 12 patients) (C). Gray lines near the start of each trace show the baseline pupil diameter and the timing of the stimulus onset. (D, E) Families of TPLRs recorded with increasing luminance of green and orange stimuli in a representative normal subject (D) and CEP290-LCA patient P17 (E). Vertical gray line shows the fixed time of 0.9 second after stimulus onset when the response amplitude was measured. Stimulus monitor is shown. Interruptions of the traces are due to blinks. (F, G) Chromatic luminance-response functions in the normal (F) and CEP290-LCA patient (G) shown above. Horizontal gray dashed lines represent the criterion (0.3 mm) response amplitude, and vertical gray continuous lines represent the criterion threshold for each stimulus color. (H) Scotopic criterion threshold (upper) and green-orange threshold difference (lower) for the subset of responding CEP290-LCA patients (n = 9). (I) Loss of pupillary light sensitivity as a function of perceptual light sensitivity.
gaze along the four cardinal meridians result in approximately 4-mm movements of the center of pupil away from the center (Fig. 5B). To a first approximation, the location of the center of the pupil can thus be used to quantify the gaze position and its stability over time. As demonstrated in a representative subject, normal eyes tend to be very stable with or without fixation (Fig. 5C, upper traces). In a group of normal eyes (10 eyes of five subjects), the oculomotor instability parameter is 0.36 (±0.20) mm without fixation and 0.21 (±0.15) mm with fixation; the mean gaze offset value, on the other hand, is 1.48 (±0.64) mm without fixation and 1.36 (±0.63) mm with fixation.

CEP290-LCA patients as a group (n = 32 eyes of 16 subjects) had a significantly greater OCI parameter compared with normal; however, individual patients could have a wide spectrum ranging from reliable control of gaze position with small amplitude nystagmus in some patients to complete lack of oculomotor control with wandering eyes in other patients.

**Figure 5.** Oculomotor instability in CEP290-LCA. (A) Upper: Schematic representation of the coordinate system centered at the medial (nasal) canthus and the center of pupil (white cross) at primary gaze. Lower: Individual data from left eye and right eye of all normal subjects at primary gaze. Mean value is also shown (circle). (B) Schematic representation of eyes fixating 30° eccentric along the four cardinal directions, and relative offsets of the center of pupil measured from the primary gaze locus. (C) Chart records showing the radial offset of the center of pupil from the mean normal primary gaze locus (thick gray line) during a 30-second-long recording epoch in a representative normal subject and two CEP290-LCA eyes. Two records shown are with (right column) and without (left column) fixation. (D) Oculomotor instability plotted against mean gaze offset in individual CEP290-LCA eyes (triangles; n = 32 eyes of 16 patients) recorded with and without fixation. Equivalent results from normal eyes are also shown (gray circles). Gray lines demarcate the upper (mean ± 2 SD) limits of normal for each parameter.
P8 represents CEP290-LCA patients with partially retained perception. With fixation off in a dark room, there was a mean gaze offset of 2.63 mm and an instability of 0.72 mm (Fig. 5C, middle trace). With fixation on, the mean gaze offset value reduced to 0.79 mm and the instability reduced to 0.58 mm. P9, on the other hand, represents CEP290-LCA patients with NLP. With and without fixation, there was severe instability of ocular motor behavior (Fig. 5C, lower trace) with a mean gaze offset of larger than 2 mm and an instability of larger than 2 mm. Quantitative summary of all CEP290-LCA eyes (n = 32 eyes of 16 subjects) shows a range of ocular motor instability from 0.3 to 3.5 mm without fixation and 0.2 to 3.6 mm with fixation; similarly, mean gaze offset ranged from 0.6 to 5.5 mm without fixation and 0.2 to 6.2 mm with fixation (Fig. 5D). The eyes with measurable VA had statistically significantly better ocular motor instability (0.6 vs. 2.1 mm with fixation off, P < 0.001; 0.5 vs. 1.8 mm with fixation on, P < 0.001) and mean gaze offset (1.7 vs. 3.0 mm with fixation off, P = 0.012; 1.3 vs. 2.8 mm with fixation on, P = 0.010) compared with eyes with LP or worse.

Test-retest variability of the novel OCI measure developed here was evaluated on two consecutive days (Supplementary Fig. S4). Right eye of P5 is representative of CEP290-LCA results with overall OCI measures of 3.61 and 4.18 mm without fixation on days 1 and 2, respectively, and similar results with fixation (Supplementary Fig. S4A). For the ocular motor instability parameter, there were minor biases between the days (mean day 1 minus day 2 differences of −0.06 and −0.08 mm, without and with fixation, respectively) and their 95% limits of agreement were −0.97 to 0.86 and −1.17 to 1.01 mm, respectively (Supplementary Fig. S4B). For the mean gaze offset parameter, there were also minor biases between the days (mean day 1 minus day 2 differences of −0.10 and 0.12 mm, without and with fixation, respectively) and their 95% limits of agreement were −2.33 to 2.13 and −1.56 to 1.80 mm, respectively (Supplementary Fig. S4C).

Obstacle Course and Quality-of-Life Questionnaire

Results of testing 16 CEP290-LCA patients in the obstacle course we designed (Supplementary Fig. S1) are tabulated (Supplementary Table S1). The number of incidents observed across all sessions ranged from 0 to 15 with a median of 10 (n = 48 sessions for right eye, left eye, and both eyes; 16 patients). There were no significant differences between eyes (P = 0.1, paired t-test). There was evidence of monotonic relationships between mobility score and VA, and between mobility score and cone sensitivity (Spearman’s ρ = +0.52 and −0.54, with P = 0.002 and P = 0.001, respectively; n = 32 eyes), suggesting that patients with a greater amount of retained vision had fewer incidents.

Scores of the NEI-VFQ, a quality-of-life questionnaire, that we performed in 19 of our CEP290-LCA patients ranged from 34.9 to 75.5, with a mean score of 49.7 (SD 10.1). These results are similar to those obtained in previous studies of severe retinal diseases, including forms of retinitis pigmentosa (RP) and LCA.

CEP290-LCA Genotypes

All 22 patients had at least one allele with the common splice site mutation (Supplementary Table S1). Four patients were homozygous for this mutation. Of the remaining 18 patients, the large majority had a second CEP290 mutation that would be predicted to lead to a premature termination codon (seven patients with a nonsense mutation and eight patients with a small frameshift deletion). The other three patients had intron (IVS) mutations. The mutation distribution in the present report is consistent with previously reported CEP290-LCA patient cohorts (for example, Ref. 6). Given further detailed studies of phenotype as in the present work, it would be worthwhile to determine if any observed phenotypic variability was associated with specific genotypes.

Discussion

The results from the current study of a cohort of CEP290-LCA patients with the common intronic mutation confirm and extend our previous observations in this ciliopathy. Most patients retain a central retinal island of photoreceptor nuclei with indistinct distal lamination implying abnormal photoreceptor inner and outer segments, and there is impaired vision that is disproportionate to the remaining foveal ONL. The goal of the current study was not to advocate for any specific mode of therapy or method of delivery, but to identify potentially useful outcomes for this form of LCA no matter what therapy is proven safe and sufficiently valuable to consider for a clinical trial in these patients. The limited central island of structure in these patients, however, would seem ideal for an intravitreal approach; inducing a foveal-off detachment could risk further loss of outer retinal structure.

A key question concerning future treatment strategies is whether the central island of cone nuclei is retained or eventually lost to progressive degeneration; and if lost, over how long an interval? The current results indicate that retinal structure, as quantified by OCT across the horizontal meridian and NIR-RAFI in the macular region, showed a measurable constriction of the central island over time, albeit at a very slow rate. Vision, as quantified by FST, did not show major negative change in the small cohort of patients we were able to follow longitudinally. A parsimonious interpretation of these results taken together would be that the cone-based vision in CEP290-LCA derives from the very central few degrees of the residual island and the progressive reduction of the extent of the island does not impair the peak sensitivity at the center.

A protracted natural history of retained central ONL has not been documented in other forms of LCA. To our knowledge, this is the first study with longitudinal retinal structural data spanning a decade in CEP290-LCA patients. The extent of centrally retained ONL region studied longitudinally in other genetic forms of retinal degeneration with similar methods have shown substantially greater rates of constriction measurable by structure and/or function.

Quantitation of Severe Oculomotor Abnormalities

CEP290-LCA cohorts in general, and those with intronic mutations in particular, demonstrate a wide spectrum of oculomotor abnormalities. At the severe end of this spectrum are patients who report being congenitally blind and having never experienced visual perception. Lack of visual experience in early childhood leads to the failure to develop normal oculomotor control and results in severe abnormalities in voluntary eye positioning and eye movements. At the milder end of the CEP290-LCA spectrum are patients with measurable visual acuity and ability to control eye position albeit with nystagmus. Here we developed a method that allowed quantification of OCI across the full spectrum of CEP290-LCA using a simple infrared imaging system that is readily available in most retinal clinics, free of calibration required for corneal glint imaging-based techniques, and free of the retention of a corneo-retinal standing potential required for electro-nystagmography techniques. Test-retest variability of the new technique was defined in our cohort of CEP290-LCA.
LCA. Not unexpectedly, patients with measurable VA had significantly less OCI compared with those with LP or worse VA. Thus, improvement of vision could potentially result in changes to OCI measures.

**What Efficacy Outcomes Would Be Most Useful in a Clinical Trial of CEP290-LCA?**

Among the assays of phenotype that we investigated, there were those that would not be useful in a clinical trial setting for CEP290-LCA patients. Although traditionally used in IRDs as a retina-wide objective assay, the ERG was nondetectable in all the patients at all ages (for which we had records) and would represent an unknown baseline for any trial. Submicrovolt flicker ERGs could be obtained, given the residual central island of cones, but the nystagmus and the wandering eyes would complicate the recordings with movement artifacts. Another traditional measure not valuable in these patients is the visual field (whether kinetic, static, or with fundus imaging); most of the patients would have no measurable fields at baseline. The rare patient with a measurable field had only a small central island that would not be quantifiable as a measurable outcome in an LCA subgroup, considering the unstable fixation.

What outcomes could be useful? FST, dark-adapted with a red stimulus, should be one of the main subjective outcomes used to follow the residual cone function in these patients; there is experience not only with the technique in assessing patients with LCA but also in clinical trials. Intervisit variability has been established and there is no requirement for stable fixation. Traditional Early Treatment Diabetic Retinopathy Study VA measurement would be expected as another outcome. OCT, as a horizontal cross-section through the fovea, certainly should be used as an inclusion criterion. Although there is difficulty in obtaining such scans in patients with eye movement abnormalities, OCT also could be an objective measure of efficacy. The abnormal distal lamination in the foveal region in untreated patients and their untreated contralateral eyes would be worth assessing and comparing pre- and post-therapy, either qualitatively or quantitatively. En face autofluorescence imaging, such as NIRM-HRFL, would be a safety assay rather than an objective outcome of efficacy. There is experience with the evaluation of ocular motor-driven changes in a clinical trial of LCA. However, the previously used method based on retinal imaging is not appropriate for CEP290-LCA. The novel method developed in this article to measure ocular control and instability would be valuable as a secondary outcome to determine if a therapy led not only to better cone sensitivity by FST and VA but also to greater ocular motor control of gaze direction and stability. Pupilometry, which was used in recent gene therapy trials of the RPE65 form of LCA, provided no detectable response to a maximal achromatic stimulus in more than half of our cohort of CEP290-LCA patients. In responding patients, pupilometry can provide objective evidence of the transmission of retinal signals to higher visual centers. In nonresponders, future investigations with brighter and longer stimuli may provide evidence of melanopsin-based signaling.

**Mobility Testing and Quality-of-Life Questionnaires in Very Low Vision Patients**

Do mobility testing and quality-of-life questionnaires have a place in early-phase clinical trials of novel therapies for LCA? There is a considerable literature about quantifying the ability of visually impaired patients to function in everyday life (for example, Refs. 53–55). If a clinical trial treatment protocol of a severe form of LCA is looking ahead to phase 3, there may be an attempt to include secondary outcomes to try to determine if the patient’s daily life was altered by therapy. In the ideal scenario, a therapy would lead to major improvements in vision, and certain behavior dependent on vision would now be possible or at least easier. Obstacle courses have thus been used more commonly in protocols now that treatments for patients with severely impaired vision have begun to emerge.

Early-phase clinical trials for LCA due to RPE65 mutations designed and used mobility performance tasks to be able to determine whether subretinal gene augmentation therapy altered the ability of patients to negotiate obstacles differently after treatment versus before, or comparing treated and untreated eyes (for example, Refs. 20, 56). Some of these courses tested performance under different levels of ambient illumination, considering preclinical evidence that rod-mediated vision could be affected by therapy.

It has been our experience to date that one mobility test does not fit all LCA phenotypes. For example, in RPE65-LCA, the choice of a single standard light level (100–400 lux) would have made the test insensitive to change when applied to these patients; however, lower light levels permitted identification of those patients with rod vision improvement. Subsequently, we showed that the mobility course used for RPE65-LCA was not transferrable to another form of LCA (caused by mutations in GUCY2D) because of the preserved rod vision despite severely impaired cone vision in many of the patients. CEP290-LCA patients also have severe cone visual consequences, but unlike GUCY2D-LCA there is essentially no measurable rod-based vision. The CEP290-LCA phenotype calls for a test that is sensitive to more central cone-function change in the presence of very severe peripheral visual dysfunction. With the mobility design that we used, this CEP290-LCA cohort showed a range of results that correlated with other visual function metrics, yielded negligible differences between eyes, and showed reasonable repeatability. By using three-dimensional obstacles with high contrast against the floor and end wall, color differences and positioning at different heights, the design allowed for the contribution of different visual mechanisms to achieve object detection, some of which may potentially be enhanced by future treatment. The replication scheme chosen is a compromise providing some reduction in variability while keeping a short session duration. The NEI-VFQ data from our cohort of CEP290-LCA, as a group, fall within the published ranges for widespread retinal degenerations. Such quality-of-life estimates can complement other measures of safety and efficacy in a CEP290-LCA clinical trial.

**Should NLP Patients Be Included in Clinical Trials?**

Approximately 35% of the patients in this cohort had NLP (Supplementary Table S1); most the NLP patients in our cohort reported lack of vision from early life. In a sample of the literature, NLP has been reported in patients with CEP290-LCA and the intronic mutation in 8 of 55 patients, approximately 15%. Should CEP290-LCA patients with congenital absence of LP be considered candidates for inclusion in a clinical trial to restore retinal function? This question about congenitally blind patients has been asked in clinical trials of retinal prosthesis implants and, for the most part, inclusion criteria have required patients to have a history of visual perception in early life that progressively was lost to an outer retinal degeneration, such as RP. This presumes that the amount of visual experience preceding blindness relates to the potential for efficacy of a restorative therapy, and congenitally blind patients would thus show little or no efficacy. Absence of early visual input has been considered to lead to permanent damage to visual cortical organization.
well-known neuroscience indicating that permanently impaired vision in animals can result from absence of visual input during development (for example, Refs. 61, 62). Confirmation and extension of such observations to humans have occurred.63–66 However, the complexity is far greater. In blind subjects, there can be cross-modal neuroplasticity; visual cortical areas can be reorganized to serve other sensory modalities or recruited for other brain tasks, such as language processing.67–69 Whereas cross-modal plasticity is the functional and structural reorganization that occurs because of visual deprivation, there is also supramodal plasticity and this occurs despite the lack of vision.70 The latter is a more abstract representation of a stimulus and not dependent on a specific sensory input.71 How cross-modal and supramodal plasticity would complicate or enhance a retinal recovery from therapy is unknown.72

We may not know enough to exclude NLP patients with CEP290-LCA from trials of therapy for the retinal defect. There also may be oversimplification of congenital blindness when it comes to those retinal conditions like CEP290-LCA with residual photoreceptor structure. We are uncertain how much visual experience or light perception is needed to maintain visual cortical architecture, the threshold is not known. Other sources of “light” may also influence visual development and thereby alter the potential for therapy in congenital blindness; there is spontaneous retinal activity that guides activity-dependent development prenatally, before vision is experienced,73 melanopsin-dependent signals throughout life,74 and the presumed phosphes commonly elicited by blind patients as a stereotyped behavior known as eye poking or pressing.75,76 Of interest, of the six CEP290-LCA intronic mutation patients with NLP, all had a history of eye poking in early infancy and childhood.

The hypothesis that NLP CEP290-LCA patients would not respond to retinal therapy should be tested in possibly later cohorts of early-phase trials considering the relatively large percentage of patients with this level of vision.

Ordering of Cohorts for a Phase 1 Clinical Trial of CEP290-LCA

After identifying molecularly clarified patients who meet inclusion criteria, there should be a strategy for how to order patients into cohorts in an early-phase clinical trial. Earliest cohorts in previous LCA gene therapy trials have been adults (>18 years of age), and later cohorts have included children. Results of the current study indicate that there is a wide age spectrum in which there is preserved central photoreceptor structure and there is very slow disease progression with age. These data lead to the conclusion that an early-phase clinical trial of this disorder does not require inclusion of very young children, at least in the first few cohorts, but should be performed at ages when patients are better able to cooperate, not only for safety assays but also for quantitative outcome measures of efficacy, such as in adults and adolescents (12–17 years).

Given five cohorts of three patients each, for example, what would be the order of inclusion of patients with an OCT-proven central retinal photoreceptor nuclear layer? Assuming a strategy of dose escalation over the first three cohorts, some homogeneity of phenotype of these nine patients would seem appropriate. For example, these cohorts can be within a range of measurable vision from 1.0 to 2.0 logMAR. Pending the results of safety and possible efficacy in these nine patients, two further cohorts should include those patients with HM and LP vision, and finally, NLP patients. The conclusions from a trial in adults and adolescents can then be applied to a pediatric trial (or additional pediatric cohorts of a single trial), which may require a different strategy based on tested hypotheses rather than assumptions.

Acknowledgments

Supported by ProQR Therapeutics, The Chatlos Foundation, and Research to Prevent Blindness.

Disclosure: S.G. Jacobson, None; A.V. Cideciyan, None; A. Sumaroka, None; A.J. Roman, None; J. Charrng, None; M. Lu, None; W. Choi, None; R. Sheplock, None; M. Swider, None; M.S. Kosyk, None; S.B. Schwartz, None; E.M. Stone, None; G.A. Fishman, None

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