Treatment of Leber Congenital Amaurosis Due to RPE65 Mutations by Ocular Subretinal Injection of Adeno-Associated Virus Gene Vector: Short-Term Results of a Phase I Trial

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Abstract

Leber congenital amaurosis (LCA) is a group of autosomal recessive blinding retinal diseases that are incurable. One molecular form is caused by mutations in the RPE65 (retinal pigment epithelium-specific 65-kDa) gene. A recombinant adeno-associated virus serotype 2 (rAAV2) vector, altered to carry the human RPE65 gene (rAAV2-CBSB-hRPE65), restored vision in animal models with RPE65 deficiency. A clinical trial was designed to assess the safety of rAAV2-CBSB-hRPE65 in subjects with RPE65-LCA. Three young adults (ages 21–24 years) with RPE65-LCA received a uniconal subretinal injection of 5.96 × 10^10 vector genomes in 150 μl and were studied with follow-up examinations for 90 days. Ocular safety, the primary outcome, was assessed by clinical eye examination. Visual function was measured by visual acuity and dark-adapted full-field sensitivity testing (FST); central retinal structure was monitored by optical coherence tomography (OCT). Neither vector-related serious adverse events nor systemic toxicities were detected. Visual acuity was not significantly different from baseline; one patient showed retinal thinning at the fovea by OCT. All patients self-reported increased visual sensitivity in the study eye compared with their control eye, especially noticeable under reduced ambient light conditions. The dark-adapted FST results were compared between baseline and 30–90 days after treatment. For study eyes, sensitivity increases from mean baseline were highly significant (p < 0.001); whereas, for control eyes, sensitivity changes were not significant (p = 0.99). Comparisons are drawn between the present work and two other studies of ocular gene therapy for RPE65-LCA that were carried out contemporaneously and reported.

Introduction

Incurable genetic retinal blindness became treatable for the first time when early results of ocular gene therapy were reported for two clinical trials, each treating three adult subjects with Leber congenital amaurosis (LCA) caused by mutations in the RPE65 gene (Bainbridge et al., 2008; Maguire et al., 2008). RPE65 (retinal pigment epithelium-specific 65-kDa) is the isomerase enzyme in the RPE that catalyzes a critical step in the visual (retinoid) cycle that permits photoreceptor visual pigments to absorb photons and maintain sight (reviewed in Travis et al., 2007). These clinical trial announcements followed years of fundamental and applied science in animal models of RPE65-LCA (e.g., Redmond et al., 1998; Acland et al., 2001, 2005; Dejneka et al., 2004; Pang et al., 2005; Jacobson et al., 2005), dose-efficacy and safety studies (Jacobson et al., 2006a,b), and retinal function-structure and visual pathway investigations of the human disease (Jacobson et al., 2005, 2007, 2008; Aguirre et al., 2007).

Despite similarities in disease diagnosis and age of the subjects in the two published clinical trials, there were important differences in the results and their interpretations.
According to clinical Good Manufacturing Practices, cobson LCA.

Study eligibility and protocol
tions to be formed and posed.

sents at least 2 weeks before formal enrollment to enhance received paper and recorded versions of the informed con-

Because of their severe visual abnormalities, all patients performed consent was obtained and subjects spoke with a De-

the National Institutes of Health, monitors the trial. The UF. A Data and Safety Monitoring Committee, appointed by the Vice Provost Research Review Committee of UP, the Institutional Biosafety Committees (IBCs) of UP and UF, and Institutional Review Boards (IRBs) were obtained from the Institutional Review Boards (IRBs) and Institutional Biosafety Committees (IBCs) of UP and UF, the Vice Provost Research Review Committee of UP, the Western IRB, and the General Clinical Research Center of UF. A Data and Safety Monitoring Committee, appointed by the National Institutes of Health, monitors the trial. The tenets of the Declaration of Helsinki are being followed. Informed consent was obtained and subjects spoke with a Decision Monitor to ensure the voluntary nature of participa-

tion. Because of their severe visual abnormalities, all patients received paper and recorded versions of the informed consents at least 2 weeks before formal enrollment to enhance detailed understanding of the trial and to permit all ques-

tions to be formed and posed.

Study eligibility and protocol

The three young adult subjects had a clinical diagnosis of LCA. RPE65 mutations were determined by the John and Marcia Carver Nonprofit Genetic Testing Laboratory at the University of Iowa (Iowa City, IA). Inclusion and exclusion criteria are listed in Supplementary Table 1 (see www.liebertonline.com/hum). A summary of the protocol study visits is to be found in Supplementary Table 2 (see www.liebertonline.com/hum).

cGMP vector production, purification, and titering

The recombinant adeno-associated viral (rAAV) vector, AAV2-CB68-hRPE65, has previously been described (Jacobson et al., 2006a). The vector was packaged and purified according to clinical Good Manufacturing Practices (cGMP). Briefly, HEK-293 cells (adenovirus serotype 2 [Ad2], E1A´E1B`) were cotransfected with the pDG packaging plasmid, which encodes three other adenoviral genes required for packaging (E2A, VA, and E4) and the AAV2 rep and cap genes, and with the AAV vector plasmid as pre-

viously published (Grimm et al., 1998). Downstream purifica-

tion of vector was done by all-column purification methods (Snyder and Flotte, 2002; Snyder and Francis, 2005; Zolotukhin et al., 2002). The titer of the vector, expressed as vector genomes (VG), was determined by dot-blot assay (Zolotukhin et al., 2002).

Vector administration

The eye with worse visual function was chosen for vector administration in each subject. After mild intravenous seda-
tion, the surgical eye received retrobulbar anesthesia and was then prepped and draped in a standard sterile fashion. A standard three-port 23-gauge core and peripheral vitrectomy was performed. The conjunctiva over the right-sided sclerotomy was dissected with Westcott scissors and .3 for-
ceps. Hemostasis was maintained by eraser-tipped cautery. The sclerotomy was enlarged with a 20-gauge MVR blade so that the subretinal cannula could easily be inserted into the eye. The vector was drawn into a 39-gauge injection cannula (Synergetics, O’Fallon, MO) and was introduced into the subretinal space. At the end of the procedure, the sclerotomy sites were secured with 7.0 Vicryl sutures and the conjunctiva was closed with interrupted sutures. Subconjunctival antibodies and steroids were administered. Topical antibi-
tics and steroids were used for 20 days after surgery.

Safety parameters

Ocular safety was assessed with standard eye examina-
tions at two baseline visits, daily for the first 10 days post-
treatment, and on days 14, 20, 30, 60, and 90. To quantify the severity of inflammatory response, standard grading sys-
tems were used (Hogan et al., 1959; Nussenblatt et al., 1985; Ladas et al., 2005). To document fundus appearance, fundus photographs (using an infrared camera to avoid excess visible light exposure) were taken at baseline and at posttreat-
ment visits. Systemic safety was evaluated with a physical examination at both baseline evaluations, daily after admin-
istration for 10 days, and on days 30 and 90 postadminis-
tration. Routine hematology, serum chemistry, prothrombin time (with international normalized ratio [INR]), partial thromboplastin time, and urinalysis were performed as part of baseline evaluations and 1, 3, 10, 30 and 90 days after vec-

tor administration.

Anti-AAV2 antibody titers

Serum samples from the patients were assayed for circu-
lating antibodies to the AAV2 capsid proteins at baseline and on days 14 and 90. Briefly, 96-well plates were coated with 1.2 × 10⁶ AAV2 particles per well overnight at 4°C. A wash with phosphate-buffered saline (PBS)–TWEEN was followed by blocking for 2 hr at 37°C with 10% fetal bovine serum (Cellgro; Mediatech, Herndon, VA). After a 1× wash with PBS–TWEEN, samples and a known positive human standard were diluted between 1:10 and 1:10,240 and allowed to bind overnight at 4°C. Each sample was run in duplicate. Washing was followed by addition of the detection antibody at a dilution of 1:50,000 (goat anti-human immunoglobulin, con-
jugated with horseradish peroxidase [HRP]; Biosource In-
ternational/Invitrogen, Camarillo, CA) for 2 hr at 37°C. Finally, the plate was washed and exposed to tetramethyl-
benzidine (TMB) peroxidase detection substrate (KPL, Gaithersburg, MD) and read at 450 nm with an enzyme-linked immunosorbent assay (ELISA) plate reader (Molecular Devices, Sunnyvale, CA). Sample anti-AAV2 titers were then read relative to a human standard curve derived from the same plate. We did not run an assay for antibody against the vector transgene. Because no human antibody to human
RPE65 was available to use as a positive control with which to construct the relevant calibration curve in an ELISA for antibody against the vector transgene, negative results from patient samples could not be confidently interpreted.

**Antigen-specific response**

Anti-AAV2 antigen-specific lymphocyte proliferation responses were assessed as previously described (Hernandez et al., 1999). After isolation and purification from blood, lymphocytes were cultured at $1 \times 10^9$ cells per well in 200 µl of RPMI 1640 medium (10% human serum) in 96-well round-bottom plates. Lymphocytes were separated into four groups with three (controls) and six (unknowns) cultures per group: unstimulated (as negative control), stimulated with AAV2 (5000 particles/cell), stimulated with AAV2 (500 particles/cell), and stimulated with AAV2 (50 particles/cell). After 5 days of incubation the stimulation index (SI) was defined as: (mean counts per minute of $[^3H]$thymidine from stimulated cells)/(mean counts per minute of $[^3H]$thymidine from unstimulated cells). On the basis of antigen-specific lymphocyte proliferation response (ASR) results at baseline in another (nonocular) study, SI values greater than 2.0 (Brantly et al., 2006) or 3.0 (Hernandez et al., 1999) have been considered significant. The viability of each lymphocyte culture was confirmed by positive controls with mitogen-induced proliferation in response to phytohemagglutinin (PHA) (10, 1, 0, and 0.1 µg/ml) and *Candida albicans*.

**AAV DNA in peripheral blood**

Procedures for biodistribution studies have been described (Song et al., 2002). In brief, 1 µg of extracted genomic DNA was used in all quantitative polymerase chain reactions (PCRs); reaction conditions followed those recommended by Applied Biosystems (Foster City, CA) and included 50 cycles of 94°C for 40 sec, 37°C for 2 min, 55°C for 4 min, and 68°C for 30 sec. Primer pairs were designed to the cytomegalovirus (CMV) enhancer/chicken β-actin promoter as described (Donsante et al., 2001) and standard curves were established by spike-in concentrations of a plasmid DNA (CBAT) carrying the same promoter as mentioned previously and the α1-antitrypsin cDNA (Song et al., 2002). DNA samples were assayed in triplicate. The third replicate was spiked with CBAT DNA at a ratio of 100 copies/µg of genomic DNA. If at least 40 copies of the spike-in DNA were detected, the DNA sample was considered acceptable for reporting vector DNA copies. When the copy number of the vector DNA found in that sample was $>100$ copies/µg, the sample was considered positive and the measured copy number per microgram was reported. The sample was considered negative if $<100$ copies/µg were present. When less than 1 µg of genomic DNA was analyzed to avoid PCR inhibitors copurifying with DNA in the extracted tissue, the spike-in copy number was reduced proportionally to maintain the 100 copies/µg DNA ratio. Biodistribution in blood was determined at the first baseline visit and on days 1, 3, and 14 postinjection.

**Interferon-γ enzyme-linked immunospot assay**

Blood samples were collected by venipuncture, using heparin as anticoagulant, and either shipped overnight (baseline and day 14 samples) or delivered immediately (day 90 samples) to the testing laboratory at UP. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque gradient centrifugation (GE Healthcare Life Sciences, Piscataway, NJ). All samples were processed on receipt and either evaluated in enzyme-linked immunospot (ELISpot) assays (see below) as fresh samples or cryopreserved in liquid nitrogen to be tested later. If cryopreserved, the PBMC samples were recovered and cultured in complete R10 medium (RPMI 1640 with 10% FBS ) at 37°C overnight before being assayed.

Interferon (IFN)-γ ELISpot assays were performed according to previously described protocols (Fu et al., 2007). PBMCs were added at a density of $2 \times 10^5$ cells per well and stimulated for 18–20 hr at 37°C with peptide libraries derived from the AAV2 VP1 protein at a concentration of 2 µg/ml. Medium alone served as a negative control, and PHA as a positive control. In addition, responses to CEF (Mabtech, Stockholm, Sweden), which contains 23 MHC class I-restricted peptides (Mabtech) that represent T cell epitopes of viruses common in the human population (cytomegalovirus, Epstein–Barr virus, and influenza) and is able to bind to a broad range of HLA molecules, served as references. The peptide libraries were synthesized as 15-mers with a 10-amino acid overlap with the preceding peptide (Mimotopes, Clayton, Victoria, Australia). The AAV2 VP1 peptide library was divided into three pools such that pool 2A contained the first 50 peptides of AAV2 VP1, pool 2B contained peptides 51–100, and pool 2C contained peptides 101–145. Spots were counted with an ELISpot reader (AID Diagnostika, Strassberg, Germany). Peptide-specific cells were represented as spot-forming cells (SFCs) per 10⁶ PBMCs. A positive response to any peptide pools was arbitrarily defined as three times over background (medium alone control) and the response was more than 55 SFCs per 10⁶ PBMCs.

**Visual function and retinal structure**

Visual function was measured using ETDRS visual acuity (Ferris et al., 1982) and dark-adapted full-field sensitivity testing (FST; Roman et al., 2005, 2007b). Briefly, FST was performed with blue stimuli after subjects were dark-adapted for 1 hr. In each patient, baseline data were available for four pretreatment visits obtained on different days within a 6-month window before surgery. Posttreatment data were obtained up to 3 months after surgery. For each session, the average FST sensitivity was defined as the mean value of the four highest recorded sensitivities. FST sensitivity change was defined as the signed difference between each session and the mean baseline sensitivity for each eye; a positive change indicates improvement. Overall statistical analyses were performed separately in the study and control eyes by t test between pooled post- and pretreatment sessions. When the overall difference post- and pretreatment was statistically significant, further post hoc pair-wise comparisons were performed for each patient.

Retinal structure was assessed by cross-sectional imaging, using optical coherence tomography (OCT). Data were acquired using ultrahigh-speed and high-resolution OCT imaging with a Fourier domain (FD) OCT instrument (RTVue-100; Optovue, Fremont, CA). The high-definition line protocol (HD line) of the FD-OCT system was used to...
obtain overlapping 4.5-mm-long horizontal scans composed of 4096 A-scans acquired at 26,000 A-scans per second. Lateral sampling density of the line scans were reduced by averaging groups of eight neighboring A-scans to increase the signal-to-noise ratio, aligned using a dynamic cross-correlation algorithm (Huang et al., 1998), and digitally stitched (Aleman et al., 2008). Foveal thickness measurements were performed as described (Sandberg et al., 2005).

Results

Study population

The three subjects ranged in age from 21 to 24 years and all had a clinical diagnosis of LCA. Molecular studies indicated that two subjects were homozygotes for point mutations in the RPE65 gene and one subject was a compound heterozygote (Table 1). The mutations were previously reported to be associated with LCA (Morimura et al., 1998; Thompson et al., 2000; Lorenz et al., 2000; Simovich et al., 2001; Felius et al., 2002; Yzer et al., 2003; Booij et al., 2005) and showed little or no RPE65 isomerase activity by in vitro studies (Redmond et al., 2005; Takahashi et al., 2006).

Vector administration and safety

An infrared view of a normal fundus is shown and major retinal landmarks consisting of the optic nerve head, fovea, and macula, defined as the central 5.5–6 mm (diameter) of retina (Hogan et al., 1971), are indicated (Fig. 1). The locations of the retinal detachments created by subretinal injection in each subject are also shown. The treated eyes were the left eye in P1, and the right eye in P2 and P3; to permit comparisons in Fig. 1, all images are shown as left eyes. P1 had two attempted injections: the first, a retinotomy near the superior macular region, did not cause a retinal detachment; the second, a retinotomy inferior and temporal in the macula, achieved the goal of delivering the 150 µl of vector. The retinal detachment involved the inferior macular region (including the fovea) and extended beyond it inferiorly. P2 had a single subretinal injection attempt at a site superior and temporal to the macula. The retinal detachment extended away from the fovea and into the superotemporal retina, not including the fovea. P3 had three attempted injections: two attempts were at the same retinotomy site in the superotemporal retina outside the macula and both failed to cause a retinal detachment; the third injection site was inferior to the original sites and almost directly temporal. The retinal detachment extended away from the fovea and into the temporal extramacular retina. P2 had laser treatment applied to a vitreoretinal tag in the far peripheral superior retina. P3 had very light laser treatment applied to the retinotomy site that did not produce retinal detachment.

For all three patients, there was absorption of most subretinal fluid by 5–6 hr postsurgery. There were no retinal tears or other complications from the surgery. Retinotomy sites continued to be visible by ophthalmoscopic examination for at least 1 month. There was no clinical evidence of intraocular inflammation in any patient; inflammation of the

| Current study | Patient 1  | 24/M  E417Q/E417Q | 3     | L    | 5.96 × 10^{10} | 0.15  | M   | No   | FT   | 20/240 a | 20/317 | No | +    |
| P1           | 23/F  R44Q/R91W    | 3     | L    | 5.96 × 10^{10} | 0.15  | ST  | No   | No   | 20/195 a | 20/138 | No | +    |
| P2           | 21/M Y368H/Y368H   | 3     | L    | 5.96 × 10^{10} | 0.15  | T   | No   | No   | 20/283 a | 20/191 | No | +    |
| P3           | 20/2000 | 20/1050 | Yes | <20/2000 | 20/1050 | Yes | +    |
| Patient 2b   | 26/F  E102K/E102K   | 4.75  | G    | 1.5 × 10^{10} | 0.15  | SN  | Yes  | No   | <20/2000 | 20/710 | Yes | +    |
| Patient 3b   | 26/M  E102K/E102K   | 2.75  | G    | 1.5 × 10^{10} | 0.15  | M   | Yes  | MH  | <20/2000 | 20/710 | Yes | +    |
| Patient 3c   | 19/F  R234X/R234X   | 1.25  | G    | 1.5 × 10^{10} | 0.15  | M   | Yes  | 60/400 | 20/290 | Yes | +    |

**Abbreviations:** LCA, Leber congenital amaurosis; L, local; G, general anesthesia; M, macula including fovea; ST, superotemporal retina; SN, superonasal retina; T, temporal retina; MH, macular hole; FT, foveal thinning; NC, no change; NM, not measured; +, patient-reported improvement.

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**TABLE 1. COMPARISON OF RESULTS OF RPE65-LCA CLINICAL TRIALS**

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"Average of two baseline measures. Refractive errors: P1 = −0.25 + 1.50 × 090; P2 = −1.50 + 0.50 × 075; P3 = −2.70 + 1.75 × 100."
conjunctiva diminished slowly over the first month and the study eyes were quiet on evaluation at 60 days and thereafter.

Physical examinations were unchanged from baseline and there were no clinically significant abnormalities in hematology, serum chemistry, coagulation parameters, and urinalysis after retinal gene transfer in all subjects.

**Immune response assays and vector biodistribution to the peripheral blood**

Humoral immune responses were monitored by measuring levels of circulating antibody to AAV serotype 2 capsid at baseline and on days 14 and 90 posttreatment (Fig. 2A). For P1 and P3 at all time points there were no significant changes in AAV2 antibody titers. P2 experienced a 4.5- to 7.5-fold increase in titer on day 90 compared with baseline or day 14, but her day 90 titer (168,700 mU/ml) was similar to the baseline value for P3 (118,861 mU/ml) and ~16% of the mean titer (1,042,089 mU/ml) determined contemporaneously from 79 independent samples.

The AAV2 capsid-specific reactivity of peripheral lymphocytes (ASR) was also monitored at baseline and on posttreatment days 14 and 90 (Fig. 2B). Again P1 and P3 showed no significant rise in stimulation index (SI) at any time point. P2 on day 90 showed a marginally increased SI of 2.10 from a baseline SI of 1.89 (minimal level of significance for SI ranges from 2 to 3).

The T cell immune response to AAV2 capsid was monitored by IFN-γ ELISpot assays. PBMCs from subjects at baseline and on days 14 and 90 after treatment were stimulated with AAV2 peptide library pools and assayed for IFN-γ secretion. There were no positive responses to AAV2 peptide pools at any time points tested in the three subjects (Fig. 2C).

Biodistribution of vector in the peripheral blood was monitored by quantitative PCR (Poirier et al., 2004) at baseline and on days 1, 3, and 14 posttreatment. For all patients at all time points, there were no vector genome copies detectable.

**Visual function and retinal structure**

All three patients noted increased light sensitivity in the study eye but to different degrees. These observations were most evident to the patients under low ambient light conditions and when using their control eyes as comparators. P1 described small regions of higher light sensitivity around the center of vision in the study eye, and also reported decreased clarity in this eye. P2 noted a region of increased light sensitivity below the point of fixation in the study eye. P3 described considerable improvement in light sensitivity across a wide region in the nasal visual field of the study eye.

Visual acuities in study and control eyes were plotted in all patients (Fig. 3A and C). P1, the only subject with an in-
jection that included the fovea, showed the longest recovery time of visual acuity after surgery. At 90 days, the results remained three letters below the lowest of the baseline measurements. P1 has returned to baseline, using a conventional statistical limit of 3 lines (corresponding to 15 letters) of visual acuity loss or gain as significant (Sieving et al., 2006). P2 showed a more rapid time course of recovery of visual acuity and returned to baseline. P3 had almost no visual acuity loss postoperatively and remains at baseline (Fig. 3A). All control eyes were unchanged over the 90 days after surgery (Fig. 3C).

The retinal anatomic basis of the visual acuity measurements in all subjects was studied by OCT (Fig. 3B and D). Differences in foveal thickness between pretreatment and 90 days posttreatment in control eyes of all subjects varied from 1.1 to 6.2 μm, which are all within published 98% confidence limits of intervisit variability in a retinal degeneration population (±16.7 μm; Sandberg et al., 2005). In the study eyes of P2 and P3, there was also no significant difference between pretreatment and 90 days posttreatment (P2, 4.1 μm; P3, 8.2 μm). P1, however, showed a significant difference in the study eye of 80.3 μm between pre- and posttreatment foveal thickness. Inspection of the scans shows that the foveal architecture in the study eye of P1 before surgery did not show the typical normal depression. This abnormality may have been due to an epiretinal membrane or, as observed in other retinal degenerations, possible swelling of central Muller glial cells (Jacobson et al., 2006c). There was evidence, however, of epiretinal membranes in all eyes of all patients pre- and postoperatively (arrows, Fig. 3B and D). At 90 days after surgery, the fovea of P1 had a more typical depression but thickness was reduced overall.

To quantify the patients’ observations of increased light sensitivity in their study eyes (under low light conditions), we measured dark-adapted visual sensitivity with FST (Fig. 4), a psychophysical test designed for patients with severe visual loss (Roman et al., 2005, 2007b). In an earlier study, untreated RPE65-LCA subjects showed visual sensitivities in response to full-field light stimuli that were 3–4 log units decreased compared with normal (Aguirre et al., 2007); the three patients in the current study showed similar pretreatment results (Fig. 4A). After gene therapy, control eyes did not show consistent sensitivity changes; study eyes showed reductions in sensitivity immediately after the surgery which recovered generally within the first week (data not shown). Sensitivities obtained on days 30, 60, and 90 were pooled and compared with pooled sensitivities obtained pretreatment. Study eyes showed consistent increases in sensitivity compared with baseline; changes in control eyes were not consistent (Fig. 4A). To normalize for pretreatment differences between eyes, sensitivity changes were calculated from mean pretreatment baselines. Sensitivity changes were highly significant (p < 0.001) for all study eyes as a group (Fig. 4B, right); there were no obvious trends for sensitivity change.
between days 30 and 90. For the control eyes (Fig. 4B, left), sensitivity changes were not significant (p = 0.99). Post hoc analysis in individual patients showed no significant changes in the control eyes of three patients; study eyes of P1 and P2, however, showed significant (p = 0.04 and p = 0.002, respectively) positive changes from mean baseline sensitivity after treatment; P3 changes were not significant (p = 0.07).

FIG. 3. Central vision and retinal structure in the RPE65-LCA patients. (A) Visual acuity as a function of time before (baseline) and after the day of surgery (0) in the study eyes of P1, P2, and P3. (B) OCT scans along the horizontal meridian before surgery and at 90 days after surgery in study eyes. (C) Visual acuity and (D) OCT scans in control eyes for comparison. Arrows, epiretinal membranes (detectable in all scans).
slightly higher than at baseline and its relevance is unclear at present. This patient’s ASR stimulation index will be reassessed at follow-up (see Supplementary Table 2) to understand better these results and their variation with time. Overall, however, the fact that little if any immune response was measurable in any patient may not be surprising given the small doses delivered subretinally in each of these studies relative to rAAV2 vector doses commonly administered in nonocular gene therapy trials; these are typically 2–4 orders of magnitude higher. In addition, the relative immune privilege of posterior ocular spaces is well appreciated (Streilein, 2003) and may serve to further limit any potential immune response to subretinal rAAV2 vector.

A key question for future RPE65 LCA clinical trials concerns how the outcomes of the three current trials relate to the respective titers of vector delivered in each case. Unfortunately, there are at least four levels of uncertainty directly related to the vector in such an analysis that largely preclude any clear conclusions. First, each study made, purified, and titered their vector in a different facility using different protocols without reference to a common standard or to each other’s vector. Thus, each stated titer cannot be related with confidence to the other two. Second, the area of RPE/neural retina exposed to vector within the injection bleb, necessary to estimate an effective “multiplicity of infection” for vector particles per RPE cell, is not identical, or even knowable, in all three studies. This trial and Maguire and coworkers (2008) delivered a 150-μl volume, and might be compared (but see preceding and following information). In Bainbridge and coworkers (2008), 1 ml of vector was delivered subretinally and the resultant bleb was then moved by gas manipulation over an unknown, and perhaps unknowable, retinal area in order to expose the fovea to vector in each patient. Such manipulation of the vector bleb after injection will in effect reduce the vector titer within any repositioned bleb by an unknown amount due to vector binding to RPE cells and photoreceptors in the initial detached area. In addition, as the initial bleb is relocated to its final position, vector binding to newly exposed RPE and photoreceptors will occur as the bleb transits between them. Thus the effective amount of vector in the bleb’s final position is difficult to estimate. Third, each of the three vectors was constructed with different regulatory elements controlling expression of the human RPE65 cDNA. Maguire and coworkers (2008) employed a CBA promoter, like the present trial, but inserted a modified Kozak translational initiation sequence before the RPE65 cDNA (Bennicelli et al., 2008), unlike the natural initiation sequence used in this trial. Although asserted to optimize transgene expression, side-by-side experiments with a similar vector containing the natural initiation sequence was not reported. Bainbridge and coworkers employed a short RPE65 promoter that, although targeting expression primarily to RPE cells, is of unknown transcriptional strength relative to the CBA promoter-driven expression in RPE cells. Thus, the relative expression strengths of each of the three vectors on a per-vector genome basis cannot be confidently assessed. Fourth, the large differences in visual function at baseline among the cohorts of patients in each trial, as discussed previously, might suggest that the relative availability of RPE cells versus photoreceptors to interact with vector in the subretinal vector bleb could be significantly different from cohort to cohort, particularly if the ratio of
RPE cells to photoreceptors varies progressively with loss of visual function. Thus, it is not currently possible to reliably comment on how the amount of vector delivered relates to any differences in clinical outcome among the three trials.

Retinal complications did occur. These included a full-thickness macular hole in patient 2 of Maguire and coworkers (2008) and foveal thinning defined by high resolution optical imaging in P1 of the current study. Both of these patients had vector injections into areas that included the fovea; and both retinotomy sites were within 1–1.5 mm of the foveal region (Maguire et al., 2008; Fig. 1). Other subretinal injections with retinal detachments that included the fovea (n = 4) had retinotomies at further eccentricities, near the superior retinal vessel arcade. No measurements of foveal thickness before and after these injections, however, were reported (Bainbridge et al., 2008; Maguire et al., 2008). Retinotomies near the fovea would appear (on current evidence) to be an ill-advised strategy that may lead to disruption of foveal structure. Although subretinal injection is intricate and complex microsurgery, the procedure has many currently uncontrolled variables that could affect outcomes. This includes the alignment of the fluid stream during vector injection from the small-gauge needles in relation to the fovea, the exact trajectory of the fluid stream, and the degree of frailty of foveal photoreceptors and RPE that are chronically stressed by degenerative retinopathy. Even in normal retinas, there can be incomplete recovery of foveal vision after successful anatomic repair of fovea-off retinal detachments (Burton, 1982; Ross and Kozy, 1998; Shocket et al., 2006). Subretinal injections in the proximity of the fovea are expected to cause the largest separation between the neural retina and the RPE, one of the factors related to visual acuity recovery after retinal detachment with foveal involvement (Ross et al., 2005). Epiretinal membrane contraction, a common feature of retinal degenerations (Milam et al., 1998), has been mentioned as a possible cause of the macular hole in patient 2 of Maguire and coworkers (2008) but is unlikely to be the sole determinant of this complication (Moshefgi et al., 2003).

Efficacy in these early safety studies, although a secondary outcome, is of high interest to the field of gene therapy and to those seeking treatment for otherwise incurable hereditary retinal degenerations (Bok, 2004). The standard outcome measure in ocular studies is visual acuity (Ferris et al., 1982). High-resolution visual acuity is subserved by the fovea, the primate-specific central retinal region with the greatest density of cone photoreceptors and unique neural connectivity (Provis et al., 2005). There was no proven increase in foveal visual acuity in any of the nine patients, despite the fovea being included in the subretinal injections in six of the nine patients. The only study to report improved visual acuity is that of Maguire and coworkers (2008), but these three patients had the lowest visual acuities at baseline of all the studies and the improvements did not increase visual acuity even to the level of the baseline acuities in the other two studies. The fact that a macular hole resulted from surgery but failed to be visually significant confirms the conclusion that any positive change due to treatment was from extremely severe vision loss to less severe vision loss. At lower resolutions (typically worse than 20/200), visual acuity may also be subserved by extrafoveal retina. The Maguire and coworkers (2008) result, although suggested to be a placebo effect due to the low level of vision being measured (Miller, 2008), is more likely real and attributable to para- or perifoveal increases in visual sensitivity.

Another measure of efficacy, dark-adapted sensitivity, targets the characteristic visual deficit in RPE65-LCA patients: the >4-log unit reduction in light sensitivity in this disease in humans and in animal models (Jacobson et al., 2005; Aguirre et al., 2007; Roman et al., 2007a). All three patients in the Maguire and coworkers study (2008) self-reported “improved vision in dimly lit environments” but dark-adapted visual measurements were not reported. Bainbridge and coworkers (2008) performed dark-adapted perimetry in all three patients and found increased function only in their patient 3. In the present study, all patients also self-reported an increase in vision under dim light conditions and there was a statistically significant increase in measured dark-adapted sensitivity posttreatment compared with baselines. None of the studies to date have determined whether an increment in sensitivity was mediated by rod or cone photoreceptors or both. The visual cycle subserving rod and cone photoreceptors appears to differ (Travis et al., 2007) and it is uncertain how RPE65 gene replacement will differentially affect this chronically abnormal and key retinoid pathway in rods versus cones.

What is the next step for this clinical research? Conclusions from the previous two studies suggest that any lack of efficacy is due to the advanced stage of retinal disease at baseline and there is thus a need for both higher doses within the fovea and prompt advance to children with RPE65-LCA (Bainbridge et al., 2008; Maguire et al., 2008). More recent work, however, has indicated that there is no strong relationship between age and retinal structure or function until after the fourth decade of life (Jacobson et al., 2007, 2008). The present study suggests a need for greater understanding of the positive and negative effects of subretinal injection on retinal structure and visual function. Items worthy of attention in the group of nine patients already treated include the following: identification of the precise retinal location of any positive treatment effects, measurement of the magnitude of the effect across the treated zone, determination of the rod and cone photoreceptor contributions to the observed treatment effects, establishment of the relationship between the magnitude of any effect on baseline measures of photoreceptor and RPE integrity, and full explanation of any treatment failures revealed by sensitive noninvasive testing in this era of sophisticated high-resolution ophthalmic imaging.

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Author Disclosure Statement

B.J.B., W.W.H., and the University of Florida have a financial interest in the use of AAV therapies, and own equity in a company (AGTC Inc.) that might, in the future, commercialize some aspects of this work. The University of Penn-
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