Title: Bilateral Visual Improvement with Unilateral Gene Therapy Injection for Leber Hereditary Optic Neuropathy

Authors: Patrick Yu-Wai-Man,[1,2,3,4 *], Nancy J. Newman[5], Valerio Carelli[6,7], Mark L. Moster[8], Valerie Biousse[5], Alfredo A. Sadun[9], Thomas Klopstock[10,11,12], Catherine Vignal-Clermont[13,14], Robert C. Sergott[8], Günther Rudolph[15], Chiara La Morgia[6,7], Rustum Karanjia[9], Magali Taiel[17], Laure Blouin[17], Pierre Burguière[17], Gerard Smits[18], Caroline Chevalier[17], Harvey Masonson[18], Yordak Salermo[18], Barrett Katz[18], Serge Picaud[19], David J. Calkins[20], José-Alain Sahel[14,19,21,22 *]

Affiliations:

[1]Cambridge Centre for Brain Repair and MRC Mitochondrial Biology Unit, Department of Clinical Neurosciences, University of Cambridge, Cambridge, CB2 0PY, UK.

[2]Cambridge Eye Unit, Addenbrooke's Hospital, Cambridge University Hospitals, Cambridge, CB2 0QQ, UK.

[3]Moorfields Eye Hospital, London, EC1V 2PD, UK.

[4]UCL Institute of Ophthalmology, University College London, London, EC1V 9EL, UK.

[5]Departments of Ophthalmology, Neurology and Neurological Surgery, Emory University School of Medicine, Atlanta, Georgia 30322, USA.

[6]IRCCS Istituto delle Scienze Neurologiche di Bologna, UOC Clinica Neurologica, 40139 Bologna, Italy

[7]Unit of Neurology, Department of Biomedical and Neuromotor Sciences (DIBINEM), University of Bologna, 40139 Bologna, Italy

[8]Departments of Neurology and Ophthalmology, Wills Eye Hospital and Thomas Jefferson University, Philadelphia, PA 19107, USA

[9]Doheny Eye Institute and UCLA School of Medicine, Los Angeles, CA 90086, USA

[10]Friedrich Baur Institute at the Department of Neurology, University Hospital, LMU Munich, , 80336 Munich, Germany

[11]German Center for Neurodegenerative Diseases (DZNE), 80336 Munich, Germany

[12]Munich Cluster for Systems Neurology (SyNergy), 80336 Munich, Germany

[13]Department of Neuro-Ophthalmology and Emergencies, Rothschild Foundation Hospital, 75019 Paris, France

[14]Centre Hospitalier National d'Ophtalmologie des Quinze Vingts, FOReSIGHT, INSERM-DGOS CIC 1423, 75012 Paris, France

[15]Department of Ophthalmology, University Hospital, LMU Munich, 80336 Munich, Germany

[16] Ottawa Hospital Research Institute and University of Ottawa Eye Institute, Ottawa, Ontario K1H 8L6, Canada

[17] GenSight Biologics, 75012 Paris, France.

[18] GenSight Biologics, New York, NY 10016, USA.

[19] Sorbonne Université, INSERM, CNRS, Institut de la Vision, 75012 Paris, France

[20]The Vanderbilt Eye Institute, Vanderbilt University Medical Center, Nashville, TN 37232, USA.

[21] Fondation Ophtalmologique A. de Rothschild, 25-29 Rue Manin, 75019 Paris

[22] Department of Ophthalmology, The University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA

Corresponding Authors :

*José-Alain Sahel, Institut de la Vision, Sorbonne Université, 75012 Paris, France and University of Pittsburgh Medical Center; email: sahelja@upmc.edu

*Patrick Yu-Wai-Man, Cambridge Centre for Brain Repair, Department of Clinical Neurosciences, University of Cambridge, Cambridge, CB2 0PY, United Kingdom; email: py237@cam.ac.uk

One Sentence Summary:

At 96 weeks after unilateral intravitreal injection of rAAV2/2-*ND4,* vision improved in both eyes in 78% of subjects affected with LHON.

Abstract:

REVERSE is a randomized, double-masked, sham-controlled, multicenter, phase III clinical trial that evaluated the efficacy of a single intravitreal injection of rAAV2/2-*ND4* in subjects with visual loss from Leber hereditary optic neuropathy (LHON). A total of 37 subjects carrying the m.11778G>A (*MT-ND4*) mutation and with duration of vision loss between 6 to 12 months were treated. Each subject's right eye was randomly assigned in a 1:1 ratio to treatment with rAAV2/2-*ND4* (GS010) or sham injection. The left eye received the treatment not allocated to the right eye. Unexpectedly, sustained visual improvement was observed in both eyes over the 96-week follow-up period. At Week 96, rAAV2/2-*ND4*-treated eyes showed a mean improvement in best-corrected visual acuity (BCVA) of -0.308 LogMAR (+15 ETDRS letters). A mean improvement of -0.259 (0.068) LogMAR (+13 ETDRS letters) was observed in the sham-treated eyes. Consequently, the primary endpoint, defined as the difference in the change in BCVA from baseline to Week 48 between the two treatment groups, was not met ($p = 0.894$, ANCOVA). At Week 96, 25 subjects (68%) had a clinically relevant recovery in BCVA from baseline in at least one eye and 29 subjects (78%) had an improvement in vision in both eyes. A non-human primate study was conducted to investigate this bilateral improvement. Evidence of transfer of viral vector DNA from the injected eye to the anterior segment, retina and optic nerve of the contralateral non-injected eye supports a plausible mechanistic explanation for the unexpected bilateral improvement in visual function after unilateral injection.

Introduction

Leber hereditary optic neuropathy (LHON) is a maternally-inherited blinding bilateral optic neuropathy (*1*). It is the most common primary mitochondrial DNA (mtDNA) disorder, affecting approximately 1 in 30,000 to 1 in 50,000 people, particularly young adult males (*2*). The pathophysiology of LHON is characterized by selective loss of retinal ganglion cells (RGCs) and their axons, which leads to rapidly progressive bilateral vision loss. The visual prognosis is poor and most patients progress to vision worse than 20/200 within the first year following disease onset (*3*). Three mtDNA point mutations account for approximately 90% of all LHON cases, namely m.3460G>A (*MT-ND1*), m.11778G>A (*MT-ND4*), and m.14484T>C (*MT-ND6*), with m.11778G>A being the most common mutation worldwide (*1,3,4*). These mutations affect complex I subunits of the mitochondrial respiratory chain, impairing mitochondrial function and increasing the production of reactive oxygen species. RGCs appear to be selectively vulnerable to mitochondrial dysfunction resulting in apoptotic cell death, optic nerve degeneration and the development of optic atrophy (*3*).

The current treatment for LHON remains limited (*5*). Idebenone (Raxone, Santhera) was granted market authorization in the European Union for treatment of LHON under exceptional circumstances (*6*). LHON is especially amenable to gene therapy as it has a well-defined onset with an expected natural history. Furthermore, the biological targets, the RGCs, are easily accessible for gene delivery through standard intravitreal injection (*7*).

Over the past decade, substantial progress has been made in the application of gene therapy to monogenic blinding diseases, with the first treatment approved by both American and European regulatory agencies for an inherited retinal degenerative disorder, namely Leber congenital amaurosis caused by recessive *RPE65* mutations (*8*). Gene therapy in mitochondrial

disorders is challenging as the wild-type protein needs to reach the mitochondrial matrix compartment by crossing the mitochondrial outer and inner membranes. The allotopic expression strategy involves the nuclear expression of the wild-type recoded replacement mitochondrial gene, which having been engineered to carry a mitochondrial targeting sequence (MTS), results in mRNA translation and co-translocation of the protein into mitochondria (*9*). This strategy has been successfully applied in cell models and safely translated in induced rodent LHON models with preservation of RGCs and visual function (*10-14*).

rAAV2/2-*ND4* (GS010) is a recombinant replication-defective adeno-associated virus, serotype 2, which contains a modified cDNA encoding the human wild-type mitochondrial ND4 protein and a specific MTS for translocation of the protein into the mitochondrial matrix. rAAV2/2-*ND4* has been shown to efficiently rescue an induced defect in mitochondrial respiratory chain complex I in a rat model of the disease (*13*), and it was able to restore complex I activity and ATP synthesis in cultured LHON fibroblasts carrying the m.11778G>A mutation (*14*). Based on these preclinical studies, a first-in-human trial (GS-LHON-CLIN-01) showed that a single intravitreal administration of increasing doses of rAAV2/2-*ND4* was safe and well-tolerated in LHON subjects (*15,16*). Three ongoing clinical trials are examining the efficacy of intravitreal injection of rAAV2/2-*ND4* (GS010) in LHON subjects with the m.11778G>A mutation and with vision loss within one year (RESCUE NCT02652767, REVERSE NCT02652780 and REFLECT NCT03293524). Here, we report the 96-week results of the phase 3 REVERSE trial in 37 LHON subjects carrying the m.11778G>A mutation and with duration of vision loss between 6 and 12 months upon inclusion.

REVERSE was designed with unilateral intravitreal injection of rAAV2/2-*ND4* and sham-treated fellow eye, under the assumption that the therapeutic effects of treated versus

untreated eyes would be compared. The unexpected observation of a bilateral improvement of visual function prompted us to further investigate the possible transfer of rAAV2/2-*ND4* viral vector to the contralateral sham-treated eye by conducting a non-human primate (NHP) study equivalent to the human REVERSE study. We confirmed the presence of viral vector DNA in the contralateral visual pathway, providing a plausible explanation for the clinical results observed in REVERSE.

Results

Demographics and Baseline Characteristics of the Study Population

Thirty-seven subjects with LHON due to the m.11778G>A mutation and duration of vision loss between 6 to 12 months were enrolled in the multicenter REVERSE trial between February 2016 and February 2017. Subjects were predominantly males (78.4%) with a mean age of 34.2 years at enrollment (Table S1). Mean duration of vision loss for rAAV2/2-*ND4*-treated and sham-treated eyes was 8.8 months and 9.3 months, respectively. At baseline, the mean (standard deviation [SD]) best-corrected visual acuity (BCVA) for rAAV2/2-*ND4*-treated and sham-treated eyes was 1.67 (0.50) and 1.55 (0.42) logarithm of the minimal angle of resolution (LogMAR), respectively, and this difference was not significant ($p = 0.152$ by paired t-test) (Table 1). At enrollment, 54% of all eyes scored 0 logarithm contrast sensitivity (LogCS) on the Pelli-Robson chart, meaning that no more than one out of 3 letters was read correctly at highest contrast (100%). The mean (SD) baseline score for contrast sensitivity for rAAV2/2-*ND4*-treated and sham-treated eyes was 0.25 (0.40) and 0.35 (0.46) LogCS, respectively, with sham-treated eyes reporting significantly better contrast sensitivity ($p = 0.032$ by paired t-test) (Table S2). Fourteen subjects had prior use of idebenone and they all had discontinued this medication at least 7 days prior to enrollment.

Efficacy Data at Week 96

The mean (SD) change in BCVA from baseline to Week 48 was -0.218 (0.055) and -0.211 (0.055) LogMAR in rAAV2/2-*ND4*-treated and sham-treated eyes, respectively. The difference of the change in BCVA between these two groups at Week 48 was -0.007 LogMAR ($p = 0.894$, analysis of covariance, ANCOVA). The primary endpoint, which was defined as a clinically significant difference of 0.3 LogMAR between treated and sham-treated eyes, was, therefore, not met. At Week 96, rAAV2/2-*ND4*-treated eyes showed a mean (SD) improvement in BCVA of -0.308 (0.068) LogMAR, equivalent to a gain of 15 Early-Treatment Diabetic Retinopathy Study (ETDRS) letters ($p < 0.0001$ for change from baseline) (Table 1). An average improvement of -0.259 (0.068) LogMAR was observed in the sham-treated eyes, equivalent to a gain of 13 ETDRS letters ($p = 0.0001$ for change from baseline). The mean change from baseline in BCVA increased continuously and bilaterally over the 96-week period following treatment (Fig. 1A).

The proportion of eyes with a clinically relevant response (CRR) in the rAAV2/2-*ND4*-treated eye group (62%) was significantly higher than in the sham-treated eye group (43%) ($p = 0.0348$). Twenty-five subjects (68%) showed a CRR in at least one eye at Week 96 and 23 of these 25 subjects (92%) had a CRR in the rAAV2/2-*ND4*-treated eye (Table S3). Fourteen (38%) subjects showed a CRR in both eyes and 9 (24%) subjects in the rAAV2/2-*ND4*-treated eye only (Fig. 1B). Two subjects (5%) showed a CRR in the sham-treated eye only. In a second responder analysis, an eye responder at Week 96 was defined as an on-chart improvement of at least -0.3 LogMAR (3 ETDRS lines) or an improvement from off-chart to on-chart vision with a final BCVA of 1.4 LogMAR or better (equating to at least the first 3 ETDRS lines of the chart being read at a distance of 1 meter). Using these criteria, the eye responder rate was 35% for

rAAV2/2-*ND4*-treated eyes and 27% for sham-treated eyes (Table S3). Fifteen subjects (41%) had this response in at least one eye. Based on a generalized estimating equation model to assess treatment effect, rAAV2/2-*ND4*-treated eyes were 3.6 times more likely to achieve a BCVA better than 20/200 ($p = 0.0032$). Based on the identical statistical model, rAAV2/2-*ND4*-treated eyes were 2.8 times more likely to achieve a BCVA better than or equal to 20/200 ($p = 0.0094$). Most subjects showed similar BCVA changes in both eyes, as indicated by the distribution of data points close to the bisector, and a bilateral improvement in BCVA was observed in 29 (78%) subjects (Fig. 1B). Amongst subjects with a bilateral improvement in BCVA from baseline, 44% showed an earlier improvement in the rAAV2/2-*ND4*-treated eye than in the sham eye, 15% showed an earlier improvement in the sham eye, and 41% had a simultaneous improvement in both eyes. On average, rAAV2/2-*ND4*-treated eyes improved 66 days earlier than the sham eyes.

The mean improvement from the nadir (worst BCVA for each eye of each subject) for rAAV2/2-*ND4*-treated eyes and sham-treated eyes was -0.570 LogMAR (28.5 ETDRS letters) and -0.490 LogMAR (24.5 ETDRS letters), respectively (Table 2). In total, 78% of REVERSE subjects presented a CRR from the nadir in at least one eye.

Contrast sensitivity improved in rAAV2/2-*ND4*-treated and sham-treated eyes with a mean (SD) increase from baseline to Week 96 of 0.22 (0.06) and 0.12 (0.06) LogCS, respectively (Fig. S1 and Table S2). The mean deviation on automated perimetry showed a mean (SD) improvement of 2.70 (0.90) and 2.57 (0.90) dB in rAAV2/2-*ND4*-treated and sham-treated eyes, respectively (Table S2). There were no differences in the change of spectral-domain optical coherence tomography (SD-OCT) parameters from baseline to Week 96 in rAAV2/2-*ND4*-treated and sham-treated eyes (Table S2).

Patient-reported outcome measures were evaluated using the National Eye Institute Visual Function Questionnaire-25 (NEI VFQ-25). The change in quality of life was assessed and compared with the baseline values before treatment. The composite score, which is an average of the vision-targeted subscales scores excluding the general health rating question, showed a mean improvement of 9.5 points. Improvements were reported for the following subscales (*22*): dependency (+18.5, +130.2%), mental health (+16.1, +108.2%), role difficulties (+15.9, +78.9%), near activities (+13.3, +78.1%), peripheral vision (+10.8, +41.0%), distance activities (+10.7, +47.4%), color vision (+6.9, +21.6%), general vision (+6.5, +32.4%), and social functioning (+4.7, +32.8%) (Table S4).

Safety Data at Week 96

Treatment with viral vector was tolerated well, without any occurrences of discontinuation over the 96 weeks of follow-up. One serious adverse event (a retinal tear requiring hospitalization) was reported in the sham-treated eye of one subject, which was deemed clinically unrelated to the study drug or procedure. No prophylactic oral or topical steroids were provided before or immediately after the intravitreal injection. In rAAV2/2-*ND4*-treated eyes, the most frequent ocular adverse event was intraocular inflammation, which was documented in 34 (92%) eyes and assessed as mild for 28 (76%) eyes. Anterior and intermediate uveitis was reported in 27 (73%) and 25 (68%) eyes, respectively, with no posterior uveitis affecting the retina and optic nerve reported. The anterior uveitis was graded as mild in 23 eyes and moderate in 4 eyes. There was no incidence of severe anterior uveitis. The intermediate uveitis was graded as mild in 21 eyes and moderate in 3 eyes. One eye developed severe intermediate uveitis. Intraocular inflammation resolved without sequelae following standard therapy in all patients. For the 34 eyes that developed intraocular inflammation, 29 (85%) eyes received topical steroids for a mean duration

of 211 days. A course of oral steroids was prescribed for 9 (26%) patients based on the clinician's judgement. An increase in intraocular pressure was reported in 27% of eyes and was mostly mild elevation, resolving with standard therapy. No subject developed chronic ocular hypertension or glaucoma during follow-up. Viral vector biodissemination was assessed for all patients at 2 weeks post-treatment. All the tested samples showed negative quantitative PCR (qPCR) results, demonstrating no systemic diffusion of GS010.

Biodistribution of rAAV2/2-ND4 (GS010) DNA Following a Single Eye Intravitreal Injection in Cynomolgus Monkeys

We conducted a study using non-human primates to probe a possible mechanism underlying the unexpected improvement in visual function in the contralateral untreated eye following unilateral intravitreal administration of the viral vector in the REVERSE clinical study. Viral DNA was detected and quantified using *ND4* transgene-specific qPCR three months following unilateral intravitreal injection of rAAV2/2-*ND4* in three animals and vehicle formulation in one control animal. The qPCR protocol used specifically targeted the *ND4* transgene of rAAV2/2-*ND4*. Viral DNA was neither detected (below the limit of detection, [BLD]) nor quantified (below the limit of quantification, [BLQ]) in the 13 sentinel control samples that were concomitantly extracted, indicating no cross-contamination during the extraction and the qPCR analysis. All the tissue samples from both eyes of the control animal (Group 1) showed no detection (BLD) of viral DNA three months after unilateral intravitreal injection of vehicle formulation in the right eye (Table 3).

Three months after unilateral intravitreal injection of rAAV2/2-*ND4* in the right eye, rAAV2/2-*ND4* DNA was detected in all the tissue and fluid samples tested for the 3 animals (Table 3 and Fig. 2). When quantified, the mean quantity of rAAV2/2-*ND4* ranged from

$2.84x10^2$ to $3.21x10^6$ vg/µg of DNA in tissue samples, and $1.65x10^3$ and $8.70x10^4$ copy/µL in the aqueous and vitreous humors, respectively. rAAV2/2-*ND4* DNA was detected in the contralateral non-injected eyes and visual pathways in the following tissues: anterior segment of the eye (all 3 animals; quantifiable for the 3 animals), retina (all 3 animals; quantifiable for 2 animals, BLQ for 1 animal), optic nerve (all 3 animals; quantifiable for the 3 animals), optic tract (2 animals; BLQ for the 2 animals), and lateral geniculate nucleus (2 animals; BLQ for the 2 animals). Viral DNA was detected in the optic chiasm of all 3 animals (Table 3 and Fig. 2). No viral DNA was detected in the contralateral visual cortex. When quantified, the mean quantity of rAAV2/2-*ND4* in the contralateral non-injected eyes ranged from $3.39x10^3$ to $1.00x10^4$ vg/µg of DNA. No PCR inhibition was detected in any sample.

Discussion

REVERSE is the largest phase III clinical trial of gene therapy in LHON for which results are now reported. The subjects recruited were representative of the LHON population in terms of demographics and baseline visual characteristics (*1-4*). Both the rAAV2/2-*ND4*-treated and sham-treated eyes showed an improvement in BCVA when compared with baseline and nadir measurements. The sustained improvement observed in the contralateral sham-injected eyes was unexpected and resulted in the primary endpoint at Week 48 not being met. At Week 96, the change from baseline of -0.308 LogMAR (+15 ETDRS letters) in rAAV2/2-*ND4*-treated eyes and -0.259 LogMAR (+13 ETDRS letters) in sham-treated eyes represents a clinically meaningful bilateral improvement of vision. As some subjects were still in the dynamic phase of the disease process upon enrollment, the visual gain from the nadir was of an even larger magnitude, reaching 28.5 ETDRS letters for rAAV2/2-*ND4*-treated eyes and 24.5 ETDRS letters for sham-treated eyes. At the time of study design, it was assumed that the BCVA in sham-

treated eyes would act as the placebo arm, remaining stable over time or worsening if the nadir of vision had not been reached at baseline. As a result, REVERSE lacks a true internal control group, resulting in our dependence on comparisons with previously published natural history figures. In the RHODOS study, which was a randomized controlled trial comparing idebenone with placebo in LHON patients treated within 5 years of the onset of visual loss, an improvement from baseline of -0.139 LogMAR (+6 ETDRS letters) was noted at Week 24 in the cohort of 35 patients with the m.11778G>A mutation treated with placebo (*17*). Similar natural history results were reported in a larger retrospective study of a LHON cohort of idebenone-treated patients compared with those untreated (*18*). In a retrospective study that included all three common mtDNA LHON mutations, 82% of patients had a BCVA of 20/200 or worse at the last available data point, which was on average 14.9 months after the onset of vision loss (range from 2.3 to 56.7 months) (*19,20*). In that study, spontaneous CRR was defined as an improvement from baseline either from off-chart BCVA to reading at least 5 letters on the ETDRS chart at 1 meter, or as an on-chart improvement of at least 10 letters on the ETDRS chart. The analysis was run on a cohort of 86 subjects, including childhood-onset cases. For the subgroup of 61 subjects that carried the m.11778G>A mutation, 15% (9/61) experienced a spontaneous CRR from baseline in at least one eye at the last follow-up visit. In comparison, 68% of subjects (25/37) in REVERSE showed a similar improvement 2 years after treatment.

In a prospective natural history study of LHON, 12 subjects with LHON due to the m.11778G>A mutation and with vision loss of up to 12 months had no improvement of their ETDRS score after 12 months of follow up, and a slight decrease in ETDRS score at 24 months in 9 subjects (*21*). Including patients with vision loss of more than 12 months, 13 out of 88 eyes (15%) showed a spontaneous improvement of at least 15 ETDRS letters during follow-up,

accounting for 18% of enrolled subjects. Childhood-onset LHON carries a better prognosis for spontaneous clinical recovery of vision when compared with adult-onset cases (*23*). The prospective natural history study included 8 subjects who were below the age of 15 years at the time of onset and 3 (38%) of those subjects were reported as being responders in at least one eye at their last follow-up visit. Direct comparisons between the Lam *et al*. study and our study are problematic because of the relatively small numbers in the subgroups in the Lam *et al*. study and the potential confounders of inclusion of subjects with young age at onset, enrollment of some patients with visual loss duration of less than 6 months, and possible patient concurrent use of idebenone *(21)*. However, 38% of rAAV2/2-*ND4*-treated eyes and 32% of sham-treated eyes in REVERSE showed an improvement from baseline of at least 15 ETDRS letters at week 96, representing 46% of the entire study cohort, suggesting a possible better responder rate than in any subgroups of the Lam *et al*. study.

The bilateral gain in BCVA in the REVERSE study was consistent with the trend observed for other visual parameters, including contrast sensitivity and visual field perimetry. The NEI VFQ25 questionnaire has been validated as a responsive and sensitive measure of vision-related quality of life (*22*). In REVERSE, the composite NEI VFQ25 score showed a mean improvement of 9.5 points, which is more than the clinically relevant threshold estimated at between +3.9 and +4.3 points (*22*). LHON has a major detrimental impact on activities of daily living and the substantial improvement in composite score suggests a clinically meaningful improvement in patient-reported outcome measures (*24*).

The unexpected bilateral improvement in vision observed in REVERSE is in contrast to the results obtained in other gene therapies targeting photoreceptors or the retinal pigment epithelium with a surgical subretinal injection of the viral vector (*7*,*25*). However, an

improvement in visual function in the untreated eye following unilateral intravitreal administration of the viral vector has been demonstrated in other gene therapy trials for LHON (*26-29*).

Regarding potential mechanisms for the observed contralateral improvement, the most intriguing explanation would be the inter-eye transfer of the rAAV2/2-*ND4* viral vector. In one rodent study, fluorogold nerve tracer dye injected intravitreally into rat eyes was detected in the optic nerves of the contralateral non-injected eyes, implying axonal or glial transfer through the anterior visual pathways (*30*). There is also evidence supporting the transneuronal spread of AAV, possibly through synaptic transfer mechanisms (*31*). Mitochondria have been shown to migrate long distances in axons to distribute energy and allow for distal neuronal activity (*32*). Mitochondria within RGC axons can also be engulfed into vesicles and exported to astrocytes that are found at a high density in the optic nerve head and then stored in endosomes (*33,34*). The dense network of interconnected astrocytic processes could, therefore, allow for the long-distance cell-to-cell transfer of cytoplasmic elements via membrane junctions (*35*). Another hypothetic underlying mechanism for the contralateral improvement is brain plasticity with reorganization of the visual areas contributing to the visual improvement in the contralateral non-injected eyes (*36,37).*

To explore potential inter-eye transfer, we conducted an equivalent biodistribution study in healthy *cynomolgus* monkeys. Viral vector DNA was detected in quantifiable amounts in the anterior segment, retina and optic nerve of the non-injected eye three months following unilateral injection of rAAV2/2 *ND4*. Although the presence of viral vector DNA in the optic chiasm provides a possible clue to the diffusion pathway, our study was not designed to address the exact mechanisms underlying this transfer. The detection of viral vector DNA in the anterior

segment of the non-injected eye is also interesting. Additional work is needed to clarify these important observations that have broader relevance to the design of gene therapy trials for optic neuropathies.

The results of REVERSE raise a number of fundamental questions about the prospect of gene therapy for LHON and the best approach to correct the underlying pathogenic mtDNA mutation. The extent of visual recovery seen in patients treated with rAAV2/2-*ND4* is more than what one would have expected based on the analysis of the published natural history data, which shows a much lower rate of recovery for this specific mutation *(38)*. However, we did not have a prospective control group of untreated patients carrying the m.11778G>A mtDNA mutation assessed using the same comprehensive protocol with whom to make a direct comparison. If we consider that the improvement of vision does not reflect the natural history of LHON, but a true biological effect, the mechanisms that underpin the rescue of RGCs warrant further mechanistic investigations. Although speculative, it is also possible that the enhanced survival of RGCs could arise, at least partly, from the secondary effects of the intravitreal injection with the resulting inflammatory response increasing the expression of coactivators and transcription factors that upregulate mitochondrial biogenesis, which has been shown to be neuroprotective in the presence of a LHON mtDNA mutation *(39,40,41).* In turn, these mediators could find their way to the non-injected eye accounting for the unexpected bilateral improvement in visual function seen with a unilateral injection of rAAV2/2-*ND4*. Investigating this hypothesis would require a trial design that assigns one arm to receiving placebo intravitreal injection, which entails a number of safety and ethical considerations, in addition to patient acceptability. Furthermore, the intraocular inflammation seen with rAAV2/2-*ND4* is generally mild and transient and it is

unlikely to fully account for the sustained enhanced visual rescue. Another hypothesis, besides gene transfer, is the possible exchange of metabolic resources between the optic pathways *(42).*

In conclusion, the REVERSE study showed bilateral improvement of visual function in LHON subjects treated with a unilateral intravitreal injection of rAAV2/2-*ND4*. The unexpected visual improvement observed in the contralateral non-injected eyes may reflect transfer of viral vector DNA from one eye to the other as demonstrated in our complementary NHP study. However, further investigations are needed to confirm these findings and their underlying mechanisms. This study, providing both clinical and preliminary experimental evidence for a bilateral effect of unilateral intravitreal injections targeting RGCs suggests that interocular diffusion of viral DNA vector could occur. These findings could have major implications for gene therapy clinical trial design and outcome measures.

Materials and Methods

Study Design

REVERSE (NCT026527080) was a randomized, double-masked, sham-controlled, phase III clinical trial to evaluate the efficacy of a single intravitreal injection of rAAV2/2-*ND4* in LHON subjects with the m.11778G>A mutation and vision loss occurring in both eyes in the prior 6 to 12 months. A total of 37 subjects were enrolled in seven centers (France, Germany, Italy, United Kingdom and United States of America). The objective was to evaluate the efficacy of rAAV2/2-*ND4* compared with a sham injection at Weeks 48 and 96, with the change from baseline in visual acuity expressed as the LogMAR as the primary efficacy endpoint. An interim analysis was planned at Week 72 to bring additional insights to Week 48 results.

The right eye of each subject was randomly allocated to receive either treatment with rAAV2/2-*ND4* via intravitreal ($9E^{10}$ viral genomes in 90 µl per eye) or sham-treatment. The fellow (left) eye received the treatment not allocated to the right eye, in a 1:1 ratio. Treatment with prophylactic oral or topical steroids was not provided. Both eyes received standard antiseptic preparation with administered topical ocular anesthetic agent and underwent pupillary dilation. Before treatment, an intraocular pressure lowering agent was administered. The viral vector rAAV2/2-*ND4* was administered in a single intravitreal injection. Following the same preparation steps preceding an intravitreal injection, a sham injection was performed on the sham-treated eye using the blunt end of a syringe and applying pressure to the eye at a typical injection site. Only the pharmacy team, the injecting physician and the medical team assisting in treatment were unmasked to treatment allocation. The unmasked study team performed the first assessment the day after treatment, whereas a separate medical team masked to treatment allocation performed all following ocular examinations.

The protocol was reviewed and approved by independent ethic committees at all sites. The study was conducted in accordance with the principles and requirements of the International Conference on Harmonization Good Clinical Practice. An independent Data Safety Monitoring Board periodically reviewed study data to ensure the continued safe conduct of the trial and protection of subjects.

To be enrolled, LHON subjects had to be 15 years or older, with duration of vision loss in both eyes between 6 and 12 months and visual acuity of at least counting fingers in each eye. Documented genotyping was required to confirm the presence of the m.11778G>A mutation in the *MTND4* gene and the absence of other primary LHON-associated mutations (m.3460G>A in *MTND1* or m.14484T>C in *MTND6)*. The main exclusion criteria were known mutations in

other genes involved in pathological retinal or optic nerve conditions, previous treatment with an ocular gene therapy product, glaucoma, optic neuropathy other than LHON, history of amblyopia, previous vitrectomy in either eye or ocular surgery of clinical relevance within 90 days. Any prior use of idebenone was required to have ceased at least 7 days prior to enrollment.

Outcome Measures

Ophthalmic evaluations included assessment of BCVA using the ETDRS chart at 1 or 4 meters, assessment of contrast sensitivity using the Pelli-Robson chart, Humphrey Visual Field (HVF) 30-2 testing, Farnsworth-Munsell 100-Hue Color Vision testing, slit-lamp biomicroscopy, Goldmann applanation tonometry, fundoscopy SD-OCT, and color fundus photography.

When subjects could not read any letters on the ETDRS chart, they were asked to count the assessor's fingers or to detect hand motion. An off-chart Snellen equivalent was derived using both the distance at which the assessment was made and the size of the assessor's fingers, as described by Karanjia et al (*43*). The method was also adapted to hand motion visual acuity to provide conversion into a LogMAR value. Light perception and no light perception visual acuities were assigned a value of 4.0 and 4.5 LogMAR, respectively.

Contrast sensitivity (CS) – the reciprocal of contrast threshold – was measured using the Pelli-Robson chart at 1 meter, performed according to test instructions and expressed as a logarithm (LogCS). Subjects who could not read any letter on the Pelli-Robson chart were assigned the worst possible score (0 LogCS). Intraocular inflammation was assessed and graded according to the Standardization of Uveitis Nomenclature (SUN) (*44*) and the National Institutes of Health Grading Scale for Vitreous Haze (*45*).

Spectral domain-OCT was performed with the Spectralis OCT (Heidelberg Engineering). Parameters were measured for the optic nerve (Retinal Nerve Fiber Layer [RNFL], Ganglion Cell Layer [GCL], RNFL of the Papillo Macular Bundle [PMB]) and posterior pole per standard protocols included in the Spectralis software. At pre-specified visits, certified technicians performed one "Optic Nerve Head – Radial Scan and Concentric Circle Scan" (ONH-RC) and one "Posterior Pole N Scan" (PPoleN) for each eye, after maximal dilation. A reading center masked to treatment allocation - the Optic Nerve Research Center (ONRC) - performed quality control, analysis, and interpretation of all SD-OCT data.

The standardized automated HVF 30-2, Central Threshold, SITA-FAST procedure was performed with the HVF Analyzer II. The reliability of the HVF test results were quality controlled by the ONRC reading center and the HVF test was repeated if considered unreliable by that center (defined as fixation losses ≥ 15%, false positive errors ≥ 20%, or false negative errors ≥ 33%).

Quality of life was assessed at enrolment and Week 96 using the NEI VFQ-25 (*22*). The VFQ-25 consists of 25 vision-targeted questions representing 11 vision-related constructs, and a General-Health rating question. All items were scored so that a high score on a 0 to 100 scale represents better functioning. An overall Composite Score was calculated as the average of the vision-targeted subscale scores, excluding the General-Health rating question. For each subscale, change from baseline was calculated in terms of average score increase/decrease, and as the average of percent changes in scores.

Statistical Analyses

The primary endpoint for the REVERSE study was the change from baseline to Week 48 of LogMAR BCVA following treatment. A difference of -0.3 LogMAR (15 ETDRS letters

equivalent) between the change from baseline in the rAAV2/2-*ND4*-treated eyes and the sham-treated eyes was considered clinically significant based on the U.S. Food and Drug Administration (FDA) recommendations. The sample size calculation was based on the primary endpoint and on the paired comparison of rAAV2/2-*ND4*-treated and sham-treated eye. The sample size calculation through paired comparison required assumptions due to the absence of published data on the within-subject correlation between right and left eyes and on the within- and between-subject variance of the LogMAR acuity. Based on available data (*14*), the sample size required to have a power of 85% was 36 subjects.

The baseline value of functional endpoints (BCVA, CS, HVF 30-2) was defined as the last reported value prior to treatment administration, considering the subacute progression of visual signs. For OCT parameters, baseline was defined as the average value of screening and baseline visits assessments. We defined the "nadir" during the course of the study as the worst BCVA for each eye of each subject from baseline to Week 96 (including baseline and Week 96 values). By definition, the change in BCVA from nadir for each eye results in no change or improvement. A statistical analysis plan was prepared after the study protocol was approved before the database lock.

The efficacy analyses were run using the intent-to-treat population including all subjects who were randomized and received the actual study treatment (rAAV2/2-*ND4*). The safety analyses were run using the safety population including all subjects who received study treatment (rAAV2/2-*ND4*).

The change of LogMAR BCVA from baseline to Week 96 in rAAV2/2-*ND4*-treated eyes was compared to that in sham-treated eyes (intra-subject comparison) using a mixed-effects analysis of covariance (ANCOVA) model with subject and eyes of the subject as random factors,

treatment as a fixed effect, and baseline LogMAR BCVA as a covariate. The difference in the mean change from baseline between the two treatment groups and associated 95% confidence interval were reported. Statistical significance was assessed using an alpha of 0.05.

In order to compare our results with previously published data on the natural history of LHON, two responder analyses were performed using different responder definitions. In the first responder analysis, a CRR at Week 96 was defined as either an eye that was on-chart (able to see letters on the chart) at baseline and that showed an improvement of at least 10 ETDRS letters, or an eye that was off-chart (not able to see letters on the chart) at baseline and that became able to read 5 ETDRS letters on-chart at 1 meter *(6,19,20)*. A subject responder was defined as having this response in at least one eye at Week 96. In the second responder analysis, an eye responder at Week 96 was defined as an on-chart improvement of at least -0.3 LogMAR (3 ETDRS lines), or an improvement from off-chart to on-chart vision with a final BCVA of 1.4 LogMAR or better (able to read at least the first 3 ETDRS lines of the chart at 1 meter) (*21)*. A subject responder was defined as having this response in at least one eye at Week 96. We opted for the first responder analysis, CRR, as this approach was considered by the European Medicines Agency (EMA) in its assessment of the efficacy of idebenone (Raxone, Santhera) in LHON (*6*). A group of experts have also endorsed the use of CRR as a valid outcome measure when assessing the effect of treatment with idebenone in LHON (*1*).

Non-Human Primate Study

Methods

Four healthy male purpose-bred cynomolgus monkeys (*Macaca fascicularis*) were allocated to groups using a computerized stratification procedure so that the average body weight of each group was similar. At the beginning of the treatment period, the animals were at least 24 months

old and with body weights between 3 and 5 kg. Formulations were administered by unilateral intravitreal injection in the right eye on Day 1 under a volume of 90 µL. Following injection, the animals were kept for an observation period of 3 months ± 2 days (with day 1 corresponding to the day of treatment). Group 1 included one control animal receiving unilateral intravitreal injection of vehicle, composed of formulation buffer (balanced sterile saline solution [BSSS buffer] supplemented with 0.001% Pluronic F68). Group 2 included 3 test animals receiving unilateral intravitreal injection of rAAV2/2-*ND* (S222/DP0014/FC003 batch, Novasep), formulated in BSSS buffer supplemented with 0.001% Pluronic F68 to reach a dose of $4.3x10^{10}$ viral genomes (vg) in 90 µL injected in the right eye. The $4.3x10^{10}$ vg dose was determined based on animal toxicology studies previously conducted and it corresponds to the human injected rAAV2/2-*ND4* dose, proportional to the vitreous volume of the cynomolgus monkeys.

At the end of the 3-month observation period, the animals were necropsied, and the following tissues were sampled from each animal (with right and left sides being collected and preserved separately): vitreous humor, aqueous humor, anterior segment of the eye (including cornea, iris, lens and choroid), lacrimal gland, retina, optic nerve, optic chiasm, lateral geniculate nucleus, optic tract and visual cortex. Analyses consisted of qPCR (DNA) targeting the *ND4* transgene of rAAV2/2-*ND4*. Special attention was given to sample collection to avoid cross-contamination. Tissues and fluids were collected first for Group 1 control animals. Amongst the treated animals, tissues and fluids related to the non-treated eye were collected first. A clean set of disposable sterile instruments was used for each animal and for each tissue collected. The precise times of sacrifice of the animals and of snap freezing of the tissues were recorded.

DNA from tissues was extracted using the Nucleospin Tissue kit (Macherey-Nagel). Tissue samples, except the anterior segment of the eye samples, were homogenized in 1 mL of

T1 buffer. The anterior segment of the eye was entirely sampled and weighed. All the samples were homogenized into 2 mL of T1 buffer. For each tissue sample, 400 µL of the homogenized tissue were used for the extraction. The remaining homogenized tissue was kept at -80°C until further notice. Each tissue was weighed before the DNA extraction step. To the extent possible, tissue samples of minimum 20-30 mg (around 3 x 3 x 3 mm for small organs) and tissue sample of minimum 80 mg (around 5 x 5 x 5 mm for larger organs) were used. The volume of each fluid was measured before the DNA extraction step. Sentinel controls are samples composed by the first reagent that are usually added onto the biological material to monitor the potential cross-contamination between samples during the extraction process. Sentinel controls were included within each DNA extraction batch. DNA concentration and purity were determined by UV spectrophotometry. DNA was then stored at -20°C until analysis.

The extracted DNA was used as a template for qPCR amplification. The TaqMan qPCR protocol specifically targets the *ND4* transgene of rAAV2/2-*ND4*. The qPCR protocol was validated in a previous dedicated study and the analytical parameters for sensitivity, specificity and accuracy were determined. Each qPCR contained 5 µL of DNA corresponding to 400 ng of DNA when feasible (or 400 ng of herring sperm for NTC sample, or 5 µL of ultrapure nuclease-free water for no DNA sample or 5 µL of eluate from sentinel controls), and 20 µL of the qPCR master mix was composed of PCR Master Mix 2X (12.5 µL), forward-primer and reverse-primer (300 nM each), TaqManProbe (150 nM), and ultrapure nuclease-free water (qsp 20 µL). PCR plates were run on an Applied Biosystems 7900HT.

References

1. V. Carelli, M. Carbonell, I.F. de Coo, A. Kawasaki, T. Klopstock, W. A. Lagrèze, C. La Morgia, N.J. Newman, C. Orssaud , J.W.R. Pott, A.A. Sadun, J. van Everdingen, C. Vignal-Clermont, M. Votruba, P. Yu-Wai-Man, P. Barboni. International consensus statement on the clinical and therapeutic management of Leber hereditary optic neuropathy. *J Neuroophthalmol*. **37**, 371-381 (2017).
2. P. Yu-Wai-Man, P.G. Griffiths, D.T. Brown, N. Howell, D.M. Turnbull, P.F. Chinnery. The epidemiology of Leber hereditary optic neuropathy in the north east of England. *Am J Hum Genet*. **72**, 333–339 (2003).
3. P. Yu-Wai-Man, M. Votruba, F. Burté, C. La Morgia, P. Barboni, V. Carelli. A neurodegenerative perspective on mitochondrial optic neuropathies. *Acta Neuropathol.* **132**, 789-806 (2016).
4. N. J. Newman, M. T. Lott, D. C. Wallace. The clinical characteristics of pedigrees of Leber's hereditary optic neuropathy with the 11778 mutation. *Am J Ophthalmol.* **111**,750-762 (1991).
5. N. Jurkute, J. Harvey, P. Yu-Wai-Man. Treatment strategies for Leber hereditary optic neuropathy. *Curr Opin Neurol*. **32,** 99-104 (2019).
6. EMA/480039/2015 Committee for Medicinal Products for Human Use (CHMP) – Assessment Report Raxone (Idebenone); 25 June 2015. https://www.ema.europa.eu/en/documents/assessment-report/raxone-epar-public-assessment-report_en.pdf
7. L. Yin, K. Greenberg, J.J. Hunter, D. Dalkara, K.D. Kolstad, B. D. Masella, R. Wolfe, M. Visel, D. Stone, R.T. Libby, D. Di Loreto, D. Schaffer, J. Flannery, D. R. Williams, W.H. Merigan.

Intravitreal Injection of AAV2 Transduces Macaque Inner Retina. *Investigative Ophthalmology & Visual Science*, April 2011, Vol. 52, No. 5 (2011).

8. S. Russell, J.A. Wellman, J. Bennett, D.C. Chung , Z.F. Yu, A. Tillman, J. Wittes, J. Pappas, O. Elci, S. McCague, D. Cross, K.A. Marshall, J. Walshire, T.L. Kehoe, H. Reichert, M. Davis, L. Raffini, L.A. George, F.P. Hudson, L. Dingfield, X. Zhu, J.A. Haller, E.H. Sohn, V.B. Mahajan, W. Pfeifer, M. Weckmann, C. Johnson, D. Gewaily, A. Drack, E. Stone, K. Wachtel, F. Simonelli, B. P. Leroy, J. F. Wright, K. A. High, A. M. Maguire. Efficacy and safety of voretigene neparvovec (AAV2-HRPE65V2) in patients with RPE65-mediated inherited retinal dystrophy: a randomized, controlled, open-label phase 3 trial. *Lancet*. **390**, 849-860 (2017).
9. R.E. Gray, R. H. Law, R. J. Devenish, P. Nagley. Allotopic expression of mitochondrial ATP synthase genes in nucleus of Saccharomyces cerevisiae. *Methods Enzymol*. **264**, 369-389 (1996).
10. J. Guy, X. Qi, F. Pallotti, E. A. Schon, G. Manfredi, V. Carelli, A. Martinuzzi, W. W. Hauswirth, A. S. Lewin. Rescue of a mitochondrial deficiency causing Leber hereditary optic neuropathy. *Ann Neurol*. **52**, 534-542 (2002).
11. R. Koilkonda, H. Yu, V. Talla, V. Porciatti, W.J. Feuer, W.W. Hauswirth, V. Chiodo, K.E. Erger, S.L. Boye, A.S. Lewin, T.J. Conlon, L. Renner, M. Neuringer, C. Detrisac, J. Guy. LHON gene therapy vector prevents visual loss and optic neuropathy induced by G11778A mutant mitochondrial DNA: biodistribution and toxicology profile. *Invest Ophthalmol Vis Sci*. **55**, 7739-7753 (2014).
12. R. D. Koilkonda, T. H. Chou, V. Porciatti, W.W. Hauswirth, J. Guy. Induction of rapid and highly efficient expression of the human *ND4* complex I subunit in the mouse visual

system by self-complementary adeno-associated virus. *Arch Ophthalmol*. **128**, 876-883 (2010).

13. H. Cwerman-Thibault, S. Augustin, C. Lechauve, J. Ayache, S. Ellouze, J.A. Sahel, M. Corral-Debrinski. Nuclear expression of mitochondrial *ND4* leads to the protein assembling in complex I and prevents optic atrophy and visual loss. *Mol Ther Methods Clin Dev.* **2**, 15003 (2015).
14. C. Bonnet, S. Augustin, S. Ellouze, P. Bénit, A. Bouaita, P. Rustin, J.A. Sahel, M. Corral-Debrinski. The optimized allotopic expression of *ND1* or *ND4* genes restores respiratory chain complex I activity in fibroblasts harboring mutations in these genes. *Biochim Biophys Acta*. **1783**, 1707-1717 (2008).
15. C. Vignal, S. Uretsky, S. Fitoussi, A. Galy, L. Blouin L, J.F. Girmens JF, S. Bidot, N. Thomasson, C. Bouquet, S. Valero, S. Meunier, J.P. Combal, B. Gilly, B. Katz, J.A. Sahel. Safety of rAAV2/2-*ND4* gene therapy for Leber hereditary optic neuropathy. *Ophthalmology*. **125**, 945-947 (2018).
16. C. Bouquet, C. Vignal Clermont, A. Galy, S. Fitoussi, L. Blouin, M.R. Munk, S. Valero, S. Meunier, B. Katz, J.A. Sahel, N. Thomasson. Immune response and intraocular inflammation in patients with Leber hereditary optic neuropathy treated with intravitreal injection of recombinant adeno-associated virus 2 carrying the *ND4* gene: a secondary analysis of a phase 1/2 clinical trial. *JAMA Ophthalmol*. **137**, 399-406 (2019).
17. T. Klopstock, P. Yu-Wai-Man, K. Dimitriadis, J. Rouleau, S. Heck, M. Bailie, A. Atawan, S. Chattopadhyay, M. Schubert, A. Garip, M. Kernt, D. Petraki, C. Rummey, M. Leinonen, G. Metz, P.G. Griffiths, T. Meier, P.F. Chinnery. A randomized placebo-

controlled trial of idebenone in Leber's hereditary optic neuropathy. *Brain*. **134**, 2677–2686 (2011).

18. V. Carelli, C. La Morgia, M.L. Valentino, G. Rizzo, M. Carbonelli, A. M. De Negri, F. Sadun, A. Carta, S. Guerriero, F. Simonelli, A. A. Sadun, D. Aggarwal, R. Liguori, P. Avoni, A. Baruzzi, M. Zeviani, P. Montagna, P. Barboni. Idebenone treatment in Leber's hereditary optic neuropathy. *Brain*. **134(Pt 9)**, e188 (2011).
19. M. Silva, X. Llòria, C. Catarino, T. Klopstock. Natural history of Leber's hereditary optic neuropathy (LHON): findings from a large patient cohort. Poster presented at 45th annual meeting of the North American Neuro-Ophthalmology Society. Las Vegas, NV, 16 to 21 March 2019
20. M. Silva, X. Llòria, C. Catarino, T. Klopstock. Natural history findings from a large cohort of patients with Leber's hereditary optic neuropathy (LHON): new insights into the natural disease course. *Acta Ophthalmol*. **96**, 117 (2018).
21. B.L. Lam, W.J. Feuer, J.C. Schiffman, V. Porciatti, R. Vandenbroucke, P.R. Rosa, G. Gregori, J. Guy. Trial end points and natural history in patients with G11778A Leber hereditary optic neuropathy. *JAMA Ophthalmol*. **132**, 428-436 (2014).
22. I. J. Suñer, G. T. Kokame, E. Yu, J. Ward, C. Dolan, N. M. Bressler. Responsiveness of NEI VFQ-25 to changes in visual acuity in neovascular AMD: validation studies from two phase 3 clinical trials. *Invest Ophthalmol Vis Sci*. **50**, 3629-3635 (2009).C. M. Mangione, P.P. Lee, P. R. Guttierrez, K. Spritzer, S. Berry, R.D. Hays, National Eye Institute Visual Function Questionnaire Field Test Investigators. Development of the 25-Item National Eye Institute Visual Function Questionnaire. *Arch Ophthalmol*. **119**, 1050-1058 (2001).

23. Majander A, Bowman R, Poulton J, et al. Childhood-onset Leber hereditary optic neuropathy. *Br J Ophthalmol*. **101**, 1505-1509 (2017).

24. M. A. Kirkman, A. Korsten, M. Leonhardt, K. Dimitriadis, I. F. De Coo, T. Klopstock, P. G. Griffiths, G. Hudson, P. F. Chinnery, P. Yu-Wai-Man. Quality of life in patients with Leber hereditary optic neuropathy. *Invest Ophthalmol Vis Sci*. **50**, 3112-3115 (2009).

25. A.V. Cideciyan. Leber congenital amaurosis due to *RPE65* mutations and its treatment with gene therapy. *Progress in Retinal and Eye Research.* **29**: 398-427 (2010).

26. J. Guy, W.J. Feuer, J.L. Davis, V. Porciatti, P.J. Gonzalez, R.D. Koilkonda, H. Yuan, W.W. Hauswirth, B.L. Lam. Gene therapy for Leber hereditary optic neuropathy: low- and medium-dose visual results. *Ophthalmology*. **124**, 1621-1634 (2017).

27. S. Yang, S.Q. Ma, X. Wan, H. He, H. Pei, M.J. Zhao, C. Chen, D.W. Wang, X.Y. Dong, J.J. Yuan, B. Li. Long-term outcomes of gene therapy for the treatment of Leber's hereditary optic neuropathy. *EBioMedicine*. **10**, 258-268 (2016).

28. Hong-li Liu, Jia-jia Yuan, Yong Zhang, Zhen Tian, Xin Li, Dan Wang, Yang-yang Du, Lin Song and Bin Li. Factors associated with rapid improvement in visual acuity in patients with Leber's hereditary optic neuropathy after gene therapy. *Acta Ophthalmol.* Online ahead of print (2020).

29. J. Yuan, Y. Zhang, H. Liu, D. Wang, Y. Du, Z. Tian, X. Li, S. Yang, H. Pei, X. Wan, S. Xiao, L. Song. Seven-Year Follow-up of Gene Therapy for Leber's Hereditary Optic Neuropathy. *Ophthalmology.* Online ahead of print (2020).

30. S. Yang, H. He, Y. Zhu, X. Wan, L.F. Zhou, J. Wang, W.F. Wang, L. Liu, B. Li. Chemical and material communication between the optic nerves in rats. *Clin Exp Ophthalmol.* **43**, 742-748 (2015).

31. B. Zingg, X. Chou, Z. Zhang, L. Mesik, F. Liang, H. Whit Tao and L. Zhang. AAV-Mediated Anterograde Transsynaptic Tagging: Mapping Corticocollicular Input-Defined Neural Pathways for Defense Behaviors. *Neuron*. **93**, 33-47 (2017).

32. A. Mandal, C. M. Drerup. Axonal transport and mitochondrial function in neurons. *Front Cell Neurosci*. **13**, 373 (2019).

33. T. C. Burdett, M. R. Freeman. Neuroscience. Astrocytes eyeball axonal mitochondria. *Science*. **345**, 385-386 (2014).

34. A. M. Falchi, V. Sogos, F. Saba, M. Piras, T. Congiu, M. Piludu. Astrocytes shed large membrane vesicles that contain mitochondria, lipid droplets and ATP. *Histochem Cell Biol*. **139**, 221-231 (2013).

35. J. Ariazi, A. Benowitz, V. De Biasi, M.L. Den Boer, S. Cherqui, H. Cui, N. Douillet, E.A. Eugenin, D. Favre, S. Goodman, K. Gousset, D. Hanein, D. I. Israel, S. Kimura, R.B. Kirkpatrick, N. Kuhn, C. Jeong, E. Lou, R. Mailliard, S. Maio, G. Okafo, M. Osswald, J. Pasquier, R. Polak, G. Pradel, B. de Rooij, P. Schaeffer, V.A. Skeberdis, I.F. Smith, A. Tanveer, N. Volkmann, Z. Wu, C. Zurzolo. Tunneling nanotubes and gap junctions-their role in long-range intercellular communication during development, health, and disease conditions. *Front Mol Neurosci*. **10**, 333 (2017).

36. S. Hrvatin, D.R. Hochbaum, M.A. Nagy, M. Cicconet, K. Robertson, L. Cheadle, R. Zilionis, A. Ratner, R. Borges-Monroy, A.M. Klein, B.L. Sabatini, M.E. Greenberg.

Single-cell analysis of experience-dependent transcriptomic states in the mouse visual cortex. *Nat Neurosci*. **21**, 120-129 (2018).

37. J. A. Sabel, J. Flammer, L. B. Merabet. Residual vision activation and the brain-eye-vascular triad: dysregulation, plasticity and restoration in low vision and blindness – a review. *Restor Neurol Neurosci*. **36**, 767–791 (2018).

38. N.J. Newman, V. Carelli, M. Taiel, P. Yu-Wai-Man. Visual outcomes in Leber hereditary optic neuropathy patients with the m. 11778>A (MTND4) mitochondrial DNA mutation. *J Neuroophthalmol.* In press (2020).

39. C. Giordano, L. Iommarini, L. Giordano, A. Maresca, A. Pisano, M.L. Valentino, L. Caporali, S. Deceglie, M. Roberti, F. Fanelli, F. Fracasso, P. D'Adamo, G. Hudson, A. Pyle, P. Yu-Wai-Man, P.F. Chinnery, M. Zeviani, S.R. Salomao, A. Berezovsky, R. Belfort Jr., D.F. Ventura, M.N. Moraes, M.N. Moraes-Filho, P. Barboni, F. Sadun, A. De Negri, F.N. Ross-Cisneros, A.A. Sadun, A. Tancredi, G. d'Amati, P.L. Polosa, P. Cantatore, V. Carelli. Efficient mitochondrial biogenesis drives incomplete penetrance in Leber's hereditary optic neuropathy. *Brain.* **137**, 335-353 (2014)

40. A. Pisano, C. Preziuso, L. Iommarini , E. Perli , P. Grazioli, A.F. Campese, A. Maresca, M. Montopoli, L. Masuelli, A.A. Sadun, G. d'Amati, V. Carelli, A. Ghelli, C. Giordano. Targeting estrogen receptor β as preventive therapeutic strategy for Leber's hereditary optic neuropathy. *Hum Mol Genet.* **24**, 6921-6931 (2015)

41. A.D. Cherry, C.A. Piantadosi. Regulation of mitochondrial biogenesis and its intersection with inflammatory responses. *Antioxid Redox Signal.* **22**, 965-976 (2015)

42. M.L. Coopera, S. Pasinia, W.S. Lamberta, K.B. D'Alessandroa, V. Yaoa, M.L. Risnera, D.J. Calkins. Redistribution of Metabolic Resources through Astrocyte Networks Mitigates Neurodegenerative Stress. *Proc Natl Acad Sci U S A.* In press (2020)

43. R. Karanjia, T.J. Hwang, A.F. Chen, A. Pouw, J.J. Tian, E.R. Chu, M.Y. Wang, J.S. Tran, A.A. Sadun. Correcting finger counting to Snellen acuity. *Neuroophthalmology*. **40**, 219-221 (2016).

44. D.A. Jabs, R.B. Nussenblatt, J.T. Rosenbaum, Standardization of Uveitis Nomenclature (SUN) Working Group. Standardization of uveitis nomenclature for reporting clinical data. Results of the First International Workshop. *Am J Ophthalmol.* **140**, 509–516 (2005).

45. R.B. Nussenblatt, A.G. Palestine, C.C. Chan, F. Roberge. Standardization of vitreal inflammatory activity in intermediate and posterior uveitis. *Ophthalmology*. **92**, 467-471 (1985).

46. J.T. Holladay. Proper method for calculating average visual acuity. *J Refrac Surgery.* **13,** 388-391 (1997).

Acknowledgments:

We are grateful to the study teams that have contributed to the conduct of REVERSE in the various recruitment centers (supplementary materials). We would also like to thank the patients who took part in this gene therapy study.

Funding:

GenSight Biologics fully funded and sponsored the study. PYWM is supported by a Clinician Scientist Fellowship Award (G1002570) from the Medical Research Council (UK), and also receives funding from Fight for Sight (UK), the Isaac Newton Trust (UK), the National Eye Centre UK), the UK National Institute of Health Research (NIHR) as part of the Rare Diseases Translational Research Collaboration, and the NIHR Biomedical Research Centre based at Moorfields Eye Hospital NHS Foundation Trust and UCL Institute of Ophthalmology. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health. VC is supported by grants from the Italian Ministry of Health (RF-2018-12366703), the Italian Ministry of Research (20172T2MHH), and Telethon-Italy (GUP15016). VC is also supported by patients' organizations MITOCON and IFOND, and patients' donations. TK is supported by the German Federal Ministry of Education and Research (BMBF, Bonn, Germany) through grants to the German Network for Mitochondrial Disorders (mitoNET, 01GM1906A) and to the E-Rare project GENOMIT (01GM1920B). JAS and SP are supported by the Agence Nationale de la Recherche within the Programme Investissements d'Avenir, Institut Hospitalo-Universitaire FOReSIGHT [ANR-18-IAHU-0001].

Clinical Trial Number:

NCT026527080

Competing Interests:

L Blouin, P Burguière, M Taiel, C Chevalier, H Masonson, Y Salermo, B Katz are employees of GenSight Biologics. G Smits is a consultant for GenSight Biologics. P Yu-Wai-Man is a consultant for GenSight Biologics and Stealth BioTherapeutics, and has received research support from GenSight Biologics and Santhera Pharmaceuticals. M Moster is a consultant for GenSight Biologics and has received research support from GenSight Biologics. NJ Newman is a consultant for GenSight Biologics, Santhera Pharmaceuticals and Stealth BioTherapeutics, has received research support from GenSight Biologics and Santhera Pharmaceuticals, serves on the Data Safety Monitoring Board for Quark NAION study, and is a medical legal consultant. V Biousse is a consultant for GenSight Biologics, Santhera Pharmaceuticals and Stealth BioTherapeutics, has received research support from GenSight Biologics and Santhera Pharmaceuticals, serves on the Data Safety Monitoring Board for Quark NAION study, and is a medical legal consultant. AA Sadun is a consultant for Stealth BioTherapeutics. T Klopstock is a consultant for GenSight Biologics and Santhera Pharmaceuticals, and has received research support from GenSight Biologics and Santhera Pharmaceuticals. C Priglinger has received research support from GenSight Biologics and Santhera Pharmaceuticals. C Vignal Clermont is a consultant for GenSight Biologics and Santhera Pharmaceuticals. V Carelli is a consultant for GenSight Biologics, Santhera Pharmaceuticals and Stealth BioTherapeutics, and has received research support from Santhera Pharmaceuticals and Stealth BioTherapeutics. RC Sergott is a consultant for GenSight Biologics. D Calkins is a consultant for GenSight Biologics and Stuart

Therapeutics. S Picaud is a consultant for GenSight Biologics, co-founder and shareholder of GenSight Biologics. JA Sahel is the co-founder and shareholder of GenSight Biologics, and the patent co-author on allotopic transport.

Data and Materials Availability:

All data associated with this study are available in the main text or the supplementary materials.

Fig 1. (A) Mean Change from Baseline in BCVA up to 96 Weeks Post-Administration of rAAV2/2-*ND4* Gene Therapy. Error bars: ±1 standard error. The Y-axis was inverted to represent BCVA improvement going upward. The asterisks indicate a statistically significant change from baseline (*p<0.05, **p<0.01, ***p<0.001). **(B) Individual Changes in LogMAR BCVA from Baseline to Week 96 in REVERSE Subjects.** CRR: clinically relevant response at Week 96. Subject with a CRR in at least one eye: defined as at least one eye that was on-chart at baseline and had an improvement of at least 10 ETDRS letters, or was off-chart at baseline and became on-chart with at least 5 ETDRS letters read. Data labels represent subject ID numbers. The X and Y-axes were inverted to represent BCVA improvement going right for sham-treated eyes and upward for rAAV2/2-*ND4* eyes. The diagonal bisector indicates equal change in BCVA in both eyes of a subject. In the REVERSE study, 68% of the subjects experienced a CRR in at least one eye (points colored in green, red and blue), and 78% of the subjects experienced a bilateral improvement of BCVA (points located in the right upper quadrant).

Table 1. Change of Best-Corrected Visual Acuity from Baseline to Week 96

Best-Corrected Visual Acuity (LogMAR)		rAAV2/2-*ND4* Eyes	Sham-Treated Eyes
At Baseline	n	37	37
	Mean (SD)	1.67 (0.50)	1.55 (0.42)
	Min, Max	0.80, 3.17	0.70, 2.81
Change from Baseline	n	37	37
	LS Mean (SE)	-0.308 (0.068)	-0.259 (0.068)
	95% CI	-0.446, -0.170	-0.396, -0.121
	p-value	*< 0.0001*	*0.0001*
Between-eye Difference in Change from Baseline [(1)]	n	37	
	LS Mean (95% CI)	-0.049 (-0.144, 0.046)	
	p-value	*0.3019*	

(1) A mixed-effects analysis of covariance (ANCOVA) model was used with change from baseline at as the response, and subject and eyes of the subject as random factors, treatment as a fixed effect, and the baseline LogMAR value as covariate. P-value is used to assess the significance of the difference between All-rAAV2/2-*ND4* and All-Sham with respect to change from baseline.

CI = confidence interval; LogMAR = logarithm of the minimal angle resolution; LS = least square; SD = standard deviation; SE = standard error

Table 2. Change of Best-Corrected Visual Acuity from Nadir to Week 96

Best-Corrected Visual Acuity		rAAV2/2-*ND4* Eyes	Sham-Treated Eyes
	n	37	37
Change from Nadir	Mean LogMAR	-0.570	-0.490
	Letters Equivalent	+28.5	+24.5

The mean improvement in BCVA from nadir to Week 96 was converted from LogMAR to "letters equivalent" by multiplying the LogMAR value by -50 *(46)*. Nadir is defined as the worst BCVA recorded in any of the visits in REVERSE, including the baseline visit immediately prior to the injection. BCVA = best-corrected visual acuity; LogMAR = logarithm of the minimal angle resolution.

Table 3. rAAV2/2-*ND* DNA Detection and Quantification in Tissues from the Eye and the Central Nervous System

	3 Months											
	Group 1 – Control (Vehicle IVT Right Eye)						Group 2 – Test (rAAV2/2 *ND4* IVT Right Eye)					
	Left Side/Eye (Contralateral)			Right Side/Eye (Injected)			Left Side/Eye (Contralateral)			Right Side/Eye (Injected)		
	BLD	BLQ	Mean Copy*	BLD	BLQ	Mean Copy*	BLD	BLQ	Mean Copy*	BLD	BLQ	Mean Copy*
Lacrimal gland	1			1			2	1			1	2 (4.35×10^2)
Anterior segment of the eye	1			1					3 (3.39×10^3)			3 (3.21×10^6)
Aqueous humor	1			1			3				1	2 (1.65×10^3)
Vitreous humor	1			1			3					3 (8.70×10^4)
Retina	1			1				1	2 (5.99×10^3)		1	2 (2.70×10^6)
Optic nerve	1			1					3 (1.00×10^4)		1	2 (1.45×10^3)
Optic chiasm	1			1				3			1	2 (3.28×10^4)
Optic tract	1			1			1	2			3	
Lateral geniculate nucleus	1			1			1	2			2	1 (2.84×10^2)
Visual cortex	1			1			3				2	1 (2.05×10^3)

*Quantity in copy/µg of DNA for tissue samples and copy/ µL for aqueous and vitreous humors.

BLD: Below Limit of Detection (15.6 copies/µg of DNA); BLQ: Below Limit of Quantification (250 copies/µg of DNA); IVT: Intravitreal injection.

Figure 2. Detection of rAAV2/2-*ND4* DNA in Tissues from the Eye and the Central Nervous System

The bar graph indicates the number of animals (out of 3) in which rAAV2/2-*ND4* DNA was present above the limit of detection (15.6 copies/μg of DNA).

SUPPLEMENTARY FIGURES AND TABLES

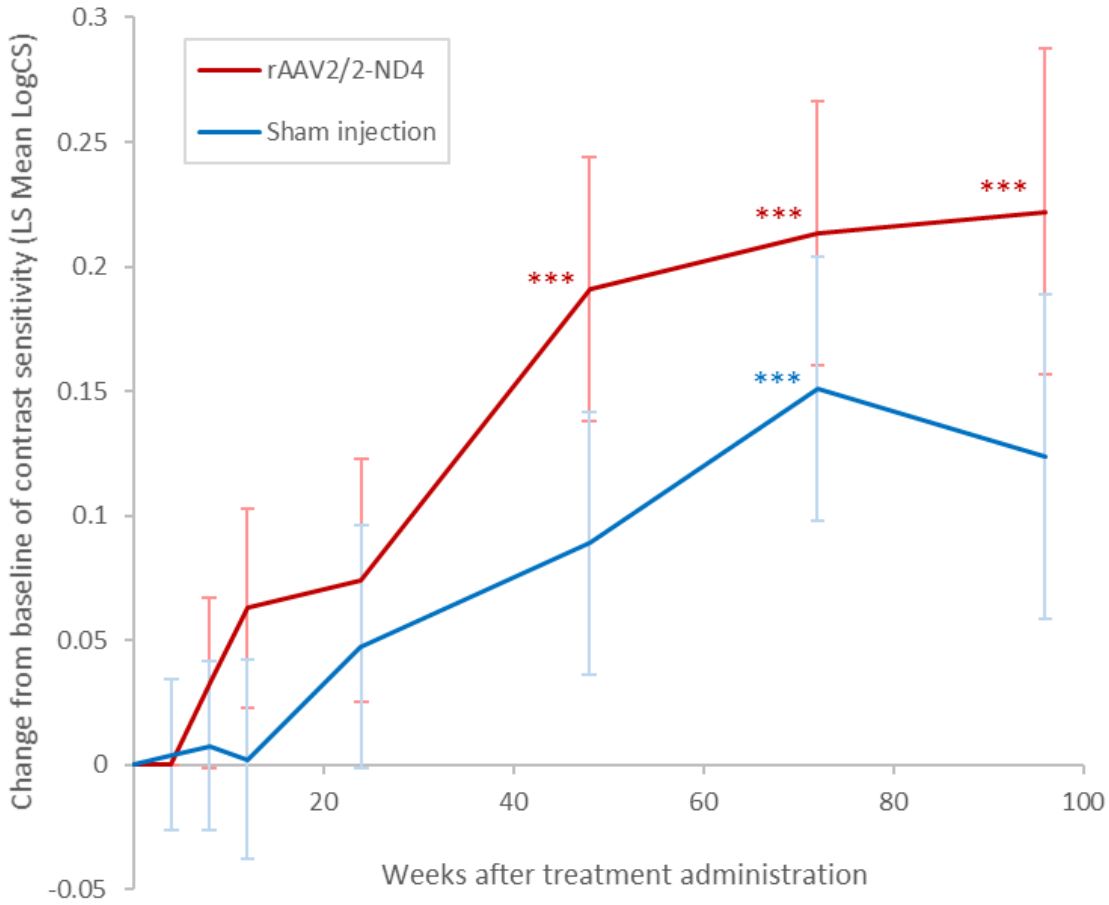

Fig. S1. Mean Change from Baseline in Contrast Sensitivity up to 96 Weeks Post-Administration of rAAV2/2-*ND4* Gene Therapy

Error bars: ±1 standard error. Asterisks indicate a statistically significant change from baseline (***p<0.001).

Table S1. Baseline Characteristics of Subjects Enrolled in the REVERSE Study

		All Subjects (N = 37)
Age (Years)	Mean (SD)	34.2 (15.2)
	Min, Max	15, 67
Gender		
Female	n (%)	8 (21.6)
Male	n (%)	29 (78.4)
Duration of Vision Loss (Days)		
rAAV2/2-*ND4* eyes	Mean (SD)	263.1 (53.9)
	Min, Max	181, 362
Sham-Treated eyes	Mean (SD)	278.8 (64.5)
	Min, Max	181, 364

Table S2. Change from Baseline to Week 96 for Contrast Sensitivity, Humphrey Visual Field Perimetry and OCT Parameters

		rAAV2/2-*ND4* Eyes	Sham-Treated Eyes
Contrast Sensitivity (LogCS)			
At Baseline	n	37	37
	Mean (SD)	0.25 (0.40)	0.35 (0.46)
	Min, Max	0.00, 1.50	0.00, 1.35
Change from Baseline	n	37	37
	LS Mean (SE)	0.22 (0.06)	0.12 (0.06)
	95% CI	0.09, 0.34	-0.01, 0.25
Between-eye Difference in Change from Baseline [(1)(2)]	n	37	
	LS Mean (95% CI)	0.10 (-0.02, 0.21)	
	p-value	*0.1036*	

		rAAV2/2-*ND4* Eyes	Sham-Treated Eyes
Mean Deviation (dB) HVF			
At Baseline	n	37	37
	Mean (SD)	-25.99 (8.37)	-24.94 (9.70)
	Min, Max	-34.64, -1.80	-34.64, -3.20
Change from Baseline	n	37	37
	LS Mean (SE)	2.70 (0.90)	2.57 (0.90)
	95% CI	0.89, 4.50	0.76, 4.37
Between-eye Difference in Change from Baseline [(1)]	n	37	
	LS Mean (95% CI)	0.13 (-1.32, 1.59)	
	p-value	*0.8539*	

		rAAV2/2-*ND4* Eyes	Sham-Treated Eyes
RNFL Thickness - PMB (µm)			
At Baseline	n	37	37
	Mean (SD)	23.1 (6.2)	23.6 (7.2)
Change from Baseline	n	35	36
	LS Mean (SE)	1.2 (1.3)	0.7 (1.3)
	95% CI	-1.4, 3.8	-1.9, 3.2
Between-eye Difference in Change from Baseline [(1)]	n	35	
	LS Mean (95% CI)	0.6 (-2.8, 3.9)	
	p-value	*0.7412*	
RNFL Thickness - Temporal Quadrant (µm)			
At Baseline	n	37	37
	Mean (SD)	27.5 (7.4)	28.9 (8.5)
Change from Baseline	n	35	36
	LS Mean (SE)	-1.8 (1.0)	-2.0 (0.9)
	95% CI	-4.2, 0.6	-4.4, 0.3
Between-eye Difference in Change from Baseline [(1)]	n	35	
	LS Mean (95% CI)	0.2 (-2.2, 2.7)	
	p-value	*0.8465*	

		rAAV2/2-*ND4* Eyes	Sham-Treated Eyes
GCL Macular Volume (mm^3)			
At Baseline	n	37	37
	Mean (SD)	0.534 (0.063)	0.526 (0.069)
Change from Baseline	n	36	36
	LS Mean (SE)	-0.018 (0.012)	-0.031 (0.012)
	95% CI	-0.041, 0.006	-0.054, -0.008
Between-eye Difference in Change from Baseline [(1)]	n	36	
	LS Mean (95% CI)	0.013 (-0.016, 0.042)	
	p-value	*0.3528*	

(1) A mixed-effects analysis of covariance (ANCOVA) model was used with change from baseline at as the response, and subject and eyes of the subject as random factors, treatment as a fixed effect, and the baseline LogMAR value as covariate. P-value is used to assess the significance of the difference between All-rAAV2/2-*ND4* and All-Sham with respect to change from baseline.

(2) Subjects who could not read any letter on the Pelli-Robson chart were assigned the worst possible score (0 LogCS).

CI = confidence interval; GCL = ganglion cell layer; HVF = Humphrey visual field; LS = least square; PMB = papillo-macular bundle; RNFL = retinal nerve fiber layer; SD = standard deviation; SE = standard error

Table S3. Visual Responders at Week 96

	Eye Responders		Subject Responders
	rAAV2/2-*ND4*	**Sham**	
Clinically relevant response (CRR) [1]			
Responder	23 (62%)	16 (43%)	25 (68%)
Non-Responder	14 (38%)	21 (57%)	12 (32%)
	p = 0.0348 [2]		
BCVA improvement by at least 3 ETDRS lines [3]			
Responder	13 (35%)	10 (27%)	15 (41%)
Non-Responder	24 (65%)	27 (73%)	22 (59%)
	p = 0.2568 [2]		

(1) CRR was defined per treatment group as either an eye which is on-chart at baseline with an improvement at Week 96 of at least 10 ETDRS letters, or an eye which is off-chart at baseline that became on-chart with at least 5 letters read at Week 96. A subject responder was defined as having a CRR in at least one eye at Week 96.

(2) p-value from McNemar test compares the rates of eye responders between treatments.

(3) Responder was defined as an on-chart improvement by at least -0.3 LogMAR (3 ETDRS lines), or an improvement from off-chart to on-chart vision with a final BCVA of 1.4 LogMAR or better (i.e. at least the first 3 ETDRS lines of the chart were read at 1 meter). A subject responder was defined as having this response in at least one eye at Week 96.

CRR: clinically relevant response.

Table S4. Change in Vision-Related Quality of Life at Week 96

VFQ-25 Subscales [1]	Baseline Score	Change from Baseline	
	Mean (SD)	Mean (SD)	Mean %[2]
Dependency	31.8 (24.7)	+18.5 (28.9)	130.2%
Mental Health	32.1 (24.6)	+16.1 (18.9)	108.2%
Role Difficulties	34.5 (28.3)	+15.9 (28.4)	78.9%
Near Activities	23.7 (14.8)	+13.3 (22.0)	78.1%
Peripheral Vision	59.5 (27.2)	+10.8 (26.0)	41.0%
Distance Activities	35.5 (18.7)	+10.7 (18.6)	47.4%
Color Vision	68.1 (29.6)	+6.9 (21.2)	21.6%
General Vision	30.8 (13.0)	+6.5 (17.0)	32.40%
Social Functioning	49.0 (23.5)	+4.7 (24.4)	32.80%
Ocular Pain	84.8 (18.4)	-1.0 (20.7)	1.60%
Composite Score [3]	42.1 (15.5)	+9.5 (12.7)	+28.8%

(1) Subscales not reported in this table: General Health (missing values), Driving (not applicable to LHON).

(2) The mean percent change from baseline was calculated from individual percent changes from baseline.

(3) The Composite Score is the average of vision-targeted sub-scale scores, excluding the General Health rating question.