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## Supplementary appendix

This appendix formed part of the original submission and has been peer reviewed. We post it as supplied by the authors.

Supplement to: MacLaren RE, Groppe M, Barnard AR, et al. Retinal gene therapy in patients with choroideremia: initial findings from a phase 1/2 clinical trial. *Lancet* 2014; published online Jan 16. http://dx.doi.org/10.1016/S0140-6736(13)62117-0.

# Appendix

Appendix to: Retinal gene therapy for choroideremia: Initial findings from a phase 1/2 clinical trial. MacLaren RE, Groppe M, Barnard AR, Cottriall CL, Tolmachova T, Seymour L, Clark KR, During MJ, Cremers FPM, Black GCM, Lotery AJ, Downes SM, Webster AR, Seabra MC

**Supplementary Methods**

*Patient selection and study design*

Male patients aged 18 or over with a clinical diagnosis of choroideremia and a confirmed *CHM* mutation were eligible to take part with informed consent. Since the fellow eye was to be used as a control, patients needed to have symmetrical disease with no other co-pathologies (e.g. macular hole). Best corrected visual acuity (BCVA) needed to be good enough to read letters on a standard ETDRS (early treatment for diabetic retinopathy study – a vision standard) vision chart and to perform reliable microperimetry. There was no upper limit of visual acuity, the aim being to treat patients with 6/6 vision, as long as reliable and consistent defects in microperimetry could be identified within the macular area. Patient (P) preference was considered in deciding which eye to treat, which was subjectively the slightly worse eye in all cases, although in two (P3 and P6) the treated eye could see 4 more ETDRS letters than the fellow eye at baseline (Table 1). Efficacy of treatment following treatment with the AAV.REP1 vector was assessed by improvements in visual acuity and microperimetry measured six months after surgery and correlated to the dose of vector applied per $mm^2$ of surviving retinal pigment epithelium (RPE)-retina measured at baseline.

The multicentre study was approved by the UK Gene Therapy Advisory Committee (GTAC 171) and the local ethics committees for each of four participating sites: Oxford Eye Hospital, Moorfields Eye Hospital, Manchester Royal Eye Hospital and the Eye Department of Southampton General Hospital. The study was sponsored by the University of Oxford. Molecular testing of REP1 took place in the Genetics Department at the University of Manchester. Patient selection was agreed between the study clinical investigators (REM, GCB, AJL, SMD, ARW, MG) who reviewed scans, molecular diagnoses and clinical data. Data on the AAV vector was reviewed by REM, MJD, KRC, TT, ARB and MCS. Generation and validation of the REP1 sequence and protein expression was performed by FPMC, REM, MCS and TT. Immunology testing was overseen by ARB, MG, REM and LS. Baseline characteristics of the six patients are shown in Table 1 below. Three patients were recruited from Oxford (P1, P3 and P6) and three were recruited from London (P2, P4 and P5).

*Table 1 – Patient demographics*

| | Age | *CHM* mutation identified | ETDRS BCVA RE | ETDRS BCVA LE | Treated eye |
|---|---|---|---|---|---|
| Patient 1 | 63 | NM_000390.2:c.940+2T>C | 58 | 23 | LE |
| Patient 2 | 47 | NM_000390.2:c.189+1G>C | 82 | 79 | LE |
| Patient 3 | 35 | NM_000390.2:c.492_493delGA | 85 | 89 | LE |
| Patient 4 | 57 | NM_000390.2:c.535_538delGAAA | 76 | 53 | LE |
| Patient 5 | 41 | NM_000390.2:c.529delG | 79 | 83 | RE |
| Patient 6 | 56 | NM_000390.2:c.189+1G>C | 84 | 88 | LE |

NM_000390.2:c.940+2T>C is predicted to cause a skip of exon 8 which would lead to a frameshift, NM_000390.2:c.189+1G>C is predicted to cause a skip of exon 3 which would lead to a frameshift. NM_000390.2:c.492_493delGA, NM_000390.2:c.535_538delGAAA and NM_000390.2:C.529delG create frameshifts in exon 5.

*Vector production*

The vector genome comprised of a strong constitutive expression cassette, a hybrid CBA promoter, the human CHM cDNA (encoding REP1), a modified WPRE sequence and a bovine poly-adenylation signal flanked by AAV2 ITRs, AAV2-CBA-hREP1-WPRE-bGH, and hereafter referred to as AAV2.REP1. It has been tested for efficacy in preclinical studies. The CHM cDNA fragments were originally isolated from a human retinal cDNA library created by Jeremy Nathans from several unaffected individuals.[1] The AAV2.REP1 vector for clinical use was made to current good manufacturing practice (cGMP) and consistent with the US Food and Drug Administration's "Guidance for Industry – cGMP for Phase 1 Investigational Drugs", at the Clinical Manufacturing Facility of the Nationwide Children's Hospital (NCH), Columbus Ohio. A qualified person (QP) acting on behalf of the UK Medicines and Healthcare products Regulatory Agency (MHRA) inspected the GMP facility at NCH in advance, to ensure that protocols were aligned with both FDA and UK MHRA recommended standards.

The manufacturing process includes procedures to ensure the safety, identity, quality, purity and strength of the manufactured biologic. The AAV2.REP1 vector was produced using standard triple DNA plasmid transfection composed of the REP1 vector plasmid, AAV2 rep-cap helper plasmid and an adenovirus helper plasmid containing E2A, E4ORF6, and VA RNA ORFs. All plasmid DNA was manufactured as GMP-S™ grade by Aldevron Inc. (3233 15th Street South Fargo, ND 58104, USA). Fargo, ND). HEK 293 cells were expanded from a qualified Master Cell Bank (MCB) in Corning Cell Stacks and transfected using standard calcium phosphate co-precipitation. The HEK 293 MCB met all FDA and ICH guidelines and testing requirements for generation of MCBs (i.e., FDA Guidance for Industry: Guidance for Human Somatic Cell Therapy and Gene Therapy Section II-C). Cells and media were harvested 72 hour post-transfection and single-pass microfluidization was used for cell disruption in the presence of benzonase nuclease. Cellular debris was removed by sequential depth and bioburden reduction (0.22 um) capsule filters. Tangential flow filtration using a disposable 100 kD hollow fiber membrane (Spectrum Laboratories, Inc.) was used to achieve approximately 50-fold volume reduction and then the product was run over an iodixanol step gradient as previously described [2] to enrich for full particles present in the 40% iodixanol layer. The product was further polished using Heparin column chromatography using a NaCl salt step gradient similar to that previously described.[3] Lastly, the AAV2 vector product was formulated in 20 mM Tris (pH 8.0), 1 mM $MgCl_2$ and 200 mM NaCl containing 0.001% (v/v) PF68 (Spectrum Chemical with a Certificate of Analysis showing compliance with the pharmacopoeial monograph National Formulary grade – i.e. excipient is not of animal or human origin), to a final titre of $1x10^{12}$ gp/ml, sterile filtered and manually filled into sterile polypropylene gasketed vials in 100 µl volumes and stored at ≤ $-60^{o}$ C as a frozen liquid. In-process, Bulk Intermediate and final Drug Product release testing met all pre-determined testing specifications in accordance to guidance from the MHRA. Importation, storage and QP release was organized by the MHRA-approved Clinical Biomanufacturing Facility at the University of Oxford.

A previous study highlighted the possibility of AAV particles sticking to plastic inside the injection system and recommended us of surfactant.[4] In order to test the dose emerging from the syringe through the tip of the Dorc 41G cannula and hence delivered into the subretinal space, the injection equipment was shipped to the NCH Vector Core in Columbus Ohio for testing. The assays were performed on 11-14 October 2010, by Dr K. Reed Clark (KRC), Director of the NCH Vector Core following detailed discussions with the Chief Investigator (REM) in relation to the precise steps and timings that would be involved in the operating theatre. The titre of genomic resistant particles (gp/ml) was assessed by quantitative polymerase chain reaction (qPCR) on the vector at baseline, after injection through the Dorc 41G cannula and on the vector remaining in the syringe. Two samples were tested from each group and at two separate dilutions, with forward and reverse primers directed against the CBA promoter sequence. The doubling cycle threshold (CT) was generated for each sample and the results shown in Table 2 show a mean titre of $3.69 \times 10^{10}$ gp/ml in the vector suspension in the injection system without surfactant and a mean dose of $2.00 \times 10^{11}$ gp/ml with 0.001% PF68 surfactant. This difference, equivalent to an approximate 81% loss of vector particles in the absence of PF68 surfactant was highly significant ($p<0.0001$). Hence PF68 0.001% was included in the final GMP vector suspension at the time of filling.

*Table 2 – Validation of vector dose delivered*

| *No.* | *Description* | *Dilution* | *Detector* | *CT* | *Quantity* | *Titre (gp/ml)* | *Average Titre* |
|---|---|---|---|---|---|---|---|
| 1 | Vector injected | 1000 | CBA | 28.7069 | 7.91E+04 | 3.16E+10 | 3.19E+10 |
| 2 | Vector injected | 1000 | CBA | 28.9884 | 6.53E+04 | 2.61E+10 | |
| 3 | Vector injected | 10000 | CBA | 31.9783 | 8.59E+03 | 3.44E+10 | |
| 4 | Vector injected | 10000 | CBA | 31.9389 | 8.83E+03 | 3.53E+10 | |
| 5 | Vector in syringe | 1000 | CBA | 28.3148 | 1.03E+05 | 4.13E+10 | 4.19E+10 |
| 6 | Vector in syringe | 1000 | CBA | 28.2015 | 1.11E+05 | 4.46E+10 | |
| 7 | Vector in syringe | 10000 | CBA | 31.6966 | 1.04E+04 | 4.16E+10 | |
| 8 | Vector in syringe | 10000 | CBA | 31.7505 | 1.00E+04 | 4.01E+10 | |
| 1 | PF68+ vector injected | 1000 | CBA | 25.7056 | 6.06E+05 | 2.42E+11 | 1.87E+11 |
| 2 | PF68+ vector injected | 1000 | CBA | 26.3771 | 3.84E+05 | 1.54E+11 | |
| 3 | PF68+ vector injected | 10000 | CBA | 29.5299 | 4.52E+04 | 1.81E+11 | |
| 4 | PF68+ vector injected | 10000 | CBA | 29.5991 | 4.32E+04 | 1.73E+11 | |
| 5 | PF68+ vector in syringe | 1000 | CBA | 25.9066 | 5.29E+05 | 2.12E+11 | 2.13E+11 |
| 6 | PF68+ vector in syringe | 1000 | CBA | 25.8216 | 5.60E+05 | 2.24E+11 | |
| 7 | PF68+ vector in syringe | 10000 | CBA | 29.2757 | 5.38E+04 | 2.15E+11 | |
| 8 | PF68+ vector in syringe | 10000 | CBA | 29.3822 | 5.00E+04 | 2.00E+11 | |

*Vitreoretinal surgery*

Due to the complexity and unpredictability of detaching the retina in end-stage choroideremia, in which the retina and choroid are extremely thin and fused in places, a new technique of subretinal gene therapy was developed. This involved performing the vector delivery in two steps after core and peripheral vitrectomy. In the first step the retinal detachment procedure used balanced salt solution (BSS, Alcon Laboratories, Ft Worth, Texas) injected through a 41 gauge Teflon cannula (Figure S1) with continuous footswitch pressure (15-17 psi) from a 10 ml connecting syringe taken from the Alcon viscous fluid injection pack. This facilitated delivery of a controlled but slow jet of BSS which could be held with the 41 gauge tip embedded in the retina without vibration of the hand. The initial point of entry was chosen over an island of surviving autofluorescence as OCT scans showed thicker retina in these areas with surviving photoreceptors and a more clearly defined subretinal plane. No fluid air exchange was required to detach the retina. All surgery was performed by one surgeon (REM).

P1 and P2 required 3-4 injections to detach the fovea whereas the fovea of P3 and P5 detached on the first wave. P4 required 5 injections as the retina was difficult to separate from the underlying choroid and an initial injection created a choroidal detachment. P6 had 5-6 initial attempts to detach the fovea from outside the arcade but these were unsuccessful and eventually an injection placed over pigmented tissue close to the papillomacular bundle successfully detached the macula (Figure 1Q). With the exception of P6, care was taken to avoid injecting close to the thin fovea as this could increase the risk of developing a macular hole. Some stretching of degenerate retina around the surviving island of retina was noted in all patients, but in P6 it was of some concern as this included the papillomacular bundle. For this reason and to reduce the chance of damage caused by further stretching, a reduced vector volume was injected into this patient (see below).

Once the fovea had been detached with a sufficient volume of BSS, the retinal detachment cannula was removed from the eye and the vector was prepared for injection (see below). An advantage of a two step procedure is that any unexpected complications of retinal detachment can be managed conservatively without concerns about the vector, the injection of which could be deferred until a later date if, for instance, a macular hole was created which required treatment with gas. Also since the volume of fluid required to detach the fovea is variable, by removing the vector from this first step, a precise consistent dose in terms of genome particles can still be applied to each patient in the second injection into the subretinal bleb of fluid, which is far more predictable. P1-P5 received $10^{10}$ gp of vector whilst P6 received a reduced dose of up to 0.06 ml ($6 \times 10^{9}$ gp of AAV.REP1) as the thin retina containing the papillomacular bundle was seen to stretch noticeably during subretinal injection. The reduced dose was within the trial protocol, which allowed *up to* $10^{10}$ genome particles per patient.

The AAV.REP1 vector was supplied in labelled polypropylene vials, each containing 100 microlitres of vector suspension at a concentration of $1 \times 10^{12}$ genome particles (gp) per ml and each 0.1 ml vial contained $10^{11}$ gp of vector in total. The vector was drawn up into a 1 ml BD (Becton, Dickinson UK Ltd, Edmund Halley Road, Oxford Science Park, Oxford OX4 4DQ) syringe which has a Luer-Lok tip, through a BD white 19 gauge microlance needle. Once 0.1 ml was in the syringe the plunger was withdrawn to 1.0 ml and 0.9 ml of BSS was added from another syringe. The mixed suspension (now diluted to 1 in 10) contained $1 \times 10^{10}$ gp in each 0.1 ml. A Dorc (Dutch Ophthalmic Research Center International BV, Scheijdelveweg 2, 3214 VN Zuidland, The Netherlands) Teflon 41 gauge (41G) needle was then added to

the tip of the syringe and primed (Figure S1) prior to injecting vector suspension into the subretinal space, which had been detached including the fovea in the previous step. The residual 1:10 diluted vector in the syringe that had not been injected was refrozen and tested to confirm REP1 protein expression using Western blot analysis at a later of similar samples collected across all six patients.

*Western blot quantification of AAV.REP1*

HT1080 cells were seeded at 4 x $10^6$ cell per 10 cm dish (1 plate per patient). The following day, cells were washed twice with PBS and treated overnight with 10 mM hydroxyurea in serum free Iscove's modified Dulbecco's media (IMDM, Invitrogen, Life Technologies Corp, Carlsbad, CA). Cells were then washed twice with IMDM and infected with 100 µl of the diluted AAV2.REP1 suspension obtained from theatre immediately after treatment of each patient in 7 ml of IMDM medium for 2 hours, after which 3.5 ml of IMDM + 30% serum was added to each plate. Cells were cultured for 6 days after transduction on the same dish (without replating) with occasional change of the medium. To analyse REP1 protein expression, cells were collected by trypsinisation, washed in PBS and lysed in 50 mM Hepes, 10 mM NaCl, 1 mM dithiothreitol (DTT), supplemented with Complete Protease Inhibitor Cocktail (Roche), by sonication. Cell lysates were spun at 7000 *g* for 10 min at 4° C and loaded onto SDS-PAGE gel (30 g of lysate per lane), transferred to a polyvinylidene fluoride (PVDF) membrane and probed with 2F1 antibody (specific for human REP1)[5] and alpha-tubulin antibody as a loading control. Positive and negative antibody controls were also provided by human REP1 (hREP1) protein and a non-transduced H1080 cell lysate. For assessment of endogenous REP1 expression from HT1080 cells, the plates were exposed until a clear band could be seen in the non-transduced HT1080 lane. This was normalised to actin together with the lanes corresponding to the vector used in each of the six patients. Finally the over-expression of REP1 from the vector transduced cells was normalised to the levels of REP1 present in the cell line.

*Best corrected visual acuity testing*

Refraction was completed prior to BCVA and contrast sensitivity assessment at all relevant visits. Patients were refracted using the early treatment for diabetic retinopathy study (ETDRS) chart R at 4 m. At each follow-up visit, the refraction recorded from the previous visit was used as the start point. If less than 4 letters were read on the top line, the light box was brought to 1 metre and the refraction was continued. The test took place in two parts with initial testing at 4 metres, followed by testing at 1 metre only if an insufficient number of letters were read at the initial test distance. Wearing the distance prescription, each eye was tested separately at 4 metres using the standard ETDRS charts; Chart 1 to test the right eye and Chart 2 to test the left eye. The patient was asked to read from the top of the chart and to read slowly the letters (at a rate not faster than about one letter per second) in order to achieve the best identification of each letter and to not proceed until the participant had given a definite response. The patient was told that there are only letters on the charts and no numbers or symbols. If the patient identified a letter as one of two or more letters, they were asked to choose one letter and, if necessary, to guess even if the next letter had already been read. The examiner did not tell the patient whether a letter was correctly or incorrectly identified. It was accepted that if a patient changed their response before they had read the next letter out loud it could be counted. If the patient changed a response after they had read the next letter it was not accepted. The test was continued until the patient had read all the letters possible.

*Testing of contrast sensitivity*

Contrast sensitivity was measured at 1 metre on Pelli-Robson charts, using standard methods. Chart 1 to test the right eye and Chart 2 to test the left eye. Chart illumination was between 75-120 foot candles with no greater than 10% variance across the chart. The distance prescription was worn, with an additional +0.75 sphere to correct for the 1 metre testing distance. The patient needed to state one definitive answer per letter, beginning with the letter in the upper left hand corner of the chart and reading across each row of two groups of three letters of equal contrast. If they changed an incorrect response to a correct one before moving onto the next letter, the letter was counted as correct. The test was continued until the patient stated that they cannot see anything in the next group of three letters, even after being encouraged to guess. The faintest triplicate with 2 or more letters read was taken as the log contrast sensitivity (three letters represent each log unit).

*Microperimetry*

The testing protocol was based on the Maia system (Centervue SpA, Via Tommaseo 77, 35131, Padova, Italy) and was developed following initial examinations on choroideremia patient volunteers. A Goldman size III stimulus of 200 ms was used with a maximum intensity of 1000 apostilb (asb) equivalent to 320 candela per square metre (cd/$m^2$) and a range of 32db. The background luminance was set to 4 asb (1.3 cd/$m^2$). The maximum possible testing field was modified to 37 x 37 degree field. The image presentation of the scanning laser ophthalmoscope was modified to reduce increased brightness due to increased scleral reflectivity. This allowed a clear identification of surviving areas of retina and choroid. The testing threshold strategy was a 4-2 staircase. The Maia normative database in the inbuilt software was obtained from a multicentric international study on 494 normal eyes for patients using this machine and has been reported previously.[6] Normative data relevant to this study show a range from 30.42±1.34 dB for patients aged 30-40, to 28.39±1.56 dB for patients aged 60-70 (mean ± standard deviation).[7] This standard sensitivity curve was plotted as green columns together with the test patient results automatically by the inbuilt software. Prior to testing Marco Morales from Centervue SpA made site visits to check the calibration of the machines and adapted the autoexposure of the inbuilt camera to avoid overexposure in choroideremia patients. At the end of the eye alignment, the Maia microperimeter performs an automatic focusing process, during which the image auto-exposure is performed by evaluating the live image brightness compared to a maximum established value. If the brightness exceeds a certain threshold the MAIA reduces the light source according to the software algorithms. This maximum value was adjusted to compensate for the brighter scleral reflectance.

Each trial participant had at least three preoperative tests to reduce any learning effect. To encourage compliance, patients were also informed that reproducible microperimetry testing from both eyes was a prerequisite for entry into the trial. Patients were tested after 20 min dark adaptation (1.5 cd/$m^2$) and with the background luminance set at 4 apostilb (1.3 cd/$m^2$). The first patient was tested with a standard 10 degree grid, which was centred foveal in his right eye and onto the central area of surviving retina in the left eye. Subsequently all patients were tested with a modified grid with 1 or 2 degree regular spacing between testing points. The 2 degree grid was adapted by adding additional points centrally to the grid pattern in areas of surviving area confirmed by autofluorescence. The centre of the grid was placed at the central area of fixation. Initial tests were performed to determine regions of absolute scotoma in each patient. In order to shorten the overall test duration and therefore improve patient

compliance, some surplus testing points in areas of absolute scotoma were removed, but at least one to two rows of testing points were left around areas of light sensitive retina as some tissue could be seen in these regions. To avoid skewing data away from normality and to provide mean dB readings that were relevant to the seeing retina rather than the number of points tested, areas of absolute scotoma present in both tests were not included in the analysis of mean sensitivity. Where an absolute scotoma corresponded to a seeing point on the other test, it was included and assigned a value of -2 dB, as 0 db represents a detectable point (decibel is a log value).

*Statistical tests*

Data used for statistical analyses were checked for normality using the Anderson-Darling test and were normally distributed with regard to microperimetry change in treated eyes (p=0.53), change in control eyes (p=0.52) and log dose of vector per $mm^2$ retina (p=0.75). For microperimetry means, 95% confidence intervals were calculated using the CONFIDENCE function with alpha set to 0.05 in Microsoft Excel 2007. Mean values are shown ± the standard error of the mean (SEM), unless stated otherwise. In each patient, the number of test points (n) used to generate the mean differed between treated and control eyes, but remained constant with respect to each eye. A paired t-test was used for comparisons of retinal thickness in the same eye before and after surgery using the TTEST function. For the statistical analysis of dose-effect, the change in microperimetry (dB) was plotted against the log dose of vector administered per $mm^2$ of retina to generate the Pearson correlation co-efficient (r) using the PEARSON function. The p value of r was determined from the two-tailed t distribution generated with the following formula: $t = r\sqrt{[(n-2)/(1-r^2)]}$, with n=6 and n-2 degrees of freedom.

*Optical coherence tomography (OCT) and autofluorescence imaging*

Optical coherence tomography (OCT) and fundus autofluorescence imaging was performed on a Heidelberg Spectralis (HRA3) according to the manufacturer's protocols. OCT measurements of outer retinal thickness were taken vertically between the outer limit of the outer plexiform layer and the sharp contour of Bruch's membrane. These boundaries were chosen over total retinal thickness in order to exclude inner retinal changes in the measurements and thereby concentrate on detecting any outer nuclear layer thinning changes, which would be the layer most likely to show cell loss resulting from retinal detachment. Furthermore these anatomical parameters define the standard central retinal thickness (CRT) and central point thickness (CPT) measurements for this OCT machine, thereby allowing comparisons of our dataset to the published data on foveal thickness measurements, including the known standard deviations for repeated measurements.[8,9] For assessing areas of autofluorescence, images were imported into Adobe Photoshop (Version 12, Adobe Systems Inc, San Jose, CA) and adjusted to similar levels of contrast. Only autofluorescent areas exposed to vector were included to generate an automated pixel count using the Histogram function at 400% zoom, which was then converted into area measurements in $cm^2$ using the imported scale bar. Standard operating procedures (SOPs) were established for all tests in order to harmonise data collected across different sites.

*Immunology tests*

Blood from participants was collected in heparinised vacutainer tubes (BD, Oxford, UK). The peripheral blood mononuclear cell (PBMC) fraction was isolated using Leucosep tubes (Greiner Bio-One Ltd,

Stonehouse, Gloucestershire, UK) pre-filled with Ficoll Paque PLUS (GE Healthcare, Buckinghamshire, UK) according to manufacturer's instructions and without delay. Cells were manually counted and promptly cryopreserved in FBS (Life Technologies Ltd, Paisley, Renfrewshire, UK) with 10% DMSO (Sigma-Aldrich Company Ltd, Dorset, UK) at a concentration of $5x10^6$ cells/mL. Cells were cooled at a controlled rate (StrataCooler Cryo Presevation Module, Agilent Technologies UK Limited, Stockport, Cheshire, UK) to -80°C and then transferred to vapour phase liquid nitrogen storage until use. *Ex vivo* cell mediated immune responses against AAV2 capsids and overlapping 15-mer peptides of the REP1 protein were assessed using an Interferon-gamma (IFN-γ) ELISpot assay (HRP Pro-Kit, Mabtech AB, Stockholm, Sweden). The IFN-γ ELISPOT assays were performed according to the manufacturer's instructions and following previously described protocols.[10,11,12]

On the day of testing PBMCs were thawed using RPMI-1640 media (Sigma-Aldrich) supplemented with antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin, Sigma-Aldrich), L-glutamine solution (final concentration 2 mM, Sigma-Aldrich), 20% FBS (Life Technologies) and 50 U/mL Benzonase nuclease (Novagen by Merck Chemicals Ltd, Nottingham, UK). Cells were washed once and re-suspended with a similar media (without Benzonase nuclease and only 10% FBS). Cells were counted and viability assessed using Trypan blue solution (Sigma-Aldrich). Viability after thawing was always over 80%. Cells were rested for 90 min in a humidified 37°C 5% $CO_2$ incubator before testing.

To test cellular responses to AAV2 particles, patient-specific vector (excess therapeutic vector collected from each surgery and stored at -80°C) was thawed on the day of the test and $10^8$ intact capsids added to each test well (calculated based on genome particle titre). To investigate responses to the therapeutic transgene, a 15-mer peptide library, representing sequential regions of the REP1 protein (with 12 peptide overlap) was synthesised by Mimotopes (UK) Ltd (Heswall, Wirral, UK). Peptide sets were handled and solubilised as per the manufacturer's instructions and the 214 individual peptides pooled into 3 sequential samples for testing (mode number of 72 peptides per pool). Each pool was tested on cells at a final concentration of 2 µg/mL of each peptide. Positive controls consisted of a monoclonal antibody to human CD3 (Mabtech, final concentration 100 ng/mL) and Phytohemagglutinin-M (PHA-M) from *Phaseolus vulgaris* (Sigma-Aldrich, final concentration 2 µg/mL). The CEF peptide pool (Mabtech) consists of 23 MHC class I-restricted T-cell epitopes from human CMV, EBV and influenza (flu) virus (CEF), designed to stimulate T cells from donors with a variety of HLA types and induce IFN-γ responses in almost 90% of all Caucasians. This was used as an antigen-specific control for CD8+ T cell responses at final concentration of 2 µg/mL of each peptide. Negative control wells contained medium without any antigen/mitogen/stimulant. Cell-free wells containing antigen/mitogen/stimulant were used to test for reagent contamination/non-specific spot formation. Each condition was tested in triplicate.

Cells from each participant that were collected pre-operatively and cells from 1-2 weeks and 1 month post-surgery were always tested on the same ELISpot plate. For AAV2, REP1 peptide pools, CEF peptide pool and media only controls, $2 \times 10^5$ PBMCs were added to each well. For the potent control activators (CD3 mAb and PHA-M), $1 \times 10^5$ PBMCs were added to each well to reduce confluent spot formation. Assembled ELISpot plates were placed in a humidified 37°C 5% $CO_2$ incubator for at least 22 h. Detection and development of spots was then performed using a HRP conjugated detection antibody and TMB substrate as per the ELISpot kit manufacturer's instructions (Mabtech). Spots were counted with an AID ELISpot reader (Autoimmun Diagnostika GmbH, Strassberg, Germany) and counts per well converted into spot forming units (SFU)/$10^6$ PBMCs.

**Supplementary Results**

*Surgery*

The retina detached cleanly over fluorescent areas but became increasingly difficult to detach beyond the border. The retina took several attempts to detach in Patients 1, 2, 4 and 6 whereas the retina detached on first attempt in Patients 3 and 5. In P4 the initial injection of saline created a detachment of the choroid, which due to thinning was extremely difficult to visualise and separate from the overlying thin retina. Eventually after several further attempts a subretinal cleavage plane was identified and the fovea was detached successfully. The surgery for P6 was similar to P4, except the final successful subretinal injection created visible stretching of the retina nasal to the fovea. There were concerns intra-operatively about possible damage to the papillomacular bundle from retinal stretch and a smaller volume of vector (up to 0.06 ml) was injected through the retinotomy, which resulted in a lower dose (max 6 x $10^9$ gp) in this patient. There were no retinal breaks and 23 gauge sclerostomy sites were sutured with 8/0 vicryl to prevent post-operative hypotony which might affect vector uptake from the subretinal space due to effects on choroidal fluid dynamics. In conclusion, surgery in all six patients was complex but considered to be within the predicted variations of the technique and there were no Protocol violations or serious adverse events to report.

*Vision changes*

The two patients with reduced visual acuity at baseline showed notable visual acuity gains and there was a mean improvement in maximal retinal sensitivity in the treated eyes across the whole group (see main text). Contrast sensitivity was measurably reduced at baseline only in Patient 1, in whom it increased from 0·75 to 1·05 log units in the treated eye and decreased from 0·90 to 0·75 log units in the untreated eye over the six month period (0·15 log unit = 3 letters). Hence the contrast sensitivity between his two eyes reversed, such that the treated eye became more sensitive than the control eye. In the other five patients, contrast sensitivity was 1·35 or higher at baseline and did not change by more than one letter at follow up. The following tables show the vision changes for each patient with regard to ETDRS and microperimetry data. Additional data is presented to illustrate the reduced retinal function in a young patient with choroideremia before the onset of degeneration (Figure S4) and in an unaffected adult male patient who had a short period of macula off retinal detachment six months after recovery (Figure S5).

*Table 3 – ETDRS vision changes (letters)*

| | P1 | | P2 | | P3 | | P4 | | P5 | | P6 | | MEAN Change |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 m | 6 m | 0 m | 6 m | 0 m | 6 m | 0 m | 6 m | 0 m | 6 m | 0 m | 6 m | |
| Treated eye letters | 23 | 44 | 79 | 78 | 89 | 86 | 53 | 64 | 79 | 77 | 88 | 85 | + 3.8 |
| Control eye letters | 58 | 69 | 82 | 79 | 85 | 89 | 76 | 75 | 83 | 83 | 84 | 82 | + 1.5 |

There was a mean improvement in visual acuity of 4 ETDRS letters (5 letters = 1 line) in eyes undergoing foveal detachment and vector administration but this masked two trends, with gains of two or more

lines seen in the two patients with vision worse than 6/9 at baseline (P1 and P4) and small losses of 1-4 letters in the others who read 6/9 or better before surgery.

*Table 4 – Changes in maximal sensitivity (dB)*

| | P1 | | P2 | | P3 | | P4 | | P5 | | P6 | | MEAN Change |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 m | 6 m | 0 m | 6 m | 0 m | 6 m | 0 m | 6 m | 0 m | 6 m | 0 m | 6 m | |
| Treated eye | 23 | **28** | 25 | 27 | 24 | **28** | 23 | 23 | 18 | 20 | 25 | 26 | + 2.3±0.8 |
| Control eye | 25 | 18 | 25 | 25 | 22 | 26 | 23 | 21 | 22 | 22 | 27 | 27 | - 0.8±1.5 |
| Treated change | **+ 5** | | **+ 2** | | **+ 4** | | **+ 0** | | **+ 2** | | **+ 1** | | **+ 2.3** |

Central serous retinopathy (CSR) is a condition that has similarities to the gene therapy treatment in that the fovea undergoes a limited period of detachment from the underlying RPE. In CSR reduction in retinal sensitivity can be detected in anatomically normal retina one year after resolution.[13] Hence in the absence of any vector effects and notwithstanding any effect of the underlying retinal degeneration, a reduction in the maximal sensitivity might be expected six months after foveal detachment. In contrast however the most sensitive point had improved in the treated eyes across the group by a mean of 2.3 dB (95% CI 0.8-3.8 dB). The points with maximal sensitivity (28 dB - within the normal population range for this machine) were only seen in post-treatment eyes (grey boxes). This is consistent with improved rod function as the maximal value represents the lowest light intensity that can be detected after 20 minutes of dark adaptation.

*Table 5 –Mean microperimetry changes (dB)*

| | P1 | | P2 | | P3 | | P4 | | P5 | | P6 | | MEAN Change |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 m | 6 m | 0 m | 6 m | 0 m | 6 m | 0 m | 6 m | 0 m | 6 m | 0 m | 6 m | |
| Treated eye | 6.4 | 10.8 | 11.4 | 12.1 | 11.2 | 13.1 | 10.0 | 14.5 | 9.0 | 10.1 | 13.0 | 10.7 | + 1.7±1.0 |
| Control eye | 8.0 | 5.8 | 12.9 | 9.8 | 10.0 | 12.1 | 10.4 | 6.3 | 12.3 | 11.3 | 16.1 | 15.0 | - 1.6±0.9 |
| Relative change | **+ 6.7** | | **+ 3.7** | | **- 0.2** | | **+ 8.6** | | **+ 2.2** | | **- 1.1** | | **+ 3.3** |

In contrast to measurements of maximal sensitivity, which identify the dimmest detectable stimulus and most likely rod function, mean microperimetry measurements arise from all seeing points in the test area and will include contributions from cones for the brightest stimuli. When comparing the mean microperimetry changes it is also important to consider that the doses of vector differ and the target areas vary in size considerably across this patient group. Hence the gain in the treated eyes and was also plotted against the vector dose per $mm^2$ of retina administered to each eye (see Figure 4C main text). Nevertheless the relative change in mean sensitivity between the two eyes provides a snapshot of outcomes, including unexpected surgical complications and variable presentations in the first six patients. The relative gains are greatest in P1 (6.7 dB) and P4 (8.6 dB), consistent with the visual acuity

improvements in these patients. Across the whole group there was a mean improvement of 3.3 db relative to the control eye (95% CI 0.2-6.3 dB).

*Table 6 – Changes in area of visual field (number of test points seen)*

| | P1 | | P2 | | P3 | | P4 | | P5 | | P6 | | MEAN Change |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 m | 6 m | 0 m | 6 m | 0 m | 6 m | 0 m | 6 m | 0 m | 6 m | 0 m | 6 m | |
| Treated | 26 | 28 | 27 | 27 | 33 | 42 | 13 | 15 | 15 | 19 | 41 | 33 | + 1.5±2.3 |
| Control | 21 | 19 | 28 | 25 | 39 | 43 | 18 | 12 | 16 | 17 | 36 | 34 | - 1.3±1.4 |
| Relative change | **+ 4** | | **+ 3** | | **+ 5** | | **+ 8** | | **+ 3** | | **- 6** | | **+ 2.8** |

The visual field changes represent individual points changing in sensitivity to detection of the brightest stimulus (0 dB) before and six months after surgery. The visual field enlarged by a mean of 1.5±2.3 points in the treated eyes compared to a reduction of -1.3±1.4 points in control eyes six months after surgery (95% CI of difference, -0.9 to 6.6 points). P6 who had a reduced vector dose due to retinal stretch during surgery is an outlier1 in this series and is the only one to have a reduced visual field in his treated eye.

*Table 7 –Microperimetry in a non-study control group (dB)*

| | C1 | | C2 | | C3 | | MEAN values | Study group eyes |
|---|---|---|---|---|---|---|---|---|
| | 0 m | 11 m | 0 m | 8 m | 0 m | 5 m | | |
| Mean sensitivity | 12.7 | 12.2 | 9.6 | 8.4 | 15.7 | 13.7 | 12.6 | 10.9 |
| Change over 6 m | - 0.23 | | - 0.88 | | - 2.33 | | - 1.15 | - 1.59 |

In order to investigate the possibility of any selection bias for the patients in the study with regard to baseline levels of retinal sensitivity, readings were also obtained from choroideremia patients who were not in the clinical trial. In total three patients who were in the age range of the trial participants (37, 42 and 57 years) and who had undergone baseline and repeat testing of both eyes similar to the protocol within a 12 month (m) period could be identified. The mean sensitivity in these three non-study patients (12.6±1.2 dB) was similar to the study group eyes at baseline (10.9±1.0 dB) and both were less than the general population of unaffected patients in the fifth decade tested with the standard grid (29.9±1.3 dB).[7] The three non-study choroideremia patients showed a small decline in sensitivity during this period (as expected due to the effects of the degeneration). This decrease over time was assumed to be linear and was normalised to account for the time between tests to estimate the change over 6 m. The mean change (- 1.15 dB) was similar to the study group unoperated eyes over the same period (- 1.59 dB).

*Changes in retinal structure*

Sequential optical coherence tomography (OCT) scans were taken through the fovea or thickest outer nuclear layer (ONL) region where the fovea had degenerated (Figure S2). Measurements were made

from the inner plexiform layer (IPL) / ONL junction to Bruch's membrane, both of which could be identified in degenerate areas. These parameters also allowed comparison to the published data on central retinal thickness (CRT) and test-retest variability form normal subjects measured with this particular machine (289±16 µm, n=20).[8] The mean ONL thickness at the most sensitive point was 175±27 µm before surgery and had reduced to 169±26 µm by six months, although this was not statistically significant (paired t-test, p=0.21). The numerically greatest change (20 µm, 8% reduction) was seen in Patient 3. Morphologically and accounting for test-retest variability (SD = ±16 µm) [8], the retina appeared similar six months after gene therapy in 5 of the 6 patients. Specifically there was no persistent fluid, macular edema or epiretinal membrane formation. Some inner retinal cystic changes could be seen in the overlying neurosensory retina in areas of pre-existing RPE atrophy, but no degenerative changes were noted in any regions corresponding to functional retina. In P6 however the region of retina nasal to the fovea which had been noted to stretch during subretinal injection appeared noticeably thinner at six months, but due to loss of non-functional outer retinal tissue. Subjectively this patient was unaware of any reduction in his vision and the thickness of the ONL in his region of fixation remained similar (Figure S2).

*Immune responses*

No immunological responses were seen in the first five patients with Interferon-gamma (IFN-γ) enzyme-linked immunosorbent spot (ELISPOT) testing to the REP1 peptide pools or AAV2 capsid protein. A borderline increase in T-cell activity was noted to REP1 in P6 compared to the control, but the control response was very poor in this patient at baseline and overall the activity was low (Figure S3).

*Subjective changes in vision*

Subjectively Patient 1 describes 'substantial improvements' in the vision in his treated eye since the operation. He reports some aspects of his vision are now clearer in the treated eye than in the control eye which has a better visual acuity. Patient 2 describes seeing blue colours more clearly in his treated eye, but no acuity change. Patient 3 reports colours as being 'more intense' in the treated eye, but no acuity change. Patient 4 describes improvements in acuity when playing tennis and when reading, to the point where he now benefits again from a prescription near addition lens to his spectacles. Patients 5 and 6 are not aware of any subjective changes from baseline. No patients reported any serious adverse events but Patient 2 has a posterior subcapsular cataract developing that may require surgery in future.

*Validation of visual acuity data*

Both patients P1 and P4 had visual acuity gains in their injected eyes of over 4 and 2 ETDRS lines, respectively. This degree of improvement would be highly unusual in a patient with choroideremia in the absence of any reversible pathology, such as cataract or macular edema and extremely unlikely in any patient with a retinal degeneration undergoing surgery to induce foveal detachment. An alternate explanation, however, might be that the lower baseline measurement was an artefact, perhaps due to patient compliance. After the six month follow up and with the permission of these patients, we therefore contacted their home optometrists to request their historical visual acuity records.

P1 could not see any test letters of the eye chart at 6 m in his left eye in 2009 and on retesting in 2011 prior to enrolment, but had read 6/15 letters in his unoperated eye before the trial started (data provided by Jan Juul of Juul & Payne Opticians, 70 Alma Road, Clifton, Bristol UK BS8 2DJ). His recent visual acuity in the operated eye measured by his optician had increased to 6/36 and he was issued with a contact lens in this eye for the first time in many years. These results are entirely consistent with the study findings. Similarly, for P4 the best corrected visual acuity readings in the treated eye were as follows: 6/9 (1999), 6/36 (2009), 6/36+1 (2010), 6/36+1 (2011). In 2012 and unknown to the study team he returned to his local optician eight weeks after undergoing gene therapy for his regular check up (Figure S6). The optician recorded his vision at 6/18+1 and this was doubled checked as it was noted to be inconsistent with his visual acuity record (data provided by Derek Dorman of Ultimate Eyewear, 25a High Street, Chesham, UK HP5 1BG). These vision tests are entirely consistent with the ETDRS readings obtained using the clinical trial protocol and were made years before the patients would have known about the gene therapy programme. The independent tests support the accuracy of the visual acuity measurements in these patients and therefore provide additional validation of a real gain resulting from gene therapy, which is consistent with their improvement in microperimetry and subjective experiences.

*Safety reporting*

Adverse events reported included those related to eye surgery in general and applied to all patients (red eye, blurred vision, ocular discomfort) but none more than would be expected for a standard vitrectomy procedure. Adverse events specifically related to retinal detachment included mild distortion of central vision (metamorphopsia) which had resolved by 6 months (P3 and P5). P1 and P4 had no symptoms of metamorphopsia whereas P2 and P6 had mild metamorphopsia before surgery as expected due to splitting of the fovea by the degeneration and this had returned to baseline by six months. All patients had some sub-clinical progression of lens opacity visible on the slit lamp by six months and the cataract in P2 had progressed the most. Two subjects reported increased appetite due to the perioperative steroids. Two patients (P2 and P3) described ‘more vivid colours’ in their treated eyes by six months and we are currently investigating if this is an adverse event or whether it simply reflects a treatment effect due to improved cone function. There were no serious adverse events reported.

**Supplementary Figures**

*Figure S1 - Device for administering vector*

The 41G Dorc needle tip (arrow) attached to 1 ml BD syringe facilitates entry into the subretinal space with minimal trauma to the overlying retina. The injection device included 0.001% PF68 surfactant to prevent vector particles sticking to plastic surfaces inside the syringe and was calibrated prior to use.

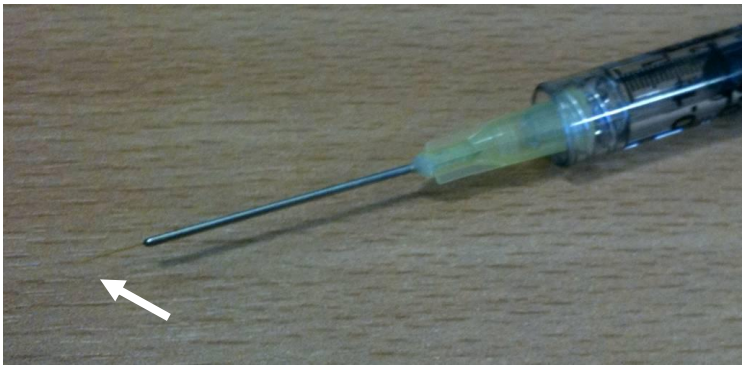


*Figure S2 – Retinal structural changes at baseline and six months after gene therapy*

Anatomical changes in the outer retina as measured by optical coherence tomography (OCT) in patients 1-6 at baseline (A, C, E, G, I, K) and six months after surgery (B, D, F, H, J, L). There was a trend towards thinning, with a mean outer retinal thickness of 175±27 µm at baseline, falling to 169±26 µm (3.4% loss) at 6 months. The contour of the nasal para-foveal tissue in P6 that was noted to undergo stretching during surgery showed the most significant change, with thinning of the outer retina visible at six months compared to baseline (K: arrow). This was however in a non-seeing area beyond the limits of the RPE and outside the region of outer retina used for fixation. There was no subjective change in this patient's vision and his ETDRS score returned to only two letters less than his control eye six months after retinal re-attachment.

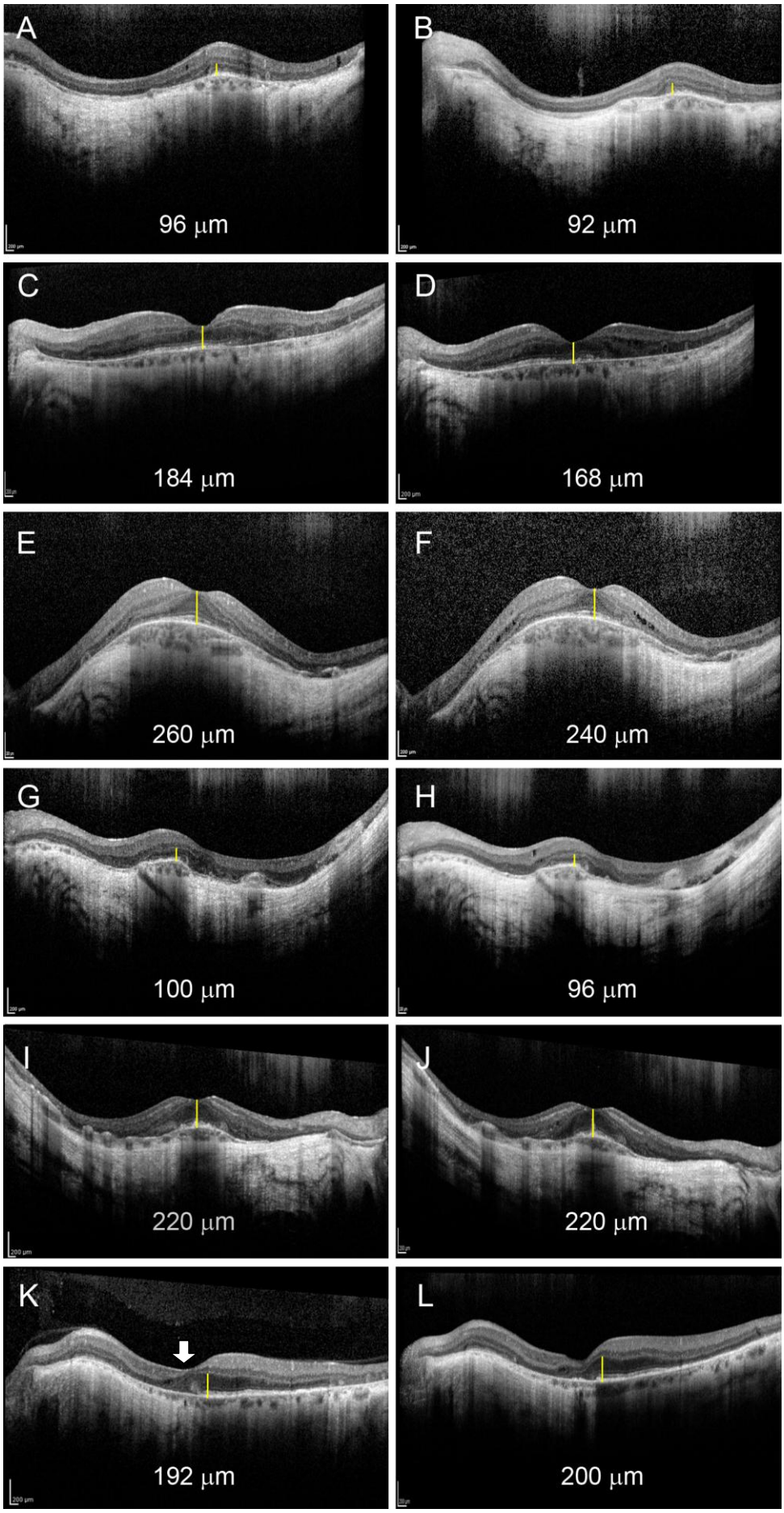

A
96 μm
B
92 μm
C
184 μm
D
168 μm
E
260 μm
F
240 μm
G
100 μm
H
96 μm
I
220 μm
J
220 μm
K
192 μm
L
200 μm

*Figure S3 – Enzyme-linked immunosorbent spot (ELISpot) assays*

ELISpot data presented as spot forming units (SFU) per $10^6$ peripheral blood mononuclear cells (PBMC) at baseline, one week after surgery and one month (range 5-6 weeks) after surgery. Peptide pools include 15-mer overlapping REP1 peptides. AAV2 capsids were derived from the vector suspension used. In patients P1-5 there is no significant reaction compared to negative control (Media) and positive control (Human CMV, EBV and influenza virus - CEF). P6 shows a mild elevation in PBMC activity compared to CEF at one month, but the baseline data show the CEF response is lower than the peptide pools before gene therapy. Hence this may just represent a generally low T-cell response in this patient. A representative series of test plates (from P4) is shown below, together with other positive controls to phytohemagglutinin (PHA) and anti-human CD3 monoclonal antibody (mAb CD3).

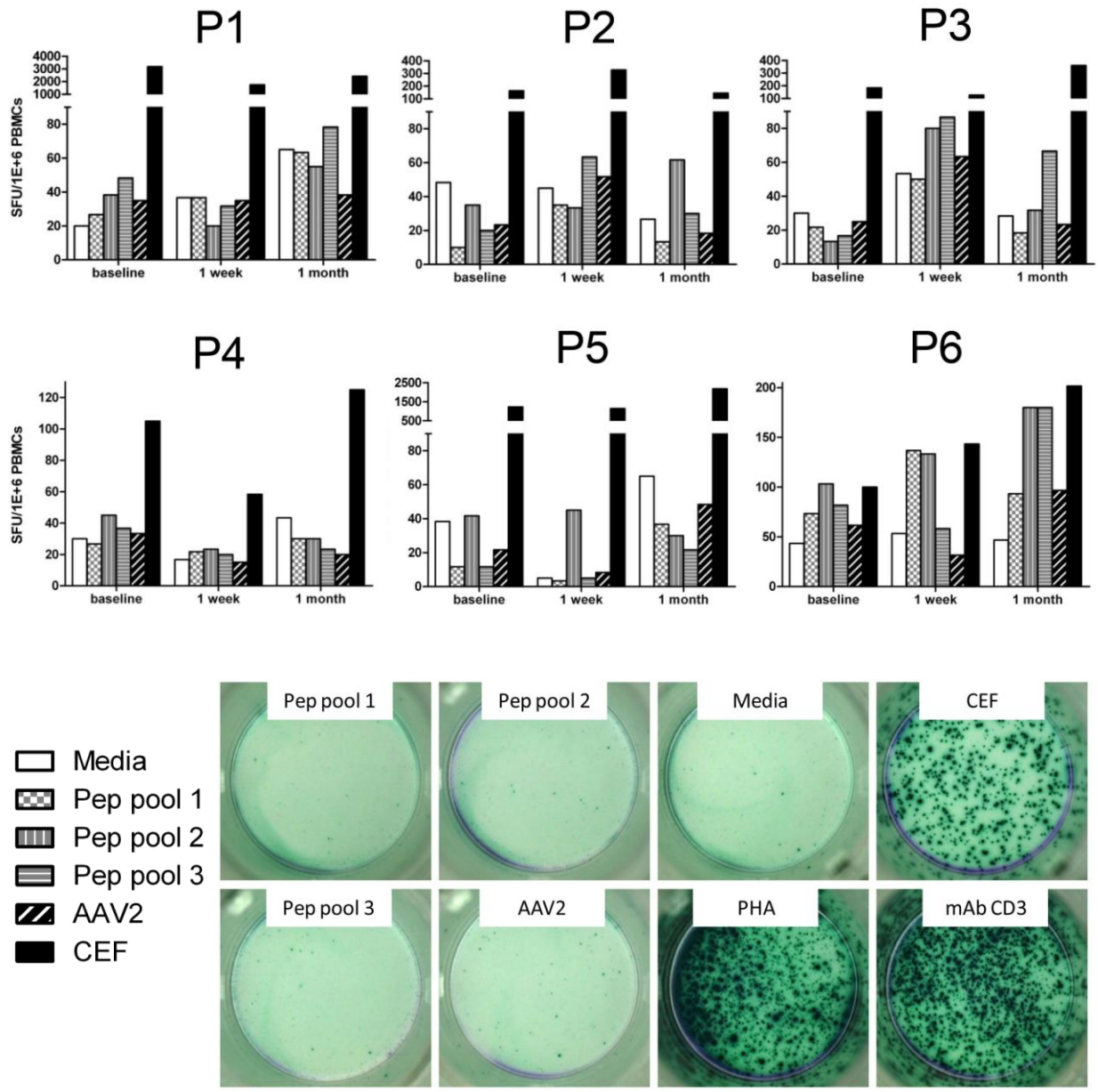


*Figure S4 – Microperimetry reveals functional loss in early choroideremia*

The retina in choroideremia undergoes a considerable amount of remodelling resulting in reduced retinal sensitivity by the second decade, long before the onset of any significant retinal thinning or visual acuity loss.[14] A clear functional defect early in the disease process has previously been described by measuring psychophysical thresholds at 1.7° diameter, 200-ms duration stimuli spaced at 2° intervals, which is similar to the testing strategy we employ in this study with the Maia. In that study,[14] defects in retinal function of up to 15 dB (approx 42-52 dB normal range) were detected in parafoveal locations in 10 of 12 patients below 40 years of age. Although a normative database of microperimetry in choroideremia patients is not yet available (and would most likely be highly variable), we were keen to see if our testing protocol could detect functional loss early in the disease process before anatomical degeneration, as it would provide the rationale for the functional improvement after gene therapy.

We therefore identified our youngest patient with genetically confirmed choroideremia who could perform reliable microperimetry plots and OCT scans (age 10). (A, B) Although he maintains 6/6 vision in both eyes, he has significant functional defects detected by microperimetry. His only symptom is poor night vision. (C, D) The normal distribution for his retinal sensitivity lies outside the population mean for his age group (30.5 ±1.6 dB, n=123 test subjects age under 30).[7] The mean values across the whole grid are 19 dB in the right eye and 18 dB in the left eye. The reduction in sensitivity is throughout the central macula and not just at the edges. (E, F) At this stage retinal structure is still relatively well preserved in the centre as seen on OCT. (G, H) To exclude the possibility of a poor test or poor compliance, microperimetry was repeated 9 months later. (I, J) This showed similar readings at 18.9 dB right eye and 19.2 dB left eye with uniform reduced sensitivity across the central macula. At this young age, there is very limited degeneration in the central macular area and so the decline in retinal sensitivity seen in the older patients would not be expected. At both time points his retinal sensitivity is approximately 6-7 standard deviations lower than the normative data for his age group. Hence the microperimetry protocol used in this study provides a reliable method for quantifying the reduced retinal function that exists before the onset of irreversible retinal cell loss.

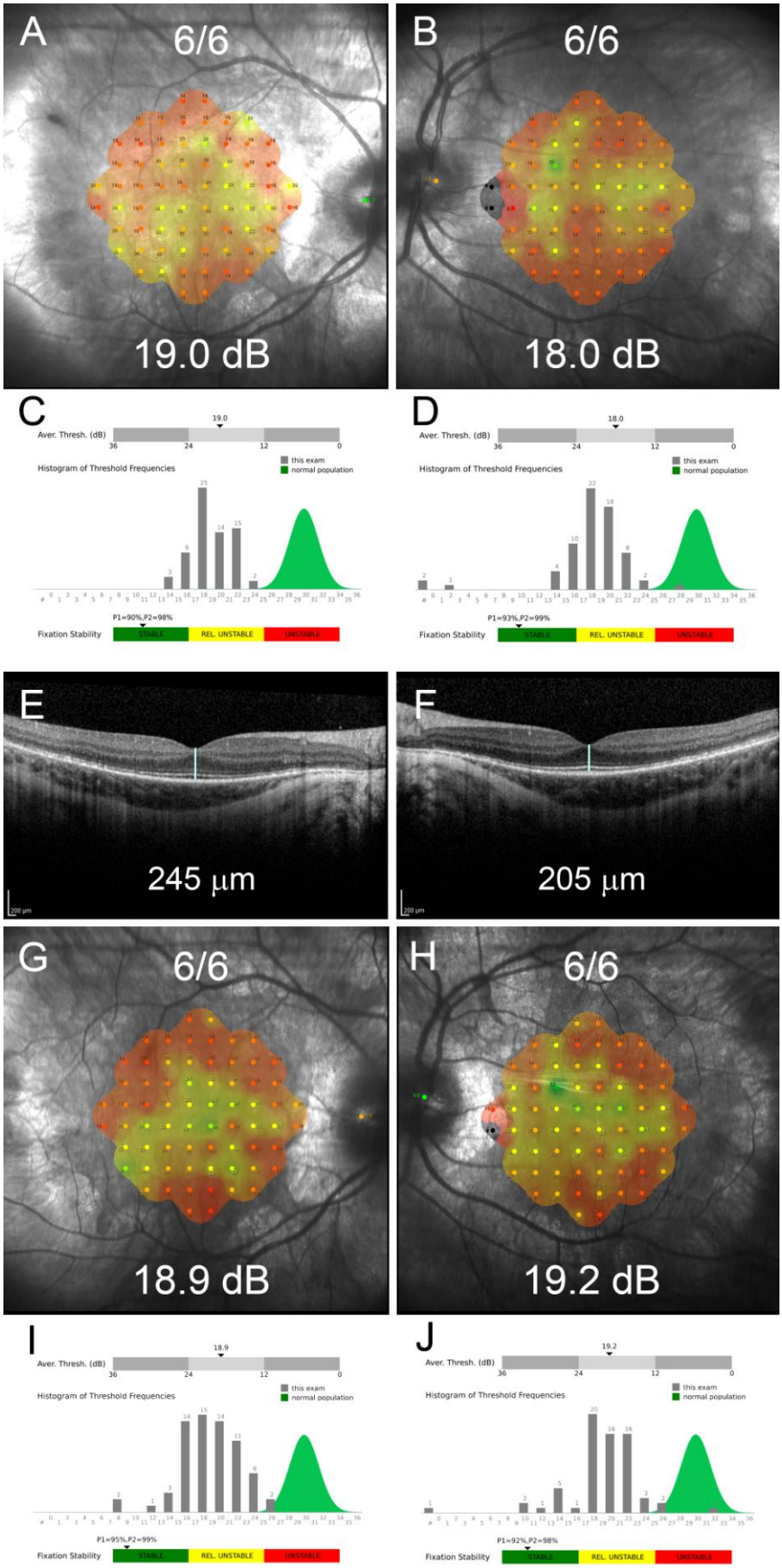
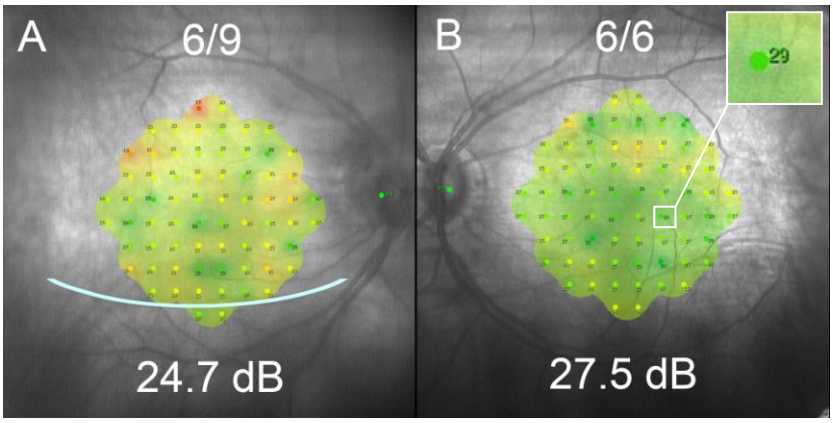
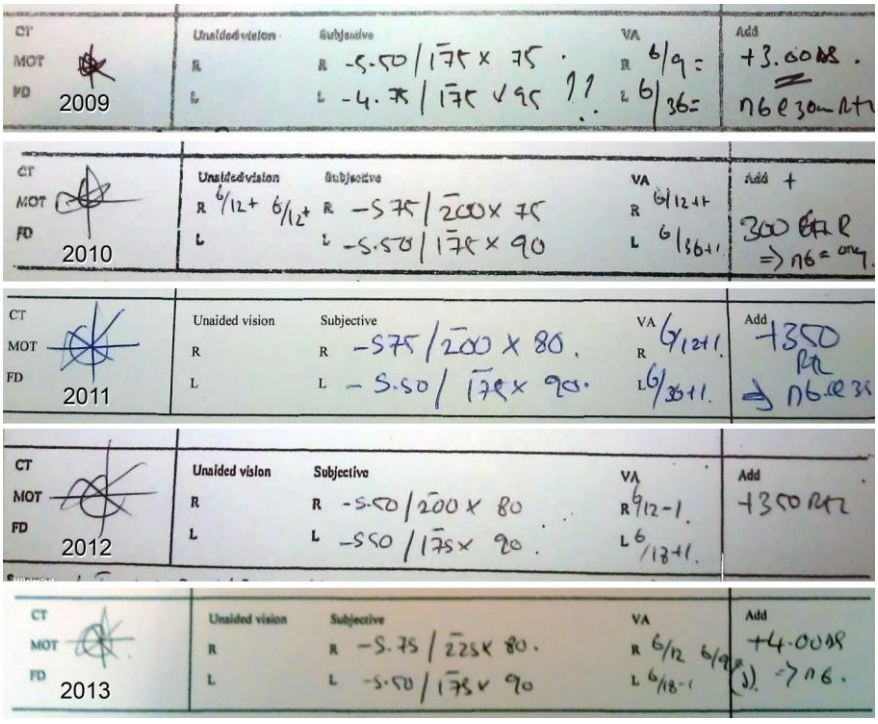

A
6/6
19.0 dB
B
6/6
18.0 dB
C
Aver. Thresh. (dB)
19.0
36
24
12
0
Histogram of Threshold Frequencies
this exam
normal population
P1=90%,P2=98%
Fixation Stability
STABLE
REL. UNSTABLE
UNSTABLE
D
Aver. Thresh. (dB)
18.0
36
24
12
0
Histogram of Threshold Frequencies
this exam
normal population
P1=93%,P2=99%
Fixation Stability
STABLE
REL. UNSTABLE
UNSTABLE
E
245 µm
F
205 µm
G
6/6
18.9 dB
H
6/6
19.2 dB
I
Aver. Thresh. (dB)
18.9
36
24
12
0
Histogram of Threshold Frequencies
this exam
normal population
P1=95%,P2=99%
Fixation Stability
STABLE
REL. UNSTABLE
UNSTABLE
J
Aver. Thresh. (dB)
19.2
36
24
12
0
Histogram of Threshold Frequencies
this exam
normal population
P1=92%,P2=98%
Fixation Stability
STABLE
REL. UNSTABLE
UNSTABLE

*Figure S5 – Microperimetry reveals functional loss from retinal detachment*



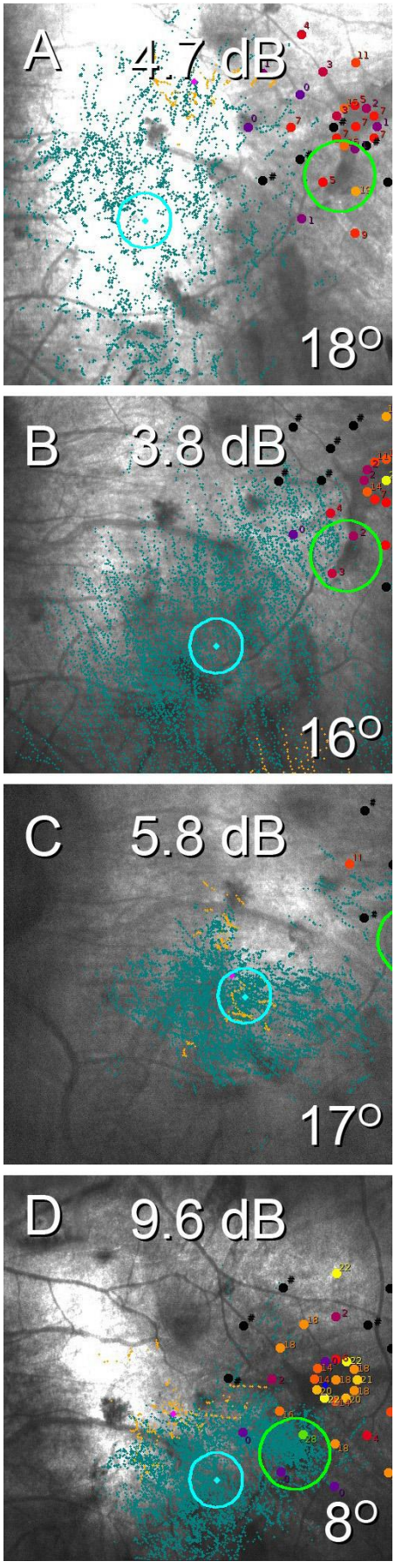

(A) Visual acuity and microperimetry readings taken from a 57 year old man 6 months after successful vitrectomy surgery for an acute macula off retinal detachment. The blue line shows the limit reached by subretinal fluid in his right eye as indicated by a 'tide mark' on the RPE. (B) The left eye is healthy and the point corresponding to the maximal sensitivity of P1is highlighted to illustrate that his sensitivity (28 dB) had almost reached the expected range at this location (29 dB) following gene therapy. Microperimetry was performed using the same settings as applied to this clinical trial. In order to align results more closely to the study group, only the central 16 spots are used for calculation of the mean values. In most people these values would be similar and if so in this case, would predict an approximate 2-3 dB mean reduction in retinal sensitivity caused by the subretinal injection. dB = decibels.

*Figure S6 – Independent validation of visual acuity gain in P4*




Unknown to the investigators and outside of the study protocol, P4 continued to visit his local optometrist for his annual check up before and after gene therapy. After noting the visual acuity gain at the six month follow up the patient mentioned that a similar gain had already been noted by his optician and with his permission, a copy of the historical records from the optician was requested. The readings for the left (treated) eye were consistently 6/36+1 between 2009 and 2011, but in 2012 had improved to 6/18+1, representing a two line gain. The reading taken in 2012 was only eight weeks after the gene therapy treatment, which is consistent with the time required for AAV transduction and recovery from retinal detachment. A recent 2013 test shows the left visual acuity gain maintained one year later. Photographs of clinical history from optometry clinic notes were provided by Derek Dorman of Ultimate Eyewear, 25a High Street, Chesham, UK HP5 1BG.

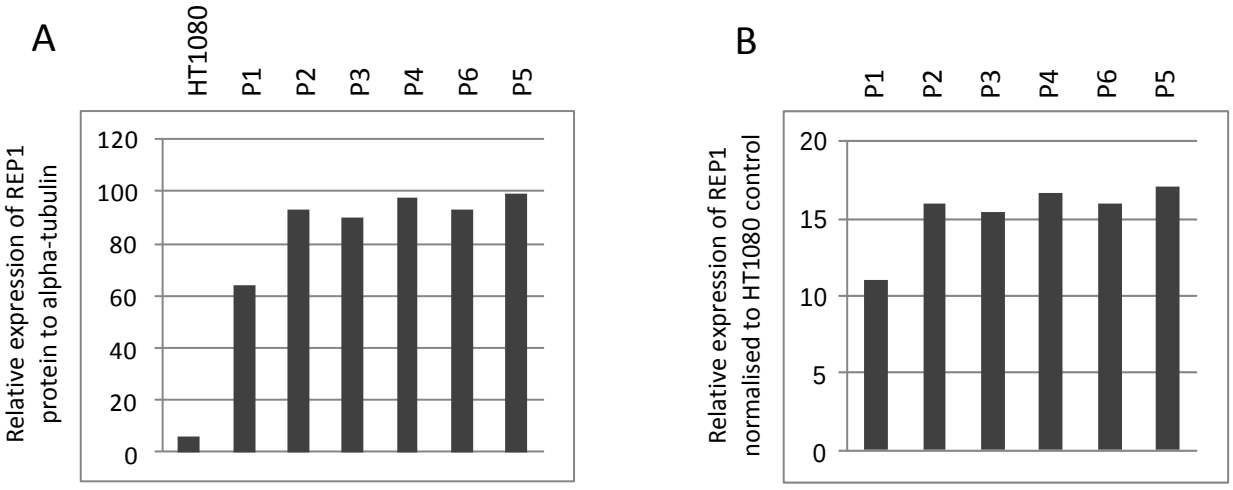
*Figure S7 – Analysis of fixation changes after gene therapy in P1*




Detailed analysis of fixation changes are shown in P1 during the three tests prior to surgery and compared to the follow-up at six months after gene therapy surgery. The cyan ring shows the mean centre of preferential retinal locus (PRL) and the green ring identifies the part of the retina closest to the PRL that was exposed to gene therapy. The angle separating the centre of these two points is shown in the lower right corner of each frame. (A) In the first 'familiarisation' test performed three months prior to surgery, the fixation in this patient is highly variable as evidenced by the scatter of blue dots. He can however see the test points in the eccentric area corresponding to his surviving retina with a mean sensitivity of 4.7 dB. (B) The second 'familiarisation' test performed one month prior to gene therapy shows a slight improvement in fixation stability, but at a similar locus in the non-seeing central area with 3.8 dB mean sensitivity. (C) The third test performed 1 week prior to gene therapy shows 5.8 dB mean sensitivity and 17 degree separation. This test was used as the baseline against which to assess the subsequent effects of retinal detachment and gene therapy. (D) Six months after gene therapy the mean sensitivity has increased to 9.6 dB and the patient is now using the eccentric target area at times for fixation. Although still variable, a second preferential retinal locus can be seen emerging within the retina exposed to gene therapy as evidenced by the cluster of blue dots which has appeared within this area (green ring). The overall mean position of fixation has shifted from 16-18 degrees to within 8 degrees of the area exposed to gene therapy. Note that although (C) and (D) represent the same tests used to generate the before and after retinal sensitivity data in the main text, the mean values shown here differ slightly. This is because customised maps were used to generate the study data for which points lying in areas of absolute scotoma were excluded from analysis. Here all points within the test circle are included and the mean values are therefore comparable across (A-D). As per the protocol, only the third test (C) was used as the baseline for subsequent follow up examinations in this patient. The Image in (A) is over-exposed because it was taken before the auto-exposure function was adjusted for choroideremia patients. All images have purple points at dB=0 as the maps were printed after the recent Maia software (version 1.7.0) upgrade – these points appear red in the other figures. Only the circular region of retina shown by the coloured dots was tested, which did not include the island of retina discussed in Figure 3.

*Figure S8 – Quantification of REP1 expression in HT1080 cells*

At the end of surgery, the syringe and diluted aliquots of vector were frozen and stored for subsequent analysis as a single batch in a Western blot on human HT1080 cells, as detailed above. In order to quantify REP1 expression from the vector relative to non-transduced HT1080 cells, the Western blot was exposed for a longer period (3 minutes) such that a band could be seen in the non-transduced HT1080 column. (A) Shows the overall levels of REP1 protein generated by each vector on the transduced HT1080 cells relative to alpha-tubulin. (B) Shows levels of REP1 protein relative to non-transduced HT1080 cells. The vector from all patients yielded at least a ten-fold increase in REP1 over endogenous protein in the HT1080 cells. There appears to be slightly less from P1, but this surplus vector had been stored and frozen for considerably longer than the other aliquots. The ratios in (B) confirm a similar range of potencies between the vectors in this in vitro assay (mean 15 times higher than baseline).



**Acknowledgements and funding statements**

This clinical trial was funded by the Health Innovation Challenge Fund (HICF-1009-006), a parallel funding partnership between the Wellcome Trust and the UK Department of Health. Additional funding was provided from the Health Foundation, Fight for Sight, the Lanvern Foundation, the Special Trustees of Moorfields Eye Hospital, the Royal College of Surgeons of Edinburgh, the Choroideremia Research Foundation and the NIHR Biomedical Research Centres at the Oxford University Hospitals and Moorfields Eye Hospital NHS Trusts.

We are grateful to the following who provided administrative support or advice at various stages of the trial: *Data Monitoring Committee*: Prof Alan Wright, Prof Bal Dhillon, Mr David Charteris; *Patient care and surgery*: Sr Alexina Fantato, Anna Rudenko, Sr Jeanette Mitchell, Dr Martin Zinkernagel; ELISPOT Immunology advice: Lili Wang, Dr Shisong Jiang; *Imaging and photography*: Lewis Smith, Dr Mandeep Singh, Prof Peter Charbel Issa; *Regulatory issues*: Dr Michelle McClements, Dr Marion Watson, Dr Eleanor Berrie, Gillian Traynor, Mike Breese, Dr Caroline Jenkins; *Background optometry data*: Mrs Jan Juul (Juul and Payne Opticians, Bristol), Mr Derek Dorman (Ultimate Eyewear, Chesham); *Statistical advice*: Prof Doug Altman (University of Oxford).

"This publication presents independent research commissioned by the Health Innovation Challenge Fund (HICF-1009-006), a parallel funding partnership between the Wellcome Trust and the Department of Health. The views expressed in this publication are those of the authors and not necessarily those of the Wellcome Trust or the Department of Health." – Health Innovation Challenge Fund.

"This research is supported by the National Institute for Health Research (NIHR) Oxford Biomedical Research Centre based at Oxford University Hospitals NHS Trust and University of Oxford and the Ophthalmology Biomedical Research Centre based at the UCL Institute of Ophthalmology and Moorfields Eye Hospital NHS Foundation Trust. The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health." - National Institute for Health Research.

REM, MG, CLC, ARB, TT, MS, ARW, MD, GCMB, KRC, FPMC, SMD, AJL contributed to data collection and analysis. REM, ARB, MG. SMD, GCMB, CLC, ARW, AJL contributed to study design. REM and MCS obtained funding. LS, KRC provided regulatory advice. REM performed surgery. REM, MG, ARB, TT, CLC, GCMB, AJL, FPMC, SMD, ARW, MD, MS contributed to writing the report.

**References**


1. Nathans J, Thomas D, Hogness DS. Molecular genetics of human color vision: the genes encoding blue, green, and red pigments. Science 1986 232:193-202.
2. Zolotukhin S, Byrne BJ, Mason E, et al. Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. Gene Ther 1999 6:973-985.
3. Clark KR, Liu X, McGrath, JP, Johnson PR. Highly purified recombinant adeno-associated virus vectors are biologically active and free of detectable wild-type viruses. Hum. Gene Ther 1999 10:1031-1039.
4. Bennicelli J, Wright JF, Komaromy A, et al. Reversal of blindness in animal models of leber congenital amaurosis using optimized AAV2-mediated gene transfer. Mol Ther 2008 16:458-65.
5. Anand V, Barral DC, Zeng Y, et al. Gene therapy for choroideremia: in vitro rescue mediated by recombinant adenovirus. Vision Res. 2003 43:919-26.
6. Smolek MK, Notaroberto NF, Fereday LE, Jaramillo A. Normative distribution indices for microperimetry analysis. IOVS 2012 53: ARVO Abtract 4367.
7. Smolek MK, Lebow K, Notaroberto N, Pallikaris A, Vujosevic S. Neural network algorithms for a device to measure macular visual sensitivity. IOVS 2010 51: ARVO Abstract 3550.
8 . Wolf-Schnurrbusch UE, Ceklic L, Brinkmann CK, et al. Macular thickness measurements in healthy eyes using six different optical coherence tomography instruments. Invest Ophthalmol Vis Sci. 2009 50:3432-7.
9. Grover S, Murthy RK, Brar VS, Chalam KV. Comparison of retinal thickness in normal eyes using Stratus and Spectralis optical coherence tomography. Invest Ophthalmol Vis Sci. 2010 51:2644-7.
10. Bainbridge JW, Smith AJ, Barker SS, et al. Effect of gene therapy on visual function in Leber's congenital amaurosis. N Eng J Med 2008 358:2231-9.
11. Maguire AM, Simonelli F, Pierce EA, et al. Safety and efficacy of gene transfer for Leber's congenital amaurosis. N Engl J Med 2008 358:2240–2248.
12. Hauswirth WW, Aleman TS, Kaushal S, et al. 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. Hum Gene Ther 2008 19: 979–990.
13. Ojima Y, Tsujikawa A, Hangai M, et al. Retinal sensitivity measured with the micro perimeter 1 after resolution of central serous chorioretinopathy. Am J Ophthalmol. 2008 146:77-84.
14. Jacobson SG, Cideciyan AV, Sumaroka A, et al. Remodeling of the human retina in choroideremia: rab escort protein 1 (REP-1) mutations. Invest Ophthalmol Vis Sci. 2006 47:4113-20.