Intravitreous injection of AAV2-sFLT01 in patients with advanced neovascular age-related macular degeneration: a phase 1, open-label trial

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Summary

Background **Long-term intraocular injections of vascular endothelial growth factor (VEGF)-neutralising proteins can preserve central vision in many patients with neovascular age-related macular degeneration. We tested the safety and tolerability of a single intravitreous injection of an AAV2 vector expressing the VEGF-neutralising protein sFLT01 in patients with advanced neovascular age-related macular degeneration.**

Methods **This was a phase 1, open-label, dose-escalating study done at four outpatient retina clinics in the USA. Patients were assigned to each cohort in order of enrolment, with the first three patients being assigned to and completing the first cohort before filling positions in the following treatment groups. Patients aged 50 years or older with neovascular age-related macular degeneration and a baseline best-corrected visual acuity score of 20/100 or less in the study eye were enrolled in four dose-ranging cohorts (cohort 1, 2×10^8 vector genomes (vg); cohort 2, 2×10^9 vg; cohort 3, 6×10^9 vg; and cohort 4, 2×10^{10} vg, n=3 per cohort) and one maximum tolerated dose cohort (cohort 5, 2×10^{10} vg, n=7) and followed up for 52 weeks. The primary objective of the study was to assess the safety and tolerability of a single intravitreous injection of AAV2-sFLT01, through the measurement of eye-related adverse events. This trial is registered with ClinicalTrials.gov, number NCT01024998.**

Findings **19 patients with advanced neovascular age-related macular degeneration were enrolled in the study between May 18, 2010, and July 14, 2014. All patients completed the 52-week trial period. Two patients in cohort 4 (2×10^{10} vg) experienced adverse events that were possibly study-drug related: pyrexia and intraocular inflammation that resolved with a topical steroid. Five of ten patients who received 2×10^{10} vg had aqueous humour concentrations of sFLT01 that peaked at 32·7–112·0 ng/mL (mean 73·7 ng/mL, SD 30·5) by week 26 with a slight decrease to a mean of 53·2 ng/mL at week 52 (SD 17·1). At baseline, four of these five patients were negative for anti-AAV2 serum antibodies and the fifth had a very low titre (1:100) of anti-AAV2 antibodies, whereas four of the five non-expressers of sFLT01 had titres of 1:400 or greater. In 11 of 19 patients with intraretinal or subretinal fluid at baseline judged to be reversible, six showed substantial fluid reduction and improvement in vision, whereas five showed no fluid reduction. One patient in cohort 5 showed a large decrease in vision between weeks 26 and 52 that was not thought to be vector-related.**

Interpretation **Intravitreous injection of AAV2-sFLT01 seemed to be safe and well tolerated at all doses. Additional studies are needed to identify sources of variability in expression and anti-permeability activity, including the potential effect of baseline anti-AAV2 serum antibodies.**

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Introduction

Age-related macular degeneration is a complex disease in which multiple gene defects and environmental exposures result in retinal degeneration and gradual loss of central vision. In a subgroup of patients with neovascular age-related macular degeneration, subretinal neovascularisation is superimposed, and these patients experience a rapid reduction in visual acuity due to leakage of plasma from incompetent new vessels, which compromises retinal function. Vision loss is reversible with timely fluid elimination, but permanent loss of central vision can occur from chronic persistent or recurrent fluid, subretinal fibrosis, or both.

Vascular endothelial growth factor (VEGF) plays a central role in the development of subretinal neovascularisation and excessive plasma leakage into and under the retina. Intraocular injections of VEGF-neutralising proteins reduce leakage, allowing fluid resorption and improvement in visual acuity;[1] however, repeated intraocular injections are needed in most patients. When patients with neovascular age-related macular degeneration who participated in clinical trials with monthly injections of a VEGF-neutralising protein were later enrolled in long-term studies with less frequent visits and injections, much of the visual gains obtained during intensive treatment were lost.[2] Clinical trials randomly assigning patients with neovascular age-related macular degeneration to monthly injections of an antibody that neutralises VEGF versus monthly visits with injections only when intraretinal or subretinal fluid was present showed only a small decrease in overall number of

Research in context

Evidence before this study

We searched PubMed for reports of all interventional clinical trials in neovascular age-related macular degeneration and all completed ocular gene therapy trials for any indication. ClinicalTrials.gov was searched for all ongoing ocular gene therapy trials. We searched for studies containing the search terms "neovascular AMD" and "clinical trials", "gene therapy" and "eye" and "clinical trial", or "ocular gene therapy" in English on June 15, 2009. Large multicentre trials have shown that intraocular injections of VEGF-neutralising proteins cause substantial visual improvement in patients with neovascular age-related macular degeneration, but often require frequent injections for the remainder of the patient's life to maintain benefits. Numerous studies in both mice and non-human primates relevant to neovascular age-related macular degeneration have shown that gene transfer of VEGF-neutralising proteins have provided measurable benefit. A clinical trial has shown that intravitreous injection of an adenoviral vector expression pigment epithelium-derived factor resulted in benefit for patients with neovascular age-related macular degeneration. This provided proof of concept for use of gene transfer to treat neovascular age-related macular degeneration, but also showed that a vector that provided long-term expression would be needed. Studies in animals and humans have shown that subretinal injection of AAV2 vectors is safe and well tolerated with long-term expression in retinal pigmented epithelium cells and photoreceptors. Finally, studies in animals have shown that intravitreous injection of AAV2-sFLT01 is well tolerated, transduces a subpopulation of ganglion and transitional epithelial cells in the pars plana, and provides long-term expression of sFLT01.

Added value of this study

We have shown the safety of intravitreous injection of AAV2-sFLT01. These data also support intravitreous injection of AAV2 vectors for delivery of other secreted transgenes. To our knowledge, this study provides the first direct measurement of transgene expression after injection of an AAV vector in human eyes. Additionally, we have shown that there is heterogeneity in expression of sFLT01 after intravitreous injection of AAV2-sFLT01 and that dose is one factor for consideration in future studies. We have shown that the pre-injection titre of anti-AAV2 serum antibodies might be a second factor influencing transgene expression after intravitreous injection of an AAV vector. In this study, an optimal outcome was achieved in some patients despite aqueous concentrations of sFLT01 being below the lower limit of quantification. We conclude that sFLT01 expression was sufficient in some patients, but not all, and additional studies are needed to explore ways to boost expression.

Implications of all the available evidence

Intravitreous injection of AAV2-sFLT01 is safe and results in good transgene expression in some patients and a good anti-permeability effect in some patients. Intravitreous injection of AAV2-sFLT01 might be promising new therapy for patients with chronic retinal or choroidal vascular diseases, but additional studies are needed to test higher doses of intravitreous AAV2-sFLT01 and elucidate the role of anti-AAV2 serum antibodies on expression.

injections in the second group, and mean improvements in visual acuity after 2 years of treatment were significantly better in patients receiving monthly injections.[3] These data suggest that sustained suppression of VEGF is likely to provide the best long-term outcomes in patients with neovascular age-related macular degeneration.

One strategy to provide long-term sustained suppression of VEGF is ocular gene transfer aimed at producing a VEGF-neutralising protein. This approach has been shown to provide good suppression of retinal or subretinal neovascularisation in rodent and macaque models of choroidal neovascularisation.[4–8] Adeno-associated viral (AAV) vectors are advantageous because they transduce non-dividing cells (including neurons), provide long-term protein expression, and induce minimal immune responses.[9] AAV2 vectors strongly transduce photoreceptors and retinal pigmented epithelial (RPE) cells when injected into the subretinal space, and transduce a subpopulation of ganglion cells and transitional epithelial cells of the pars plana when injected into the vitreous cavity.[10] In primates, the internal-limiting membrane provides a barrier to AAV penetration into the retina, but the internal-limiting membrane is thin in the region surrounding the fovea, overlying and adjacent to retinal blood vessels, and in the far periphery of the retina, and ganglion cells in these areas are transduced after intravitreous injection of AAV2 vectors.[11,12] Intravitreous injection of a construct with a ubiquitous promoter driving expression of a chimeric VEGF-neutralising protein consisting of domain 2 of Flt-1 (VEGF receptor 1) linked by a polyglycine 9-mer to human IgG1-Fc (sFLT01) packaged in AAV2 (AAV2-sFLT01) resulted in good expression in ganglion cells in mice and suppressed ischaemia-induced retinal neovascularisation.[13] 22 weeks after intravitreous injection of 2×10^{10} vector genomes (vg) of AAV2-sFLT01 in macaques, high concentrations of sFLT01 were found in the aqueous humour.[14] A long-term safety and pharmacokinetic study[15] in non-human primates showed mild to moderate vitreous cells and haze in several eyes injected with $2{\cdot}4\times10^{10}$ vg that lasted for several months; however, no histological or functional evidence of damage to the retina or other intraocular tissues was observed. Although aqueous humour concentrations of sFLT01 varied between eyes injected with $2{\cdot}4\times10^{10}$ vg, concentrations were above the limit of quantification (LOQ) in six of seven eyes, and these

concentrations were stable between 6 and 12 months ranging from about 60 to 200 ng/mL. After euthanasia at 12 months, vitreous humour concentrations of sFLT01 were measured in two monkeys and were five-to-ten times higher than those found in aqueous humour. The one monkey that had aqueous sFLT01 concentrations below the LOQ beyond month 3 was euthanised at month 15 and the vitreous concentration of sFLT01 was 100 ng/mL. The efficacy and safety of AAV-sFLT01 shown in preclinical models supported the initiation of clinical trials. Herein we report the results of a phase 1 dose-ranging study investigating the effects of intravitreous injection of AAV2-sFLT01 in patients with advanced neovascular age-related macular degeneration.

Methods

Study design and participants

This was a phase 1, open-label, safety and tolerability study in which 19 patients with advanced neovascular age-related macular degeneration received a single intravitreous injection of AAV2-sFLT01 at four outpatient retina clinics in the USA. There were four dose-ranging cohorts in which patients received a single injection (cohorts 1–4, n=3 per group) and one maximum tolerated dose (MTD) cohort (cohort 5, n=7). Patients in the first four cohorts had to have poor visual potential due to subretinal fibrosis, but this criteria was not required for cohort 5. The duration of the core study was 52 weeks, after which time patients were encouraged to enrol in an extended follow-up study.

The structure of AAV2-sFLT01 has been published[13] and is described in the appendix. Institutional Review Board approval was obtained at each site. Written informed consent was obtained from all participants and the study complied with the Declaration of Helsinki and the Health Insurance Portability and Accessibility Act.

Eligible patients were 50 years of age or older with neovascular age-related macular degeneration and best-corrected visual acuity (BCVA) of 20/100 or worse in the study eye and 20/400 or better in fellow eye; showed intraretinal or subretinal fluid in the macula on optical coherence tomography (OCT); had adequate pupillary dilation to permit thorough ocular examination and testing; and were able to understand and comply with the clinical protocol and provide written informed consent. Full inclusion and exclusion criteria are given in the appendix. In the dose-escalation cohorts, patients who were enrolled were required to have advanced neovascular age-related macular degeneration with subretinal fibrosis in the study eye. Patients were eligible for the MTD cohort (cohort 5) if they had shown responsiveness to anti-VEGF therapy (defined as reduction of intraretinal or subretinal fluid on OCT within 6 weeks of an intraocular injection of a VEGF-neutralising protein) during the 12 months preceding the study screening visit. The presence of subretinal fibrosis was not required.

Procedures

Patients were assigned to each cohort in sequential order of enrolment, with the first three patients being assigned to and completing the first cohort before filling positions in the second cohort and so on for the remaining three treatment groups.

Eligible patients used a broad spectrum topical antibiotic solution in the study eye for 3 days before the injection and then received a single intravitreal injection into the study eye on day 0 using standard antiseptic techniques and topical anaesthesia procedures. AAV2-sFLT01 (Sanofi Genzyme, Framingham, MA, USA) was administered by a single intravitreal injection at a fixed volume of 100 µL at a dose of 2×10^8 vg (cohort 1), 2×10^9 vg (cohort 2), 6×10^9 vg (cohort 3) or 2×10^{10} vg (cohorts 4 and 5). Briefly, a lid speculum was inserted and 4% proparacaine was applied. The conjunctiva was treated with 5% povidone iodine and a cotton tip soaked in 4% proparacaine was held on the injection site for 60 s. A 30-gauge needle was passed through the conjunctiva, sclera, and pars plana 4 mm posterior to the limbus and 100 µl of vector was injected into the vitreous cavity. The eye was washed with sterile saline to remove all povidone iodine and the fundus was examined to ensure good retinal perfusion. Intraocular pressure was measured 30 min after injection. Patients continued to use the same broad spectrum topical antibiotic solution in the study eye for 2 days after injection.

Viral vector concentrations were measured using a Taqman quantitative PCR assay (Thermofisher Scientific, Pleasanton, CA, USA).[15] We optimised extraction procedures for each of the matrices—whole blood, nasopharyngeal swabs, and urine. Aqueous humour was of limited volume so was prioritised for transgene expression and antibody testing; no vector shedding was assessed in this matrix. sFLT01 transgene concentrations were measured by ELISA using a method developed at Genzyme Sanofi.

See Online for appendix

Assessment of CST does not account for changes in subretinal neovascular tissue and therefore could underestimate vector-related effects. To address this underestimation, masked graders at the Digital Angiography Reading Center (DARC, New York, NY, USA) assessed central retinal lesion thickness (CRLT), the thickness of subretinal tissue and fluid, and the retina in the fovea for all OCT scans at baseline (day 0), day 7, and at weeks 2, 4, 8, 12, 18, 26, 38, and 52. The results were reported as percentage change from baseline CRLT. The timing of other study assessments and all protocol amendments are listed in the appendix.

Outcomes

The primary objective of this study was to assess the safety and tolerability of a single intravitreous injection of AAV2-sFLT01 in one eye of patients with neovascular age-related macular degeneration by assessing the incidence of adverse events with careful monitoring for ocular inflammation, change in intraocular pressure, and change

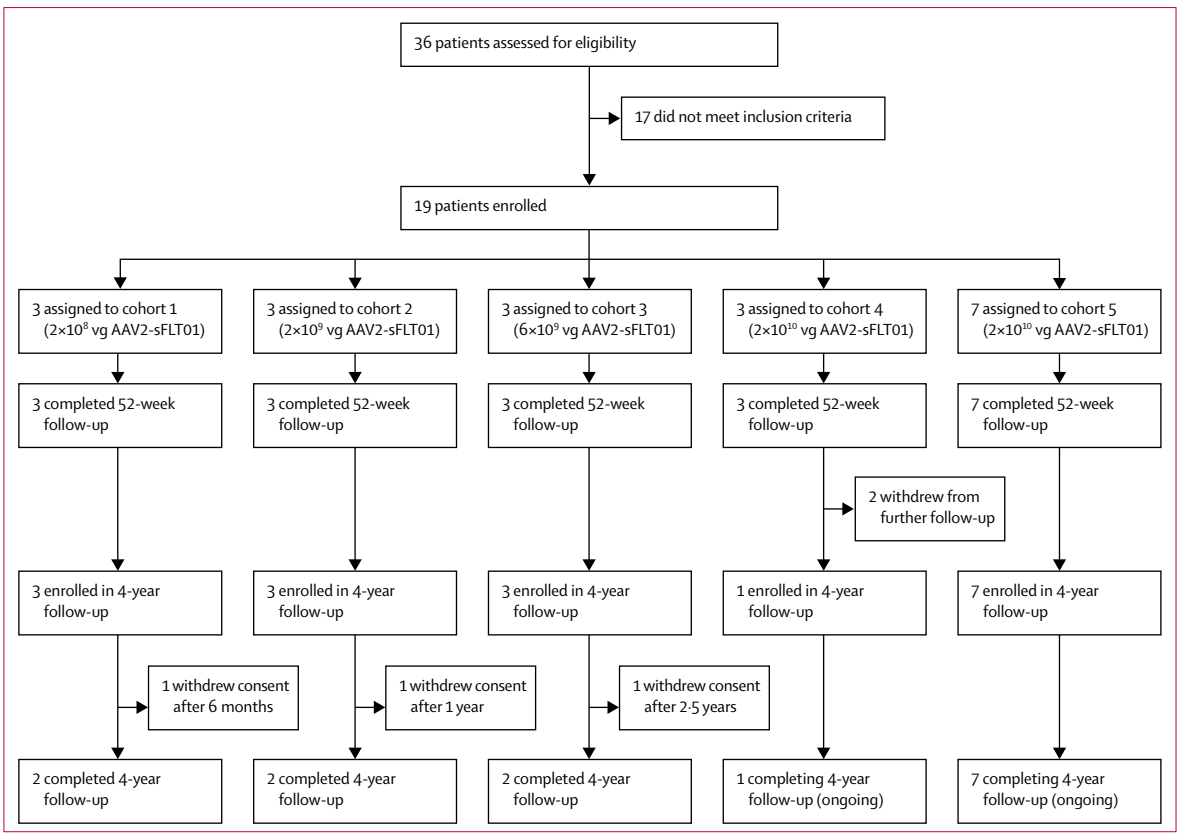

Figure 1: **Trial profile**

from baseline BCVA measured by the Early Treatment Diabetic Retinopathy Study protocol.[16] Concentrations of AAV2-sFLT01 vector DNA were measured in blood, nasopharynx, urine, and semen (in consenting male subjects only). Neutralising AAV2 antibody titres and sFLT01 antibody titres were measured at baseline, and several timepoints after injection (appendix). Secondary objectives were to assess transgene expression by measuring sFLT01 in aqueous fluid at several timepoints and to assess biological activity based on change from baseline BCVA and change from baseline central subfield thickness (CST) measured by OCT. Spectral domain-OCT (SD-OCT) was used in most patients but time domain OCT was used in four patients for whom SD-OCT was not available at the time of enrolment.

Statistical analysis

Given that this was a phase 1 study, no power calculation was done to determine cohort size, which instead was decided by safety considerations. We calculated frequencies of continuous and categorical variables using SPSS v.22.0 software (IBM Corp, Armonk, NY, USA). We did not do any statistical tests to assess differences between the cohorts. After the third patient enrolled in the first cohort had completed 8 weeks in the study, safety data were sent to data monitoring committee (DMC), which identified whether it was safe to proceed to the next cohort. This procedure was repeated for each subsequent cohort. No MTD was identified and therefore the highest dose was used for the seven patients enrolled in cohort 5. After the first three patients in cohort 5 had completed 4 weeks of follow-up, the DMC analysed safety data and decided if it was safe to proceed with the final four patients. After the last patient in cohort 5 completed 12-week assessments, safety data from all study patients were reviewed by the DMC. The DMC did an end-of-study analysis of all safety data after the last treated patient completed 52 weeks of study assessments. This study is registered with ClinicalTrials.gov, number NCT01024998.

Role of the funding source

Genzyme Corporation, A Sanofi Company (Cambridge, MA, USA) provided the funding for the study and, with input from the investigators, designed the trial and employed an independent clinical research organisation to monitor study sites, collect data, and maintain a secure database. JSH and PAC were responsible for the decision to submit the manuscript and had access to all data, helped to analyse and interpret data, and wrote the first draft of the manuscript. The manuscript was edited for factual accuracy by SHC, CD, AP, SR, AL-H, RV, RB, and AS and approved by the funder.

Results

A total of 36 patients were screened for eligibility, of whom 17 patients were ineligible for the following reasons: three patients did not meet the BCVA inclusion criteria of 20/100 or worse in the study eye, five patients did not have intraretinal or subretinal fluid at baseline, three patients had concomitant ocular pathology at baseline that could have affected the visual accuity or OCT findings, three patients had systemic diseases; one patient had an aflibercept injection in the proposed study eye within 4 months before the commencement of the study, one patient had a submacular haemorrhage between screening and baseline that made the patient ineligible for the study, and one patient who was screened for the dose-escalation phase had an absence of subretinal fibrosis at baseline. 19 patients with advanced neovascular age-related macular degeneration (53% men, mean age of 76·2 years, standard deviation [SD] 8·6) were enrolled in four dose-ranging cohorts (n=3 for each) and one MTD cohort (n=7; figure 1). The first patient was enrolled on May 18, 2010, and all 19 patients completed the 52-week core study by July 15, 2014. Mean BCVA at baseline was 25·5 Early Treatment Diabetic Retinopathy Study (ETDRS) letter score (20/320 Snellen equivalent) and seemed to be poorer in cohorts 1 and 4 than in the other cohorts (table 1). 17 patients enrolled in a 4-year extended follow-up study, but three subsequently withdrew consent with stated reasons failure to thrive, wish to enrol in another study, and too aged to travel. Reported here are the safety, anatomical, and visual outcomes of all patients during the 52-week core study, as well as all available safety data obtained during extended follow-up until Aug 13, 2014, the date of database lock. Duration of follow-up for each patient is shown in the appendix.

There were no dose-limiting toxic effects during the dose escalation phase and since no MTD was identified, the highest dose, 2×10^{10} vg, was used in MTD cohort resulting in a total of ten patients treated with 2×10^{10} vg (cohort 4 and cohort 5). Two patients who received 2×10^{10} vg experienced adverse events that were likely to be study-drug-related: pyrexia and intraocular inflammation. Pyrexia began 5 h after intravitreous injection of vector and resolved in 3 h. The second patient reported vitreous floaters after vector injection, which resolved in 2 days; 1 month later, this patient developed moderate intraocular inflammation consisting of iritis, vitritis, and keratic precipitates. Treatment with a topical steroid (difluprednate eye drops) was prescribed, and the inflammation resolved in 5 weeks with no recurrence after the steroid was stopped. One patient in cohort 5 had a large pigment epithelial detachment at baseline that resolved by week 18, after which time there was modest reduction in BCVA (appendix), followed by a large reduction from a letter score of 44 at week 38 to a letter score of 14 at week 52. There had been substantial progression of cataract during that time period, but after subsequent cataract surgery, BCVA only improved to a letter score of 27, suggesting that a decrease in retinal function also contributed to the reduced BCVA score. This reduction in BCVA was not attributed to AAV2-sFLT01 because resolution of pigment

	Cohort 1 2×10^{8} vg AAV2-sFLT01 (n=3)	Cohort 2 2×10^{9} vg AAV2-sFLT01 (n=3)	Cohort 3 6×10^{9} vg AAV2-sFLT01 (n=3)	Cohort 4 2×10^{10} vg AAV2-sFLT01 (n=3)	Cohort 5 2×10^{10} vg AAV2-sFLT01 (n=7)
Median age, years	71 (69–86)	78 (57–79)	77 (74–85)	83 (64–89)	75 (67–89)
Male sex	3 (100%)	1 (33%)	1 (33%)	1 (33%)	4 (57%)
Ethnic origin					
White	3 (100%)	3 (100%)	3 (100%)	3 (100%)	6 (86%)
African American	0	0	0	0	1 (14%)
Median duration of AMD, years	6·0 (5·4–8·8)	5·6 (5·3–8·0)	6·6 (6·0–18·0)	2·0 (1·0–5·4)	3·0 (2·0–8·0)
Median BCVA (ETDRS letter score; Snellen equivalent; IQR	15 (13–18)	27 (24–32)	23 (18–33)	18 (17–21)	27 (22–46)
ETDRS letter score	20/500	20/320	20/400	20/500	20/320
Median CST, µm	459 (434–495)	470* (368–571)	623 (547–947)	442 (228–453)	449 (324–716)

Data presented are n (%) or median (range) unless otherwise indicated. AMD=age-related macular degeneration. BCVA=best corrected visual acuity. CST=central subfield thickness. ETDRS= Early Treatment Diabetic Retinopathy Study. *One patient in cohort 2 had time domain-OCTs on a Stratus 3 machine and the scans that were stored on the machine were inadvertently deleted during a software upgrade; therefore values are shown for only two patients in cohort 2.

Table 1: **Baseline characteristics of patients**

	Cohort 1 (n=3)		Cohort 2 (n=3)		Cohort 3 (n=3)		Cohorts 4 and 5 (n=10)	
	Events n	Patients n (%)	Events n	Patients n (%)	Events n	Patients n (%)	Events n	Patients n (%)
Conjunctival haemorrhage*	0	0	0	0	1	1 (33%)	6	6 (60%)
Retinal haemorrhage	0	0	2	2 (67%)	2	1 (33%)	1	1 (10%)
Conjunctival hyperaemia*	0	0	0	0	3	3 (100%)	0	0
Conjunctival oedema*	0	0	0	0	0	0	2	2 (20%)
Eye discharge	0	0	1	1 (33%)	0	0	1	1 (10%)
Subretinal fibrosis	0	0	1	1 (33%)	0	0	1	1 (10%)
Cataract	1	1 (33%)	0	0	0	0	1	1 (10%)
Vitreous floaters	0	0	0	0	1	1 (33%)	1	1 (10%)
Corneal deposits	0	0	0	0	0	0	1	1 (10%)
Iritis	0	0	0	0	0	0	1	1 (10%)
Vitritis	0	0	0	0	0	0	1	1 (10%)
Eye pain*	0	0	0	0	1	1 (33%)	0	0
Eyelids pruritus*	0	0	0	0	1	1 (33%)	0	0
Retinal tear	0	0	0	0	0	0	1	1 (10%)
Subretinal fluid	0	0	1	1 (33%)	0	0	0	0
Visual impairment	0	0	0	0	0	0	1	1 (10%)
Vitreous detachment	0	0	1	1 (33%)	0	0	0	0
Total reported ocular treatment-emergent adverse events	2	2 (67%)	7	3 (100%)	10	3 (100%)	25	8 (80%)

Data are number of events or number of patients (%). *Study procedure related adverse events (only 1 of 3 patients with conjunctival hyperaemia were reported to be related to study procedure).

Table 2: **Summary of important treatment-emergent adverse events reported in all patients**

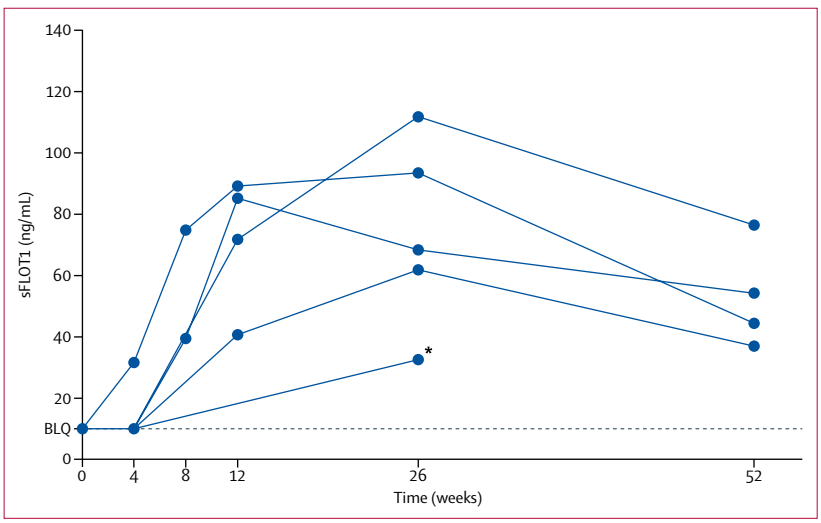

Figure 2: **Aqueous sFLT01 concentrations after intravitreous injection of 2×10^{10} vg AAV2-sFLT01**
Aqueous samples were obtained at several timepoints after intravitreous injection of AAV2-sFLT01 and sFLT01 concentrations were measured by ELISA. Each line represents a different patient (two patients from cohort 4 and three from cohort 5). Measurements at each timepoint were below the limit of quantification for all patients in the first three cohorts and in five of the ten patients in cohorts 4 and 5 who had an injection of 2×10^{10} vg AAV2-sFLT01. Aqueous sFLT01 concentrations for the other 5 patients injected with 2×10^{10} vg AAV2-sFLT01 are shown. *Patient had aqueous samples obtained at three timepoints; day 0 and week 4 samples were below the limit of quantification, so the only measurable concentration was at week 26.

epithelial detachments is often associated with visual loss in the absence of any treatment.[17–19] 14 mild or moderate adverse events were likely to be related to the intraocular injection procedure, the most common being conjunctival haemorrhage, hyperaemia, and oedema (table 2). Five retinal haemorrhages reported in four patients and subretinal fibrosis in two patients were likely to be related to disease progression.

Ten serious adverse events occurred in five patients including one death of a 91-year-old patient 1 year after study completion and 2 years after vector injection. The patient chose not to enter the extended follow-up study and information regarding the cause of death was unobtainable. The only serious adverse event to occur more than once was retinal tear, which occurred three times in three different locations in the same patient approximately 1 year after injection and was not thought to be caused by the procedure or vector. Asthenia, staphylococcal pneumonia, hip fracture, dehydration, failure to thrive, and transient ischaemic attack occurred once and were considered to be unrelated to study drug or procedure (appendix).

AAV2-sFLT01 vector DNA sequences were not detected in the blood, nasopharynx, urine, or semen of any patients after intravitreous injection of AAV2-sFLT01 at any timepoint throughout the study.

12 of the 19 patients had detectable anti-AAV2 antibodies at screening (appendix). None of the patients in cohorts 1 and 2 showed an increase in antibody titre after intravitreous injection of AAV2-sFLT01, whereas eight (62%) of 13 patients in cohorts 3–5 showed an increase after injection, although in some cases the increase was modest (appendix). Five of ten patients injected with the highest dose (2×10^{10} vg) had no anti-AAV2 antibodies at baseline, and two of these five patients remained negative throughout the study.

None of the patients in cohorts 1–3 had detectable concentrations of sFLT01 protein in aqueous humour, but five of ten patients in cohorts 4 and 5 who received an injection of 26×10^{10} vg had concentrations of sFLT01 above the LOQ at one or more timepoints after injection through week 52 (figure 2). As is typical for single-stranded AAV vectors, expression increased throughout the first several weeks and peaked at weeks 12 or 26. The five detectable aqueous sFLT01 concentrations at week 26 peaked at 32·7–112·0 ng/mL (mean 73·7 ng/mL, SD 30·5). There was a small decrease in aqueous sFLT01 between weeks 26 and 52 in each of the four patients who had measurements at both timepoints resulting in a decrease in mean concentration from 84·0 to 53·2 ng/mL (range 37·1–76·6 ng/mL, SD 17·1). Four of the five patients who had measurable concentrations of sFLT01 in aqueous had no detectable anti-AAV2 serum antibodies at baseline and the fifth had a low titre of 1:100. Of the four patients who showed a reduction in aqueous sFLT01 concentration between weeks 26 and 52, only one showed an increase in anti-AAV2 antibody titre (appendix).

Measurement of CST on OCT scans is a measure of retinal thickness that is increased from normal by the presence of intraretinal fluid; reduction from baseline CST after an intervention indicates a reduction of intraretinal fluid and possible anti-permeability activity. Of four of the nine patients in cohorts 1–3 who showed a reduction in CST, two showed large, sustained reductions in CST with the first patient showing a reduction in CST of about 170 μm at week 4, which was sustained until week 52 (cohort 1, figure 3A); in the second patient, we observed an increase in CST that then decreased, resulting in a sustained reduction from baseline of about 130 μm between weeks 26 and 52 (cohort 2, figure 3B). The other two patients showed some reduction in CST, but these reductions were not sustained through week 52 and one patient received an anti-VEGF rescue injection at week 38 (cohort 3, figure 3C). Since the administration of anti-VEGF injections makes interpretation difficult, the ten patients in cohorts 4 and 5 who received a dose of 2×10^{10} vg were analysed as two separate groups: the six patients who received no anti-VEGF rescue injections (figure 3D) and the four who received rescue injections (figure 3E). Two of the six patients who received no rescue injections had large reductions in CST sustained until week 52, three showed little change, and one showed a substantial increase in CST (figure 3D). One patient in the group of four had a reduction from baseline until week 38 but, despite this finding, received a rescue injection (figure 3E). There was little mean change from baseline CST for the entire group of patients in cohorts 4–5 (figure 3F). Thus, at week 52, four of 19 patients showed reductions of CST (with reductions of 128 μm, 172 μm, 172 μm, and 443 μm), indicating sustained vector-related

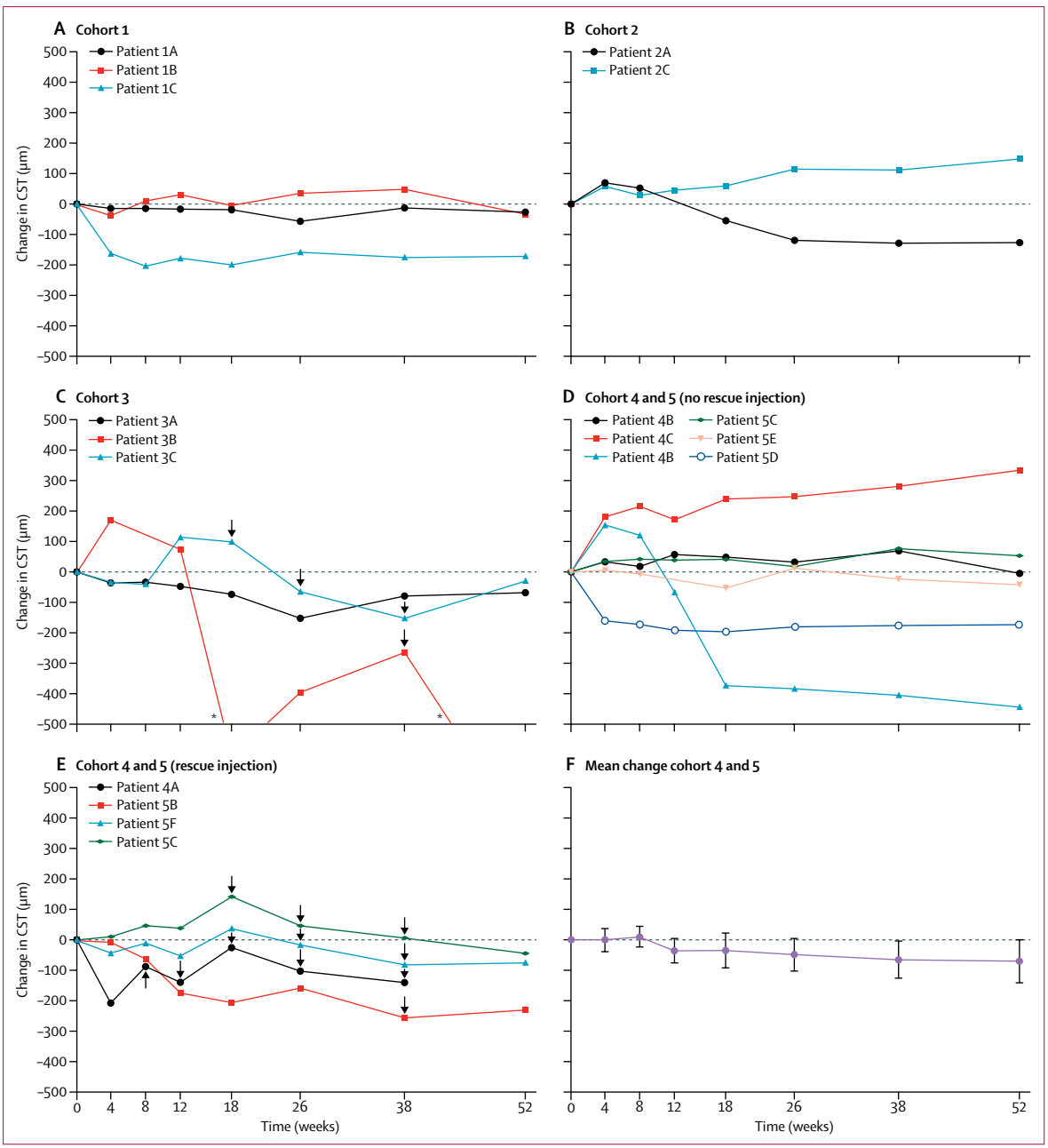

Figure 3: **Reduction from baseline central subfield thickness (CST) after intravitreous injection of AAV2-sFLT01**
Patients had CST measured by optical coherence tomography at baseline and at each study visit after injection of 2×10^{8} vg (A, cohort 1), 2×10^{9} vg (B, cohort 2), 6×10^{9} vg (C, cohort 3), or 2×10^{10} vg (D–F, cohorts 4 and 5 combined). Each line represents a different patient. Patients in cohorts 4 and 5 who did not receive any anti-VEGF rescue injections (D) and those that received rescue injections (E) are shown. The mean change from baseline CST for all ten patients in cohorts 4 and 5 who received 2×10^{10} vg is shown in (F). Arrows indicate a rescue anti-VEGF injection was given at that timepoint. One patient in cohort 2 had data that were inadvertently deleted during a software upgrade; therefore values are shown for only two patients in cohort 2.*CST was reduced from baseline by 603 µm at week 18 and 862 µm at week 52.

anti-permeability activity, and two patients showed weaker evidence because CST reduction was not stable through week 52 or a rescue injection was given at week 38.

Similar to change in CST, changes in CRLT showed considerable heterogeneity among patients, with four of 19 showing substantial, sustained reductions from baseline, with two other patients showing large reductions that were not completely sustained through week 52 (appendix).

Assessment of reduction in CST or CRLT is only useful if patients have intraretinal or subretinal fluid at baseline that can be reduced by therapeutic intervention. Although investigators were instructed to enrol patients with fluid

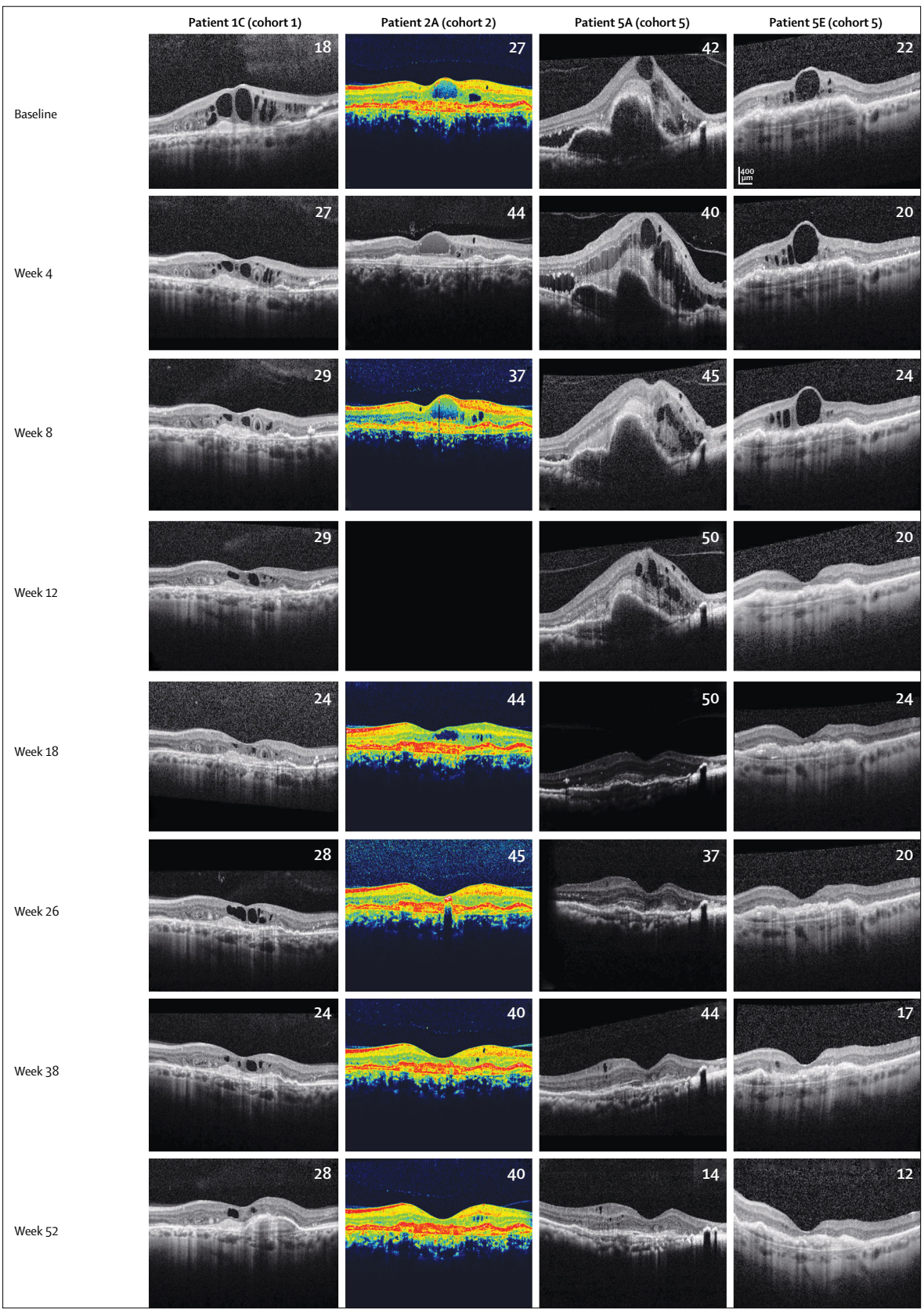

Figure 4: **Sequential horizontal optical coherence tomography scans through the fovea in four patients who showed dramatic, sustained reduction of macular fluid after intravitreous injection of AAV2-sFLT01**

At baseline, there was substantial intraretinal fluid in three patients and in the fourth there was a large pigment epithelial detachment as well as intraretinal and subretinal fluid (patient 5A). In each of the patients there was gradual fluid reduction, which is consistent with the expression pattern of single stranded AAV vectors, which require 8–12 weeks to reach peak expression. BCVA in Early Treatment Diabetic Retinopathy letter score is shown in the upper right of each panel. Reduction in best corrected visual acuity at week 52 in patient 5A was due to a combination of cataract and reduced macular function. Patient 2A missed the week 12 visit and therefore no scan or BCVA score is available. BCVA=best corrected visual acuity

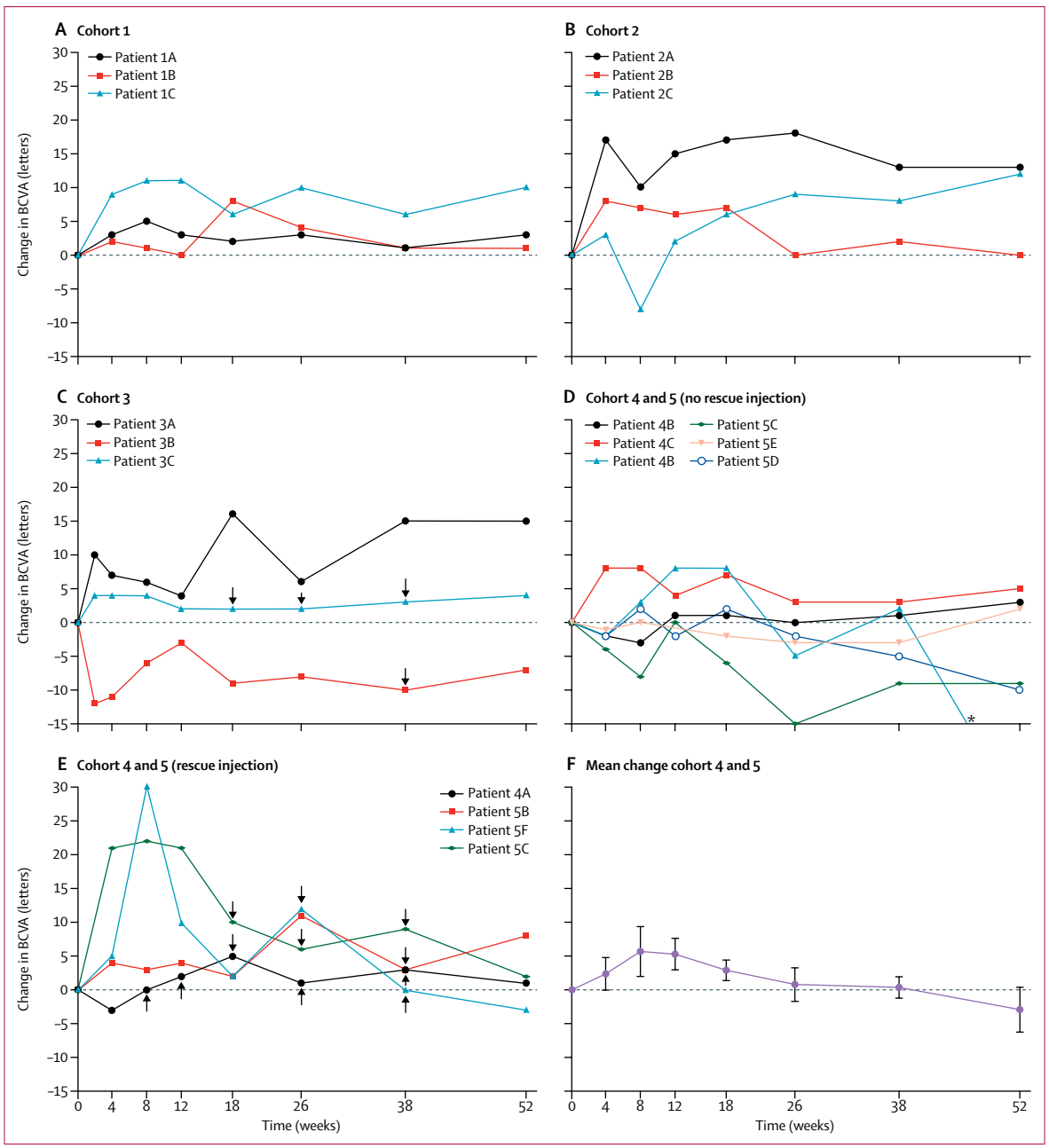

***Figure 5*: Change from baseline best corrected visual acuity after intravitreous injection of AAV2-sFLT01**
Patients had measurement of BCVA at baseline and each study visit after injection of 2×10^8 vg (A, cohort 1), 2×10^9 vg (B, cohort 2), 6×10^9 vg (C, cohort 3), or 2×10^{10} vg (D–F, cohorts 4 and 5). Patients in cohorts 4 and 5 who did not receive any anti-VEGF rescue injections are shown in (D) and those that received rescue injections are shown in (E). The mean change from baseline BCVA for all ten patients in cohorts 4 and 5 who received 2×10^{10} vg is shown in (F). Arrows indicate an anti-VEGF injection was given at that timepoint. BCVA=best corrected visual acuity.

in the macula, baseline OCT scans showed that some patients had little or no fluid. A panel of masked reading centre graders, investigators, and clinicians examined OCT scans and classified patients as to whether or not they would be expected to exhibit an anti-VEGF response consisting of a reduction in intraretinal or subretinal fluid based on whether this fluid was present at baseline and could be reduced. 11 of 19 patients were classified as those who had fluid and would be expected to respond to anti-VEGF therapy (appendix). After intravitreous injection of AAV2-sFLT01, four of these 11 patients showed substantial reduction of fluid sustained through week 52 without any rescue injections, two showed partial reductions in fluid that were not completely sustained through 52 weeks, and five showed minimal reduction in fluid. Figure 4 shows the horizontal OCT scan through the fovea at each study

visit for the four patients who showed substantial, sustained fluid reduction through week 52. These four patients had baseline anti-AAV2 antibody titres of 0, 1:100, 1:200, and 1:400; the two patients with a partial response had baseline titres of 0 and 1:400; and the five patients with no anatomic response (reduction of intraretinal or subretinal fluid) had titres of 0, 0, 1:400, 1:1600, and 1:3200 (appendix). One of four patients with a substantial response and one with a partial response had high aqueous humour concentrations of sFLT01 and the other four patients with a substantial or partial response had undetectable aqueous humour concentrations; conversely, of the other three patients with high aqueous concentrations of sFLT01, two had non-gradable anatomic responses and one had a poor response.

Many of the patients had restricted visual potential due to subretinal fibrosis, absence of fluid (no chance for improvement), or very large cystoid spaces that are often accompanied by macular damage. Two of the patients with a substantial anatomical response and one with a partial response were in cohorts 1–3 and showed large improvements from baseline BCVA (figure 5A–C). In the ten patients who received a high dose of AAV2-sFLT01 in cohorts 4 and 5, visual results were difficult to interpret because two patients had no fluid at baseline, three had large cystoid spaces with thinning of remaining retina, and one received rescue injections starting at week 8. The other four patients showed some improvements from baseline BCVA at early timepoints and gradual reduction thereafter (figure 5D and E). The mean change from baseline BCVA in patients in cohorts 4 and 5 showed a slight early improvement followed by gradual decline, but overall little change (figure 5F). There was one patient in the high-dose group who had a large pigment epithelial detachment and intraretinal or subretinal fluid at baseline and showed initial improvement in BCVA, which was associated with improvement in intraretinal or subretinal fluid, or both, followed by resolution of the pigmented epithelial detachment and loss of initial visual gains, and then a large reduction in BCVA between weeks 38 and 52 due to a combination of cataract progression and reduced macular function (figure 4 column 3).

Discussion

To our knowledge, AAV2 vectors seem to be safe and well tolerated when given by intravitreous injection. However, intraocular inflammation was seen in one of ten patients who received the highest dose of 2×10^{10} vg of AAV2-sFLT01, which resolved after administration of topical steroids, and did not recur after withdrawal of treatment. There were no systemic adverse events that were likely to be related to AAV2-sFLT01 injection.

AAV2-sFLT01 drives expression of sFLT01, a secreted VEGF-neutralising protein, and measurement of sFLT01 in aqueous humour at several timepoints after injection of AAV2-sFLT01 provides crucial information for dose selection and interpretation of visual and anatomical outcomes. No sFLT01 was detectable in aqueous humour at any timepoint after injection of 2×10^{8}, 2×10^{9}, or 6×10^{9} vg, but five of ten patients who received an injection of 2×10^{10} vg had detectable amounts of sFLT01 that peaked at 12–26 weeks and appeared to decrease between weeks 26 and 52. Since this finding is based on measurements in only four patients, we are not confident that reduction in expression over time definitely occurs, but if it is a real finding, one possible explanation is a dose effect. In cynomolgus monkeys,[15] there were reductions in aqueous humour concentrations of sFLT01 between 6 and 12 months after intravitreous injections of $2\cdot4\times10^{9}$ vg, but sFLT01 concentrations were stable between 6 and 12 months after injection of $2\cdot4\times10^{10}$ vg.[15] Since human eyes are substantially larger than eyes of non-human primates, a dose of 2×10^{10} vg in human beings is equivalent to a lower dose in non-human primates. Our data suggest that an intravitreous dose of 2×10^{10} vg AAV2-sFLT01 is not maximal on the dose-response curve; since the concentration used was not associated with dose-limiting toxicity, it would be reasonable in subsequent studies to test higher doses that could potentially overcome reduction in expression over time and might also reduce variability in expression among patients.

An important question is whether the presence of serum antibodies directed against AAV2 before intravitreous injection of AAV2-sFLT01 has a negative effect on transgene expression. Our data suggest that this could be true, because five of ten patients injected with the highest dose of AAV2-sFLT01 had no detectable anti-AAV2 serum antibodies and four of those five patients had detectable sFLT01 in aqueous humour after injection. The only patient with measurable sFLT01 in aqueous humour who had anti-AAV2 serum antibodies at baseline had a very low titre (1:100). The five patients injected with 2×10^{10} vg AAV2-sFLT01 who failed to show detectable sFLT01 in aqueous humour had baseline anti-AAV2 titres of 0, 1:400, 1:400, 1:3200, and 1:3200. The finding of one patient who received the highest dose and showed no sFLT01 in aqueous humour despite undetectable anti-AAV2 antibodies indicates that anti-AAV2 antibody titre and vector dose cannot be the only factors affecting transgene expression, but the remaining data are suggestive that baseline anti-AAV2 antibody titres could play a role. It is not possible to make firm conclusions from data obtained from only ten patients, but preclinical data in expression studies in non-human primates also suggest that serum antibodies directed against an AAV serotype could impair expression after intravitreous injection of an AAV vector of that same serotype.[15,26,27] Thus, we preliminarily conclude that patients with high anti-AAV2 titres might not be good candidates for gene therapy involving intravitreous injection of an AAV2 vector, although we concede that additional human data would be useful to be sure that this is the case. A related issue is whether or not a patient who has received an intravitreous injection of an AAV2 vector in one eye can be considered for an injection in the fellow eye. Even if

future studies confirm that serum antibodies impair expression after intravitreous vector injection, it might be possible to do an intravitreous injection of an AAV vector in both eyes within 2 weeks. If this is not done and anti-AAV2 antibodies develop, it might be possible to do a subretinal injection in the fellow eye for which it appears that serum antibodies do not impair expression.[26–28]

Four of 11 patients with intraretinal or subretinal fluid had considerable, sustained reduction of the fluid, and two patients had a substantial partial response. This finding is encouraging, but why did five patients show minimal fluid reduction? Variability in sFLT01 expression might be part of the answer, but is unlikely to be the entire cause, because our data show that there was not a good correlation between sFLT01 concentrations and anatomical response. Three patients with fluid elimination had undetectable sFLT01 in aqueous and although two patients with high expression of sFLT01 showed a good anatomical response, one showed a poor response and the other two were uninformative because of a low amount of fluid at baseline. There is considerable heterogeneity among patients with neovascular age-related macular degeneration. In some patients, subretinal or intraretinal fluid cannot be eliminated with monthly injections of a VEGF-neutralising protein, in other patients, the fluid is eliminated but monthly injections are needed to prevent its recurrence, and in others fluid is eliminated and recurrences are prevented by very infrequent injections. Thus, in some patients, low-level VEGF suppression is sufficient to achieve an optimal response, although in others strong, sustained suppression of VEGF is insufficient. It appears that despite undetectable sFLT01 in aqueous, the concentrations in retina, which should be substantially higher than those in aqueous, were sufficient for sustained elimination of fluid in three patients and substantial reduction in another. In the three patients with macular fluid at baseline who had measurable concentrations of sFLT01 in aqueous humour and hence high concentrations of the protein in retina, there was elimination or marked reduction of fluid in two patients and a poor anatomical response in the third. Higher vector doses and better patient selection based on greater understanding of the effect of baseline anti-AAV2 serum antibodies could potentially increase the number of patients with neovascular age-related macular degeneration that could benefit from this approach.

Other studies have reported anatomical and visual improvements in patients with neovascular age-related macular degeneration given subretinal injection of an AAV2 vector expressing native sFLT01.[25,29] Subretinal injection of AAV vectors has potential advantages over intravitreous injection, such as high transduction efficiency of the retinal pigment epithelium and photoreceptors and reduced effect of anti-AAV2 serum antibodies potentially leading to high intraocular amounts of transgene product regardless of baseline antibody titres. These potential advantages are balanced by some potential disadvantages. Subretinal injections of vector require an operative procedure in which the vitreous gel is removed followed by injection of vector through the retina into the subretinal space. The opening in the retina is self-sealing and the procedure is quite safe and reliable, but every procedure has potential complications, in this case a 1% risk of retinal detachment and a 60% risk of cataract progression severe enough to require cataract surgery within a year. Additionally, surgical procedures have inherent variability and there might be some patients in whom less than the intended volume of vector is successfully injected into the subretinal space. Intravitreous injections are less invasive and less expensive than subretinal injections, and are the current mode of delivery for VEGF-neutralising proteins. Our data suggest that this less invasive and widely accepted injection approach could be feasible for gene delivery aimed at achieving long-term expression of therapeutic proteins in some patients, but additional studies are needed to define the optimal patient population with regard to anti-AAV2 serum antibodies and vector dose.

This study has weaknesses that are inherent in phase 1 studies, such as a small study population, no control group, and the need to avoid patients with good visual potential who stand to benefit from standard care. By comparison with intravitreous injections of anti-VEGF proteins for which injection frequency can be adjusted on the basis of individual need, the gene transfer approach has less flexibility, but the potential of reducing treatment burden which is an obstacle to good long-term outcomes[2] is an important advantage.

This study shows safety and tolerability of AAV-sFLT01 after intravitreous injection, and has important implications. It provides important information for other investigators considering an intravitreous delivery route for an AAV vector to treat other diseases. It also provides useful information regarding expression of a VEGF-neutralising protein in the eye, and specifically for potential future development of AAV2-sFLT01. Our findings allow for more liberal eligibility criteria including patients with BCVA of 20/40–20/200 and good visual prognosis with no vision limitation for the fellow eye. In such patients, it will be necessary to give an anti-VEGF protein injection at baseline to allow time for transgene expression to occur. It will also be important to stratify patients on the basis of the presence of anti-AAV2 neutralising antibodies at baseline to definitively identify if pre-existent antibodies preclude an intravitreous route of delivery for AAV ocular gene therapy. We hope that future trials will build on these findings to further identify applications and limitations of intravitreous injection of AAV vectors for gene transfer in the eye.

Contributors

JSH, CD, AL-H, JC, SCW, RB, AS, and PAC contributed to trial design. CD, AL-H, JC, SCW, RV, RB, and AS were involved with trial management. JSH, PD, SKa, and PAC enrolled patients, did study procedures, and collected data. JSH, SKh, CD, AP, SR, AL-H, JC, SCW, RV, RB, AS, and PAC contributed to data analysis. JSH, SKh, SD, and PAC wrote the manuscript. SKh drafted the figures. PD, SKa, SHC, CD

AP, SR, AL-H, JC, SCW, RV, RB, and AS edited the manuscript. All authors approved the final manuscript for publication.

Declaration of interests

JC, SHC, CD, SCW, RB, AP, SR, AL-H, RV and AS are current employees or former employees of Genzyme Corporation or Sanofi Genzyme, which funded this study. JSH reports grants from Genzyme during the study; and personal fees from Acucela, Adverum, Aerie, Aerpio Therapeutics, Alcon, Allegro Ophthalmics, Allergan, Daiichi, EyeGate Pharmaceuticals, Genentech/Roche, Janssen R&D, Kala Pharmaceuticals, Kanghong, Notal Vision, Ocudyne, Regeneron, Regenxbio, RetroSense, Scifluor Life Sciences, Shire, Stealth Biotherapeutics, Valeant Pharmaceuticals, Voyager Therapeutics, grants from Acucela, Apellis, Astellas, Bayer, Corcept, Daiichi, Eyegate, Genentech, Genzyme, Janssen R&D, LPath, Neurotech, Novartis, Ocudyne, Ophthotech, Regeneron, SciFluor, Thrombogenics, Tyrogenex, from Ocular Therapeutics, outside the submitted work. PD reports grants from Genzyme, during the study; personal fees from Abbott/AMO, Aerpio, Alcon, Alimera Sciences, Allergan, Annidis, Acucela, ArcticAx, Avalanche Biotechnologies, Clearside Biomedical, Digisight, DOSE Medical, Genentech, Graybug Vision, Lutronic, Lux BioScience, Neurotech, Novartis, OD-OS, Omeros, Ophthotech, Opthea, Optovue, ORA, Regeneron, Roche, Pentavision, Shire Human Genetic Therapies, Stealth BioTherapeutics, Thrombogenics, Topcon; and other support from Alimera, Aerpio, Annidis, Digisight, Ophthotech, TrueVision outside the submitted work. PAC reports that Johns Hopkins University received research funding from Genzyme Corporation for the trial but reports no personal financial interests in Genzyme Corporation. PAC reports grants and personal fees from Aerpio, Alimera, Allergan, AsclipiX, Genentech/Roche, Rxi, Regenexbio personal fees from Applied Genetic Technologies; personal fees from Allegro, Intrexon, Merck, Novartis, and Bayer; , and grants from Abbvie, GlaxoSmithKline, Oxford BioMedica, and Regeneron outside the submitted work. PAC also has equity in Allegro and Graybug. SKh, SD, and SKa declare no competing interests.

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