

# Humoral Immune Response After Intravitreal But Not After Subretinal AAV8 in Primates and Patients

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See the appendix for the members of the RD-CURE Consortium.

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**PURPOSE.** To study longitudinal changes of anti-drug antibody (ADA) titers to recombinant adeno-associated virus serotype 8 (rAAV8) capsid epitopes in nonhuman primates (NHP) and patients.

**METHODS.** Three groups of six NHP each received subretinal injections (high dose:  $1 \times 10^{12}$  vector genomes [vg], low dose:  $1 \times 10^{11}$  vg, or vehicle only). Four additional animals received intravitreal injections of the high dose ( $1 \times 10^{12}$  vg). Three patients received  $1 \times 10^{10}$  vg as subretinal injections. ELISA quantified ADA levels at baseline and 1, 2, 3, 7, 28, and 90 days after surgery in NHP and at baseline and 1, 3, and 6 months after surgery in patients.

**RESULTS.** Two out of 22 animals lacked ADA titers at baseline and developed low ADA titers toward the end of the study. Titers in the low-dose group stayed constant, while two of six animals from the high-dose group developed titers that rose beyond the range of the assay. All animals from the intravitreal control group showed a rise in ADA titer by day 7 that peaked at day 28. Preliminary data from the clinical trial (NCT02610582) show no humoral immune response in patients following subretinal delivery of  $1 \times 10^{10}$  vg.

**CONCLUSIONS.** No significant induction of ADA occurred in NHP when mimicking the clinical scenario of subretinal delivery with a clinical-grade rAAV8 and concomitant immunosuppression. Likewise, clinical data showed no humoral immune response in patients. In contrast, intravitreal delivery was associated with a substantial humoral immune response. Subretinal delivery might be superior to an intravitreal application regarding immunologic aspects.

Keywords: gene therapy, vitreoretinal surgery, retina, AAV, immune response, antibodies

Adeno-associated virus (AAV)-mediated gene therapies have been shown to be clinically safe and offer new possibilities for the treatment of genetic diseases, such as blinding retinal dystrophies. However, investigators have independently found evidence of immune reactions against AAV vectors, the transgene, or the transgene product.<sup>1–4</sup> These include the full range of active defense mechanisms including innate, humoral, and cellular immunity. As such, clinical trials in hemophilia patients have shown that circulating antibodies can effectively inhibit transduction even at low titers, and that AAV-directed CD8<sup>+</sup> cells target and remove successfully transduced hepatocytes as virus-infected cells.<sup>5</sup>

In contrast to the treatment for hemophilia, where AAV vectors are injected intravenously, relatively small doses are administered in the immune-privileged space of the eye for retinal disorders like Leber's congenital amaurosis, choroideremia, or achromatopsia. In 2008, different groups independently reported a modest beneficial treatment effect of AAV2 for the *RPE65* mutation in LCA patients.<sup>6–8</sup> In none of these trials were major adverse events reported in the following 5 years.

Clinically no inflammation unresponsive to steroids was observed, and only two patients developed a transient antibody reaction.<sup>9</sup> One study reported a single case in which anti-capsid antibodies emerged in a functional assay around day 14 but declined later,<sup>6</sup> and in another study some anti-capsid antibody titers increased toward day 90 but were still low compared to the overall mean.<sup>8</sup> Amado et al.<sup>10</sup> showed that subretinal readministration of an AAV2 vector elicits a humoral immune response against the viral capsid in large animals. However, transduction was still possible under these conditions. This is in line with the observation that readministration of subretinal AAV (to the contralateral eye) in three adult patients did not lead to a rise in antibody titer.<sup>11</sup> Importantly, though, intravitreal delivery of AAV2 has been shown to induce humoral immune response in mice and block transduction in subsequent subretinal or intravitreal injections.<sup>12</sup> Others have shown that intravitreal delivery of AAV in NHP results in an increase of anti-AAV antibodies and decreased transgene expression.<sup>13</sup> Although a rise in antibody titer does not seem to be necessarily harmful, safety of the patient is of paramount importance and



the role of a potential humoral immune response should be fully understood.

To further explore this we injected 22 NHP with different doses of recombinant adeno-associated virus serotype 8 (rAAV8) as part of a formal toxicology and biodistribution assessment toward regulatory approval of a phase I/II clinical trial (NCT02610582). Subretinal or intravitreal administration routes were used in a surgical setting identical to that in human subjects (including perioperative steroids). We aimed to elucidate whether a good manufacturing practice (GMP)-grade AAV8 vector would lead to a humoral immune response in a clinical scenario and whether the route of delivery would make a difference. Additionally, the same assay was used subsequently to quantify anti-drug antibody (ADA) titers in human patients following subretinal gene therapy (NCT02610582) with the same vector.

## METHODS

### Animals and Study Design

A total of 22 NHP (*Macaca fascicularis*) were allocated into four separate groups (Supplementary Table 1). Groups 1 to 3 consisted of six animals (three males/three females). Group 1 was treated with vehicle (balanced salt solution [BSS]; Alcon, Freiburg im Breisgau, Germany) with 0.001% PF-68 (BASE, Ludwigshafen am Rhein, Germany). Animals in groups 2 and 3 received the test item (rAAV8) in the left eye only via single subretinal injection. Four animals (two males/two females) were allocated to group 4 and received the same test item via intravitreal injection. Animals in group 2 were treated with low-dose ( $1 \times 10^{11}$  vector genomes [vg]) and animals in groups 3 and 4 were treated with high dose ( $1 \times 10^{12}$  vg). Animals used in these studies were cared for and handled according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and after approval by the local authorities (Regierungspraesidium) and in full compliance with the guidelines of the European Community (EUVD 86/609/EEC) for the care and use of laboratory animals, as well as in accordance with good laboratory practice (GLP) standards as defined by German GLP monitoring authorities and in compliance with U.S. Food and Drug Administration GLP regulations.

### Vector and Vehicle

The AAV8 vector was produced according to GMP guidelines by cotransfection of human embryonic kidney cells followed by purification and concentration steps optimized for clinical use of vector solution as reported previously.<sup>14</sup>

### Surgery and Perisurgical Care of NHP

Animals received general isoflurane (Forane; Baxter GmbH, Unterschleißheim, Germany) and local 2% oxybuprocain (Conjuncain; Bausch&Lomb GmbH, Berlin, Germany) anesthesia before preparing (peri-)orbital skin with 10% povidone iodine solution and rinsing the conjunctival fornices with 1% povidone iodine solution. Sterile surgical drapes and pediatric lid specula were applied before a temporal canthotomy was performed for improved access. Three 23-gauge (G) transconjunctival sclerotomies were made approximately 1.5 mm posterior to the limbus and vitrectomy was performed as completely as possible without affecting the lens. A localized retinal detachment was induced through subretinal injection of BSS (Alcon) using a 41-G cannula (DORC 1270.EXT; D.O.R.C. Deutschland GmbH, Düsseldorf, Germany). Virus solution was injected into the preformed bleb using a foot pedal-controlled

injection system (PentaSys II; Ruck GmbH, Eschweiler, Germany). Before recovery, subconjunctival cefuroxime (125 mg; ratiopharm GmbH, Ulm, Germany) and dexamethasone (2 mg, ratiopharm GmbH) were administered to the operated eye. Postoperative prophylactic treatment consisted of antibiotic (0.5% Moxifloxacin; Pharm-Allergan GmbH, Frankfurt am Main, Germany) and anti-inflammatory (1% Prednisolone; Pharm-Allergan GmbH) eye drops given three times a day each in the treated eye for 2 weeks and prednisone (Merck Pharma GmbH, Darmstadt, Germany) 1 mg/kg intramuscularly from day -2 until day 5. In the course of the study all animals received ophthalmoscopic screening (slit lamp, fundus biomicroscopy) for signs of inflammation at days 2, 3, 7, 22, 50, and 87.

### Enzyme-Linked Immunosorbent Assay (ELISA)

Plasma samples were collected from each animal prior to dosing and at days 1, 2, 3, 7, 28, and 90 post dosing. A total of 154 samples were analyzed using a sandwich ELISA strategy utilizing a ELISA kit for the determination of AAV serotype 8 particles in cell culture supernatants or purified preparations (PROGEN Biotechnik GmbH, Heidelberg, Germany; Art. No.: PRAAV8). The microtiter strips, coated with a monoclonal antibody specific for a conformational epitope on assembled AAV8 capsids, were incubated with GMP-grade rAAV8. This procedure completed the coating for the detection of the new analyte: anti-AAV8 antibodies. Captured anti-AAV8 antibodies in plasma samples were detected using an enzyme conjugate of a rabbit anti-NHP antibody (rabbit anti human [and NHP] IgG pAb Streptavidin Peroxidase Conjugate, Cat. No. 55221; MP Biomedicals, Santa Ana, CA, USA). An anti-AAV8 biotin-conjugated antibody, together with streptavidin peroxidase, served as positive control. This antibody was used in a serial dilution of 1:3 from 250 ng/mL down to 0.34 ng/mL. The highest concentration of 250 ng/mL showed a hook effect and was therefore regarded as out of the range of the assay. The remaining seven standard dilutions covered the complete range, returned from the plasma samples. Eight negative controls were included on every ELISA plate. For the negative controls, no plasma was added and the background was calculated from the mean absorbance of these blanks. The background mean was subtracted from the plasma samples.

After addition of substrate solution the color reaction was measured photometrically at  $\lambda = 450$  nm. In order to avoid false-negative results due to very high concentrations of the analyte, the plasma samples were measured in serial dilutions (1:5, 1:25, 1:125).

To ensure the assay's validity, coating controls, a dilution sequence of positive controls, and a negative control with no analyte were included on every ELISA plate. The optimal assay setting was tested beforehand in a GLP confirming proof of principle study. The following criteria were implemented to ensure validity. Uncoated wells had to show a low absorbance value ( $OD_{STD0} \leq 0.2$ ). Coating controls ( $1 \times 10^9$ ,  $1 \times 10^8$ ,  $1 \times 10^7$  vg/mL, no coating) had to show a dose dependency in mean absorbance values. Coated wells without analyte had to show a low absorbance value and give a good signal-to-noise ratio when using a short time for color reaction and using a blocking solution ( $OD_{no\ Plasma} \leq 0.3$ ). The mean absorbance value of the positive control wells coated with  $1 \times 10^{10}$  vg/mL (standard 1, STD1) had to be  $\geq 1.0$ . The mean absorbance value of the positive controls (standards 1-8) had to show a dose dependency (STD1 > STD2 > STD3 > STD4 > STD5 > STD6 > STD7 > STD8). Plasma from seroconverted animals had to show a clear dose-dilution relationship.

As these criteria were all met, the assay was considered to be appropriate for the detection of anti-AAV8 antibodies.

## Analysis

For the NHP samples, the titer was defined as the reciprocal dilution of the plasma at which the linear, interpolated graph for individual plasma intersects a so-called titer intercept line (TIL). The range where the interpolated graph could intersect the TIL was defined between 5 and 160. In this assay, the TIL was defined as the 3.3-fold lower limit of quantification (LLOQ) of the assay. Limit of detection (LOD) and LLOQ were calculated according to German Institute for Standardization (DIN) 32654, using the standard deviation ( $\sigma(x_0)$ ) of negative controls by the following approximation: LOD:  $3 \times \sigma(x_0)$ ; LLOQ:  $k \times 3 \times \sigma(x_0)$  (with  $k=3$  at relative confidence interval  $[CI_{rel}] = 33\%$ ). In the case of clinical samples, absolute absorbance values are reported of all dilutions tested and compared in a longitudinal fashion.

Some samples did not yield quantifiable results. In these, titers exceeded or stayed below the dilution range of 1:5 to 1:160. Because we calculated the titer using the slope between different concentrations, in cases where the absorbance values did not drop in line with the dilution series (see validation criteria), the intersection with the TIL was outside the dilution range (i.e., the reading did not meet the prespecified criteria of validity). The most likely reason for this is the oversaturation of the assay due to high titer concentrations or a technical error. Likewise, plasma samples where all dilutions gave absorbance values below the TIL were considered below the range.

## Patients

Three patients (two male, one female) underwent the procedure after written informed consent was given and followed up according to the approved trial protocol (NCT02610582). The vector was applied via subretinal injection as described previously.<sup>14</sup> All three patients received  $1 \times 10^{10}$  vg of the clinical-grade vector rAAV8.hCNGA3. To monitor safety, clinical and ophthalmologic examinations were performed at screening, directly after surgery, and 1, 2, 3  $\pm$  1, 14  $\pm$  3, 30  $\pm$  5, 90  $\pm$  7, and 180  $\pm$  7 days after surgery. Blood samples were taken in all patients at screening, as well as 30  $\pm$  5, 90  $\pm$  7, and 180  $\pm$  7 days after surgery. The study was carried out in accordance with the ethical principles of the Declaration of Helsinki.

## RESULTS

### Nonhuman Primates (NHP)

For 141 of 154 NHP samples, a titer for rAAV-specific antibodies could be calculated, while 13 samples (8%) were out of the range of the assay. Of these, eight were above the range of the assay (titer  $>$  160), and five samples showed no seroconversion (titer  $<$  5). In total, 20 of 22 animals were already seroconverted before application. The two seronegative animals (28011M, 28017M), both allocated to the low-dose group, developed very low antibody titers throughout the observation period (maximum titer: 19, 28011M, day 28). No sex-specific differences in the rAAV8 specific antibody titers were observed.

In the vehicle control group (Fig. 1A), the change of titer (day  $x$  – day 1) ranged from  $-28$  in animal 28023M to  $+50$  in animal 28062F. This represents the test variability and individual titer fluctuations, which occur without an ongoing inflammatory reaction since none of the animals received vector. In the low-dose group (Fig. 1B) we observed titer

changes similar to the control group with a titer change range from  $-30$  to  $+36$ . As such, the antibody titers of the subretinal low-dose group stayed constant over the entire observation period. Although no statistical analysis is applicable, no titer changes obviously different from those of the vehicle control group were observed. Interindividual differences as well as the time curve for the titers found in the low-dose group are similar to those of the vehicle control group. In the high-dose group (Fig. 1C), two animals had titers that were above the range of the ELISA assay (animal 28060F day 28, animal 28063F day 7 and day 28). Where titers exceeded the range of the assay (160), no titer change was calculated. With the three missing values put aside, the titer change in group 3 ranged from  $-25$  to  $+68$ . The relevance of the missing values will be discussed later. In the intravitreal control group (Fig. 1D), which received the same dose as the high-dose group, the mean titer change was most pronounced compared to all other groups. All animals showed a tendency toward higher titers 7 to 28 days after surgery. In three out of four animals, titers began to rise by day 7, peaked at day 28, and declined toward day 90 but remained elevated above baseline. The titer change on day 7 ranged from  $+34$  to  $+98$  and on day 28 from  $+38$  to  $+140$ . The maximum titer change was  $+140$  (animal 28057F day 28). Individual titers for each animal and time point are shown in Supplementary Figures S1 through S4.

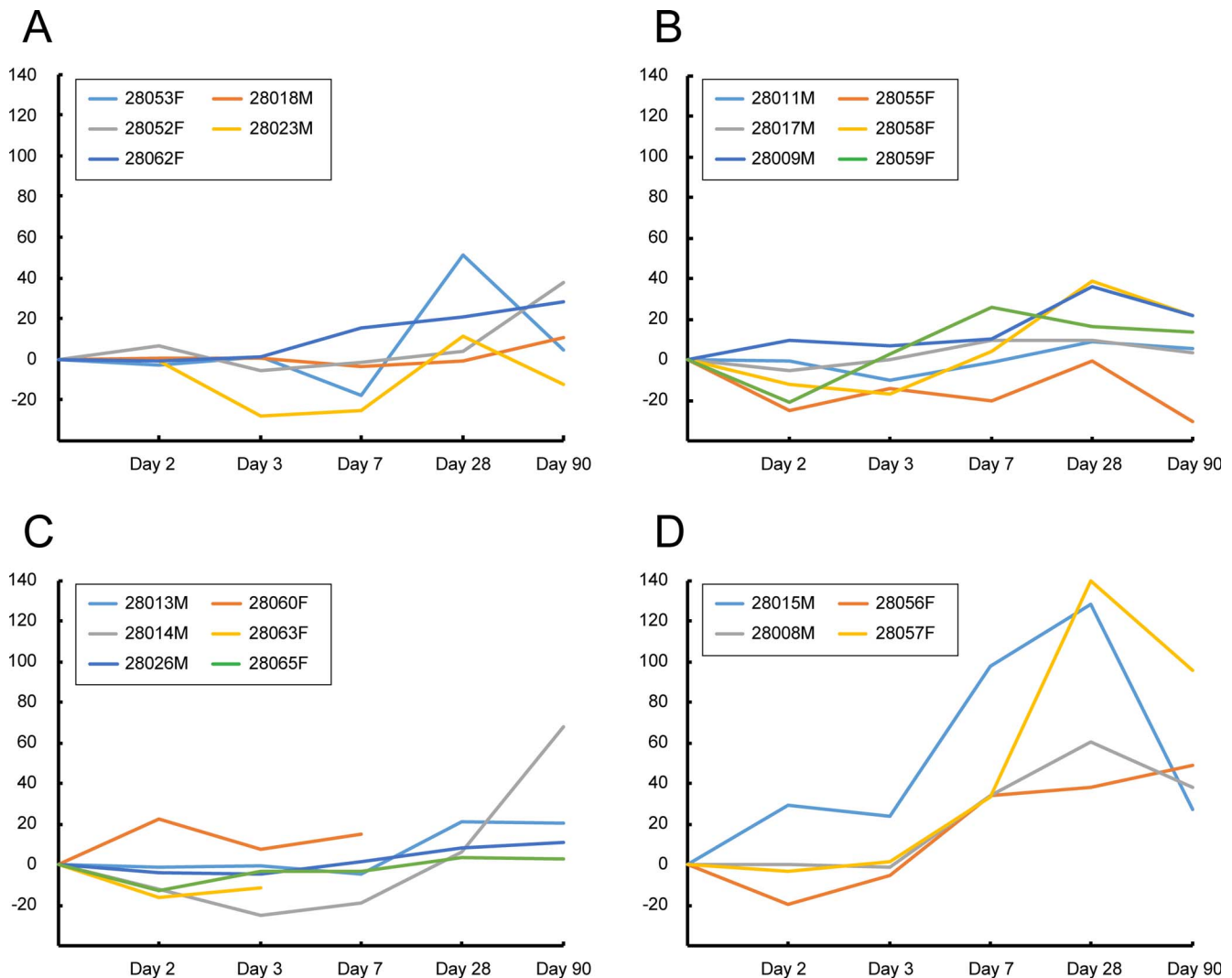
## Patient Samples

We tested plasma samples from the first three patients with CNGA3-linked achromatopsia undergoing gene therapy (NCT02610582), which applies the same vector construct used in the NHP study above. The same ADA test was applied and showed no humoral immune response within the first 6 months following subretinal delivery of  $1 \times 10^{10}$  vg (Fig. 2). All three patients had quantifiable absorbance measurements at baseline, which did not change significantly at 1, 3, or 6 months after subretinal vector delivery.

## DISCUSSION

Through contact with wild-type AAV, humans develop antibodies against the different serotypes in their first years of life.<sup>15,16</sup> Depending on the study, seroprevalence for AAV8 ranges from 15% to 30% of the population (AAV2: 30%–60%)<sup>16,17</sup> to 82% in Asian adult humans (AAV2: 97%).<sup>18</sup> Seroprevalence for AAV8 in NHP is considered to be as high as in humans or even higher.<sup>19</sup> Accordingly, in our study, 20 of 22 animals were found to be seropositive for anti-rAAV8 antibodies, indicating a seroconversion before the first treatment. Likewise, all patients from the first cohort ( $n=3$ ) of the clinical trial (NCT02610582) had quantifiable absorbance values in the ADA assay at baseline.

In general, after infection with a virus, the immune system requires a few days to develop a specific humoral immune response. Although it is difficult to exactly predict the temporal dynamics of a humoral immune response against AAV epitopes, antibody titers in a clinical gene therapy trial for hemophilia using rAAV8 rose after 1 to 2 weeks.<sup>5</sup> Antibody titers rising in a similar time frame after rAAV8 delivery can therefore be attributed to a specific humoral immune response. This is what we observed in our intravitreal control group where antibody titers began to climb on day 7 and peaked at day 28 (no samples were taken in between, e.g., on day 14). Hence, a specific humoral immune response in NHP after intravitreal delivery of rAAV8 serves as a parsimonious explanation.



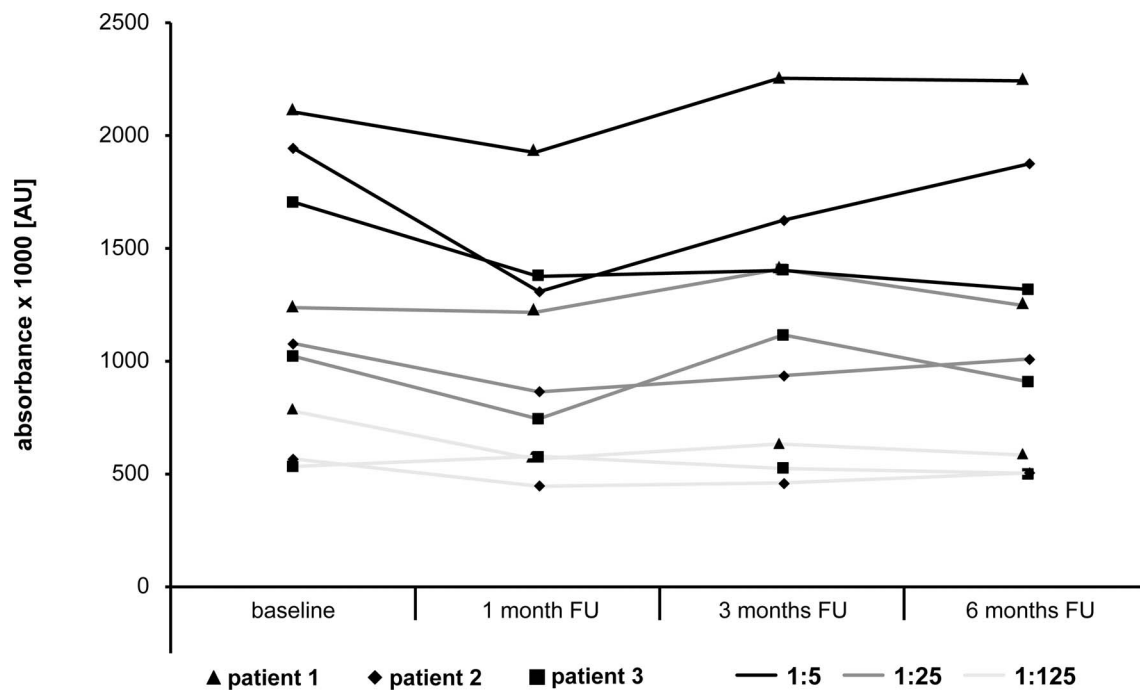
**FIGURE 1.** Individual titer change in individual animals presented as  $\Delta$  to baseline (day 1). (A) Control group, (B) low-dose group ( $1 \times 10^{11}$  vg), (C) high-dose group ( $1 \times 10^{12}$ ), (D) intravitreal high-dose group ( $1 \times 10^{12}$ ). Titers are calculated as described in Methods. Where titers exceeded the range of the assay (upper range of titer calculation: 1:160), no titer and no titer change could be calculated ([C] 28060F day 7 and 28063F days 7 and 28; [A] 28010M at all time points and therefore not included in graph).

Although no evidence is at hand for the missing values of the two animals of the high-dose group, 28060F and 28063F, having the previous considerations in mind, one could interpret these titers above the range of the assay on day 28 (28060F) and days 7 and 28 (28063F) as a humoral immune response. In both animals, titers declined toward an elevated level above baseline on day 90, which is consistent with what we observed in the intravitreal control group. Apart from these two animals, all other rAAV antibody titers in the high-dose group, as well as all rAAV antibody titers of the low-dose group, stayed constant over the 90-day observation period.

Since ELISA values for antibodies against AAV serotypes are not comparable between different studies it is difficult to make a decision on what titer change is considered a relevant change—especially since high titers do not translate into clinical findings. In our study we used the variability of control group data to assess which change in titer was numerically significant. It is important to remember, however, that this does not equate to clinical significance. Indeed, we observed no clinically relevant, test item-related changes in ophthalmologic assessment throughout the in-life phase of the study. Findings observed (limited and temporary anterior chamber

flare and cells, drusen, and pigment clumping) were either also present before dose and thus regarded as background lesions (e.g., drusen), or equally evident in groups 1 to 3 and therefore related to the surgical procedure rather than the test item. The fact that none of the 22 primates presented signs of a clinically relevant inflammation shows that a rise in antibody titer cannot be directly correlated to a clinically significant pathogenic process. It may, however, become relevant in a scenario of multiple injections and/or intravitreal applications.<sup>3,18</sup> Intravitreal may be considered the preferred route of administration when targeting inner layers or wide areas of the retina.<sup>20</sup> However, some authors have suggested that after intravitreal injections, neutralizing antibodies are more likely to be generated than after subretinal injections and that these antibodies have the potential to inhibit effective gene transfer.<sup>12,13</sup> Part of the explanation for this enhanced humoral immune response might be the fact that the shedding and biodistribution of vector after intravitreal injections is considerably higher.<sup>21</sup>

This study has certain limitations, including absence of absolute thresholds of clinical relevance for levels of antibodies against AAV8. Furthermore, there is no international standard



**FIGURE 2.** Anti-AAV8 antibody titers in patients before and after subretinal gene therapy. Samples from the first three patients were analyzed at baseline and 1, 3, and 6 months after subretinal delivery of  $1 \times 10^{10}$  rAAV8 vector genomes. Longitudinal analyses of plasma samples in serial dilution (1:5, 1:25, and 1:125) show no prominent intraindividual change in absorbance values, suggesting that there is no humoral immune response in these patients. AU, arbitrary units; FU, follow-up.

for benchmarking across studies. As such, dilution series and longitudinal follow-up are important aspects in these investigations. Another limitation is the lack of true technical repeats in the ELISA. Instead, each sample was measured in a dilution sequence and the titer calculated by using the slope instead of single values. This has the added benefit of accounting for differences in antibody affinity as a function of epitope concentration.

One needs to be cautious when extrapolating results from studies in NHP to the clinical situation because the response of the immune system of NHP to the therapeutic vector may differ from that of human subjects. However, we believe that our findings may help guide study designs of future clinical gene therapy trials. We argue that this assay can specifically detect antibodies against AAV8 epitopes and is appropriate for the comparison of pre- and postdose plasma specimens. Since our results rely on rAAV8, we can only speculate about the effects of other serotypes. It seems possible, though, that the time course of the humoral immune response as well as the effects of different routes of administration may be similar for the commonly used vector AAV2 and other serotypes.

In conclusion, our results show an excellent safety profile, especially regarding the low dose ( $1 \times 10^{11}$  vg). This is important, as this dose was chosen as the highest dose used for the clinical trial in achromatopsia patients (NCT02610582). Groups 3 and 4, having received  $1 \times 10^{12}$  vg, showed more equivocal results, with some samples exceeding the range of the ADA assay. In general, the route of administration seems to have dictated the humoral immune response against AAV8: While an intravitreal approach promises the potential of panretinal transduction without the challenges of subretinal surgery, this study adds evidence to the observation that intravitreal injections are associated with a higher risk for humoral immune responses compared to subretinal delivery of AAV vectors. An ongoing trial with

intravitreal application of AAV8 for X-linked juvenile retinoschisis (NCT02416622) will help to further clarify this observation.

More research is needed to understand the complex reaction to AAV in the immune-privileged eye. The ocular immune response against AAV beginning with innate mechanisms and leading to specific humoral/cellular immunity is still poorly understood. It is of eminent importance to gain a good knowledge of the mechanisms underlying the antiviral defense mechanisms of the visual system. This will allow further improvement of safety and enhancement of efficacy of AAV-mediated gene therapies in the eye.

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## APPENDIX

### RD-CURE Consortium

The members of the RD-CURE Consortium are the following: Bernd Wissinger, Martin Biel, Eberhart Zrenner, Karl Ulrich Bartz-Schmidt, Dominik Fischer, Susanne Kohl, Stylianos Michalakis, Francois Paquet-Durand, Tobias Peters, Mathias Seeliger, Marius Ueffing, Nicole Weisschuh, Barbara Wilhelm, Ditta Zobor, Stephen Tsang, Laura Kühlewein, Christian Johannes Gloeckner, and Nadine A. Kahle.