Prevalence and Relevance of Pre-Existing Anti-Adeno-Associated Virus Immunity in the Context of Gene Therapy for Crigler–Najjar Syndrome

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Adeno-associated virus (AAV) vector-mediated gene therapy is currently evaluated as a potential treatment for Crigler–Najjar syndrome (CN) (NCT03466463). Pre-existing immunity to AAV is known to hinder gene transfer efficacy, restricting enrollment of seropositive subjects in ongoing clinical trials. We assessed the prevalence of anti-AAV serotype 8 (AAV8) neutralizing antibodies (NAbs) in subjects affected by CN and investigated the impact of low NAb titers (<1:5) on liver gene transfer efficacy in an *in vivo* passive immunization model. A total of 49 subjects with a confirmed molecular diagnosis of CN were included in an international multicenter study (NCT02302690). Pre-existing NAbs against AAV8 were detected in 30.6% (15/49) of screened patients and, in the majority of positive cases, cross-reactivity to AAV2 and AAV5 was detected. To investigate the impact of low NAbs on AAV vector-mediated liver transduction efficiency, adult wild-type C57BL/6 mice were passively immunized with pooled human donor-derived immunoglobulins to achieve titers of up to 1:3.16. After immunization, animals were injected with different AAV8 vector preparations. Hepatic vector gene copy number was unaffected by low anti-AAV8 NAb titers when column-purified AAV vector batches containing both full and empty capsids were used. In summary, although pre-existing anti-AAV8 immunity can be found in about a third of subjects affected by CN, low anti-AAV8 NAb titers are less likely to affect liver transduction efficiency when using AAV vector preparations manufactured to contain both full and empty capsids. These findings have implications for the design of liver gene transfer clinical trials and for the definition of inclusion criteria related to seropositivity of potential participants.

Keywords: Crigler–Najjar syndrome, UGT1A1, unconjugated hyperbilirubinemia, AAV gene therapy, anti-AAV neutralizing antibodies, pre-existing immunity

INTRODUCTION

CRIGLER–NAJJAR SYNDROME (CN) is an ultrarare autosomal recessive inborn error of metabolism characterized by severe unconjugated hyperbilirubinemia, due to marked reduction or complete lack of uridine diphosphoglucuronosyl transferase 1A1 (UGT1A1) in the liver.[1,2] This enzyme deficiency restricts glucuronidation and subsequent elimination of unconjugated bilirubin (UCB). Accumulation of neurotoxic bilirubin can cause

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encephalopathy, also known as kernicterus, that leads to severe and irreversible disability or death without appropriate treatment.[3] Severely affected patients depend on phototherapy for up to 14 h a day to convert UCB into photoisomers in the skin and underlying capillaries, which can be readily excreted through bile or urine.[4,5] Currently, liver transplantation is the only curative treatment for CN and becomes inevitable at some point in life due to the burden of phototherapy and the long-term complications associated with CN.[4,6] However, liver transplantation is associated with procedure-related complications. Furthermore, the burden and risks related to lifelong immunosuppression and limited availability of donor livers underscore the need to develop novel therapeutic approaches for CN.

Adeno-associated virus (AAV) vector-mediated gene therapy is a potentially curative approach for inherited monogenic disorders and was successfully translated to clinical applications in the past decade.[7–14] Pivotal studies of gene transfer for hemophilia A (NCT03392974) and B (NCT03587116, NCT03569891) are ongoing based on earlier clinical data supporting the safety of liver gene transfer with AAV vectors and on promising preliminary efficacy data on long-term reduction of bleeding episodes.[10–12] Preclinical studies in the relevant models of CN showed complete and sustained correction of plasma bilirubin levels after a single intravenous administration of an AAV serotype 8 (AAV8) vector encoding for the human *UGT1A1* gene (AAV8–h*UGT1A1*).[15–17] Efforts to optimize and develop the vector for clinical application have resulted in a lead candidate investigational vector that is currently under clinical evaluation (NCT03466463).[18,19]

Although AAV-mediated gene therapy holds great promise for treatment of CN, not all patients are eligible candidates for this novel approach. Anti-AAV immunity, which arises after exposure to the wild-type virus, can compromise successful gene transfer after systemic administration of recombinant AAV (rAAV) vectors.[20,21] The neutralizing antibodies (NAbs) that impair the transduction are only a part of the total anti-AAV antibodies.[22,23] The prevalence of NAbs toward different AAV subtypes reported in the literature is variable, but a high prevalence is found for AAV2 ($\sim$30–60%) compared with other serotypes in the general population.[24–26] Reports about seroprevalence of AAV in patients with CN are currently lacking.

The influence of NAbs on the outcome of AAV vector-mediated gene delivery is not fully elucidated. Earlier observations already showed that even low anti-AAV NAbs decrease vector transduction efficiency[27,28]; however, follow-up studies indicate that low NAb titers (<1:5) can, at least partially, be overcome by increasing the administered dose of AAV.[29,30] Besides the NAb titer and the AAV vector dose, also the formulation of the AAV vector preparation (*e.g.*, full to empty capsid ratio) was identified as a modulating factor. Accordingly, increasing the relative amount of empty capsids in the AAV preparation, either deliberately[31] or as a consequence of the vector production protocol,[32] reduces the negative effect of NAbs on transduction efficiency. Based on these findings, here we further investigated the relevance of low NAb titers on hepatic transduction efficiency by AAV vectors to determine whether borderline seropositive subjects could also be eligible for liver-directed gene therapy.

To this aim, we first assessed the prevalence of anti-AAV8 NAbs in a cohort of 49 CN patients and then investigated the relevance of low NAb titers on gene transfer efficacy of different AAV vector preparations in a passively immunized murine model of gene transfer.

MATERIALS AND METHODS

Patient cohort study design

Patients with a genetically confirmed diagnosis of CN who have not received a liver transplant were included in an observational international multicenter study (NCT02302690). At time of inclusion, a serum sample was collected and anti-AAV immunity was assessed centrally at Genethon's laboratory (Evry). This study was reviewed and approved by the independent ethics committees of all five participating sites (Hôpital Antoine Béclère, Clamart, France; Academic Medical Center, Amsterdam, the Netherlands; Azienda Ospedaliera Papa Giovanni XXIII, Bergamo, Italy; Federico II University Hospital of Naples, Naples, Italy; Hannover Medical School, Hannover, Germany) and was carried out in compliance with the Good Clinical Practice guidelines and according to the principles of the declaration of Helsinki. All participants gave written informed consent or, in the case of children, assent and parental consent.

Production and characterization of AAV vectors

AAV vectors were produced following two different manufacturing processes in adherent and in suspension HEK293 cells, as previously described.[19] In brief, adherent HEK293 cells grown in roller bottles were transfected with the three plasmids containing the adenovirus helper proteins, the AAV Rep and Cap genes, and the ITR-flanked

transgene expression cassette. Seventy-two hours after transfection, cells were harvested, lysed by sonication, and treated with Benzonase (Merck-Millipore, Darmstadt, Germany). Vectors were then purified using two successive ultracentrifugation rounds in cesium chloride density gradients. Full capsids were collected, the final product was formulated in sterile phosphate-buffered saline (PBS) containing 0.001% of Pluronic (Sigma-Aldrich, Saint Louis, MO), and stored at –80°C.

For suspension HEK production, AAV vectors were produced in bioreactors by the adenovirus-free transient transfection method. HEK293 cells were transfected with polyethyleneimine (PEI-pro, Polyplus, France) with the same three plasmids used in adherent HEK293 cells. Vectors were purified by a single chromatography column based on AVB Sepharose immunoaffinity (GE Healthcare, Buc, France) before concentration by tangential flow filtration. Purified particles were formulated in Ringer's lactate solution containing 0.001% Pluronic (F68), vialed and stored at –80°C.

Vector titers were determined by quantitative PCR (qPCR). Specific probe and primers used for amplification and detection of viral DNA were forward 5′-GGCGGGCGACTCAGATC-3′, reverse 5′-GGGAGGCTGCTGGTGAATATT-3′, probe 5′-AGCCCCTGTTTGCTCCTCCGATAACTG-3′. Vector titers are expressed in viral genomes per milliliter (vg/mL) and are shown as average of three titration runs.

Anti-AAV NAb titer determination

For determination of anti-AAV8 NAb titer in serum, an *in vitro* reporter system was used as previously described.[33] In brief, 96-well plates were seeded with 2×10^4 2V6.11 cells/well and incubated in DMEM with 10% fetal calf serum at 37°C and 5% CO_2 for 24 h in the presence of ponasterone A (Life Technologies, Carlsbad, CA). Recombinant AAV8-CMV-luciferase (AAV8-CMV-Luc) was diluted in serum-free DMEM (Life Technologies) and incubated with threefold serial dilutions (1:1–1:3,160) of the serum samples, and then incubated for 1 h at 37°C. Subsequently, the serum–vector mixtures were added to the 2V6.11 cells, and after 24 h, cells were lysed with the Bright Glo system (Promega, Madison, WI) and the luciferase activity was measured on a luminometer (ENSPIRE™; Perkin Elmer, Waltham, MA). The NAb titer was reported as the highest serum dilution that inhibited AAV transduction by ≥50% compared with the control without serum (100% transduction). Anti-AAV8 NAb titer was assessed in two independent experiments for all subjects. Any sample with a detectable anti-AAV8 NAb titer (≥1:1) in both experiments was determined as seropositive. Also NAb cross-reactivity to AAV5 and AAV2 was assessed using this *in vitro* reporter method using AAV5-CMV-Luc and AAV2-CMV-Luc in samples that were positive for anti-AAV8 NAbs.

IgM/IgG analysis for AAV8, AAV5, and AAV2

In addition to NAb titer analysis, we assessed the prevalence of both total IgG and IgM binding antibodies to AAV using an enzyme-linked immunosorbent assay as previously described.[24] In brief, 96-well Nunc polysorp immunoplates (Dutscher, Paris, France) were coated with AAV particles to a final concentration of 1 μg/mL. A standard curve made of purified human IgG or IgM (Interchim, Montluçon, France) was added directly to the plates. Plates were coated overnight at 4°C. The next day, after blocking the plates, serum samples were added at dilution of 1:10 and 1:100 in duplicate and incubated overnight at 4°C. Monoclonal antihuman IgG or IgM HRP-conjugated (Southern Biotech, Birmingham, AL) was added to the plates. The enzymatic reaction was developed with substrate solution (3,3′,5,5′-tetramethylbenzidine from Becton Dickinson, Franklin Lakes, NJ). The reaction was stopped with H_2SO_4 3 M solution and optical density measurements were done at 450 nm using a microplate reader (ENSPIRE; Perkin Elmer). Anti-AAV antibody concentration was determined against the IgG or IgM-specific standard curve.

Animal study design

Adult male C57BL/6 mice received a single intraperitoneal administration of pooled donor-derived human intravenous immunoglobulin (IVIg; Grifols, Paris, France) at a dose of 0.5 mg ($n = 12$) or 1.5 mg ($n = 12$) or PBS control ($n = 12$) at day 0. After 24 h, animals received either the AAV8–h*UGT1A1* vector produced by triple transfection of adherent HEK293 cells (ADH) or HEK293 cells cultured in suspension (SUSP) at a dose of 2E12 vg/kg ($n = 6$ per group). At day 22, mice were sacrificed, blood was sampled, and liver tissue was snap frozen in liquid nitrogen and stored at –80°C for further analysis. All animal experiments were performed in accordance with the European Directive 2010/63/EU and with approval of the local Institutional Animal Care and Use Committee (ref.: 2014-009B). Vector genome copy number (VGCN) in liver was determined by qPCR with the same set of primers and probes described for vector titering.

Statistical analysis

Results are presented as frequency with percentage, mean with standard deviation, or median with 25–75%. Statistical methods used for data analysis are specified in the results section and figure legends. A p-value <0.05 was considered statistically significant. Analyses were performed using SPSS (version 24.0) and GraphPad Prism Software (version 7.0).

RESULTS

Baseline characteristics of participants

Between November 2014 and November 2016, a total of 49 subjects affected with CN were enrolled into the observational study. Gender was distributed equally (53.1% female). The median age of participants at inclusion was 20 years (5–26). By medical history, six subjects underwent cholecystectomy and eight subjects other surgical procedures (subtotal thyroidectomy, hysterectomy, mastectomy, or cesarean section). Genetic confirmation of the diagnosis of CN was available in all participants, with the highest allele frequency of c.1220delA (p.K407X) in 16.3% of subjects. All participants were receiving treatment to reduce serum UCB at time of inclusion and during follow-up.

Treatment comprised either phenobarbital, phototherapy, or a combination of both. Among a total of 42 participants receiving phototherapy, 20 of them used it in combination with phenobarbital (Fig. 1). Subjects affected by a milder form of CN were not receiving any phototherapy. Thirty-one participants required ≥8 h of phototherapy exposure per day, representing 63.3% of the total cohort. The mean serum total bilirubin at inclusion was 303.4 ± 108.9 μmol/L. A summary of the cohort's baseline characteristics is given in Table 1.

Prevalence of anti-AAV8 NAbs and serotype cross-reactivity

To assess what percentage of the study population was potentially eligible for inclusion in AAV gene transfer trials, a serum sample was collected from each participant at the time of inclusion. Preexisting anti-AAV8 NAbs were detected in 15 of the 49 screened participants (30.6%), with NAb titers ranging from 1:1 to 1:1,000 (Table 2). Results from two independent experiments show minor deviations of serum anti-AAV8 NAb titers in four subjects (not shown). As previously described,[31] the vast majority of AAV8 seropositive subjects have titers ≤1:100, and only three subjects presented titers of 1:1,000 (Table 2). Individuals seropositive for anti-AAV8 NAbs also presented anti-AAV5 NAb titers in serum, except for three subjects, although lower titers than those against AAV8 were measured (Table 2). As expected, based on the high

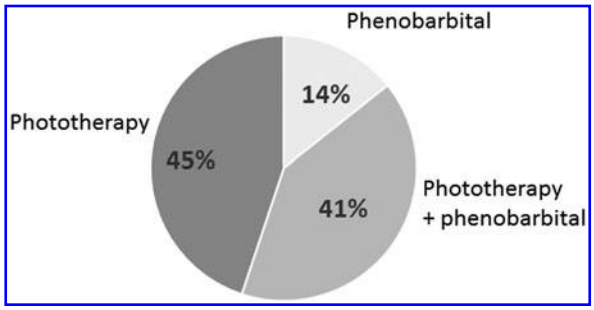

Figure 1. Management of CN in the selected population. Results are reported as percentage of total (n=49). CN, Crigler–Najjar syndrome.

Table 1. *Baseline characteristics of Crigler–Najjar subjects*

N	*49*
Female	26 (53.1%)
Age (years)	20 (5–26)
Allele frequency	
1220DelA	16 (16.3%)
1489DelG	10 (10.2%)
A(TA)7TAA	8 (8.2%)
1381T>C	6 (6.1%)
1070A>G	3 (3.1%)
Other	55 (56.1%)
Phototherapy	
None	7 (14.3%)
<8 h	11 (22.4%)
>8 h	31 (63.3%)
Total bilirubin (μmol/L)	303.4 ± 108.9
Direct bilirubin (μmol/L)	19.0 ± 12.6

Data are presented as frequency (%), median (25–75‰), or mean ± SD

Table 2. *Cross-reactivity of anti-adeno-associated virus neutralizing antibodies*

Gender	*NAb AAV8*	*NAb AAV5*	*NAb AAV2*
Female	1:1,000	1:316	>1:3,160
Female	1:1,000	1:316	>1:3,160
Female	1:1,000	1:1,000	>1:3,160
Female	1:100	1:100	1:316
Female	1:100	1:100	1:3,160
Female	1:100	1:31.6	1:1,000
Female	1:100	1:3.16	1:316
Female	1:100	1:3.16	1:316
Male	1:31.6	1:1	1:31.6
Male	1:10	1:3.16	1:316
Female	1:10	1:3.16	1:100
Male	1:3.16	1:1	1:10
Male	1:1	<1:1	1:31.6
Female	1:1	<1:1	1:31.6
Male	1:1	<1:1	1:3.16

In 15 of 49 participants, anti-AAV8 NAbs were detected and most of them showed cross-reactivity to AAV5 and AAV2. Serum anti-AAV8 NAb titers are shown for all 15 seropositive patients. Cross-reactivity against AAV5 and AAV2 was assessed. NAb titers are expressed as the highest serum dilution that inhibited AAV transduction by ≥50% compared with the control without serum.

AAV, adeno-associated virus; NAbs, neutralizing antibodies.

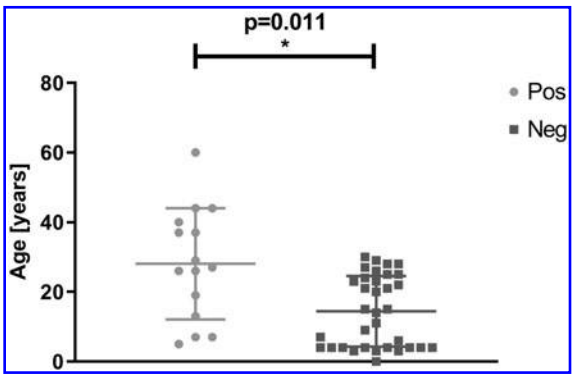

Figure 2. Subjects with pre-existing anti-AAV8 NAbs were older than seronegative subjects. The age in years at time of NAb determination of subjects with NAb titer >1:1 (positive, $n=15$) versus NAb titer ≤1:1 (negative, $n=34$). Two-tailed *t*-test was used to compare groups ($*p<0.05$). NAbs, neutralizing antibodies.

seroprevalence of AAV2 in healthy donors,[24] all anti-AAV8 NAb-positive subjects showed anti-AAV2 NAb titers in serum, which were generally higher than those against AAV8 (Table 2). Notably, subjects with pre-existing anti-AAV8 NAbs were older at time of sampling than seronegative patients ($p=0.011$; Fig. 2).

Thus, based on these results, screening for anti-AAV NAb titers to AAV8, AAV5, and AAV2 in subjects affected by CN showed both seroprevalence and antibody titers similar to those previously documented in healthy donors.

Correlation between anti-AAV NAb titers and total IgG binding antibodies

Anti-AAV NAb titers are often directly correlated to the concentration of circulating anti-AAV IgG,[34] although in some cases low anti-AAV IgG titers are found not to be neutralizing.[23] To evaluate the correlation between NAb titers and binding antibodies, total IgG antibody concentration to AAV8, AAV5, and AAV2 was determined by ELISA in all serum samples that were identified as seropositive for anti-AAV8 NAbs ($n=15/49$). Within the sample set tested, a generally good correlation between NAb and IgG levels was found (Fig. 3A, C, E). Anti-AAV8 NAb titer versus total IgG antibody concentration showed the strongest correlation ($R^2=0.88$) (Fig. 3A), with less strong correlations for AAV5 ($R^2=0.70$) and AAV2 ($R^2=0.56$) (Fig. 3C, E, respectively).

As NAb titers can also be influenced by IgM responses originated by recent exposure to AAV, total IgM antibody concentration against AAV8, AAV5, and AAV2 was also determined by ELISA. In all subjects tested ($n=49$) and for all serotypes, IgM levels were found to be below the limit of detection (Fig. 3B, D, F). These results confirm that NAb titers correlate with IgG levels across serotypes and that IgM is only rarely found.

Effect of low NAb titers on liver transfer efficiency and relationship with AAV vector purification method

AAV vectors can be manufactured according to different protocols[35] and, mainly depending on the downstream purification method, a mixture of variable proportions of empty and full capsids can be found in the final product. As empty capsids present in AAV vector preparation can greatly affect the outcome of liver gene transfer,[31] we next tested the impact of low-titer NAbs on murine liver transfer efficiency with different preparations of AAV8–h*UGT1A1* vector. AAV8–h*UGT1A1* vectors made by transfecting HEK293 cells grown in adherent cultures (AAV8 ADH) and purified by double CsCl gradient[35] were compared with preparations of the same vector construct using suspension HEK293 cell cultures (AAV8 SUSP) and column purification.[19] Although AAV8 ADH had virtually no empty capsid in the final purified product, AAV8 SUSP had a ratio of about 1:5 full to empty capsids.[19]

The impact of low anti-AAV8 NAb titers on hepatic gene transfer efficacy after systemic delivery of rAAV8–h*UGT1A1* was assessed by measuring VGCNs in liver tissue after transduction of passively immunized mice (Fig. 4A). To minimize the potential issue of differences in infectivity of the vector preparations made with the two methods, a PBS-treated control group was included for both animals infused with adherent and suspension preparations of the AAV8–h*UGT1A1* vector. One day after intraperitoneal administration of pooled human IVIg in C57BL/6 mice, low titers of anti-AAV8 NAbs up to 1:3 were measured in serum of these animals (Fig. 4B). Animals randomly received one of the two vector preparations through tail vein injection 24 h after passive immunization. VGCN in liver tissue, assessed 3 weeks after vector administration by qPCR, was decreased by 60% ($p<0.01$) in animals that were passively immunized with the highest dose of IVIg (1.5 mg) before the AAV8 ADH vector injection (Fig. 4C). Conversely, no decrease in VGCN was detected in animals receiving the AAV8 SUSP vector (Fig. 4D), suggesting that vector preparations of AAV8–h*UGT1A1* containing empty capsids have a more efficient transduction potential in the presence of NAbs.

Together, these results indicate that even seemingly small differences in vector manufacturing can potentially affect the outcome of liver gene transfer.

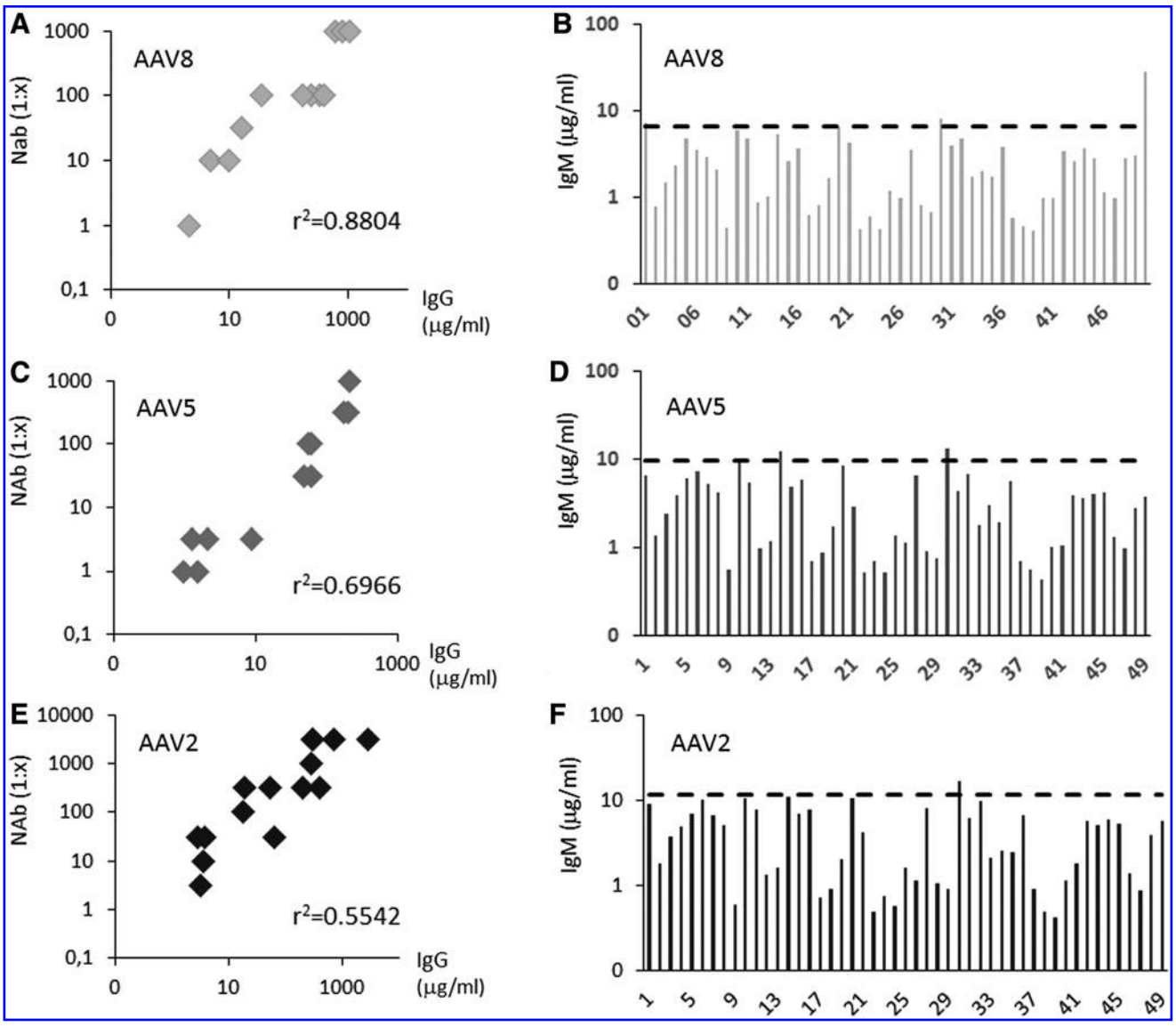

Figure 3. Correlation between anti-AAV8 NAb titers and total IgG and IgM to AAV serotypes 8, 5, and 2 in subjects affected by CN. **(A)** Anti-AAV8 NAb titer versus total IgG binding antibodies to AAV8 measured in seropositive subjects ($n=15$). **(B)** IgM binding antibodies to AAV8 ($n=49$). **(C)** Anti-AAV5 NAb titer versus total IgG binding antibodies to AAV5. **(D)** IgM binding antibodies to AAV5. **(E)** Anti-AAV2 NAb titer versus total IgG binding antibodies to AAV2. **(F)** IgM binding antibodies to AAV2. NAb titers are reported as reciprocal dilutions (1:x). The *dotted line* represents the limit of quantification for IgM.

DISCUSSION

AAV-mediated gene therapy for CN has been established in animal models supporting efforts toward translation into clinical trials. Currently, not all individuals affected by severe CN are eligible candidates for this novel approach due to preexisting immunity to AAV vectors. In this study, we assessed the prevalence of both anti-AAV NAbs and total immunoglobulin G (IgG) binding antibody concentrations in a cohort of 49 CN patients. Since we identified subjects who were borderline seropositive, with an anti-AAV8 NAb titer <1:5, we also assessed the relevance of low NAb titers on gene transfer efficacy in a murine model.

At time of inclusion, NAbs against AAV8 were detected with a frequency that was comparable with previous reports.[24,25] In addition, we observed a broad cross-reactivity with both relatively conserved (AAV2) and distant (AAV5) serotypes.[36] A broad cross-reactivity of NAbs toward different AAV subtypes has been described previously and is likely the result of the overall high amino acid sequence and structure homologies across AAV serotypes.[21] We observed a higher cross-reactivity between AAV8 and AAV2 than between AAV8 and AAV5. This is likely the result of closer structural homology between AAV8 and AAV2 than between AAV8 and AAV5, as previously described.[24] An-

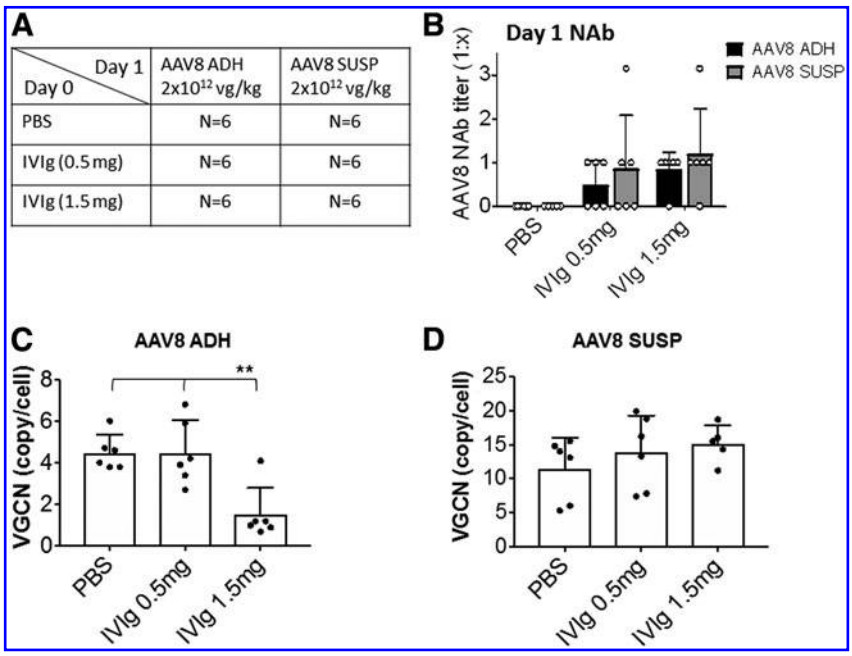

Figure 4. VGCNs in liver of passively immunized mice after receiving AAV vector preparations obtained with different manufacturing methods. **(A)** Experimental design, indicating passive immunization regimens with IVIg at day 0 followed by the administration of two different AAV vector preparations at day 1; a total of six experimental groups with $n=6$ per group were included in the study and all animals were sacrificed at day 22 postvector infusion. **(B)** Anti-AAV8 NAb titer (reciprocal dilution, 1:x) measured by an *in vitro* reporter assay, 24 h after the three immunization regimens. **(C, D)** VGCN assessed by qPCR in liver tissue at the time of sacrifice (d22) in animals receiving vectors made by triple transfection of adherent HEK293 cells (AAV8 ADH) or HEK293 cells cultured in suspension (AAV8 SUSP). Results are expressed as fold relative to PBS-treated animals. Statistical significance was determined with one-way ANOVA with Bonferroni post-test comparison of all treatment groups (**$p<0.01$). Data are represented as mean ± standard deviation. IVIg, intravenous immunoglobulin; PBS, phosphate-buffered saline; qPCR, quantitative PCR; VGCN, vector genome copy number.

other finding in this study is that older subjects appeared to have a higher chance to be seropositive. Since pre-existing anti-AAV immunity arises after exposure to the naturally occurring virus, this finding is not surprising and is aligned with previously published reports.[37,38] The lack of detection of IgM-positive subjects may be dependent on sporadic exposure to the wild-type virus and on short-term persistence of IgM that are typically produced over a brief period of time before isotype switching. In addition, longitudinal studies in humans showed that anti-AAV antibody titers tend to be very stable over time,[39] suggesting that multiple infections with wild-type AAV are uncommon, and so the appearance of IgM. As we previously described,[34] also in this study we identified a strong correlation between NAb titer and total IgG antibody concentration.

By investigating the relevance of low anti-AAV8 NAb titers to liver gene transfer efficiency after systemic delivery of AAV8–h*UGT1A1* in passively immunized mice, we identified a significant difference between the AAV vector preparations based on empty capsid content. In particular, in an effort to initiate a phase I/II trial in individuals affected by CN (NCT03466463), we developed and optimized an AAV8–h*UGT1A1* vector to correct the CN phenotype in murine models,[18] and scaled up manufacturing to provide adequate amounts of clinical-grade vector.[19] An important step in this process was changing the production method from transient triple transfection of adherent HEK293 cells to transfection of HEK293 cells in suspension grown in high-volume bioreactors.[40]

The production process using cell culture in suspension is dependent on a column-based affinity purification technique that, in contrast to the original adherent cell method, does not completely eliminate the empty particles from the final drug product. The presence of empty capsids in the AAV8 SUSP preparations does not seem to influence liver transduction efficacy.[19] However, based on previous reports on empty capsids acting as decoys for NAbs,[31] here we sought to establish the tolerance of AAV8 SUSP vectors to low levels of NAbs and provide preliminary evidence of the impact of changes in AAV vector manufacturing on the impact of gene transfer in liver. Our observation that VGCN in liver of passively immunized mice is not reduced after administration of AAV8 SUSP is in line with the hypothesis that efficacy of gene transfer can be maximized by a mixture of empty and full capsids. Whether anti-AAV NAb titers can be overcome in

human subjects by modulating the empty-to-full capsid ratio warrants further investigation, although evidence that this approach is clinically feasible comes from earlier studies of gene transfer for hemophilia B.[11] However, establishing the optimal full-to-empty capsid ratio and maximal NAb titer at which AAV vector transduction efficacy remains unaffected is challenging, as the total vector dose also plays a role in overcoming the anti-AAV NAbs. Furthermore, it is likely that this approach is only valuable as a rescue measure for individuals with low NAb titers, and other strategies will be required to circumvent the negative effects of anti-AAV NAbs in high-titer seropositive subjects. A potential disadvantage of administrating empty capsids present as contaminants of vector preparations is that this increases the total capsid dose, which may result in the activation of capsid-specific T cells, potentially limiting the duration of transgene expression.[20,41]

Alternative approaches to overcome pre-existing AAV immunity have extensively been reviewed.[21,42] One particularly promising approach, aimed at reducing anti-AAV antibodies from the circulation before vector administration, is plasmapheresis.[43,44] Extracorporeal plasma exchange techniques are routinely used in the clinical setting to remove undesired immunoglobulins from the systemic circulation. In the setting of humoral immunity to AAV, it has been demonstrated that sequential plasmapheresis cycles can lead to a substantial reduction and even depletion of anti-AAV NAbs.[44] Furthermore, studies in seropositive nonhuman primates showed that removing NAbs by plasmapheresis preserved transgene expression at levels comparable with those of treated seronegative animals after AAV vector-mediated gene transfer.[43] Plasmapheresis techniques are readily available for clinical use and are generally regarded as a safe medical procedure, rendering this method attractive to precede AAV vector administration in anti-AAV seropositive patients. However, one caveat related to plasmapheresis is that the extremely high NAb titers triggered by vector administration are likely to render the technology unuseful for vector readministration. In this setting, pharmacological blockade of antibody formation may be a better option.[45]

In summary, pre-existing anti-AAV immunity was found in ~30% of Crigler–Najjar subjects, which currently restricts enrollment of a significant proportion of patients in ongoing gene therapy trials. Although the development of strategies to circumvent the negative effects of NAbs on AAV-mediated transduction efficacy is an urgent task for the field, this study showed that low NAb titers, found in the context of natural immunity to AAV, can be overcome by administrating AAV preparations containing both full and empty capsids, offering a potential approach to treat a subgroup of borderline seropositive patients.

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AUTHOR DISCLOSURE

F.M. is an employee and equity holder of Spark Therapeutics. F.M. and F.C. are inventors in patents describing liver gene transfer approaches for metabolic diseases. L.D. has consultancy agreements with Alexion Biosciences and Vivet Therapeutics. None of the other authors declare any conflicts of financial interest.

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