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## **Prevalence and long-term monitoring of humoral immunity against adeno-associated virus in Duchenne Muscular Dystrophy patients**

Christian Leborgne<sup>1</sup>, Virginie Latournerie<sup>1</sup>, Sylvie Boutin<sup>1</sup>, Diana Desgue<sup>1</sup>, Aliénor Quéré<sup>1</sup>, Elodie Pignot<sup>1</sup>, Fanny Collaud<sup>1</sup>, Séverine Charles<sup>1</sup>, Marcelo Simon Sola<sup>1</sup>, Elisa Masat<sup>2</sup>, Fabienne Jouen<sup>3</sup>, Olivier Boyer<sup>3</sup>, Carole Masurier<sup>1</sup>, Federico Mingozzi<sup>1\*</sup>, Philippe Veron<sup>1\*</sup>

1. Genethon and INSERM U951, Evry, 91000 France
2. University Pierre and Marie Curie – Paris 6 and INSERM U974, Paris 75005, France
3. Normandie Université, UNIROUEN, Inserm, U1234, Rouen University Hospital, Department of Immunology, Rouen, France

\*Corresponding authors:

Philippe Veron, Genethon, 1, bis rue de l'Internationale, 91000 – Evry - France, [veron@genethon.fr](mailto:veron@genethon.fr)

Federico Mingozzi, Genethon, 1, bis rue de l'Internationale, 91000 – Evry - France, [fmingozzi@genethon.fr](mailto:fmingozzi@genethon.fr)

## **ABSTRACT**

Adeno-associated virus (AAV) vectors are promising candidates for gene therapy and have been explored as gene delivery vehicles in the treatment of Duchenne Muscular Dystrophy (DMD). Recent studies showed compelling evidence of therapeutic efficacy in large animal models following the intravenous delivery of AAV vectors expressing truncated forms of dystrophin. However, to translate these results to humans, careful assessment of the prevalence of anti-AAV neutralizing antibodies (NAbs) is needed, as presence of preexisting NABs to AAV in serum have been associated with a drastic diminution of vector transduction. Here we measured binding and neutralizing antibodies against AAV serotype 1, 2, and 8 in serum from children and young adults with DMD (n=130). Results were compared with to age-matched healthy donors (HD, n=113). Overall, approximately 54% of all subjects included in the study presented IgG to AAV2, 49% to AAV1, and 41% to AAV8. A mean of around 80% of IgG positive sera showed neutralizing activity with no statistical difference between DMD and HD. NAb titers for AAV2 were higher than AAV1, and AAV8 in both populations studied. Older DMD patients (13-24 years old) presented significantly lower anti-AAV8 IgG4 subclass. Anti-AAV antibodies were found to be decreased in DMD patients subjected to a 6-month course of corticosteroids and in subjects receiving a variety of immunosuppressive drugs including B cell targeting drugs. Longitudinal follow up of humoral responses to AAV over up to 6 years showed no change in antibody titers, suggesting that in this patient population, seroconversion is a rare event in humans.

**Keywords:** Duchenne Muscular Dystrophy, Gene therapy, AAV humoral immunity

## 1. Introduction

Duchenne Muscular Dystrophy (DMD) was identified as a genetic disorder by several groups in the mid-19th century [1, 2]. The disease is inherited in an X-linked recessive pattern, but one-third of all cases arise from spontaneous mutations. The phenotype of the disease is characterized by a progressive loss of skeletal muscle mass, an increasing weakness, and a late-onset cardiomyopathy. Approximately 30% of patients presents defects in cognitive function, and smooth muscle manifestations leading to gastrointestinal symptoms [1, 2]. Corticosteroids, such as prednisone [3] are commonly given to DMD patients to slow down muscle loss. More recently, an oligonucleotide therapy was approved for DMD patients with mutations amenable for exon 51 skipping [4]. DMD is among the most common single-gene disorders in humans, affecting 1 in 5,000 newborn males [5].

Among the gene transfer technologies being investigated for *in vivo* delivery, viral vectors based on adeno-associated virus (AAV) showed great promise. Adeno-associated virus (AAV) belongs to the parvovirus family and is a non-enveloped single-stranded DNA virus. The AAV capsid is composed of three proteins designated VP1, VP2 and VP3. AAV relies upon a helper virus such as adenovirus for active replication, and in the absence of a helper virus establishes a latent state in which its genome is maintained episomally or integrated into the host genome [6]. Recombinant AAV vectors are ideal gene transfer tools as, like their wild-type counterpart, they lack pathogenicity, they are replication deficient, and they do not integrate into the host genome efficiently. Additionally, AAV vectors are nonpathogenic for human, are less immunogenic than other vectors and are able to establish long-term transgene expression in both dividing and non-dividing cells from a broad host range. AAV gene transfer has resulted in several examples of success in human trials [7-14].

Several AAV serotypes have emerged as interesting candidates for muscle-directed gene transfer, in particular for the treatment of DMD [15-20]. Recently, an AAV8 vector encoding for micro dystrophin have been used to target the muscle in DMD dogs following systemic vector delivery, resulting in long-term therapeutic efficacy [21]. Together with other published results on gene transfer for DMD [15-20, 22-24],

this study provides critical data supporting the safety and feasibility of the approach in patients.

However, in translating AAV gene therapy to humans, immune responses against the vector may represent an obstacle to achieving long-term therapeutic transgene expression. Indeed, in clinical trials, cellular immune responses against the AAV capsid resulted in the loss of transgene-expressing cells [7, 25-30]. Additionally, it has been demonstrated that humoral immunity to AAV can prevent AAV vector transduction in seropositive subjects and prevents repeated vector administrations [29, 31]. In particular, published results indicate that even low levels of neutralizing antibodies (1:5-1:10) can completely abrogate transduction with AAV vectors [29, 31, 32].

Several groups reported the frequency of anti-AAV antibodies for several serotypes, including AAV1, 2, 5, 6, 7, 8 and 9. Based on these studies, 30% to 80% of healthy donors carry antibodies binding to the AAV capsid [33-43]. Similarly, prevalence of AAV antibodies in various patient populations has been reported, although no detailed reports on the AAV serology in DMD patients are available in the literature [37, 44]. Thus, the goal of this study was to determine the prevalence and profiles of serum IgG subclasses and neutralizing antibodies against AAV serotypes 1, 2, and 8 in DMD patients and compare results with an age-matched cohort of healthy donors. Results obtained highlight unique features of humoral immunity to AAV in DMD patients, and provide insights into the dynamics of anti-AAV antibody titers in humans over time.

## **2. Materials & Methods**

### ***2.1 Sample collection***

This study was conducted according to the principles of the declaration of Helsinki “Ethical Principles for Medical Research Involving Human Subjects”. Serum samples were obtained from 243 subjects (130 DMD patients and 113 healthy donors) divided into three groups: 38 younger children (3–7 years old), 45 older children (8-12 years old), and 47 adolescents and adults (>12 years old) DMD patients. As an aged matched control, 33 younger children, 40 older children, and 40 adolescent and adult healthy donors were included in the study. DMD patient and healthy donor samples were collected in different centers in Europe as part of the ADNA (Avancées Diagnostiques pour de Nouvelles Approches thérapeutiques) project ([http://www.institut-merieux.com/projetssante\\_adna.php](http://www.institut-merieux.com/projetssante_adna.php)). Samples from additional healthy donors or subjects receiving immunosuppression were obtained from Rouen University Hospital (France). All samples were de-identified and collected according to the local regulations.

Informed consent was obtained from all subjects prior to inclusion in the study. All samples were de-identified and collected according to the local regulations. After collection, samples were centrifuged immediately ( $10\,000 \times g$ , 10 min) and serum separated and stored at  $-80^{\circ}\text{C}$ . Before testing, serum samples were heat inactivated at  $56^{\circ}\text{C}$  for 30 minutes.

### ***2.2 Production of AAV vectors***

AAV vectors were generated by pseudotyping an AAV2-based recombinant genomes into AAV1, AAV2, AAV5, AAV6, AAV8 and AAV9 capsids. All the vectors used in the study were produced using a transfection protocol as previously described[45]. Briefly, HEK293 cells were triple-transfected with the adenovirus helper plasmid, a plasmid encoding for the *rep* and *cap* genes, and a plasmid encoding for the transgene expression cassette flanked by the AAV inverted terminal repeats (ITRs). Single stranded AAV vectors expressing luciferase under the transcriptional control of the cytomegalovirus immediate early (CMV IE) promoter associated with the SV40

polyA signal were produced. Recombinant vectors were purified by double cesium chloride gradient centrifugation followed by dialysis against sterile phosphate-buffered saline (PBS). Vector titers were determined by real time PCR and expressed as viral genomes per ml (vg/mL).

### **2.3 Anti-AAV antibody ELISA**

Recombinant AAV particles were diluted in coating buffer (0.1M bicarbonate, 0.034M carbonate, pH 9.5) to a final concentration of  $2 \cdot 10^{10}$  vector particles per mL. Fifty microliters were added to each well in a 96-well Nunc Maxisorp Immunoplate (ThermoFisher, Waltham, USA). Proteins corresponding to contaminants purified during the different steps of AAV production, but in absence viral particle, were plated concentration of 3.4 $\mu$ g/ml (non-specific signal). Plates were coated overnight at 4°C. The next day, plates were washed three times with blocking buffer (PBS, 6% fat-free milk) then blocked with blocking buffer for 2 hours at room temperature. Plates were again washed three times with wash buffer (PBS, 0.05% Tween-20) then incubated with heat inactivated serum serially diluted from 1:3 to 1:65610, 1h at 37°C. After 3 washes, antibody specific for total IgG and conjugated with Horseradish Peroxidase, HRP (Southern Biotech, Birmingham, USA) was incubated 1h at 37°C. After incubation, plates were washed three times with wash buffer and revealed with substrate solution (3,3',5,5'-Tetramethylbenzidine from Becton Dickinson, Franklin Lakes, USA) incubated for 30 minutes in the dark. The reaction was stopped with H<sub>2</sub>SO<sub>4</sub> 3M solution and optical density (OD) measurements were done at 450nm using a microplate reader (ENSPIRE™, Perkin Elmer, Waltham, USA). The AAV specific signal was reported as the OD from AAV coated ELISA after subtraction of the non-specific signal. Sera were considered positive for AAV-specific Ig when OD signal were  $\geq 0.5$  (cutoff based on mean OD of 50 negative healthy donors + 3 SD) at dilution  $\geq 1:3$ .

For IgG subclasses ELISA, as previously described[46], 96-well Nunc polysorp Immunoplates (Dutscher, Paris, France) were coated with rAAV, to a final concentration of 1 $\mu$ g/ml (which represent around  $5 \cdot 10^8$  vg/well). A standard curve

made of purified human IgG (Interchim, Montluçon, USA) was added directly to the plates. Plates were coated overnight at 4°C. The next day, after blocking step, serum samples were added at dilution of 1:10 and 1:100 in duplicate and incubated overnight at 4°C. An HRP-conjugated monoclonal antibody specific for each human IgG subclass (Southern Biotech, Birmingham, USA) was added to the plates. The enzymatic reaction was developed with substrate solution (3,3',5,5'-Tetramethylbenzidine from Becton Dickinson, Franklin Lakes, USA). The reaction was stopped with H<sub>2</sub>SO<sub>4</sub> 3M solution and optical density (OD) measurements were done at 450nm using a microplate reader (ENSPIRE™, Perkin Elmer, Waltham, USA). Anti-AAV IgG subclass concentration was determined against the specific standard curve using 4-parameters regression and results were expressed as µg/ml of IgG.

#### ***2.4 Neutralizing antibody assay***

The NAb assay was performed as previously described [47]. Briefly, on day 1, 96-well plates were seeded with  $2 \times 10^4$  2V6.11 cells/well for 24 hours in presence of ponasterone A (Life Technologies, Carlsbad, USA). The 2V6.11 cell line inducibly expresses the human adenovirus E4 ORF 6 34 kDa oncoprotein. This cell line was described by Mohammadi et al. [48]. The highest efficacy of AAV transduction with this cell line was stated in Meliani et al. [47] for AAV6, 8 and 9. Ellsworth et al also published data recently with this cell line in comparison to huh7 cells. In this study, we used a MOI of 200 for each serotype leading to a transduction depending on the serotype of 80, 000 RLU, 200, 000 RLU and 20, 000 RLU for AAV1, AAV2 and AAV8, respectively. Recombinant AAV-CMV-Luciferase (AAV-CMV-Luc) was diluted in serum-free DMEM (Life Technologies, Carlsbad, USA) and incubated with 2-fold serial dilutions (1:2 to 1:12800) of the serum samples, and then incubated for 1 hour at 37°C. Subsequently, the serum-vector mixtures were added to the cells incubated in DMEM with 10% FCS at 37°C and 5% CO<sub>2</sub>. Each dilution was tested in triplicate. After 24 hours, cells were lysed with the Bright Glo system (Promega, Madison, USA) and the luciferase activity was measured on a luminometer (ENSPIRE™, Perkin Elmer, Waltham, USA). Transduction efficiency was measured as Relative Light Unit



(RLU) per second. The neutralizing titer was reported as the highest serum dilution that inhibited AAV transduction by  $\geq 50\%$  compared with the control without serum (100% transduction).

## ***2.5 Statistical analyses***

Results are presented as percentages and titers. Student's t-test, unpaired parametric and Welch's t-test, were used to determine significant differences between two groups (when results are percentages) and  $X^2$  test was used to compare groups with results expressed as titers. A p value  $< 0.05$  was considered statistically significant.

### 3. Results

#### ***3.1 AAV seroprevalence in healthy donors and DMD patients is similar***

Total IgG antibodies to AAV serotype 1, 2, and 8 were determined in a cohort of 243 serum samples from healthy donors (HD) and DMD patients (Figure 1). The highest seroprevalence was observed for AAV2 (52% and 55% of IgG positive HD and DMD subjects, respectively, Figure 1A). AAV1 seroprevalence was slightly lower (46% and 52% of IgG positive HD and DMD subjects, respectively). As expected [39, 40], compared to other serotypes, a lower seroprevalence was observed for AAV8 (39% and 44% of IgG positive HD and DMD subjects, respectively). Serum samples with detectable IgG titers were analyzed for the presence of neutralizing antibodies against AAV1, AAV2, and AAV8 (Figure 1B). Sera were scored positive for neutralizing activity when at a 1:2 dilution of serum inhibited vector transduction by 50% or more. Up to ~80% of the IgG-positive samples showed neutralizing activity to AAV1 in both HD and DMD patients, while slight differences in the frequency of NAb-positive subjects were noted in HD vs. DMD patients for AAV2 and AAV8 (Figure 1B). Next, for each serotype the percentage of seropositive individuals with antibody titers below 1:10, from 1:10 to 1:270, and >1:270 for IgG, and below 1:10, from 1:10 to 1:200, and >1:200 for NAb was determined (Figure 1C-E). No differences were observed across IgG titer ranges in HD and DMD patients. Conversely, the percentage of subjects with NAb titers >1:200 was the higher for AAV1 (45% and 39% in HD and DMD patients, respectively) and AAV2 (64% and 56% in HD and DMD patients, respectively) than for AAV8 (6% and 15% in HD and DMD patients, respectively, Figure 1C, D). Even at high titers, the anti-AAV8 IgG seems to have less neutralizing abilities compared to AAV1 and AAV2 IgG. These results are consistent with published data in HD and show that anti-AAV antibody prevalence in DMD patients is similar to HD. Moreover, IgG titers and NAb titers showed a coefficient of correlation of 0.83 in 243 individuals (healthy donors and DMD patients) which is also consistent with published data [49].

### ***3.2 Age effect on seroprevalence of AAV in HD and DMD patients***

Next, we explored the effect of age on the prevalence of anti-AAV antibodies. For this purpose, IgG and NAb titers were stratified by age (Figure 2A-F). No statistical differences were found between DMD patients and healthy donors. These results confirm the age stratification of anti-AAV antibody seroprevalence in humans.

### ***3.3 AAV specific IgG subclass responses in HD and DMD patients***

We next used an ELISA to determine the relative levels of IgG subclasses specific to the different AAV serotypes in HD and DMD subjects. Serum samples from 34 HD and 47 DMD patients positive for IgG specific to AAV8 were analysed (Figure 3). Plates were coated with both AAV8 particles and a standard for each IgG subclasses allowing quantification of IgG1, IgG2, IgG3 and IgG4. IgG subclasses specific for AAV8 capsid were similar across HD and DMD patients (Figure 3). The dominant IgG subclass was IgG1 (Figure 3A), followed by IgG3 (Figure 3C). IgG2 and IgG4 (Figures 3B,D) were found at lower levels but still detectable in most samples tested. Samples from DMD patients showed a significant decrease in IgG4 titers as a function of age, with lower levels of this antibody subclass in older subjects. IgG4 titers measured in older DMD patients were also significantly lower in age-matched healthy donor (Figure 3D).

### ***3.4 Immunosuppression has limited effect on pre-existing anti-AAV antibodies***

Randomized controlled trials show that corticosteroid therapy in DMD improves muscle strength in the short-term (six months to two years) and delays the loss of ability to walk [50]. In our study, 26 DMD patients were under long-term corticosteroid treatment at the time of blood collection. We therefore investigated the effect of this immunosuppressive treatment on anti-AAV antibodies. No major difference in antibody titers against any of the serotypes tested was noted (Figure 4A, B). We next analyzed a subset of 13 DMD patients for which serum samples were available before and six month after corticosteroid treatment. In this subset of patients, a

reduction in AAV8 IgG and NAb titers was observed in 10 out of 13 subjects for IgG and 9 out of 13 subjects for NAb titer (Figure 4C, D). These results suggest that long-term treatment with corticosteroids can only partially dampen anti-AAV antibody titers. To further this finding, we surveyed NAb titers in 100 healthy donors and 100 donors who received immunosuppressive drugs as part of management of an underlying condition. While some differences in NAb titers were measured, particularly for subjects receiving B cell targeting drugs, no drug resulted in complete absence of antibodies to AAV (Figure 4E).

### ***3.5 Longitudinal follow up of AAV8-specific NAb in DMD patients reveals no change in titers over time***

No data is available in the literature on the changes in natural humoral immunity to AAV in humans over time. To address this point, we measured the variations of AAV antibody titers in the same cohort of 31 DMD patients over time for a maximum of 6 years of follow up without any immunosuppressive treatment. Interestingly, with the exception of few minor fluctuations, no change in NAb titers against AAV8 was observed in 26/31 patients (Table 1). Moreover, of the 19 seronegative patients, only two had NAb titers increasing over time from undetectable levels to a titer of 1:5. Similarly, only three subjects with detectable NAb titers returned to undetectable level. These results suggest that humoral immunity to AAV in this patient population is established very early in life and it does not frequently change over time.

## 4. Discussion

Humoral immune responses against wild-type AAV are commonly found in humans and represent a major limitation to efficient gene transfer with AAV vectors. In view of a future clinical trial, here we show a large seroprevalence study performed in DMD patients and aged matched healthy donors. Results from this study confirm previous findings and provide additional insights into antibody responses in humans triggered by the exposure to wild type AAV.

AAV vectors are one of the most promising platform for therapeutic gene transfer to treat many genetic and acquired diseases, including neuromuscular diseases [51]. For this reason, knowledge on pre-existing humoral immunity against AAV serotypes commonly adopted in clinical trials is valuable. The study presented here is relevant to the clinical translation of a gene therapy approach for DMD based on AAV as it evaluates the proportion of patients potentially eligible for gene transfer (i.e. seronegative). Results presented indicate that the prevalence of anti-AAV antibodies in patients affected by DMD is virtually identical to that found in HD and similar to that found in previously published studies [39, 43]. This was true for both binding and neutralizing antibodies, with few exceptions. In this survey, AAV8 scored as the serotype with the most favorable seroprevalence, as a lower number of individuals carry antibodies binding to this serotype and generally titers are lower than AAV1 and AAV2. Accordingly, seroprevalence of AAV2 was the highest in our study as previously published [39, 43]. Based on published work evaluating cross-reactivity of anti-AAV antibodies, subjects seropositive for AAV8 are likely to be seropositive also for AAV9, another serotype explored for gene therapy for DMD [52, 53]. No differences in AAV titers were noted in DMD patients versus healthy donors when antibody titers were stratified by age. Of note, in our study no statistical differences were noted in IgG or NAb titers in all age groups, a result in line with previously published surveys [33, 40, 54, 55].

As previously described for AAV2 [56], natural exposure to AAV types 1, 2 or 8 can result in production of antibodies from all four IgG subclasses, with a predominant

IgG1 response. Here we also detected relatively high IgG3 titers, a result not expected based on the low circulating levels of this subclass (7.3% of total IgG, compared to 66% for IgG1, 23% for IgG2, and 4% for IgG4). Of note, production of IgG3 in response to AAV exposure was previously reported ([56, 57]. Together with IgG1, the role of these effector phase antibody subclasses could be related to complement activation, targeting of AAV to macrophage through FC receptor binding, or activation of NK cells [58]. IgG4 subclass levels were found to be reduced in older DMD patients. Synthesis of IgG4 reflects long-lasting or repeated exposure to an antigen [59-63]. Additionally, this particular subclass of IgG is known to bind to mastocytes, which play an important role important in muscle inflammation and regeneration, particularly in DMD patients [64]. One point of interest of these results is that IgG subclass analysis in humans seem to correlate closely to results in mice [65].

Several strategies to overcome pre-existing immunity to AAV vectors have been described (reviewed in [66]). Among these, immunosuppression with B cell targeting drugs has shown some effect in lowering anti-AAV titers [67, 68]. As long term corticosteroids are commonly administered in DMD patients, we explored their effect on anti-AAV titers. Unexpectedly, as this class of immunosuppressive drugs does not target B cells specifically, a slight drop in titers was observed in some subjects, a result consistent with published findings [69]. A similar survey in subjects undergoing immunosuppression resulted in a similar result, mostly confirming that eradication of pre-existing immunity to AAV is not easy to achieve with pharmacological approaches.

Perhaps one of the most intriguing findings of the study presented here is the fact that anti-AAV antibody titers tend to be very stable over time. While this is expected for subjects already immunized to AAV, as the presence of antibodies would prevent reinfection, our data indicate that, even in seronegative individuals, changes in anti-AAV titers over time are rare and minimal in magnitude. Whether this finding is unique to DMD patients and to AAV8 will have to be addressed in future studies. One possibility is that exposure to AAV2 (the most prevalent serotype in humans [43], or another AAV serotype that does not cross-react to AAV8, is not detected in the NAb

assay used, thus resulting in stable titers over time. Alternatively, upon exposure to AAV, some subjects may not develop antibodies to AAV but only T cells reactive to the capsid [49].

In conclusion, this work represents a comprehensive survey of anti-AAV titers in a large population of DMD patients. Results highlight common features of humoral immunity to AAV in this patient population compared to healthy donors and confirm previous findings on the effect of immunosuppression on anti-AAV antibody titers. The stability of anti-AAV titers in humans over an extended period of time is a feature of humoral immunity to AAV that was not previously documented but that will be instrumental to guide the monitoring of NAb titers in patients prior to enrollment in gene therapy trials.

Results exposed in this article highlight unique features of humoral immunity to AAV in DMD patients, and provide insights into the dynamics of anti-AAV antibody titers in humans over time.

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## Author contributions

P.V., C.L., E.M., F.J., V.L., S.B., D.D., A.Q., and E.P. contributed to experimental activities. P.V., C.L., C.M., O.B., and F.M. provided critical insight on experimental design and data interpretation. P.V., C.L., and F.M. wrote the manuscript.

## Conflict of interest

F.M. is employee of Spark Therapeutics. All other authors declare no conflict of interest associated with the current work.

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## Figure legends

**Figure 1. Seroprevalence of AAV1, AAV2, and AAV8 in HD vs. DMD patients.** **A.** IgG levels measured in HD (n=108 for AAV1, n=113 for AAV2, n=113 for AAV8) and in DMD patients (n=124 for AAV1, n=130 for AAV2, n=130 for AAV8). Cutoff for positivity was established as 1:3. **B.** Neutralizing antibody (NAb) titers to AAV measured in anti-AAV IgG-positive samples from HD (n=36 for AAV1, n=43 for AAV2, n=43 for AAV8) and from DMD patients (n=54 for AAV1, n=62 for AAV2, n=58 for AAV8). Cutoff for positivity was established as 1:2. **C.** Ranges of antibody titers defined as low, intermediate, and high. **D.** Percentage low, intermediate, and high titer IgG and NAb titers for AAV1, AAV2, and AAV8 in HD. The same number of samples as in panels A and B were used in this analysis. **E.** Percentage low, intermediate, and high titer IgG and NAb titers for AAV1, AAV2, and AAV8 in DMD patients. The same number of samples as in panels A and B were used in this analysis. The stars indicate the statistical difference measured as a p value with \*p < 0.05 and \*\*p < 0.01 (Chi<sup>2</sup> test)

**Figure 2. Age stratification of seroprevalence for AAV1, AAV2, and AAV8 in HD vs. DMD patients.** **A.** IgG levels measured in HD (31<n<40) and in DMD patients (37<n<44) specific for AAV1. Cutoff for positivity was established as 1:3. **B.** Neutralizing antibody (NAb) titers to AAV1 measured in anti-AAV1 IgG-positive samples from HD (10<n<13) and from DMD patients (18<n<20). Cutoff for positivity was established as 1:2. **C.** IgG levels measured in HD (33<n<40) and in DMD patients (38<n<47) specific for AAV2. Cutoff for positivity was established as 1:3. **D.** Neutralizing antibody (NAb) titers to AAV2 measured in anti-AAV2 IgG-positive samples from HD (13<n<16) and from DMD patients (19<n<22). Cutoff for positivity was established as 1:2. **E.** IgG levels measured in HD (33<n<40) and in DMD patients (38<n<47) specific for AAV8. Cutoff for positivity was established as 1:3. **D.** Neutralizing antibody (NAb) titers to AAV8 measured in anti-AAV8 IgG-positive samples from HD (10<n<17) and from DMD patients (16<n<22). Cutoff for positivity was established as 1:2. Histograms represent mean with SEM and symbols represent individual values. Y axes is mentioning the precise titer obtained.

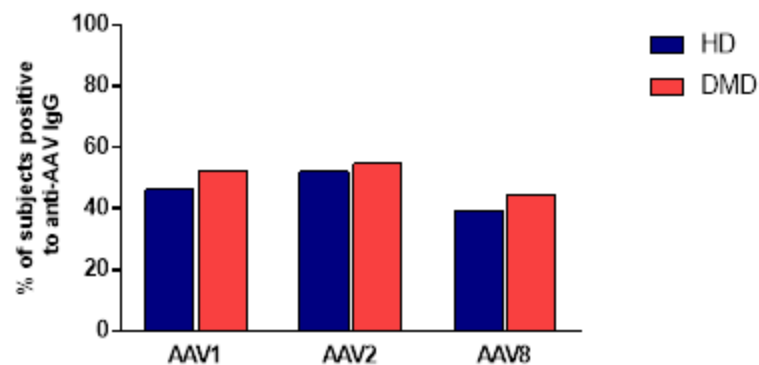
**Figure 3. AAV8-specific IgG subclasses in HD vs. DMD patients.** **A.** IgG1 levels measured in HD (n=34) and in DMD patients (n=47) specific for AAV8. **B.** IgG2 levels measured in HD (n=34) and in DMD patients (n=47) specific for AAV8. **C.** IgG3 levels measured in HD (n=34) and in DMD patients (n=47) specific for AAV8. **D.** IgG4 levels measured in HD (n=34) and in DMD patients (n=47) specific for AAV8. Histograms represent mean and symbols individuals sample values. Blue histograms represent healthy donors and are subdivided by age in light blue (3-7 yo), blue (8-12 yo) and dark blue (13-24 yo). Red histograms represent DMD patients and are also subdivided by age in light red (3-7 years old), red (8-12 years old) and dark red (13-24 years old). Stars indicate the statistical difference calculated with an unpaired parametric t-test and measured as a p value with \*p < 0.01 and \*\*\*\*p < 0.0001.

**Figure 4. Seroprevalence of AAV1, AAV2, and AAV8 in DMD patients undergoing corticoid treatment.** **A.** IgG levels specific for AAV1, AAV2 and AAV8 was measured in DMD patients treated with corticoid (23<n<26) or untreated (54<n<57). Cutoff for positivity was established as 1:3. **B.** Neutralizing antibody (NAb) titers to AAV measured in anti-AAV IgG-positive samples from DMD patients treated with corticoid (9<n<11) or untreated (20<n<23). Cutoff for positivity was established as 1:2. **C.** Follow-up of IgG levels specific for AAV in DMD patients before and after corticoid treatment (n=13). Symbols represent individual patients. **D.** Follow-up of IgG levels specific for AAV in DMD patients before and after corticoid treatment (n=11). Symbols represent individual patients. **E.** Follow-up of NAb specific for AAV8 in 100 healthy donors and 100 donors who received immunosuppressive drugs as part of management of an underlying condition. Histogram represent mean + SEM. Stars indicate the statistical difference calculated with an unpaired parametric Welch's t-test and measured as a p value with \*p < 0.01.

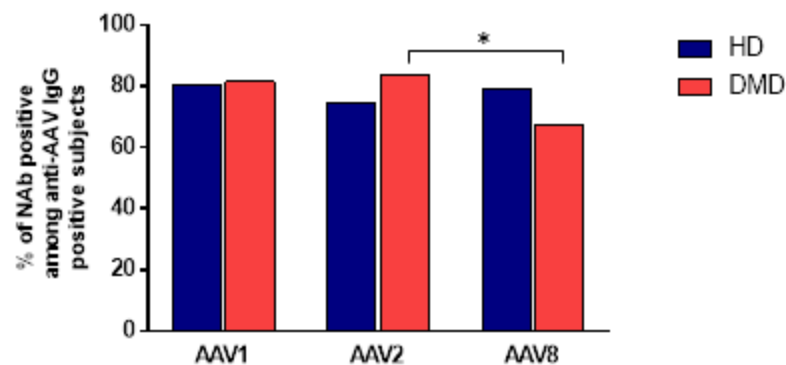
**Table 1. Seroprevalence of AAV8 in DMD patients over time.**

NAb titers specific for AAV8 capsid were measured yearly in DMD patients (n=31). Cutoff for positivity was established as 1:2. The table indicates each individual values over time. Patients highlighted in green were those found slightly positive and who became negative (n=3). Patients highlighted in orange were initially sero-negative and became positive (n=2).

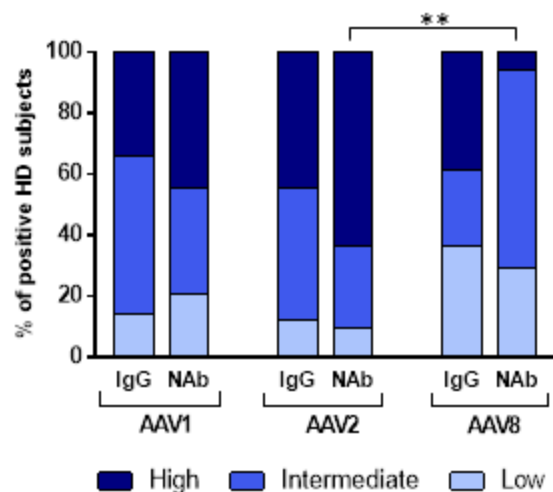
A



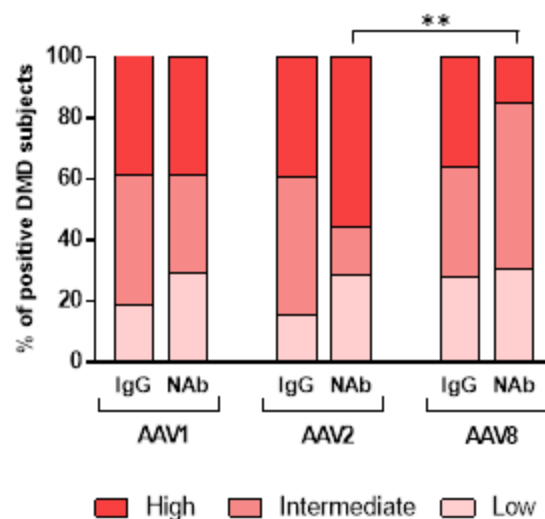
B



C



D

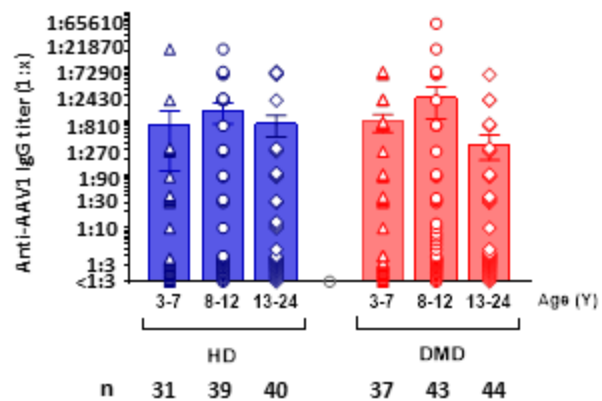
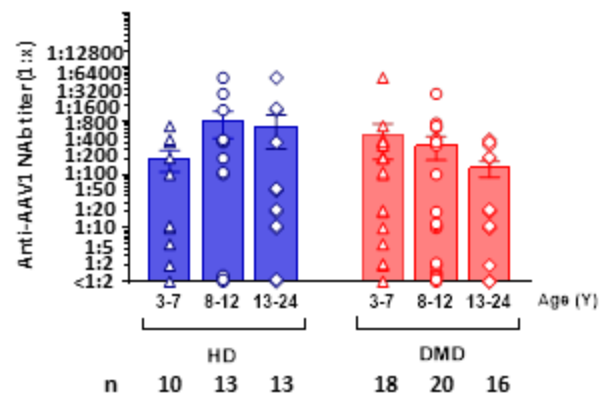
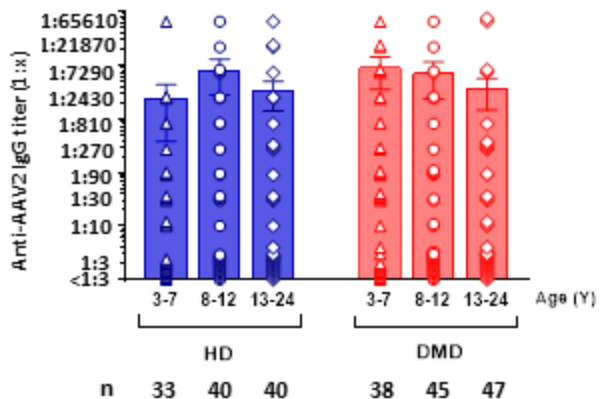
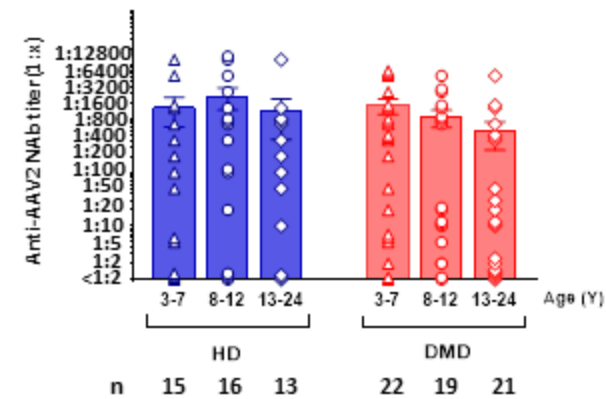
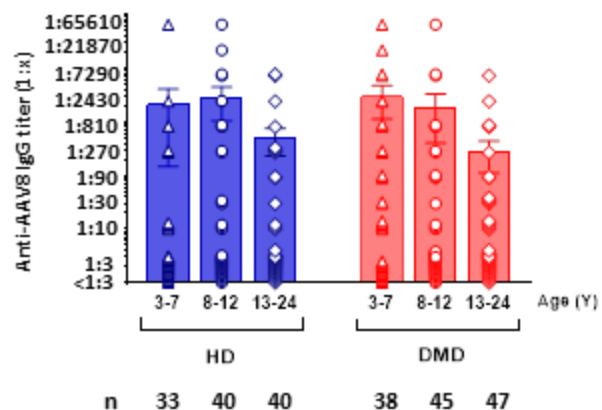
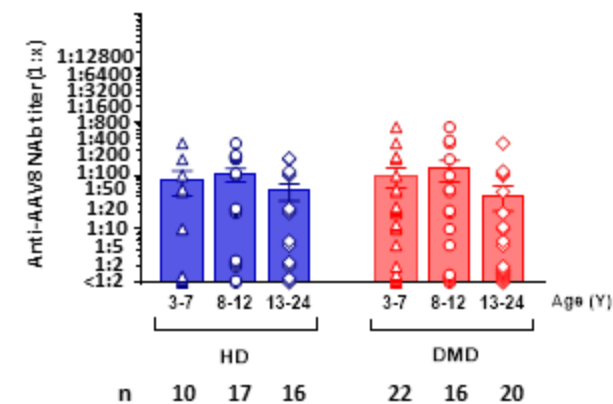


E

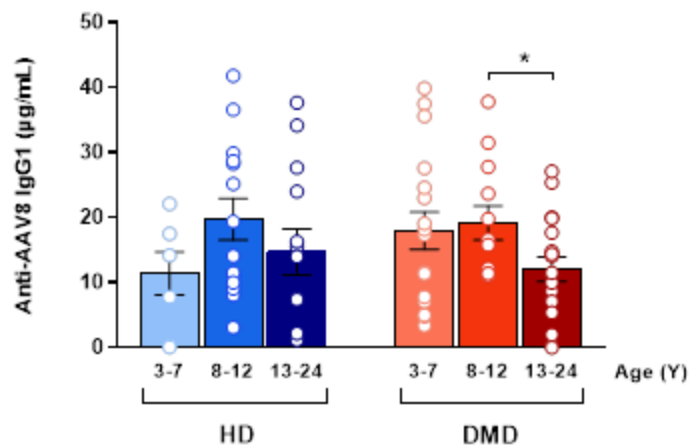
| Titer        | IgG        | NAb        |
|--------------|------------|------------|
| Low          | <1:10      | <1:10      |
| Intermediate | 1:10-1:270 | 1:10-1:200 |
| High         | >1:270     | >1:200     |

Statistical significance determined by  $\chi^2$  test  
\*,  $p < 0,05$ ; \*\*,  $p < 0,01$

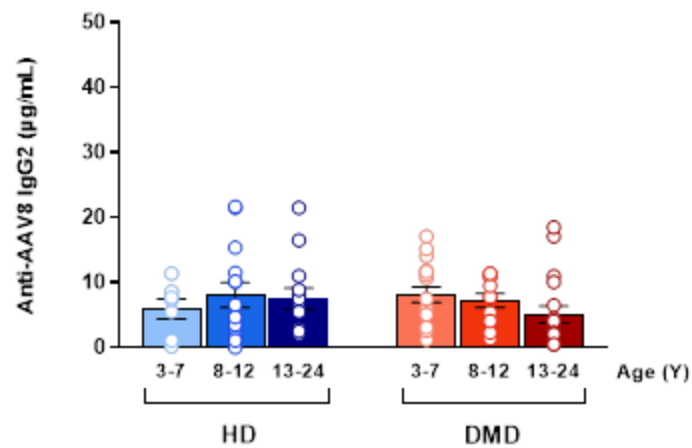


**A****B****C****D****E****F**

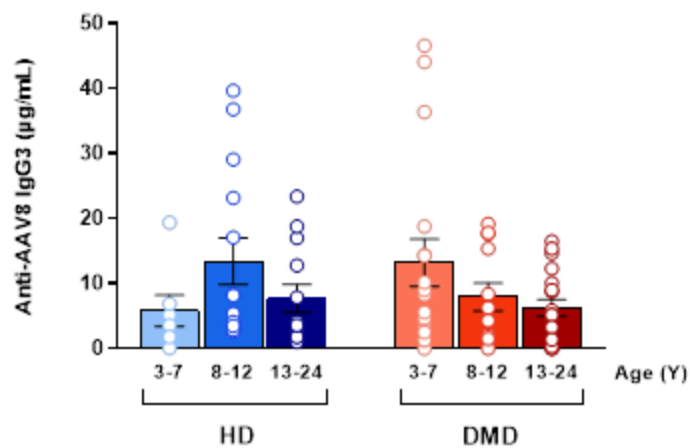
A



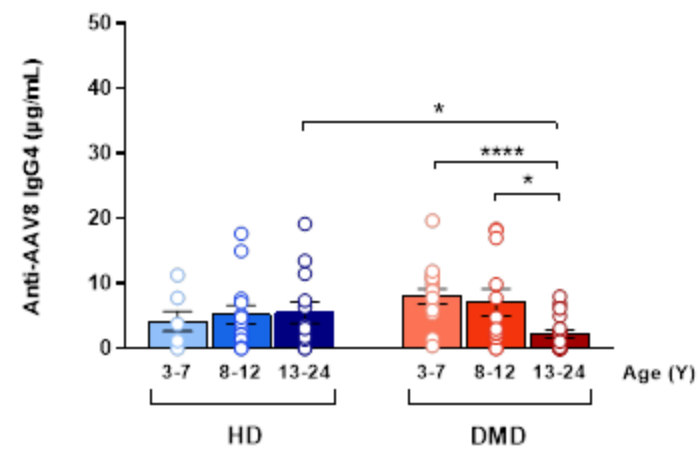
B



C

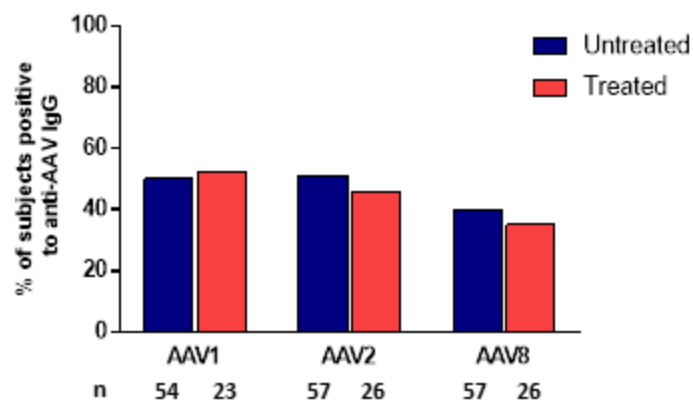


D

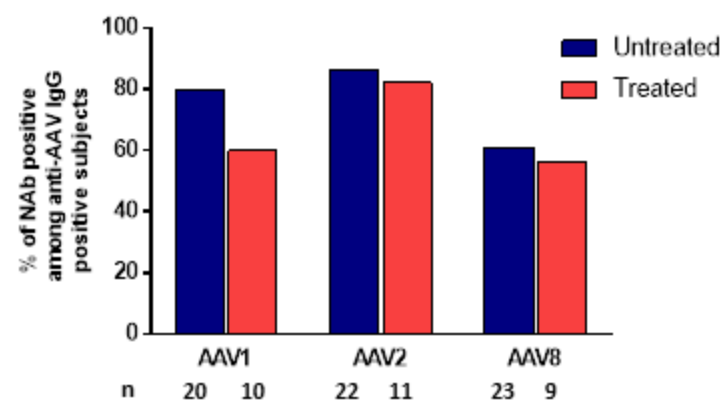


Unpaired parametric t-test  
 \*,  $p < 0,05$ ; \*\*\*\*,  $p < 0,0001$

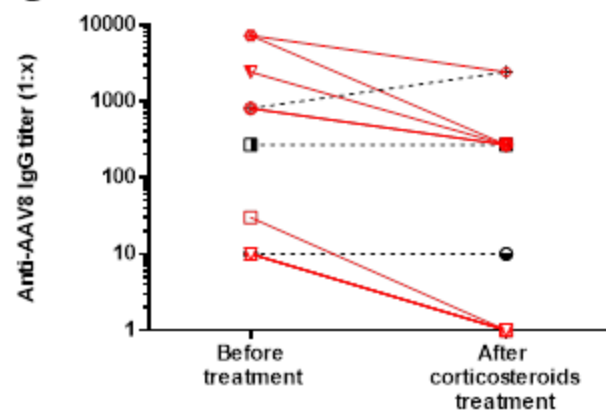
A



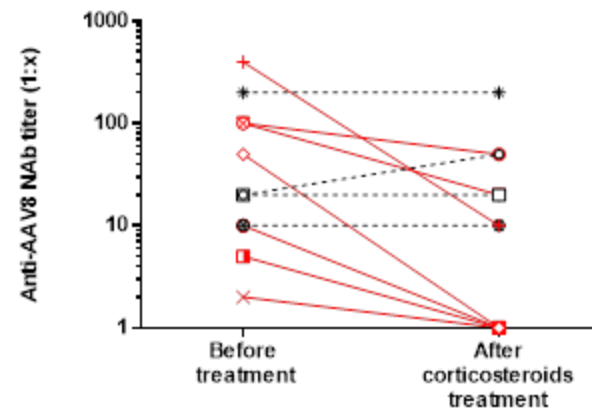
B



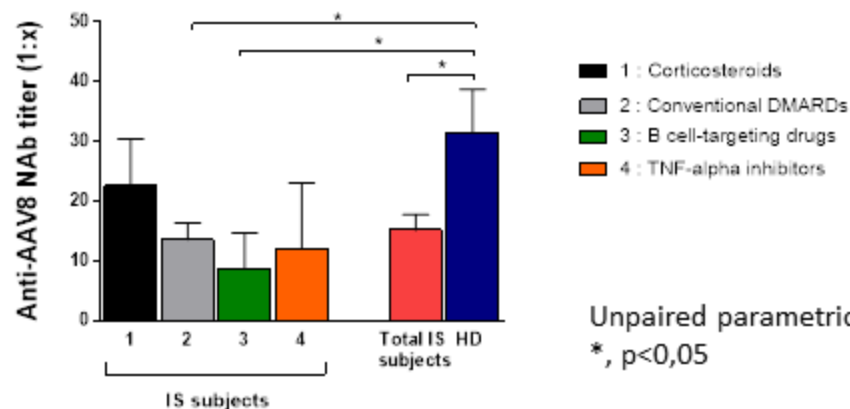
C



D



E



Unpaired parametric t-test with Welch's correction  
 \*,  $p < 0,05$

|            | anti-AAV8 NAb titers |        |        |        |        |        |
|------------|----------------------|--------|--------|--------|--------|--------|
|            | Year 1               | Year 2 | Year 3 | Year 4 | Year 5 | Year 6 |
| Patient 1  | <1:2                 | <1:2   |        |        |        |        |
| Patient 2  | 1:50                 | 1:50   | 1:20   | 1:20   | 1:50   | 1:50   |
| Patient 3  | 1:5                  | <1:2   | <1:2   | <1:2   | <1:2   | <1:2   |
| Patient 4  | <1:2                 | <1:2   |        |        |        |        |
| Patient 5  | 1:400                | 1:200  | 1:400  | 1:400  | 1:400  |        |
| Patient 6  | <1:2                 | <1:2   |        |        |        |        |
| Patient 7  | 1:20                 | 1:2    | <1:2   | <1:2   | <1:2   | <1:2   |
| Patient 8  | <1:2                 | <1:2   | <1:2   | <1:2   | <1:2   |        |
| Patient 9  | <1:2                 | <1:2   | <1:2   |        |        |        |
| Patient 10 | <1:2                 | <1:2   | <1:2   | <1:2   | <1:2   | <1:2   |
| Patient 11 | 1:2                  | 1:2    |        |        |        |        |
| Patient 12 | <1:2                 | <1:2   |        |        |        |        |
| Patient 13 | <1:2                 | <1:2   | <1:2   | <1:2   |        |        |
| Patient 14 | <1:2                 | <1:2   | 1:2    | 1:2    | <1:2   | 1:2    |
| Patient 15 | 1:10                 | <1:2   |        |        |        |        |
| Patient 16 | <1:2                 | <1:2   | <1:2   | <1:2   | <1:2   |        |
| Patient 17 | <1:2                 | <1:2   | <1:2   |        |        |        |
| Patient 18 | <1:2                 | <1:2   | <1:2   |        |        |        |
| Patient 19 | 1:800                | 1:400  | 1:1600 | 1:400  | 1:1600 |        |
| Patient 20 | <1:2                 | <1:2   | <1:2   |        |        |        |
| Patient 21 | <1:2                 | <1:2   |        |        |        |        |
| Patient 22 | <1:2                 | <1:2   | <1:2   | 1:2    | <1:2   |        |
| Patient 23 | <1:2                 | <1:2   |        |        |        |        |
| Patient 24 | <1:2                 | <1:2   | <1:2   | 1:2    | 1:5    |        |
| Patient 25 | 1:400                | 1:200  | 1:400  | 1:400  |        |        |
| Patient 26 | 1:200                | 1:200  | 1:400  | 1:400  | 1:200  | 1:400  |
| Patient 27 | 1:2                  | <1:2   |        |        |        |        |
| Patient 28 | <1:2                 | 1:5    |        |        |        |        |
| Patient 29 | 1:20                 | 1:20   | 1:20   |        |        |        |
| Patient 30 | <1:2                 | <1:2   |        |        |        |        |
| Patient 31 | 1:5                  | 1:2    |        |        |        |        |