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Pre-existing Anti-AAV9 antibodies in the Chinese healthy and rare disease populations: Implications for gene therapy

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ABSTRACT

The adeno-associated virus 9 (AAV9) vector was particularly notable for its broad tissue tropism, making it a preferred vector for gene therapy. Goals: The study aimed to investigate the patterns of pre-existing immunity against AAV9 in the Chinese population. In this study, we conducted a serological research from November 2022 to June 2024. The study included 341 participants in total with age ranged from 0 to 90 years old: 270 healthy individuals, 30 pediatric patients and 41 adults with rare diseases. Total AAV9-binding antibodies (TAbs) and neutralizing antibodies (NAbs) were measured. The seroprevalence of anti-AAV9 NAbs showed no significant differences between healthy individuals and rare disease patients across both pediatric and adult groups. Newborns exhibited a high NAb-positive rate (64.3 %), while children aged 6 months to 3 years had the lowest prevalence (7.7 %). This rate progressively increased through childhood and adolescence. Overall, 58.7 % of the Chinese population aged 0–90 years tested positive for anti-AAV9 NAbs, with adults showing a significantly higher prevalence than children (75.0 % vs. 34.3 %). Additionally, 58.1 % of the population exhibited low levels of anti-AAV9 NAb titers ($IC_{50} \leq 100$). No significant sex-specific differences were observed, and antibody titers (NAbs or TAbs) showed no strong correlation with age. A strong correlation was identified between TAb and NAb positivity rates and titers. The optimal AAV9-based GT period was between 6 months and 3 years in that patients possessed lowest pre-existing immunity. Since TAbs had a strong association with NAbs, TAbs was considered as an alternative indicator to screen rare diseases.

1. Introduction

Adeno-associated viruses (AAVs), belonging to the family *Parvoviridae* and the genus *Dependovirus*, are small, non-enveloped, single-stranded DNA viruses with a genome size of approximately 4.7 kilobases (kb) (Tenenbaum et al., 2003).These viruses have been extensively used in gene therapy for treating genetic diseases such as lipoprotein lipase deficiency (LPLD) (Scott, 2015), Leber Congenital Amaurosis (LCA) (Chiu et al., 2021), Spinal Muscular Atrophy (SMA) (Ogbonmide et al., 2023), hemophilia (Nathwani, 2022), familial amyotrophic lateral sclerosis (ALS) (Mueller et al., 2020), Pompe disease (Colella et al., 2020), alpha-sarcoglycan deficiency (Mendell et al., 2009), and familial limb-girdle myasthenia (Arimura et al., 2014). Their widespread application is attributed to their broad tissue tropism, ability to stably transduce non-dividing cells without integrating into the host genome, and relatively lower immunogenicity compared to other viral vectors (Shirley et al., 2020).

Despite these advantages, a major challenge associated with AAV-based gene therapy is the pre-existing immunity against vectors. Natural exposure to wild-type AAV (wt-AAV) typically occurs during early childhood (Blacklow et al., 1968a,b), with memory T and B cells specific to the viral capsid persisting throughout life. Upon administration of recombinant AAV (rAAV) vectors, these memory cells may become reactivated, compromising the therapy's efficacy (Vandamme et al., 2017). Even low-titer neutralizing antibodies (NAbs) against AAV can significantly reduce the transduction efficiency of rAAV vectors, thereby diminishing their therapeutic efficacy (Janelidze et al., 2014; Moskalenko et al., 2000). Additionally, antigen-binding antibodies (TAbs),

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Table 1
Demographics characteristics of participants.

Group*	0–1yrs (*N* = 20)	2–6yrs (*N* = 28)	7–18yrs (*N* = 89)	19–65yrs (*N* = 154)	>65yrs (*N* = 50)	Overall (*N* = 341)
Sex**						
Male	12 (60.0 %)	15 (53.6 %)	41 (46.1 %)	53 (34.4 %)	21 (42.0 %)	142 (41.6 %)
Female	8 (40.0 %)	13 (46.4 %)	48 (53.9 %)	101 (65.6 %)	29 (58.0 %)	199 (58.4 %)
Age						
Mean±SD	0.3 ± 0.4	4.4 ± 1.4	12.7 ± 3.1	42.7 ± 13.8	74.9 ± 7.0	33.9 ± 25.2
Median (Min, Max)	0 (0, 1)	4 (2, 6)	13 (7, 18)	41 (19, 65)	74 (66, 90)	29 (0, 90)
Health status						
Health	20 (100 %)	23 (82.1 %)	64 (71.9 %)	115 (74.7 %)	48 (96.0 %)	270 (79.2 %)
Rare diseases	0 (0.0 %)	5 (17.9 %)	25 (28.1 %)	39 (25.3 %)	2 (4.0 %)	71 (20.8 %)

*: Age was calculated in full years. 0–1yrs: 0≤age≤1, 2–6yrs: 2≤age≤6, 7–18yrs: 7≤age≤18, 19–65yrs: 19≤age≤65, above 65yrs: >65.
**: Data displayed as numbers (percentages).

regardless of their neutralizing activity, can activate immune responses and lead to adverse effects, such as hepatotoxicity (Salabarria et al., 2024). Thus, assessing the prevalence of anti-AAV antibodies in the target population is a critical prerequisite for the safe and effective application of AAV vector-based therapies.

Currently, 12 human AAV serotypes have been identified, each exhibiting unique tissue tropisms (Srivastava, 2016). The seroprevalence of anti-AAV neutralizing antibodies varies significantly with factors such as age, sex, and geographical location. Among these, anti-AAV2 neutralizing antibodies show the highest prevalence, ranging from 30 % to 60 % (Calcedo et al., 2009; Costa Verdera et al., 2020; Greenberg et al., 2016; Wang et al., 2024). The prevalence of anti-AAV9 NAbs in the general population is reported to range between 4.3 % and 47 % (Boutin et al., 2010; Fu et al., 2017; Greenberg et al., 2016; Mimuro et al., 2014; Stolte et al., 2022; Wei et al., 2024; Wang et al., 2024). Similarly, neutralizing antibodies against AAV1, AAV6, and AAV5 show prevalence rates of approximately 50.5 %, 37 %, and 3.2 %, respectively (Boutin et al., 2010), with substantial cross-reactivity observed among AAV serotypes (Calcedo and Wilson, 2016).

AAV9 vectors are particularly promising due to their ability to target neurons and efficiently cross the blood-brain barrier, making them ideal candidates for gene therapy targeting the central nervous system in conditions such as motor neuron disease and spinal muscular atrophy (Hitti et al., 2019). They are also widely utilized in developing therapeutic products for various diseases. Understanding the prevalence and titers of AAV9 neutralizing antibodies, particularly in children, is crucial for optimizing gene therapy protocols. However, studies on the prevalence of pre-existing anti-AAV9 antibodies in the Chinese population, especially in the children and patients with rare diseases, are still very limited (Wei et al., 2024).

This study aims to evaluate the prevalence of pre-existing anti-AAV9 antibodies in serum samples from various age groups (0–90 years old) within the Chinese population, including healthy individuals and patients with rare diseases. Additionally, it seeks to identify the optimal age window for AAV9-based interventions, and to explore the correlation between TAbs and NAbs. These findings aim to inform the development of AAV9 vector-based gene therapies and establish inclusion criteria for clinical trials.

2. Materials and methods

2.1. Study population

Between November 2022 and June 2024, the Clinical Pharmacology Research Center of Peking Union Medical College Hospital recruited 270 healthy volunteers from various age groups (from birth to 90 years), including 107 children (≤18 years) and 163 adults (>18 years). Participants were categorized into 5 age groups: ≤1 year, 2–6 years, 7–18 years, 19–65 years, and >65 years (age was calculated in full years). During the sample collection process, we carefully controlled the age distribution of the enrolled volunteers to minimize potential biases arising from an overrepresentation of specific age groups. Age was divided into more detailed categories to better understand the associations between NAbs, TAbs and Age. At the same time, each group was greater than 20 samples whenever feasible (≤1 year, 2–3 years, 4–6 years, 7–12 years, 13–18 years, 19–25 years, 26–35 years, 36–45 years, 46–55 years, 56–65 years, 66–75 years, and >75 years). The sex ratio (greater than 1/3) in each age subgroup were balanced. **Inclusion Criteria:** Subjects were eligible for enrollment if they met all the following criteria: (1) The subject or their legal guardian provided informed consent to participate in the study. (2) The subject had no prior exposure to any gene therapy agents. **Exclusion Criteria:** Subjects were excluded from the study if they met any of the following conditions: (1) Clinically significant abnormalities in routine blood tests, comprehensive metabolic panels, or routine urine examinations. (2) Current use of systemic immunosuppressant agents. (3) History of tumors, autoimmune diseases, severe or acute infectious diseases, hyperlipidemia, chronic kidney disease, or diabetes.

Additionally, serum samples were collected from 71 patients with rare diseases (aged 3–77 years, including 30 children and 41 adults) with conditions such as lysosomal storage disorders, spinal muscular atrophy, and hemophilia. Except for the second exclusion criterion, all other inclusion and exclusion criteria are consistent with those for healthy subjects.

Neither healthy participants nor patients were allowed to receive immunoglobulin or blood transfusion treatments within 120 days prior to blood collection. Serum samples were obtained from each participant for the detection of anti-AAV9 NAbs and/or TAbs. The study protocol was approved by the Ethics Committee of Peking Union Medical College Hospital.

2.2. Gluc (Gaussia luciferase)-Based microneutralization assay (MN) for detecting ANTI-AAV9 NAbs

The titers of anti-AAV9 NAbs in the serum of 341 Chinese individuals, comprising 270 healthy volunteers and 71 patients, were measured using a previously full-validated cell-based microneutralization (MN) assay reported by our study team (Yu et al., 2024). This assay utilized recombinant AAV9 (rAAV9) encoding a reporter gene, EGFP-2A-Gluc, for luciferase-based detection. Briefly, 50 μL of two-fold serially diluted serum was incubated with 2×10^8 viral genomes (vg) of rAAV9-Gluc in 50 μL of DMEM containing 0.1 % BSA for 1 hour. The assay was performed in triplicate. Subsequently, 20,000 HEK-293 cells in 100 μL of D10 medium were added to the 96-well plates at a multiplicity of infection (MOI) of 10^4. Plates were incubated at 37 °C with 5 % CO_2 for 48–72 h. After incubation, luciferase activity from transduced Gluc was measured using a luciferase assay system, and relative luminescence units (RLU) were recorded using a Synergy H1 microplate luminometer (Biotek, USA).

The neutralizing antibody titer was defined as the reciprocal of the serum dilution that caused a 50 % reduction in RLU compared to the rAAV9-Gluc control. Transduction inhibition titers were analyzed using Prism 8.0 software (GraphPad Software, San Diego, CA, USA), and the inhibitory concentration (IC_{50}), representing the serum dilution at which 50 % transduction inhibition occurred, was determined using nonlinear

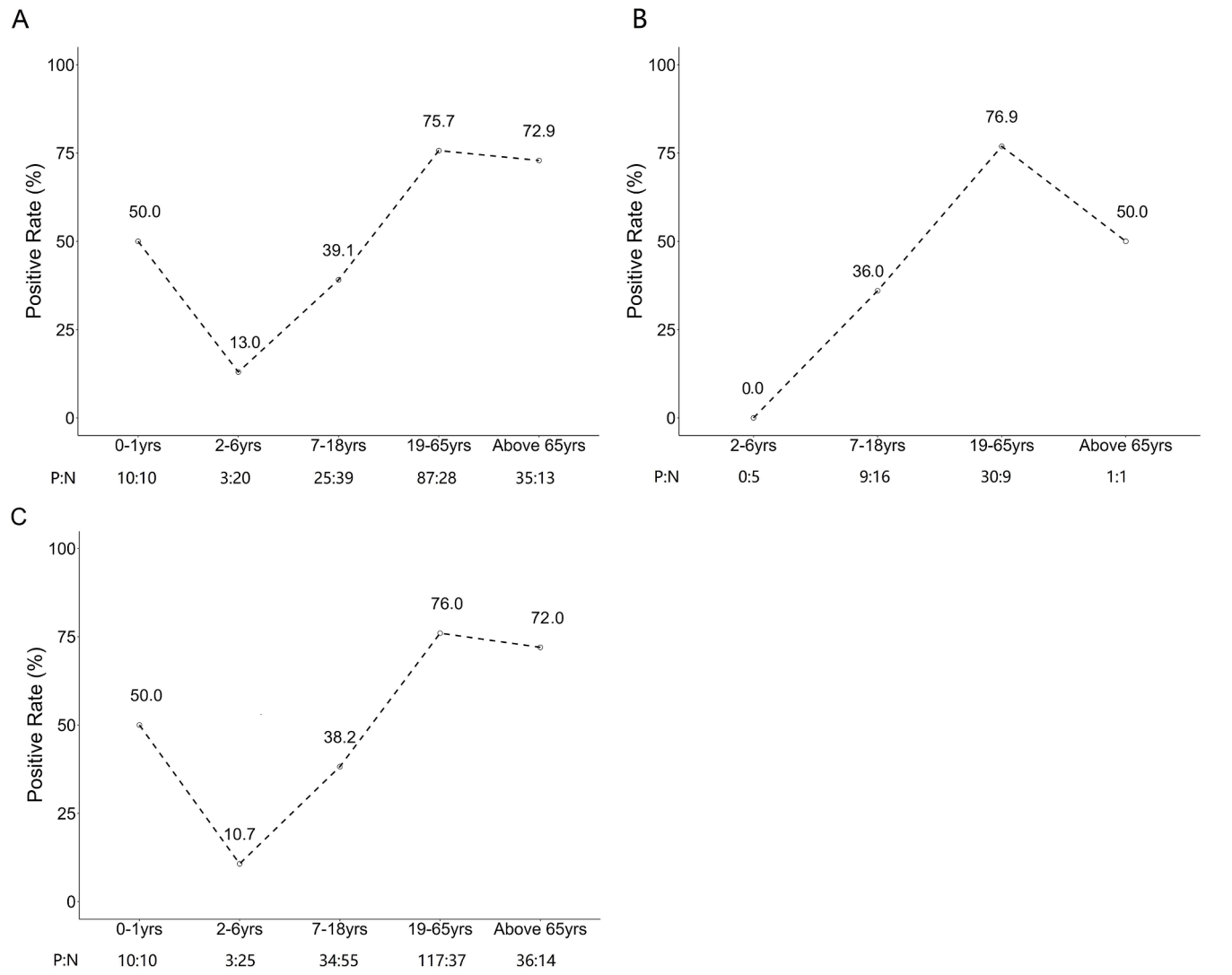

Fig. 1. Number and positivity rate of anti-AAV9 neutralizing antibodies in children and adults. A: Healthy volunteers; B: Patients with rare diseases; C: Overall volunteers. P:N: positive number : negative number. Age was calculated in full years (0–1yrs: 0≤age≤1, 2–6yrs: 2≤age≤6, 7–18yrs: 7≤age≤18, 19–65yrs: 19≤age≤65, above 65yrs: >65). The positivity rates of anti-AAV9 NAbs were 59.3 % (160/270) in the healthy population and 56.3 % (40/71) in patients with rare diseases, with no significant difference observed between the two groups. Consequently, the positivity rate was calculated based on the combined population (58.7 %, 200/341).

regression. Negative control (NC) serum was pooled from confirmed negative samples (IC_{50}<15), while positive controls (PCs) were prepared by spiking pooled negative serum (N > 10) with anti-AAV9 MoAbs. PCs were prepared at low, medium, and high concentrations (LPC, MPC, and HPC) corresponding to 200, 500, and 2000 ng/mL, respectively. The intra-assay and inter-assay variation for positive QC were 7–35 % and 22–41 %, respectively. The sensitivity of the NAb detection method was 54 ng/mL, and the cut-off value for IC_{50} was 15.

2.3. Enzyme-linked immunosorbent assay (ELISA) for detecting ANTI-AAV9 TAb titers

The titers of anti-AAV9 TAbs in the serum of 341 Chinese individuals mentioned above were determined using a fully validated ELISA method. The TAb assay followed the multi-tiered anti-drug antibody (ADA) testing approach recommended by the Food and Drug Administration (FDA), which involved a sequential testing strategy comprising a screening assay, a confirmatory assay, and a titration assay (FDA, 2019). This approach has been widely adopted in the pharmaceutical industry. Ninety-six-well plates (Costar-Corning, USA) were coated with 50 ng/well of AAV9 vector particles diluted in carbonate buffer (pH 9.6) and incubated overnight at 4 °C. The plates were then blocked for 2 h at 37 °C using phosphate-buffered saline with 0.05 % Tween-20 (PBST) containing 1 % skim milk. After washing, samples diluted to a minimal required dilution (MRD) of 1:2 in 1 % skim milk were applied to the AAV9-coated plates and incubated at 37 °C for 1 hour. Following another wash, a goat anti-human IgG antibodies conjugated with horseradish peroxidase (HRP) was added at a 1:5000 dilution and incubated for 1 hour at 37 °C. The plates were then washed again, treated with tetramethylbenzidine (TMB), and the absorbance was measured at 450 nm using an ELISA plate reader (Synergy H1, Biotek).

Anti-AAV9 specific monoclonal antibodies (MoAbs) were generated by Genecradle Therapeutics Inc. using the hybridoma technique with serotype-specific empty capsids immunized in Balb/C mice. Low- and high- PCs were prepared using mouse monoclonal anti-AAV9 antibodies at concentrations of 0.1 μg/mL and 5 μg/mL, respectively, diluted in pooled negative serum. If the detection value of a sample was below the cut-off value for the screening assay (1.90), the serum sample was defined as negative serum. Pooled negative serum (N > 10), along with low- and high- PCs, was included in each assay batch. Both controls and

Table 2
Anti-AAV9 NAbs and TAbs titers in the Chinese positive population.

	Age group*	Healthy volunteers	Patients with rare diseases	Overall
Anti-AAV9 NAbs	0–1yrs	249.5 ± 3.1 (*n* = 10)	/	249.5 ± 3.1 (*n* = 10)
	2–6yrs	258.2 ± 8.6 (*n* = 3)	/	258.2 ± 8.6 (*n* = 3)
	7–18yrs	227.4 ± 4.2 (*n* = 25)	612.83±1.74 (*n* = 9)	295.6 ± 3.8 (*n* = 34)
	19–65yrs	232.9 ± 4.1 (*n* = 87)	276.18±4.6 (*n* = 30)	243.3 ± 4.2 (*n* = 117)
	Above 65yrs	210.5 ± 3.8 (*n* = 35)	24.35±NA (*n* = 1)	198.3 ± 3.9 (*n* = 36)
Anti-AAV9 TAbs	0–1yrs	1600.0 ± 3.7 (*n* = 9)	/	1600.0 ± 3.7 (*n* = 9)
	2–6yrs	1131.4 ± 6.3 (*n* = 4)	/	1131.4 ± 6.3 (*n* = 4)
	7–18yrs	1299.6 ± 4.4 (*n* = 30)	3909.5 ± 2.1 (*n* = 9)	1675.7 ± 4.2 (*n* = 39)
	19–65yrs	893.5 ± 3.5 (*n* = 93)	771.3 ± 3.1 (*n* = 34)	865.56±3.4 (*n* = 127)
	Above 65yrs	860.55±3.8 (*n* = 40)		860.55±3.8 (*n* = 40)

*: Age was calculated in full years. 0–1yrs: 0≤age≤1, 2–6yrs: 2≤age≤6, 7–18yrs: 7≤age≤18, 19–65yrs: 19≤age≤65, >65yrs: >65.
Data displayed as geometric mean ± geometric SD
NA: Not applicable
/: No patients

test samples were pre-incubated either without (screening assay) or with (confirmatory assay) 50 ng of AAV9 vector particles for 1 hour before being added to AAV9-coated plates in duplicate. For samples that tested positive in both the screening and confirmatory assays, the titers of TAbs were determined after diluting the samples at a minimum dilution of 1:100 in 1 % skim milk.

The results were expressed as a signal-to-noise (S/N) ratio, calculated by dividing the optical density (OD) value of the sample by the OD value of the NC. The percentage of inhibition was determined using the formula: $1 - S/N$. The sensitivity of the TAb detection method reached 50 ng/mL, which was superior to the FDA-recommended level of 100 ng/mL. The cut-off values for the screening assay, confirmatory assay, and titration assay were 1.90, 56.5 %, and 2.64, respectively. The intra-assay and inter-assay variation for positive QC were 7–19 % and 9–25 %, respectively. Samples with an inhibition percentage equal to or exceeding the confirmatory cut point were classified as positive. The anti-AAV9 binding antibody titer was defined as the reciprocal of the highest serum dilution that yielded a positive signal. Based on the multi-tiered ADA testing approach, samples that had been determined to be negative by both the screening and confirmatory assays did not proceed to titration.

2.4. Statistical analysis

The raw data were processed and cleaned using the dplyr package (Version 1.1.4) in R. Statistical differences in antibody titers across age groups were assessed using the Kruskal-Wallis test. Correlations between NAb titers, IC_{50} values, and age were evaluated using Spearman's rank correlation. Fisher Exact test was used to compare the sex differences. A two-sided p-value of <0.05 was considered statistically significant. Visualizations were generated with the ggplot2 package (Version 3.5.1). Due to the similar positivity rates and titers of anti-AAV9 NAbs in the adult population aged 19–65, the data of these volunteers were merged and plotted. The geometric mean and geometric standard deviation (SD) of titers were calculated exclusively for populations with positive antibody titers and did not encompass populations with negative antibody titers. Given that different detection strategies were employed for TAbs and NAbs and the detection guidance mentioned before was from FDA, we did not obtain titer data for negative samples in the TAbs assay. Therefore, when comparing NAb IC_{50} values across different age groups and assessing the correlation between NAb IC_{50} and age, we included the IC_{50} values from NAb-negative samples. In contrast, when analyzing the correlation between TAbs titers and age, we utilized the titer data from positive samples and assigned a titer value of one-half the cut-off value to negative samples.

3. Results

3.1. The seroprevalence of ANTI-AAV9 NAbs in the Chinese population

Demographics characteristics of participants are summarized in Table 1. The positivity rates of anti-AAV9 NAbs were 59.3 % (160/270) in the healthy population and 56.3 % (40/71) in patients with rare diseases, with no significant difference observed between the two groups. Consequently, the positivity rate was calculated based on the combined population (58.7 %, 200/341). Detailed sample sizes and positivity rates for each age group are shown in Fig. 1A–C.

The distributions of the seroprevalence of anti-AAV9 NAbs was analyzed across sexes. Among the healthy population ($N = 270$), the positivity rates in males and females were 39.7 % vs. 53.3 % overall, 29.1 % vs. 42.3 % in children ($N = 107$), and 72.2 % vs.78.3 % in adults ($N = 163$). Similarly, among patients with rare diseases ($N = 71$), positivity rates in males and females were 63.8 % vs. 70.2 % overall, 30.8 % vs. 29.4 % in pediatric patients ($N = 30$), and 71.4 % vs. 76.6 % in adult patients ($N = 41$). These findings indicated no significant sex-based differences in the seroprevalence of anti-AAV9 NAbs (Supplementary Table 1).

3.1.1. Anti-AAV9 NAbs in children

The positivity rate of anti-AAV9 NAbs in children, aged from birth to 18 years ($N = 107$), was significantly lower than that in adults. The overall positivity rate for anti-AAV9 NAbs in children was 35.5 % (38/107). Among 7 healthy newborns aged ≤2 days, 5 tested positive for anti-AAV9 NAbs, corresponding to a positivity rate of 71.4 %. For newborns aged 3–18 days ($n = 7$), 4 were NAb-positive, yielding a rate of 57.1 %. Thereafter, the positivity rate declined significantly: among 6 infants aged 6 months to 1 year, only 1 tested positive (16.7 %), and all 7 children aged 2–3 years were negative for anti-AAV9 NAbs. Positivity rates began to rise again after age 3, reaching 32.9 % in children aged 3–18 years (28/85) (Supplementary Figure 1A). In the Supplementary, the age groups of volunteers are divided into more categories to better analyze the associations between IC_{50} and age and to allow readers to see more data.

We also tested anti-AAV9 NAbs in serum samples from 30 pediatric patients aged 3–18 years with rare diseases, including congenital scoliosis ($N = 16$), McCune-Albright syndrome ($N = 3$), multiple sclerosis ($N = 2$), Wilson's disease ($N = 2$), glycogen storage disease type II ($N = 2$), Castleman's disease ($N = 1$), spinal muscular atrophy ($N = 1$), glycogen storage disease type I ($N = 1$), idiopathic pulmonary hypertension ($N = 1$), and hemophilia pseudotumor ($N = 1$). The overall NAb positivity rate among pediatric patients was 30.0 % (9/30), closely aligning with the rate observed in healthy children aged 3–18 years (32.9 %). To consolidate the data, positivity rates were calculated across all children (34.3 %, 47/137). Details on the number of volunteers and positive cases in each age group are illustrated in Supplementary Figure 1B. Combined data from 107 healthy children and 30 pediatric patients are presented in Supplementary Figure 1C.

3.1.2. Anti-AAV9 NAbs in adults

The positivity rate of anti-AAV9 NAbs in 163 healthy adults aged 19–90 years was 74.8 % (122/163), significantly higher than that observed in healthy children (35.5 %). Furthermore, no decline in the positive rate or titer of NAbs was noted among elderly individuals aged over 65 years. Detailed data on the number of volunteers and positive

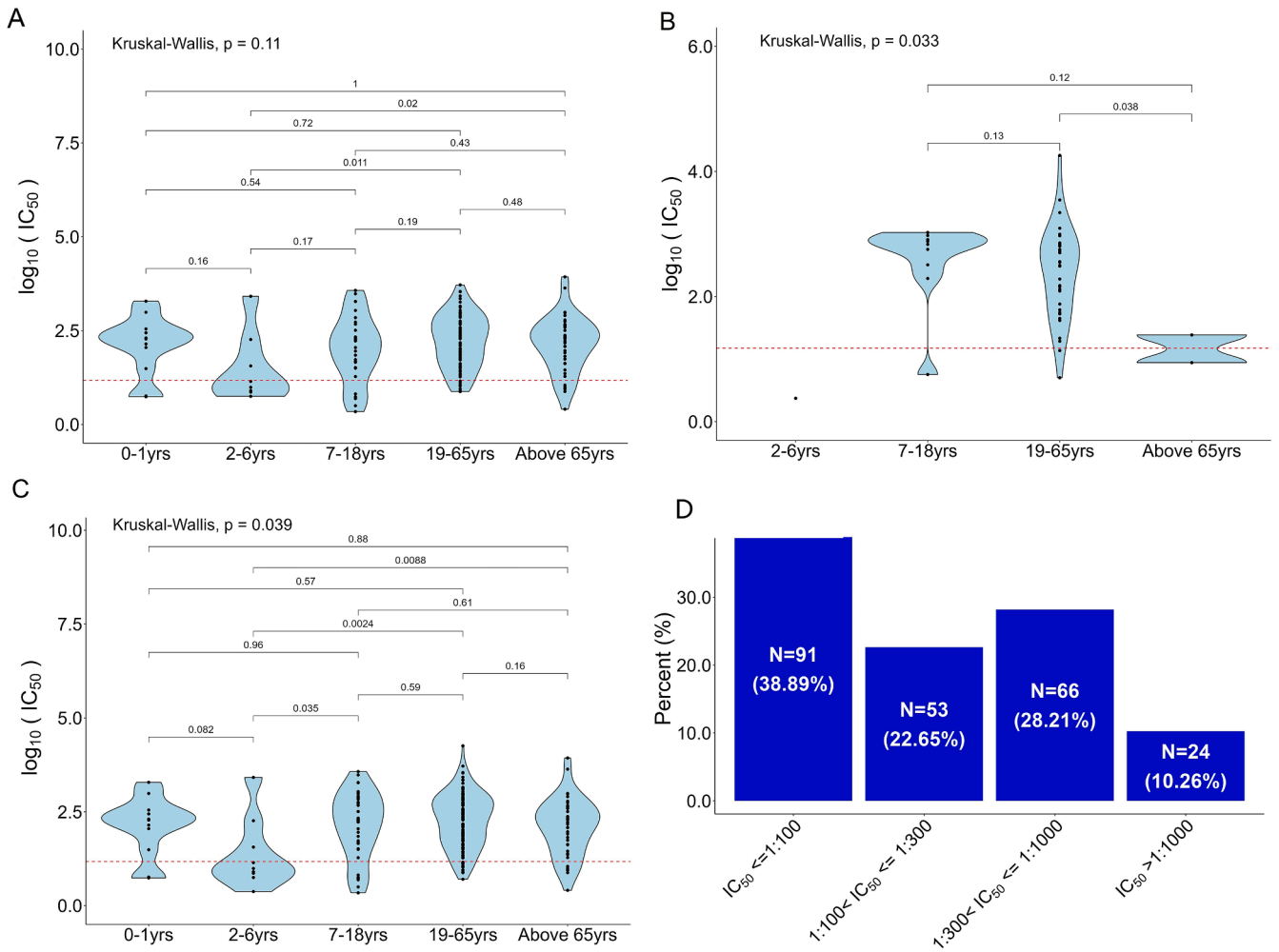

Fig. 2. IC_{50} distribution of anti-AAV9 neutralizing antibodies in children and adults. A: Healthy volunteers; B: Patients with rare diseases; C: Overall volunteers; D: The percentage of anti-AAV9 NAbs IC_{50}. The dashed line indicates the cut point of the NAbs IC_{50}. No significant differences in NAb titers were observed among the different age groups.

individuals across age groups are presented in Supplementary Figure 1D.

Additionally, serum samples from 41 adult patients with rare diseases were analyzed. These included cases of optic neuritis and myelitis spectrum disorders ($N = 8$), multiple sclerosis ($N = 7$, including 1 case of systemic sclerosis), IgG4-related diseases ($N = 6$), Castleman's disease ($N = 3$), congenital scoliosis ($N = 3$), spinal muscular atrophy ($N = 2$), idiopathic pulmonary hypertension ($N = 2$), hemophilia ($N = 2$), pulmonary alveolar proteinosis ($N = 2$), POEMS syndrome ($N = 1$), Fabry disease ($N = 1$), pulmonary cystic fibrosis ($N = 1$), Langerhans cell histiocytosis ($N = 1$), retinitis pigmentosa ($N = 1$), and mitochondrial encephalomyopathy ($N = 1$). The positivity rate of anti-AAV9 NAbs in these patients was 75.6 % (31/41), closely aligning with the rate observed in healthy adults (74.8 %). Supplementary Figure 1E provide detailed data on the number of volunteers and positive individuals in patients. Combined data from the 163 healthy adults and 41 patients are presented in Supplementary Figure 1F.

3.2. Titers of Anti-AAV9 NAbs in the Chinese population

The IC_{50} values of anti-AAV9 NAbs in the serum of 341 Chinese individuals aged 0–90 years, including 270 healthy volunteers and 71 patients with rare diseases, were measured. And the IC_{50} in the positive population are summarized in Table 2. No significant differences in NAb titers were observed among the different age groups. The distribution of IC_{50} titers for each age group are shown in Fig. 2A–C. Our previously published research findings indicated that low-level NAbs, at a titer near 1:100 (IC_{50}=100), had no effect on transduction in the heart and liver as well as cellular responses, but slightly decreased transduction in muscles (Wang et al., 2024). Therefore, based on clinically meaningful cutoff values, we categorized titers into four groups: $IC_{50} \leq 100$, $100 < IC_{50} \leq 300$, $300 < IC_{50} \leq 1000$ and $IC_{50} > 1000$. The number of positives in each group is shown in Fig. 2D. Among the 341 participants in this study, there were 141 subjects with negative anti-AAV9 NAbs and 57 subjects with positive anti-AAV9 NAbs but with IC_{50} titers <100. This meant that the potential beneficiary population for gene therapy using AAV9 as a vector could constitute 58.1 % (198/341).

3.3. The seroprevalence of Anti-AAV9 TAbs in the Chinese population

Serum samples from children and adults were also analyzed for the positive rate and titers of TAbs. The positivity rates of anti-AAV9 TAbs were 64.8 % (175/270) in the healthy population and 62.0 % (44/71) in patients with rare diseases, with no significant difference observed between the two groups. Consequently, the positivity rate was calculated based on the combined population (64.2 %, 219/341). The results exhibited trends consistent with those observed for NAbs, as detailed in Table 2 and Fig. 3. Of the 341 volunteers tested, 57.2 % (195/341) were positive for both anti-AAV9 TAbs and NAbs, while 7.0 % (24/341) were positive for TAbs but negative for NAbs. Only one child and five adults

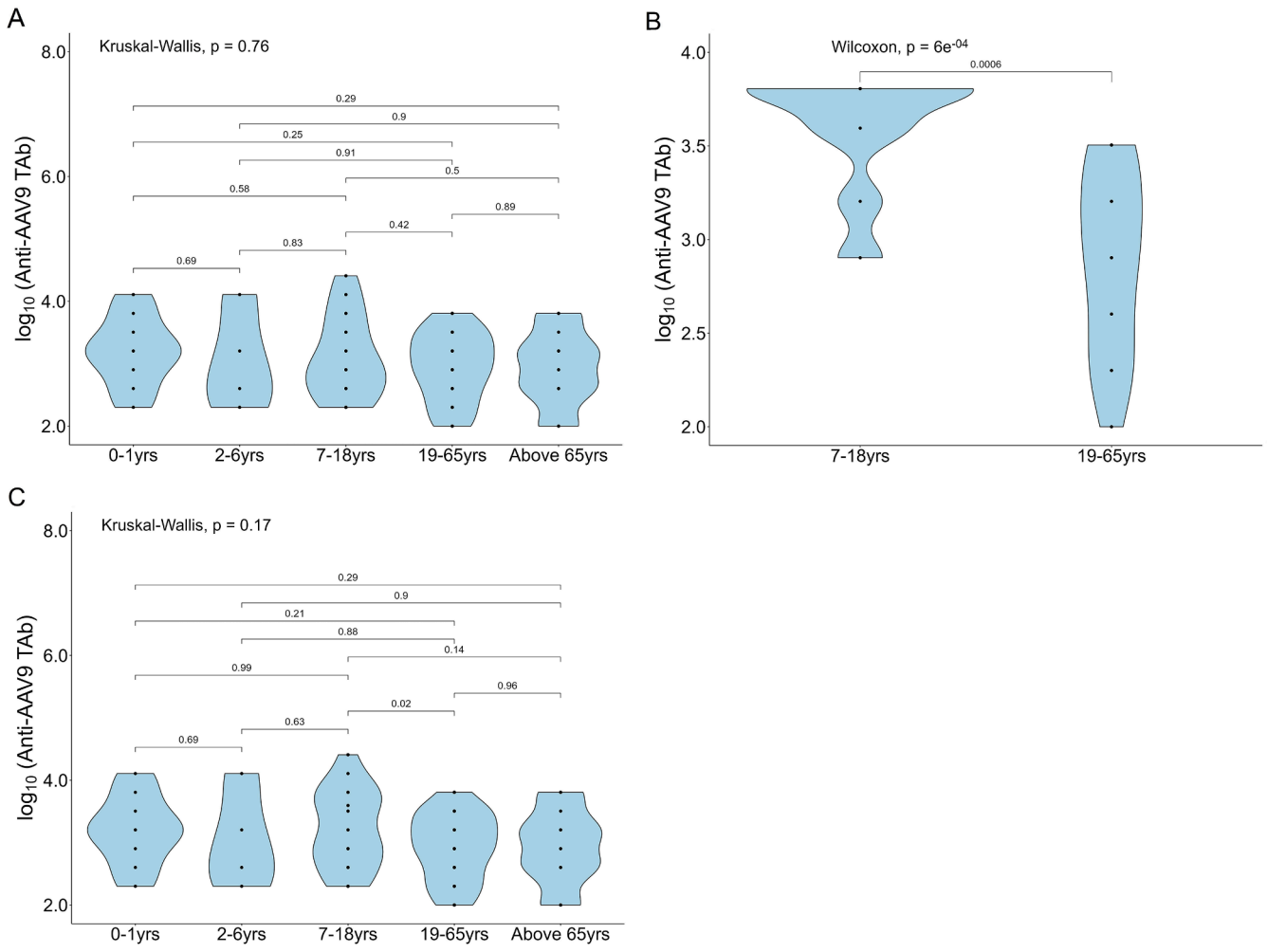

Fig. 3. Distribution of anti-AAV9 binding antibody titers in children and adults. A: Healthy volunteers; B: Patients with rare diseases; C: Overall volunteers. Serum samples from children and adults were also analyzed by an ELISA method for anti-AAV9 binding antibodies. The positive rate and titers exhibited trends consistent with those observed for anti-AAV9 neutralizing antibodies.

tested positive for NAbs with an IC_{50} ranging from 19 to 35, which were slightly above the positive cutoff for NAbs testing, while TAbs were negative.

3.4. Correlation analysis between neutralizing antibodies, binding antibodies and age

Correlation analysis of TAb titers and NAb IC_{50} values in children and adults demonstrated a significant correlation between binding antibody titers and neutralizing antibody IC_{50} (Fig. 4A).

Additionally, we analyzed the correlation between age and antibody titers. The results showed that while the positivity rate of anti-AAV9 NAbs in adults (>18 years) was significantly higher than that in children, no correlation was observed between NAb titers and age (Fig. 4B). Similarly, no correlation was found between age and TAb titers (Fig. 4C).

4. Discussion

Here, we reported the pre-existing anti-AAV9 antibodies across various age groups in the Chinese population. Our findings revealed a high positivity rate for anti-AAV9 NAbs in newborns, particularly in those <2 days old, where 5 out of 7 tested positive (71.4 %). This rate was comparable to that observed in adults (74.8 %), suggesting maternal transfer as a likely source of these antibodies. Additionally, the titers (IC_{50}) of AAV9 NAbs in newborns did not significantly differ from those in adults (240.2 ± 3.3 vs 232.9 ± 4.1). The factors contributing to stronger pre-existing immunity in newborns and adults, such as maternal antibody transmission or natural AAV infections, warrant further investigation.

The seroprevalence of anti-AAV9 NAbs reached its lowest point between 6 months and 3 years of age. This period represents a "naive" immunological status with minimal exposure to AAVs, making it potentially the optimal age range for gene therapy interventions. The seroprevalence of anti-AAV9 NAbs had a positive association with age, particularly during adolescence. A similar relationship was also found in other AAV serotypes such as AAV2 and AAV8 (Calcedo et al., 2011; Dhungel et al., 2024). This rise may result from increased opportunities for AAV exposure or repeated infections in adulthood. A global study on the seroprevalence of NAbs against six AAV serotypes across 10 countries found higher prevalence rates in Asians, with regional variations observed between Japan and South Korea. These findings highlight the influence of environmental and genetic factors on seroprevalence (Chhabra et al., 2024).

We also analyzed the relationship between age, sex, and anti-AAV9 antibody titers. It is commonly assumed that children and the elderly have weaker immune responses due to immature or declining immune systems, which might result in lower antibody titers after viral infection. However, our data showed no significant correlation between age and the titers of either anti-AAV9 Tabs or NAbs. This suggested that while

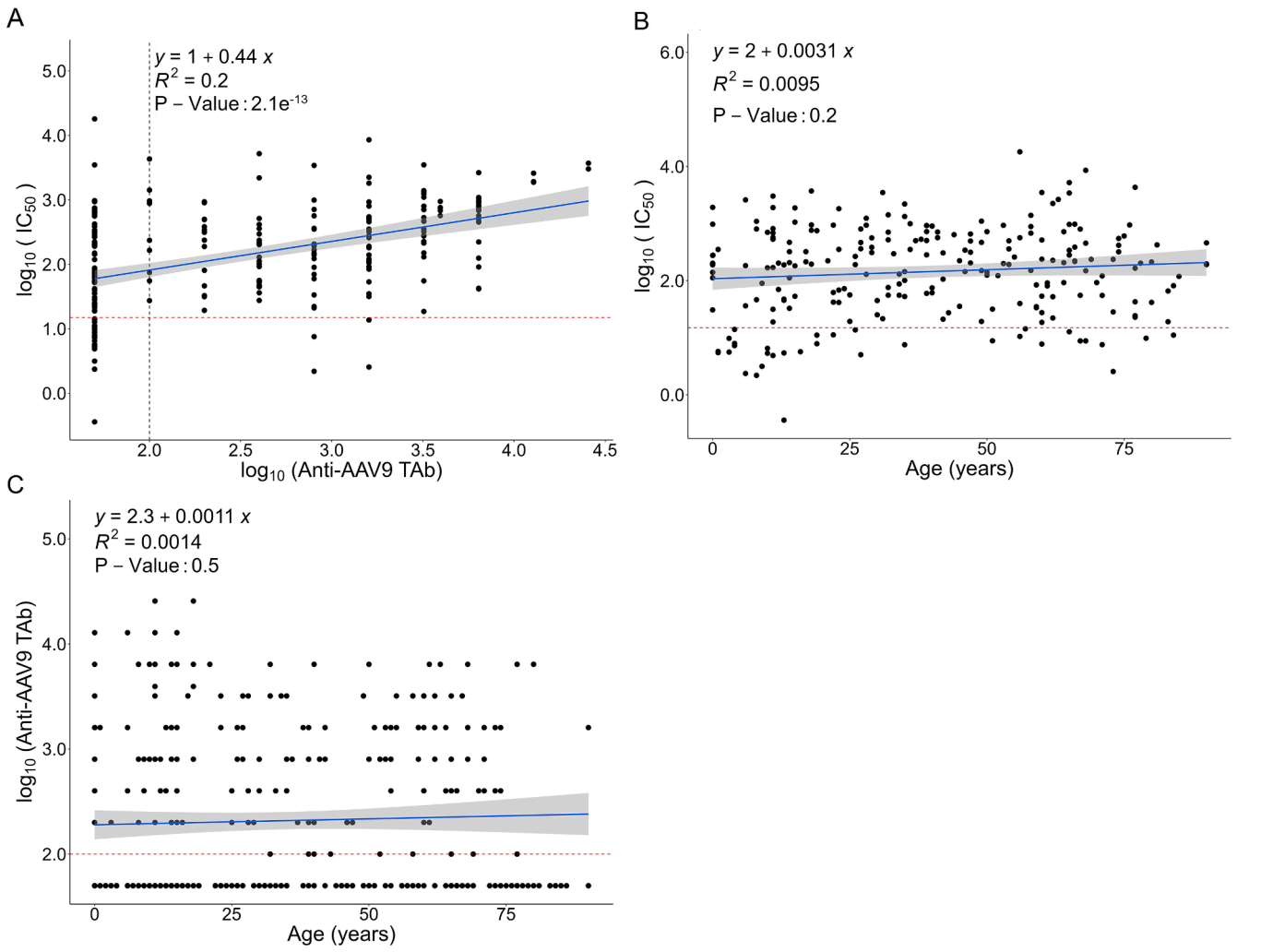

Fig. 4. Correlation analysis between neutralizing antibodies, binding antibodies and age. A: Neutralizing antibody IC_{50} vs binding antibody titer; B: Neutralizing antibody IC_{50} vs age; C: Binding antibody titer vs age. The dashed lines indicate the cut points of the neutralizing antibody IC_{50} and the binding antibody titer, respectively. Correlation analysis of binding antibody titers and neutralizing antibody IC_{50} values in children and adults demonstrated a significant correlation between binding antibody titers and neutralizing antibody IC_{50}. No correlation was observed between neutralizing antibody titers and age. Similarly, no correlation was found between age and binding antibody titers.

immune responses may vary among age groups, the production of AAV9 antibodies was comparable across children, adults, and the elderly. Interestingly, among adults with rare diseases, the titers of anti-AAV9 NAbs appeared to correlate with age ($P < 0.05$, Supplementary Figure 2), with IC_{50} values increasing as age advanced. However, this observation requires further validation due to the limited number of cases. Contrary to reports suggesting that females have stronger anti-AAV NAb responses than males (Liu et al., 2013), our study found no significant sex-based differences.

In the context of gene therapy, pre-existing NAbs against AAV can hinder vector entry into target cells, intracellular transport, and nuclear uncoating, thereby reducing transduction efficiency. Varying NAb titers can lead to diminished therapeutic efficacy or complete failure. All conducted clinical trials for gene therapy drugs have incorporated the measurement of NAb titers as one of their inclusion and exclusion criteria for subject screening. Our research has revealed a strong correlation between the titers of anti-AAV9 TAbs and Nabs, in both positivity rates and titers. Only 7.0 % (24/341) of subjects exhibited positive results for TAbs but negative for NAbs, with these subjects having very low TAb titers. By raising the TAb titer threshold for clinical inclusion, the proportion of such subjects could be further minimized. Furthermore, since TAbs detection is faster and less costly than NAbs detection, it is worthwhile to consider the use of anti-AAV9 TAbs detection as an alternative to NAbs detection for subject screening in clinical trials. This alternative strategy has been applied for patient screening in the gene therapy clinical trial of onesamnogene abeparvovec in SMA patients (Day et al., 2021).

Boutin et al. reported that the positivity rate for anti-AAV9 neutralizing antibodies was 47 % among 226 healthy French adults aged 25–64 years (Boutin et al., 2010). Kavita et al. found that among 100 American patients with heart disease, the positivity rates for anti-AAV9 neutralizing and binding antibodies were 65 % and 66 %, respectively (Kavita et al., 2018). Mimuro et al. reported that the positivity rate for anti-AAV9 neutralizing antibodies was 36.5 % among 85 healthy Japanese adults and 27.4 % among 59 patients with hemophilia (Mimuro et al., 2014). Stolte et al. reported that the positivity rate for anti-AAV9 neutralizing antibodies was approximately 46 % among 69 German adult patients with spinal muscular atrophy, aged 20–58 years (Stolte et al., 2022). These rates were all lower than the positivity rate observed in Chinese adults (75.0 %), which may be influenced by various factors, such as the detection method (e.g., sensitivity, cut-off point, and robustness), the study population, geographical distribution, disease status, and environmental influences.

Wei et al. reported that among 100 Chinese male patients with

Duchenne Muscular Dystrophy (DMD) aged 3–15 years (median age, 8 years), the positivity rate for anti-AAV9 neutralizing antibodies was 42 % (Wei et al., 2024), which was very close to the rate obtained in our study. Our data show that the positivity rates for healthy children and children with rare diseases aged 2–18 years were 47.5 % and 40.9 %, respectively. In the DMD patients, NAb titers ranged from 1.9 to 14,240, and our study demonstrated a very high degree of variability with a similar result.

The limited number of rare disease patients resulted in a small sample size for specific diseases. Consequently, while our findings showed that the positivity rates and antibody titers for AAV9 infection in rare disease patients were consistent with those in healthy individuals, the possibility of differences between specific disease populations and healthy controls cannot be excluded. Future research with larger populations of rare disease patients is needed to validate these results and explore potential disease-specific variations.

5. Conclusion

In summary, the seroprevalence of anti-AAV9 NAbs in the Chinese population aged 0–90 years was 58.7 %, with rates of 34.3 % in children (≤18 years) and 75.0 % in adults (18–90 years). The seroprevalence in adults was significantly higher than in children, with no decline observed in individuals over 65 years of age. Age between 6 months and 3 years is potentially the optimal age range for gene therapy interventions.

Both the positivity rate and titers showed a strong correlation between TAbs and NAbs. In the future, it could be considered to replace NAbs with TAbs, which are easier to test and more cost-effective, as one of the criteria for screening gene therapy subjects.

Ethics statement

This study was approved by the Ethics Committee of Peking Union Medical College Hospital (No. I-22PJ742). The participants provided written informed consent before their involvement in the study and signed agreements for the publication of this article.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2025.199549.

Data availability

Data will be made available on request.

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