Understanding pre-existing immunity against AAV: implications for hemophilia gene therapy

Gene therapies for hemophilia are currently being studied to determine their safety and efficacy.
Approved gene therapies for hemophilia may have different labelling in different countries.

Pfizer

Contents

> Synopsis

Outcomes for gene therapy in hemophilia depend on successful transduction – the delivery of a functional gene to a target cell that is then able to express the therapeutic protein of interest.[1] An engineered vector derived from the naturally occurring adeno-associated virus (AAV) is the most commonly used vector for hemophilia gene therapies currently under investigation.[2,3] During an individual's lifetime, it is possible to have natural exposure to wild-type AAV, resulting in the generation of memory B and T cells as part of an immune response.[2,4,5] AAV seroprevalence – the frequency of individuals in a population who have antibodies against a given AAV serotype – depends on many factors such as age, geographical location and AAV serotype.[5] Accurate identification of individuals with pre-existing AAV immunity is an important consideration for gene therapy as it can affect the transduction of target cells, but there are a number of variables affecting assay results.[2,6] Understanding the level of exposure to wild-type AAV within the hemophilia population may aid the evaluation of the impact of pre-existing immunity on successful transduction of AAV-mediated gene therapy.[4,7]

The information included in this brochure is accurate as of January 2023. Please visit www.genetherapyscience.com for further information and check back regularly for updates.

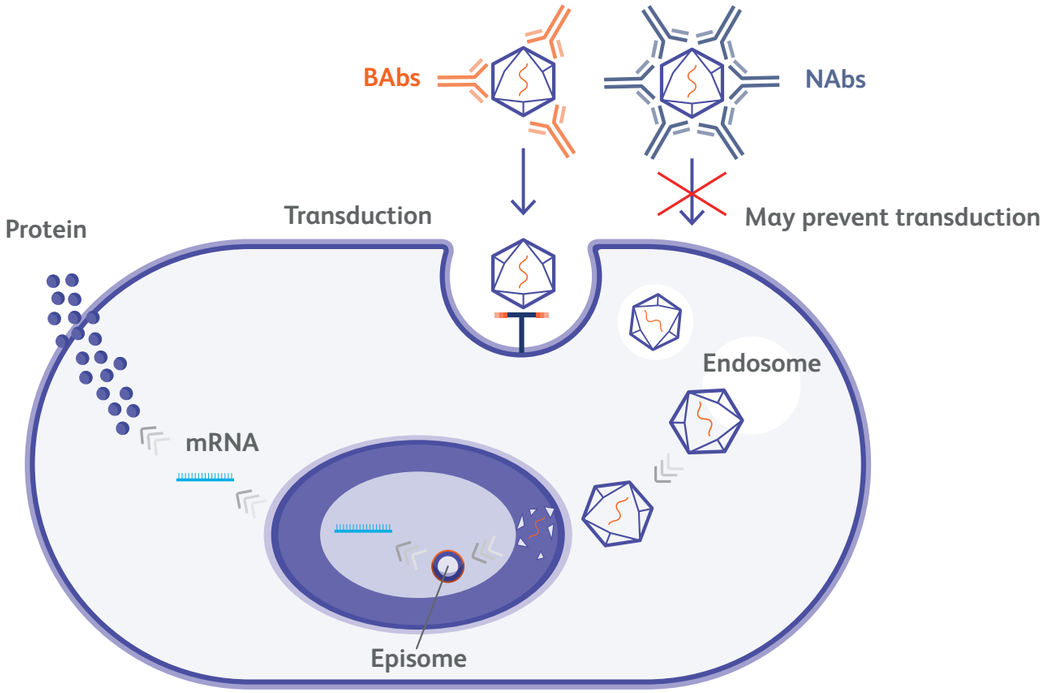
Gene therapies for hemophilia are currently being studied to determine their safety and efficacy. Approved gene therapies for hemophilia may have different labelling in different countries.

Understanding AAV serotypes and seroprevalence

Defining neutralizing antibodies and binding antibodies

Although AAV itself is not known to cause disease in humans, capsid proteins can be recognized as "foreign" by the immune system and may induce an immune response, including the production of anti-capsid neutralizing antibodies (NAbs) and binding antibodies (BAbs)[7-9]

- **NAbs** recognize AAV and may block cell transduction[8]
- **BAbs** are able to recognize the AAV capsid, but do not have the ability to prevent cell transduction[8]



Did you know?

- Antibody-dependent enhancement (ADE) occurs when antibodies bind to a pathogen but help it to enter cells, thus enhancing rather than preventing infection[10]
  - Preclinical research is currently investigating whether ADE might be exploited to improve transduction by creating complexes of AAV and anti-AAV antibodies to assist cell entry[10]

What is a serotype?

- A serotype is distinguished by **variations in the amino acid sequence of surface proteins** (antigens) on the vector, which play a key role in differing antibody responses and tissue tropism[11]
- Various serotypes of wild-type AAV have been isolated with unique tissue tropism, most of which are currently used in clinical studies for various diseases[12]

To explore the vectors used in gene therapy, see the brochure *"Recombinant AAV vectors: from virus to therapeutic vector"* or visit www.genetherapyscience.com

- Homology between AAV capsid proteins means that antibodies toward one serotype can be cross-reactive to another, recognizing and neutralizing more than one serotype[13]
  - **Cross-reactivity** is the reactivity of one antigen with antibodies developed against another antigen[14]
  - Some proteins on the surface are conserved between serotypes thus patients may exhibit an immune response to a number of serotypes[11]
  - In the context of gene therapy, at present, this suggests that re-administration of closely related AAV-derived vectors may be challenging due to AAV cross-reactivity[15]

What is seroprevalence?

- AAV seroprevalence describes the proportion of individuals in a population who have antibodies (NAbs and/or BAbs) to a given AAV serotype in their bloodstream[16]

Gene therapies for hemophilia are currently being studied to determine their safety and efficacy. Approved gene therapies for hemophilia may have different labelling in different countries.

How is seroprevalence measured?

Different assays that measure different parameters can be utilized to measure seroprevalence. The most widely used are the transduction inhibition assay and the total antibody binding assay[4]

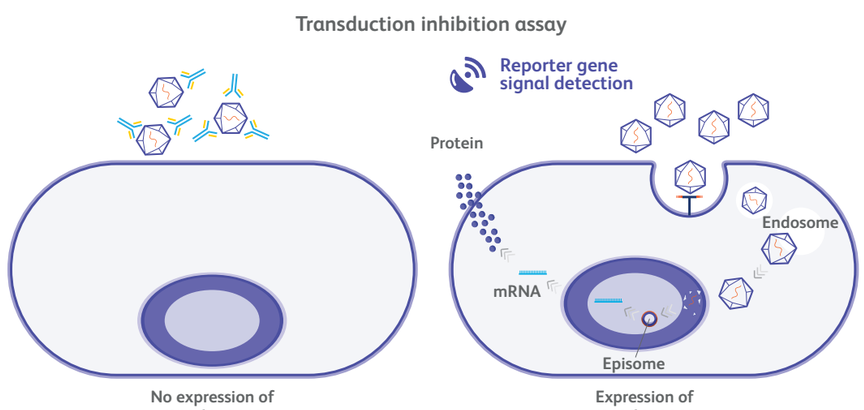
Transduction inhibition assay (NAb assay)

Cellular transduction-based *in vitro* assay[6,17]

> Measures the ability of a subject's serum/plasma samples to reduce the transduction of cells by an rAAV vector carrying the reporter gene[17]

Method

> An AAV vector expressing a reporter gene is mixed with the test serum/plasma sample, diluted and incubated with the chosen cell line, which is then analyzed for expression of the reporter gene[4,18]



Considerations

> Currently considered the standard for determining anti-AAV neutralizing factors[4]

> Highly reliant on efficient transduction *in vitro* (which for some AAV serotypes is inefficient). High multiplicity of infections (MOI) in the assay may result in lower sensitivity of detection of antibodies[18]

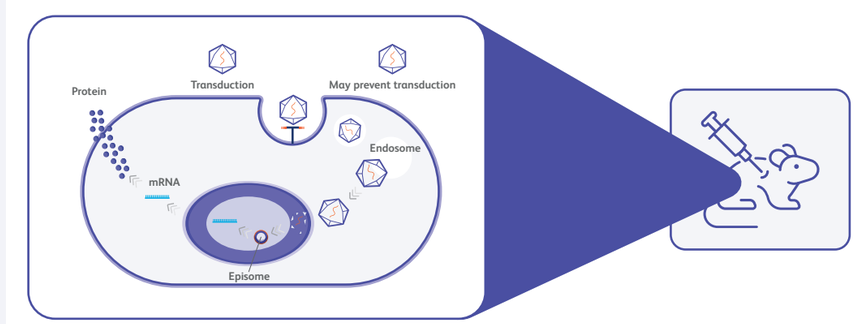
- Often utilizes reporter genes with high detection sensitivity (such as luciferase or green fluorescent protein) and cell lines that allow for AAV transduction (most often Human embryonic kidney HEK293 cells)[18,19]

> Measures anti-AAV neutralizing factors based on a reduction in transduction. These factors could encompass both NAbs as well as other non-NAb factors (e.g. inhibition of transduction due to galectin-3 binding protein)[17,19]

Cellular transduction-based *in vivo* assay (e.g. in mice)[6,19]

Method

> Involves immunization of mice with donor plasma or intravenous immunoglobulin[19,20]



Considerations

> As a sensitive assay, it can capture the low levels of inhibitors in the testing sera that affect AAV transduction[6]

> Screening for large populations is not suitable due to plasma volume requirements[19]

Variables affecting results

> Sample volume and starting dilution[4]

> Assay conditions (temperature, incubation time, culture duration)[4]

> Number of rAAV vector particles per cell and presence of empty capsids[6,19]

> Choice of cell line for *in vitro* transduction method[4]

> Choice of reporter gene (e.g. luciferase, LacZ)[4]

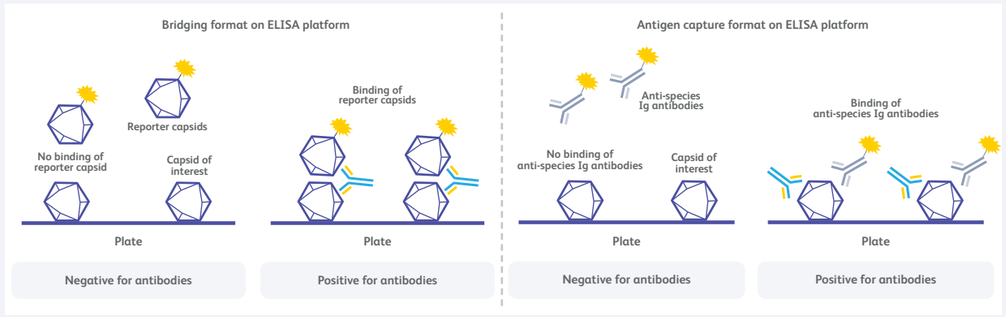
> Transduction efficiency of rAAV vector[6]

> Inactivation of complement proteins[4]

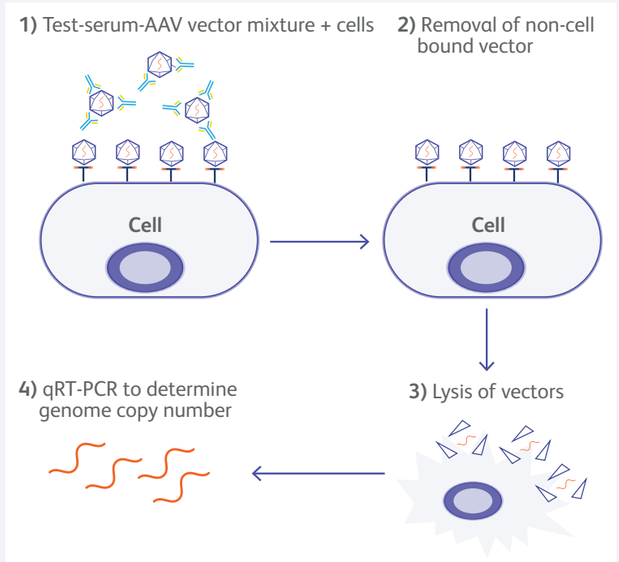
Gene therapies for hemophilia are currently being studied to determine their safety and efficacy. Approved gene therapies for hemophilia may have different labelling in different countries.

How is seroprevalence measured? (cont'd)

	Method	Considerations	Variables affecting results
Total antibody binding assay > Capture-based *in vitro* method to detect antibodies capable of binding to the AAV capsid[4]	> Test plasma/serum is added to AAV capsids coated on a plate and antibodies that bind are detected with a secondary reagent (i.e., enzyme-linked immunosorbent assay [ELISA], which can differentiate between immunoglobulin classes)[4,7,21]	> Measures total antibodies, including those with lower affinity, and does not distinguish between NAbs and BAbs[6,7,21] > Results therefore do not represent neutralizing activity[6]	> Sample volume and starting dilution[4,21] > Assay conditions (temperature, incubation time, culture duration, reagents)[4,21] > Unknown if use of empty capsids can affect results[21]



	Method	Considerations	Variables affecting results
Cell-binding assay > Cell-binding *in vitro* assay[6] > Based on AAV-target cell binding, quantifies the genome copy number of rAAV vector bound to the cell surface in the sample[6]	> Test serum and vector are mixed and incubated to allow any neutralizing activity[6] > Serum-rAAV vector mixture is incubated with chosen cell line to allow binding of rAAV vectors to cell surfaces (but not vector entry)[6] > After washing to remove unbound rAAV vector, the cells are lyzed with lysis buffer, then incubated at a higher temperature to lyze vector capsids to allow release of viral genomes[6] > Viral genome copy numbers measured via real-time qPCR assay[6]	> Only measures inhibition of receptor engagement[6,22] > May miss post-entry neutralization effects of antibodies (for instance, antibodies that block endosome escape, nuclear entry, or other post-entry vector trafficking steps)[6,22]	> Cell line, assay controls[6]



qPCR: Quantitative polymerase chain reaction.

Variations between antibody assays and lack of standardization remain a challenge for rAAV gene therapy clinical trials[4,6]

Gene therapies for hemophilia are currently being studied to determine their safety and efficacy. Approved gene therapies for hemophilia may have different labelling in different countries.

AAV seroprevalence across the globe

How many people have been exposed to wild-type AAV?

Natural exposure to wild-type AAV can occur during an individual's lifetime.[2,4,5] Since the 1960s, numerous studies have investigated the seroprevalence of AAV NAbs among the general population. Reported AAV seroprevalence rates vary between studies, depending on many factors, including: [4,7,23,24]

Assay used[4,7]

There is currently a lack of standardization of assays used to determine seroprevalence, both in the threshold for positivity (cut-off to define seropositivity), as well as the detection methods – measuring neutralizing activity vs measuring total antibodies[4,7]

> For example, if using the total antibody measure, BAbs would also be included in the measurement, but their role in the context of gene therapy regarding vector biodistribution and transgene expression is not fully understood[7,8]

AAV serotype[7,23,25]

AAV seroprevalence in the adult population is highly variable and has been reported to be as high as 95% for some serotypes[26,27]

> Neutralizing antibodies to AAV2 have been found to be the most prevalent antibodies across all regions globally, followed by antibodies to AAV1.[23] The seroprevalence of antibodies to AAV7, AAV8 and AAV9 were found to be lower than that for AAV1[7,23]

> Data are limited at present, but to date no major difference in prevalence has been found between the general population and the hemophilia population[25]

Characteristics of the study population, including age, ethnic distribution and geographical location[5,7,23–25]

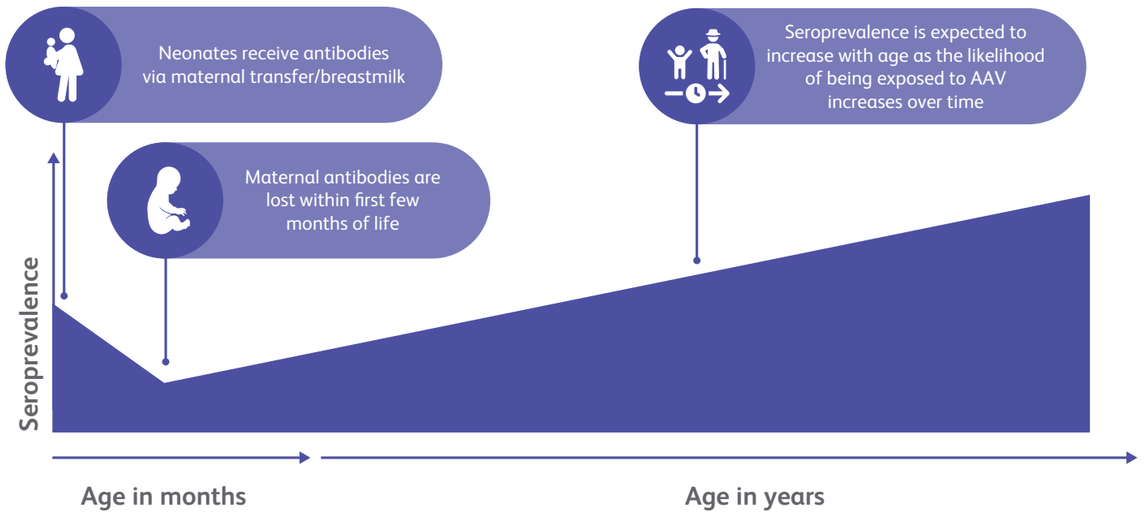


Figure developed from: Baruteau J, et al. *J Inherit Metab Dis* 2017;40:497–517.[5] Li C, et al. *Gene Ther* 2012;9:288–94.[24]

> In a recent study, inter-racial variations in AAV antibody seroprevalence have been observed, with higher prevalence reported in black and Hispanic donors compared to white donors, for most AAV serotypes[28]

Combined, these variations can greatly influence results, and lead to a high degree of uncertainty regarding actual seroprevalence levels[4]

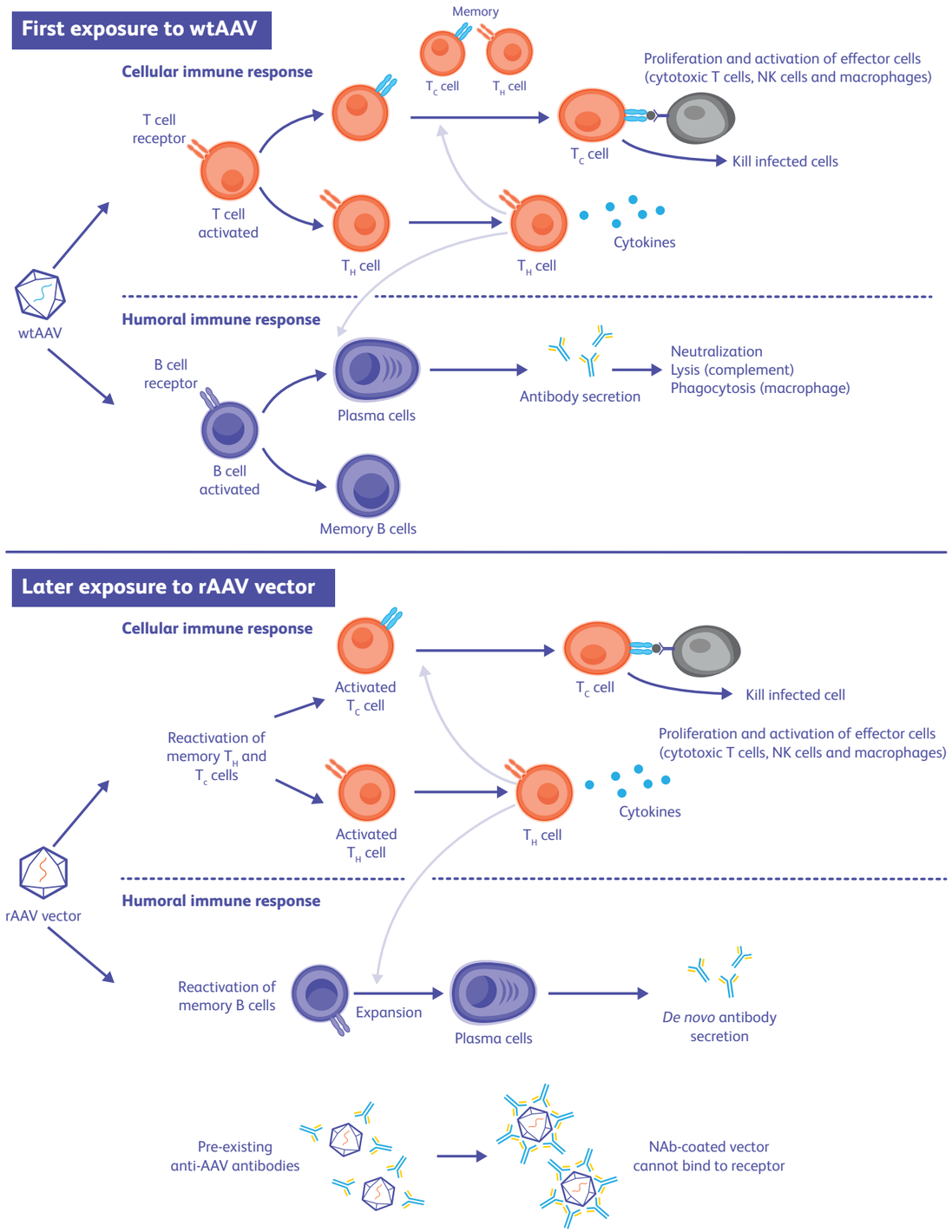
Gene therapies for hemophilia are currently being studied to determine their safety and efficacy. Approved gene therapies for hemophilia may have different labelling in different countries.

Pre-existing immunity to rAAV vector

How does exposure to wild-type AAV prime the immune system to recognize AAV antigens and impact transduction?

- An individual who has experienced natural exposure to wild-type AAV (or has pre-existing exposure to the same or a similar AAV serotype from which the rAAV gene therapy vector was engineered) can experience a rapid, highly specific adaptive immune response toward antigens on the surface of the rAAV vector following administration of gene therapy[2,5,29]
- Following exposure to AAV, **highly specific memory cell populations** and **anti-AAV antibodies** remain after the infection is resolved and can be reactivated on exposure to the same antigen,[7] thus providing long-lasting protection against AAV[30]
- The recombinant capsid of the rAAV vector used for gene therapy is a close mimic of a viral capsid (although the rAAV vector is not a virus and is not capable of inducing synthesis of viral proteins).[2] Immune responses to the vector can therefore be influenced by prior exposure to wild-type AAV from which the vector was engineered[2]
- Following administration of gene therapy, pre-existing cross-reactive anti-AAV antibodies can bind and may neutralize the rAAV vector, and the memory B cell population expands, resulting in the release of more antigen-specific antibodies[7,29]
  - The NAb-coated vector will most likely be unable to bind the receptor on the target cells, and transduction may not occur[2,29]

To explore the mechanisms of the immune response to gene therapy, see the brochure *"Immune responses associated with hemophilia gene therapy"* or visit www.genetherapyscience.com



AAV: Adeno-associated virus; NAb: Neutralizing antibody; NK: Natural killer; rAAV: Recombinant AAV; Tc cell: Cytotoxic T cell; TH cell: T helper cell; wtAAV: Wild-type AAV.

Figure developed from: Vandamme C, et al. *Hum Gene Ther* 2017;28(11):1061–74[7]; Alberts B, et al. *Molecular Biology of the Cell.* 4th edition. New York: Garland Science; 2002. Chapter 24: The Adaptive Immune System[30]; Pennock ND, et al. *Adv Physiol Educ* 2013;37(4):273–83[31]; Muhuri M, et al. *J Clin Invest* 2021;131(1):e143780[32]

Gene therapies for hemophilia are currently being studied to determine their safety and efficacy. Approved gene therapies for hemophilia may have different labelling in different countries.

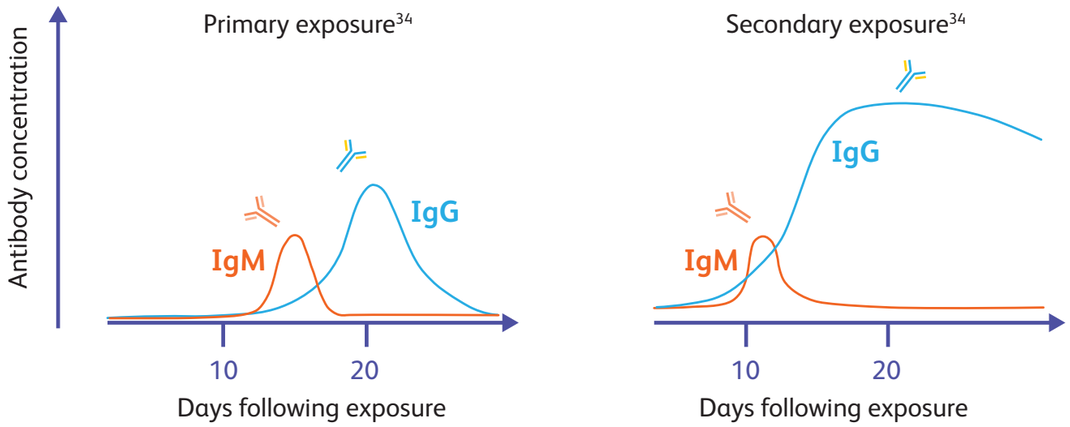
Which immunoglobulin types are involved in the antibody response to the rAAV vector following gene therapy?

IgM[33]

- Found mainly in the blood and lymph fluid
- First antibody to be made by the body to fight new infections
- IgM response declines after an initial peak[34]

IgG[33]

- Most abundant antibody type
- Found in all body fluids
- Protects against bacterial and viral infections, and other "foreign" antigens
- Dominant IgG subclass of binding antibodies: IgG1[25,26,35]
- IgG response occurs after IgM, but is higher and more sustained, particularly on subsequent exposure to an antigen[34]



Ig: Immunoglobulin.
Figure adapted under creative commons license from: Lumen Microbiology. https://courses.lumenlearning.com/microbiology/chapter/b-lymphocytes-and-humoral-immunity/ (Accessed December 2022)

- Titers of IgG1, IgG2 and IgM are correlated with neutralizing factors titers (this is not the case for IgG3 and IgG4)[25,26,35]
- rAAV vector-based gene therapy appears to be impacted by an interplay of IgG and non-IgG antibodies, that may result in neutralization or enhancement of AAV transduction[10]

Gene therapies for hemophilia are currently being studied to determine their safety and efficacy. Approved gene therapies for hemophilia may have different labelling in different countries.

Addressing pre-existing immunity to AAV

Does the presence of anti-AAV antibodies affect patient eligibility for rAAV vector-based gene therapy clinical trials?

- Most clinical studies have selected patients with low-to-undetectable anti-AAV NAbs against the recombinant vector in order to avoid adaptive immune responses to AAV[29]
  - Some hemophilia clinical trials (using AAV5-based gene therapy) have not excluded patients with pre-existing antibodies[36–40]
  - Interpretation between clinical trials is complicated by capsid design variation, vector doses, vector titer determination and vector transduction efficiency[41]
- Some antibody assays for AAV have suboptimal sensitivity (i.e., risk of false-negatives) making it difficult to exclude all patients with pre-existing anti-AAV NAbs[2]
- Studies in humans and non-human primates have shown that even low AAV2 and AAV8 NAb titers can reduce or completely block AAV liver transduction following systemic delivery,[42,43] whereas in a recent study, AAV5 NAbs do not appear to impair the efficacy of *in vivo* transduction of AAV5-based vector up to a measured titer of 340 in humans[44]
  - These findings indicate the neutralization ability of antibodies differs significantly between these AAV serotypes[45]
  - A study evaluating avidity of pre-existing anti-AAV NAbs from the healthy human population showed that anti-AAV5 NAbs formed the weakest antibody–antigen complexes, when compared with anti-AAV2 or anti-AAV8 NAbs[45]

Several different strategies have been considered or are under investigation for their application in the field of rAAV vector-mediated gene therapy – not only in hemophilia – with the aim of overcoming or evading pre-existing immune responses to AAV.

Strategies under investigation to address pre-existing immunity to AAV

Recipient-orientated strategies
(pre-clinical stage for hemophilia)

Immunosuppression[2,32,46,47]	• Aims to control the immune response to the rAAV vector[46,47] • Selective immunosuppressive drugs can have an effect on inducing immune tolerance[32]
Non-specific cleavage of circulating immunoglobulins[32,48]	• Treatment with the cysteine protease (IdeS, derived from *Streptococcus pyogenes*) can cleave IgG, preventing it from binding to the target antigen[32,48] • May provide a NAb-free period for AAV vector delivery[32]
Plasmapheresis[49]	• Repeating plasma exchange cycles to reduce the titer of circulating NAbs in a non-invasive manner[49]
Isolation of target tissue[29,32]	• Utilizing techniques such as balloon catheters followed by saline flushing to isolate the target tissue from the systemic circulation to avoid vector dilution in blood and exposure to NAbs[29,32,50]
Administration of high vector doses[2] **Administration of a decoy such as empty capsids – a by-product of rAAV vector production**[2,6,51,52]	• Using excess rAAV vectors or empty capsids to dilute the NAb response, acting as 'decoys' for AAV-specific antibodies, and allowing transduction of target cells by unbound vectors[2]

Vector-orientated strategies
(pre-clinical stage for hemophilia)

Use of AAV serotype with lower seroprevalence or use novel capsids from non-human sources[2,53,54]	• Using alternative human AAV serotypes or those isolated from other vertebrates to evade NAb responses[53,54]
Capsid engineering[2,46,55,56]	• Generation of novel serotypes by recombining existing AAV capsids to produce a large library of vectors that are prescreened and selected for low reactivity to NAbs while maintaining target cell binding specificity[46,55] • Vector engineering techniques, such as site-directed mutagenesis or masking vector epitopes, can reduce antibody responses and increase transgene expression[46,56]

AAV: Adeno-associated virus; IgG: Immunoglobulin G; NAb: Neutralizing antibody; rAAV: Recombinant AAV.

Understanding AAV seroprevalence within the hemophilia population and a deeper understanding of strategies to address pre-existing immunity to AAV will aid the evaluation of the safety and efficacy of rAAV vector-mediated gene therapy

To find out more about strategies to overcome adaptive immune response to gene therapy, see brochure *"Immune responses associated with hemophilia gene therapy"* or visit www.genetherapyscience.com

Gene therapies for hemophilia are currently being studied to determine their safety and efficacy.
Approved gene therapies for hemophilia may have different labelling in different countries.

Key take-home messages:

- Components of the rAAV vector are recognized as “foreign” by the immune system and induce an immune response, including the production of neutralizing antibodies (NAbs), which can interfere with cell transduction.[7–9] Identifying individuals with pre-existing anti-AAV immunity, or AAV seroprevalence, is therefore an important consideration for gene therapy[2,6]
- Seroprevalence can be assessed by transduction inhibition assays, total antibody binding assays and cell-binding assays; lack of assay standardization across assay results is currently a challenge for gene therapy trials using rAAV vectors [4,6]
- AAV seroprevalence varies between serotypes;[23,27] NAbs to AAV2 are the most prevalent across all regions globally, followed by antibodies to AAV1, while seroprevalence to AAV7, AAV8 and AAV9 are lower than that for AAV1[6,7,20,23]
- An individual who has pre-existing exposure to the same or a similar AAV serotype from which the rAAV vector was engineered can experience a rapid, highly specific adaptive immune response toward antigens on the surface of the rAAV vector following administration of gene therapy[2,5,29]
- Several strategies are under investigation to address pre-existing AAV immunity, which are either recipient-orientated (e.g. immunosuppression,[2,32] non-specific cleavage of circulating immunoglobulin,[32,47] plasmapheresis,[48] administration of high vector doses[2]) or vector-orientated (e.g. use of AAV serotypes with low seroprevalence, capsid engineering[2,45])

Gene therapies for hemophilia are currently being studied to determine their safety and efficacy. Approved gene therapies for hemophilia may have different labelling in different countries.

References

1. Lheriteau E, et al. *Blood Rev* 2015;29:321–8.
2. Mingozzi F, High KA. *Blood* 2013;122:23–36.
3. Nathwani A. *Hematol Am Soc Hematol Educ Program* 2019;2019(1):1–8.
4. Stanford S, et al. *Res Pract Thromb Haemost* 2019;3:261–7.
5. Baruteau J, et al. *J Inherit Metab Dis* 2017;40:497–517.
6. Guo P, et al. *Mol Ther Methods Clin Dev* 2019;13:40-6
7. Vandamme C, et al. *Hum Gene Ther* 2017;28:1061–74.
8. Fitzpatrick Z, et al. *Mol Ther Methods Clin Dev* 2018;9:119–29.
9. Shirley JL, et al. *Mol Ther Rev* 2020;28:709–22.
10. Patel N, et al. *Molec Ther* 2019;27(4S1):139.
11. Sidonio RF Jr, et al. *Blood Rev* 2021;47:100759.
12. Li C, Samulski RJ. *Nat Rev* 2020;21:255–72.
13. Grimm D, Kay MA. *Curr Gene Ther* 2003;3:281–304.
14. Meriam Webster. Available at: https://www.merriam-webster.com/dictionary/cross-reactivity (Accessed January 2023).
15. Kattenhorn LM, et al. *Hum Gene Ther* 2016:27;947-61.
16. Meriam Webster. Available at: https://www.merriam-webster.com/dictionary/seroprevalence (Accessed January 2023).
17. Falese L, et al. *Gene Ther* 2017;24:768–78.
18. Meliani A, et al. *Hum Gene Ther Methods* 2015;26:45–53.
19. Weber T. *Front Immunol* 2021; 17;12:658399.
20. Wang M, et al. *Gene Therapy* 2015;22:984–992.
21. Gorovits B, et al. *AAPS J* 2021;23(6):108.
22. Gorovits B, et al. *AAPS J* 2020;22(2):24.
23. Calcedo R, et al. *J Infect Dis* 2009;199:381–90.
24. Li C, et al. *Gene Therapy* 2012;9:288–94.
25. Kruzik A, et al. *Mol Ther Methods Clin Dev* 2019;14:126–33.
26. Boutin S, et al. *Hum Gene Ther* 2010;21:704–12.
27. Majowicz A, et al. *Blood* (2019) 134 (Supplement_1): 3353
28. Khatri A, et al. *Hum Gene Ther* 2022;33(7–8):442–50.
29. Collela P, et al. *Mol Ther Methods Clin Dev* 2018;8:87–104.
30. Alberts B, et al. Molecular Biology of the Cell. 4th edition. New York: Garland Science; 2002. Chapter 24: The Adaptive Immune System.
31. Pennock ND, et al. *Adv Physiol Educ* 2013;37:273–83.
32. Muhuri M, et al. *J Clin Invest* 2021;131:e143780.
33. Rady Children's Hospital. www.rchsd.org/health-articles/blood-test-immunoglobulins-iga-igg-igm/ (Accessed January 2023).
34. Lumen Microbiology. https://courses.lumenlearning.com/microbiology/chapter/b-lymphocytes-and-humoral-immunity/ (Accessed January 2023).
35. Murphy S, et al. *J Med Virol* 2009;81:65–74.
36. ClinicalTrials.gov. NCT03520712. www.clinicaltrials.gov/ct2/show/NCT03520712 (Accessed January 2023).
37. ClinicalTrials.gov. NCT03569891. www.clinicaltrials.gov/ct2/show/NCT03569891 (Accessed January 2023).
38. Miesbach W, et al. *Haemophilia* 2019;25:545–57.
39. Recht M, et al. Presented at the 24th American Society of Gene and Cell Therapy (ASGCT) meeting 2021; Abstract 88. Available at: https://uniqure.com/ASGCT%202021-HOPE%20B-Oral_11May_clean.pdf (Accessed January 2023).
40. Pipe SW, et al. Presented at the 62nd American Society of Hematology (ASH) meeting 2020; Abstract LBA-6. Available at: https://ash.confex.com/ash/2020/webprogram/Paper143560.html (Accessed January 2023).
41. Herzog RW, Pierce GF. *Mol Ther* 2019;27(11):1863–4.
42. Manno CS, et al. *Nat Med* 2006;12(3)342–7.
43. Jiang H, et al. *Blood* 2006;108(10):3321–8.
44. Majowicz A, et al. *Mol Ther Methods Clin Dev* 2019;14:27–36.
45. Majowicz A, et al. Abstract P054. Presented at the 13th Annual Congress of the European Association for Haemophilia and Allied Disorders (EAHAD), 5-7 February 2020, The Hague, Netherlands.
46. Wang D, et al. *Nat Rev Drug Discov* 2019;18:358–78.
47. Herzog RW. *Cell Immunol* 2019;342:103658.
48. Leborgne C, et al. *Nat Med* 2020;26:1096–101.
49. Monteilhet V, et al. *Mol Ther* 2011;19:2084–91.
50. Mimuro J, et al. *Mol Ther* 2013;21:318–23.
51. Mingozzi F, et al. *Sci Transl Med* 2013;5:194ra92.
52. Wright JF. *Mol Ther* 2014;22:1–2.
53. Selot R, et al. *Front Pharmacol* 2017;8:441.
54. Barnes C, et al. *Curr Opin Biotechnol* 2019;60:99–103.
55. Paulk NK, et al. *Mol Ther* 2018;26:289–303.
56. Sen D. *J Biomed Sci* 2014;21:103.

Gene therapies for hemophilia are currently being studied to determine their safety and efficacy.
Approved gene therapies for hemophilia may have different labelling in different countries.

For more information

The Hemophilia Gene Therapy Webinar Series explores the complex science underpinning hemophilia gene therapy. Hosted by an expert hematologist, joined by a specialist, each webinar focuses on providing a high-science review of key areas of interest in hemophilia gene therapy.

Gene Therapy
Webinar Series

Scan the QR Code and watch the webinar series, or visit **www.genetherapyscience.com/learning-center**