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REVIEW ARTICLE

Immunogenicity of Biotherapeutics: Current Industry Experience, Challenges and Opportunities

Assessment of Immune Responses Against AAV Encoded Transgene Products

Boris Gorovits[1] · Mitra Azadeh[2] · Michele Fiscella[3] · Travis Harrison[4] · Magdalena Hofer[5] · Sylvia Janetzki[6] · Vibha Jawa[7] · Brian Long[8] · Yanmei Lu[9] · Yolanda D. Mahnke[10] · Mauricio Maia[11] · Ritankar Majumdar[12] · Michelle Miller[13] · Mark Milton[14] · Robert Nelson[15] · Michael A. Partridge[1] · Saleem Shaik[16] · Veerle Snoeck[17] · Christian Vettermann[8] · Bonnie Wu[18] · An Zhao[1]

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Abstract

The number of clinical investigations and approved applications of adeno-associated virus (AAV) based transgene product (TP) delivery has grown steadily. There also has been a growing interest in understanding how anti-AAV and anti-TP immune responses affect the safety and efficacy of these gene therapy treatments. While considerations related to anti-AAV immunity have been discussed in other works, this manuscript focuses on the assessment of anti-TP immune responses, including both humoral and cellular responses. The development of anti-TP antibodies or a cytotoxic cellular response may lead to increased clearance of the TP, elimination of AAV-transduced cells, and consequently, affect the overall durability and efficacy of the treatment. Additionally, the binding and neutralization of residual endogenous protein by anti-TP antibodies might further worsen the clinical condition under treatment. Several topics are explored in this manuscript, including immunogenicity risk factors that can be considered when evaluating the overall risk and impact of anti-TP immunogenicity, potential implications of anti-TP immunogenicity, the importance of assessing anti-TP immunogenicity, and the commonly used analytical methodologies. The manuscript proposes an approach to determining the scope of anti-TP immunogenicity assessment for clinical and non-clinical studies, based on the TP nature, other intrinsic and extrinsic risk factors. Authored by a group of scientists involved in AAV-based therapeutic development from various industry organizations, the manuscript aims to provide recommendations and guidance to industry sponsors, academic laboratories, and regulatory agencies working on AAV-based modalities, with the goal of achieving a more consistent approach to the assessment of anti-TP immune response.

Keywords adeno associated virus · anti-transgene product immunogenicity · immunogenicity · transgene product

Communicated by Boris Gorovits

✉ Boris Gorovits
boris.gorovits@regeneron.com

1 Regeneron Pharmaceuticals, Inc, Tarrytown, USA
2 Progeria Research Foundation, Inc., Cambridge, Massachusetts, USA
3 REGENXBIO Inc, Rockville, USA
4 Precision for Medicine Inc., Frederick, Maryland, USA
5 Jefferson Institute for Bioprocessing, Lower Gwynedd, USA
6 ZellNet Consulting, Inc, Fort Lee, New Jersey, USA
7 EpiVax, Inc, Providence, Rhode Island, USA
8 4D Molecular Therapeutics, Emeryville, USA
9 Sangamo Therapeutics, Inc, Richmond, USA
10 FlowKnowHow LLC, New York, USA
11 South San Francisco, CA, USA
12 LabCorp Holdings, Inc, Burlington, USA
13 Miller Bioanalytical Consulting LLC, Carlsbad, USA
14 Lake Boon Pharmaceutical Consulting, LLC, Hudson, USA
15 BioAgilytix Labs, LLC, Durham, USA
16 Takeda Pharmaceutical Company Limited, Cambridge, USA
17 UCB, B-1420 Braine-l'Alleud, Belgium
18 Johnson & Johnson Innovative Medicine, Titusville, USA

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Introduction

Adeno-Associated Viruses (AAV) are considered one of the most promising classes of gene therapy vectors, demonstrating low toxicity and effective expression of the encoded transgene(s) following administration (1, 2). Recombinant AAV vectors can efficiently deliver therapeutic genes into the nuclei of target cells to directly treat a disease by correcting the genetic defects or to provide additional therapeutic benefits (3–7). In wild-type (WT) AAV vectors, the genome consists of single-stranded DNA approximately 4.7 kilobases (kb) in length (8), while in AAV vectors, the parental genes (rep, cap) and regulatory elements are replaced with a transgene cassette containing the cDNA of the therapeutic protein, flanked by two palindromic inverted terminal repeats (ITR), the only remaining nucleic acid sequences from the WT virus. A diverse range of transgenes has been expressed in clinical studies using rAAV vectors to target different tissues for the treatment of genetic disorders such as hemophilia A and hemophilia B, familial hypercholesterolemia, and Crigler–Najjar syndrome (9). Therapeutic AAV vectors may contain transgenes which encode a variety of transgene products (TP), including proteins, proteoglycans, mRNA and other classes of molecules.

Transgene products, like all biotherapeutics, may contain novel epitopes that have the potential to trigger both innate and adaptive (humoral and cellular) immunity. Activated immune responses may generate TP-specific antibodies and cytotoxic T lymphocytes (CTLs), which could prevent lasting therapeutic efficacy by inhibiting the secreted TP or clearing the transduced cells (10–12). Potential risks associated with anti-TP immune response should be considered in the context of patients' immune tolerance towards the encoded TP, the degree of homology between TP and the endogenous protein, prior exposure to the analogue of the transgene product, e.g., protein replacement therapy, comparative expression of the endogenous and transgene-encoded protein, and several other risk factors that are discussed in this publication.

Mechanisms of immune response against TP has been discussed elsewhere (11) and includes the following steps: a) cells are transduced by TP-encoding AAV vector, b) the TP is either expressed and/or internalized by antigen presenting cells (APC), c) APCs present endogenous and exogenous TP epitopes primarily in the context of MHC I and/or MHC II, respectively, and d) presentation leads to initiation of cellular and/or humoral immune response. The complex interactions between AAV vectors and the host immune system and their influence on TP expression are not yet well understood. Studies have shown that the impact of host immunity on AAV-vector based transgene expression depends on the route of administration, vector serotype, target tissue, administered dose, and the immune status of the individual (10, 13). Presentation of the TP in the context of AAV vector may also change the level of immune response generated against the TP (2, 14, 15) although it has been proposed that AAV based vectors have a lower potential to induce anti-TP immune response as compared to adenoviral or lentiviral vectors (11, 16).

Similar to evaluation of risks associated with humoral and cellular immune response against protein based biotherapeutics, anti-TP immunogenicity risk factors should be assessed based on potential impact on patients' safety and the efficacy of treatment. Humoral responses include development of binding and neutralizing anti-drug antibodies with potential to impact treatment efficacy and patient safety (17–21). Cell-mediated (cytotoxic T cell, CTL) immune responses to TP have been linked to the reduction of transgene protein expression due to destruction of AAV transduced target-cells as, for example, observed in muscle-directed gene therapy studies investigating treatment of Duchene Muscular Dystrophy utilizing AAV vector encoding mini-dystrophin (22, 23).

The following key terms will be used throughout the manuscript and are defined here for clarity. Transgene product (TP): Products encoded by the AAV vector. The TP classes include proteins with or without post-translational modifications (for example, glycosylation), modified proteins or protein derivatives such as activated zymogens as well as RNA molecules. For most of the discussion, this review is focused on adaptive immune responses against transgene proteins, including proteoglycans. Anti-transgene product antibody (ATPA) is used here to differentiate it from the term anti-drug antibody (ADA). The ATPA detecting assay may be designed as a method that determines the presence of binding or neutralizing ATPA.

The assessment of anti-TP immune responses has many similarities to the assessment of immunogenicity to protein therapeutics. However, factors involved in assessing the immunogenicity risk for an anti-TP immunity may be different and therefore applying the approach used for protein based biotherapeutics when determining anti-TP immunogenicity monitoring plan may not be suitable. This publication aims to provide (a) a review of various immunogenicity risk factors associated with the probability of inducing and level of impact of anti-TP immunity, (b) guidance for immunogenicity risk assessment of anti-TP immune responses, and (c) recommendations for the selection of methodologies needed for non-clinical and clinical in-study monitoring. Factors which underscore the importance of information obtained from anti-TP immunogenicity assessment and the types of assessment needed are discussed. This review will not discuss anti-AAV capsid immunity which has been covered previously (2, 24–27). This publication aims to outline a scientific approach to the design, implementation, and

evaluation of the immune response to AAV gene therapies with focus on the risks and implications of treatment emergent anti-TP immunity.

Humoral and Cellular Immunogenicity Risk Factors

Overview

A risk-based approach has been broadly accepted for evaluating potential immunogenicity response against exogenously introduced biotherapeutics (28–30). A similar risk-based approach could be applied when assessing immune responses against TPs encoded by AAV vectors. While many of the immunogenicity factors are similar between exogenously administered biotherapeutics and endogenously expressed TPs, there are some important differences that warrant closer analysis.

Immunogenicity risks associated with a given biotherapeutic, including risks related to immune response induction against AAV encoded TP, should be viewed as dynamic and data driven. Changes in the product characteristics, treated patient population, treatment regimen and methods used to assess immunogenicity responses may lead to a change in the projection of the overall risk level (29, 31–34). Potential changes in the Health Authorities' position and industry wide awareness on various aspects of anti-TP immunogenicity risks should also be factored in as they may lead to a change in the approach sponsors use to assess and report anti-TP immunogenicity information over time.

The risks associated with anti-TP humoral and cellular immunity should be evaluated in consideration of the potential for TP fragments to be presented in the context of major histocompatibility complex (MHC) Class I and/or Class II. It should be noted that antigen processing and presentation on MHC Class I and Class II is a complex intracellular process, yet there are some generally accepted principles applied to uptake of exogenous proteins *vs* synthesis of endogenous proteins within the cell (reviewed in (35)). De novo synthesis of proteins within the cell leads to some amount of polypeptide degradation by the proteosome into 8–10 amino acids long fragments that are actively transported into the ER for loading onto newly synthesized MHC Class I molecules (36). This MHC Class I—peptide complex is then transported through the Golgi to the cell surface for presentation to CD8 + T cells. Non-self or otherwise abnormal proteins presented on MHC Class I can stimulate activation of the appropriate CD8 + T cell population ultimately leading to generation of a cytotoxic T cells response. More simply stated, endogenous protein synthesis within the cell, such as transgene expressed proteins, results in their antigenic peptide constituents being expressed on MHC class I to potentially elicit a cytotoxic T cells response.

Conversely, exogenous proteins, such as proteins that are secreted, can be taken up into the cell by either phagocytosis or receptor mediated endocytosis, most commonly into professional antigen- presenting cells (APC) such as dendritic cells, macrophages and B cells. Uptake of exogenous proteins results in catalysis into peptide fragments, and peptides in the range of 12—20 amino acids are then loaded onto newly synthesized MHC Class II molecules (37). As with Class I, the Peptide-MHC Class II complex is trafficked to the cell surface for presentation to CD4 + T cells followed by a stimulation of CD4 + T helper response which orchestrates development of humoral (antibody) responses by activated B cells. These are B cells whose receptors have specificity for conformational epitopes on the intact protein from which the Class II peptide fragments were derived. When thinking through the immunogenicity risk assessment for TP, it's further helpful to keep in mind that all cell populations constitutively express MHC Class I (Natural Killer, NK, cells are capable of cytolytically targeting any cell that does not express MHC Class I on the cell surface), indicating that any transduced cell population is capable of stimulating a CD8 + cytotoxic T cell response under the right conditions. MHC Class II is, in general, only expressed by professional antigen presenting cells. It should be stated that dogmatic presentations in association with MHC Class I or Class II are not exclusive and cross-presentation of extracellular proteins on MHC Class I has been observed (38). Consequently, a key consideration in the immunogenicity risk assessment concerns localization of the expressed TP, particularly for expressed proteins which may remain intracellular, functioning within the transduced cell, or may be secreted or locate to the cell membrane as a transmembrane receptor. It may be considered that a TP could be primarily at risk for the MHC Class I presentation due to its intra-cellular production in transduced cells which may be a target for cytotoxic T cell (CTL) elimination (39). Expression of a cellular membrane bound TP may expose the extracellular domain to the B cell receptor, which primarily recognizes conformational epitopes, leading to the generation of a humoral anti-TP immune response. A cellular membrane expressed TP may also be presented on the MHC Class I leading to a CTL based elimination. Finally, a TP that is circulating systemically has a probability to be taken up by an antigen presenting cell (APC) and presented in the context of MHC Class II with a potential to stimulate CD4 + Th cells and drive humoral anti-TP immune response. Temporal intracellular transit of a systemically circulating TP during its translation in cells transduced by the AAV vector, or cross-presentation following endosomal uptake into professional APCs may result in MHC Class I presentation, and potential generation of a CTL response cannot be dismissed.

Various immunogenicity risk factors described for traditional protein-based biotherapeutics have been frequently combined into product specific, patient and treatment related groups (30, 40–42). Taking a similar approach, we are proposing to group anti-TP immunogenicity response related risk factors in four groups listed below.

(1) product related risks, including factors associated with the drug product (vector and related characteristics such as seroreactivity and tropism, presence of aggregates, impurities, degradation fragments, presence of unmethylated cytosine-phosphate-guanine dinucleotide (CpG) motif), transgene protein specific risk factors, such as protein structure and human sequence identity, redundancy of biological function, protein expression and site of action (e.g., intra- *vs.* extracellular).
(2) patient related risk factors, such as underlying genetic mutations and Residual Protein Expression (RPE) status, the patient's genetic background, disease-related inflammation, and presence of anti-TP pre-existing immunity due to prior administration of a replacement therapy. Note that the RPE term used in this manuscript is similar to the Cross-Reactive Immunologic Material (CRIM) used to describe patients risk factors in Pompe disease (43). CRIM terminology and its connection with potential risk of anti-therapeutic immune response has been discussed in the context of other indications in addition to Pompe disease that require a replacement therapy and, hence, justify the need for analysis of the presence of genetic mutations in the endogenously expressed protein (30).
(3) treatment related risk factors, including route of administration (systemic, direct to tissue and tissue type), and intended repeat administration. In contrast to exogenously introduced biotherapeutics, repeat administration of AAV based therapies remains a challenge and has only been used in select applications (44).
(4) risk factors related to TP expression in the context of AAV including promoter tissue specificity, strength of the promoter used in the vector, AAV capsid serotype, genome vector dose, potential for a local inflammation induced by AAV administration and potential for a continuous expression of TP. The inability to stop expression of TP after AAV vector administration could be considered as one of the most important factors that distinguishes the anti-TP immunogenicity risk *versus* that for other biotherapeutics where drug administration can be withdrawn.

Figure 1 illustrates graphical summary of the four categories and individual risk factors listed in this section. A more detailed description of risk factors is provided in the following sections of the manuscript.

Product Related Risk Factors

Immunogenicity risk factors associated with qualities and characteristics of the drug product have a critical role in determining whether the anti-TP response can be induced. These factors include characteristics of the final drug formulation administered to a patient, such as presence of large aggregates, impurities and fragments or degradants. In general, protein aggregates and other contaminants have been shown to augment induction of anti-protein biotherapeutic immune response (45, 46). Similarly, induction of a general inflammatory response or anti-viral and anti-TP immune

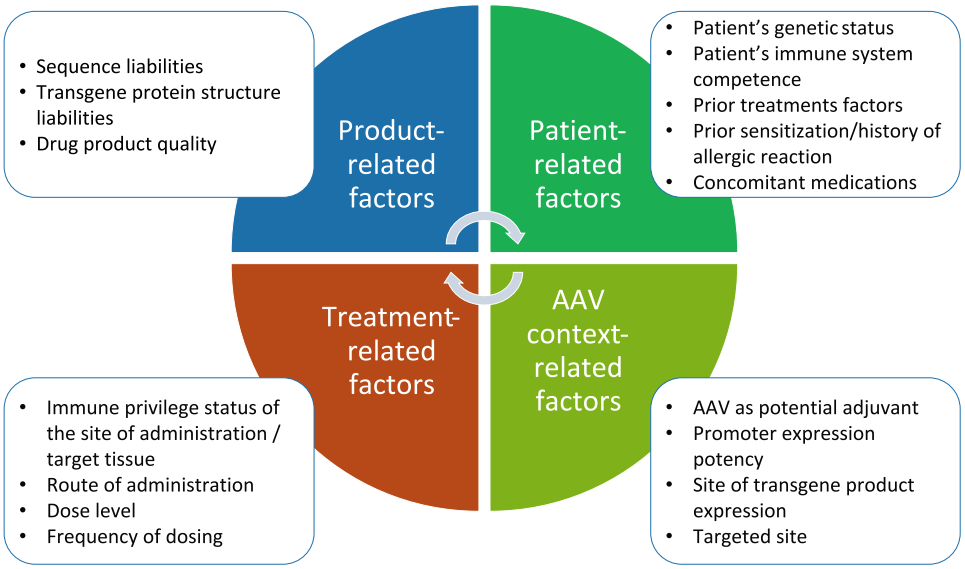

Fig. 1 Graphical depiction of risk factor categories and specific risk factors proposed for evaluation of anti-transgene product immunogenicity induction

response may be enhanced in the presence of such impurities and contaminants (47).

Quality characteristics of the AAV drug product should be considered as an element of anti-TP immunogenicity risk assessment. In one example, recognition of the CpG motif by Toll-Like receptor 9 (TLR9) was shown to trigger innate immune response and, by extension, potentiate adaptive anti-AAV immunity (48, 49). In fact, unmethylated CpG motif has been evaluated as an adjuvant in polynucleotide-based vaccines (50). The presence of unmethylated CpG was associated with formation of cytotoxic T-cell (CTL) response and elimination of AAV transduced cells (51). Low CpG content was identified as a common feature of several AAV-based trials which reported long-term FIX expression (52). Similarly, it can be proposed that induction of a general inflammatory response after AAV vector administration may result in an enhanced anti-TP immune response. The debate continues, as shown by Glenn *et al.*, who found that while CpG in AAV vectors induces early expansion of plasmacytoid dendritic-like cells, reducing CpG content decreased their count without significantly affecting anti-transgene or anti-AAV capsid antibody production (49).

Patient-related Risk Factors

Patient related immunogenicity risk factors described for protein based biotherapeutics (29, 30, 34) can be applied for the assessment of anti-TP immunogenicity potential and impact. Similar to traditional protein based biotherapeutics, anti-TP antibodies may result in significant clinical sequalae if they cross-react with and inhibit corresponding endogenous protein counterparts with a nonredundant physiological function. For endogenous proteins with redundant biological function, a neutralizing immune response may not produce an obvious clinical syndrome but consequences may become apparent when the system is stressed (30).

Immune tolerance to endogenous proteins can be shaped by various factors including a weaker immune tolerance associated with a lower abundance of protein expression or an autoimmune condition in the patient (30, 53). The patient RPE status therefore has a critical role in overall assessment of anti-TP immune response potential. Many gene therapies are designed to treat rare monogenic diseases where patients have a genetic defect resulting in lack of protein expression or expression of an inactive protein (54, 55). In these cases, the nature of genetic deficiency can impact the downstream anti-TP immunogenicity potential. For example, if the patient expresses an intact protein but at levels that are too low to perform its function, the administered therapy (either as an exogenous therapeutic protein or AAV encoded TP) is likely to be recognized as "self" and not elicit a strong immune response. Possible differences in nature or the degree of post-translational modifications (PTM) between the endogenous protein and TP, for example the type and site of protein glycosylation, may still exist. Such differences may occur because TP may be expressed in cell types that differ from those where the endogenous protein is naturally produced. In contrast, if the endogenous protein is expressed but is significantly misfolded, has large deletions or is entirely absent, the administered therapy (either as an exogenous therapeutic protein or AAV encoded TP) may be recognized as foreign with a significant potential for a robust immunogenic response (56, 57). In an example of Pompe disease, patient's CRIM status was shown to be a strong predictor of immune tolerance to the exogenously introduced protein and helped in determining the need for an immunosuppressive treatment regimen (43, 58).

Similar to exogenously introduced replacement therapeutics, the relative homology between endogenous and transgene proteins decreased the likelihood of developing ATPA (13). Conversely, lack of expression or expression of a truncated version of the endogenous protein, for example, due to a gene mutation introducing a stop codon, is more likely to result in a lack of immune tolerance and a transgene protein-specific immune response (59). Other types of mutations in the endogenous protein may also result in transgene product-specific humoral and cellular responses (22, 59–61).

Patients that are RPE negative should be expected to have an immune system that is naive towards the wild-type endogenous protein, have an elevated risk of developing higher titer antibody response, and a higher probability of a stronger T-cell response against the TP (62). In contrast, RPE positive patients may have a lower risk of developing humoral and cytotoxic CD8 + T cell responses against the transgene product (63). Overall, it appears that understanding of the patient's RPE status and its potential impact on anti-TP immunogenicity risk induction is critical.

In addition to the risk of developing new humoral responses to a TP, evidence of pre-existing anti-endogenous or anti-TP immunity may be viewed as an important immunogenicity risk factor with some trials using the presence of antibodies against endogenous protein as a part of inclusion/exclusion criteria (64). Similar to protein biotherapeutics, interaction of pre-existing anti-TP antibodies with TP may result in neutralization of the TP activity as well as in other undesirable clinical sequalae, including safety related events (65).

One common reason for the presence of pre-existing antibodies is patient's prior experience with a replacement therapy, for example, enzyme replacement therapy (ERT), that resulted in an induction of anti-ERT immune response (66, 67). The specific incidence rate of an anti-ERT immune response differs greatly between various indications and may range from a fraction of patients to the entire cohort (100%) in a clinical study (43, 68–72). The impact of an anti-ERT immune response also varies from benign, as was reported

for Elosulfase Alfa (68), to critically adverse, as was reported for infantile Pompe disease treatment using rhGAA (73). Development of coagulation factor VIII (FVIII) specific neutralizing antibody response (inhibitors) in hemophilia A patients after FVIII treatment can greatly reduce efficacy of the treatment (74). Information developed for a given indication based on the importance of the anti-ERT immune response impact is useful when aiming to understand the potential implications of anti-TP immune response after *in vivo* AAV-based gene therapy treatment.

The potential for pre-existing immunity against TP to impact clinical outcome was investigated in a clinical study (64, 75) in which participants with a history or evidence of active anti-FVIII inhibitors (neutralizing antibodies) were treated with AAV based FVIII encoding therapy. While study participants had not developed serious adverse events, it was reported that the titers of FVIII inhibitor increased in both patients with pre-existing FVIII inhibitors soon after the AAV vector administration and remained high in one while reducing in another participant (64, 76). It was suggested that such varied responses underline the complexities associated with the treatment of patients with pre-existing anti-TP immunity and call for additional investigations.

The anti-TP CTL data obtained during development of Elevidys®, a Duchenne Muscular Dystrophy (DMD) treatment, presents an important example of potential criticality to understand cause for anti-TP cellular response (77). Correlation between anti-TP CTL response and immune-mediated myositis was observed in at least one patient. This information was critical to formulate one of the parameters for the patient treatment exclusion criteria. Specifically, Elevidys® is contraindicated in patients with any deletion in exon 8 and/or exon 9 in the DMD gene as it may lead to the lack of transgene protein immunologic tolerance (77, 78).

The immune competency status of patients should also be considered. Risks associated with development of anti-TP immunity should be expected to differ between fully immunocompetent patients with developed and functional immunity and patients with the immune system that is compromised due to age or prior treatment related factors (79, 80). The impact of patient-related risk factors is therefore diverse and indication specific.

Treatment-related Risk Factors

Treatment related risk factors may include route of administration, the nature of the administration site and intent to conduct a repeat treatment. The route of administration of the AAV therapeutic should be viewed as one of the important determinants of the anti-TP immunogenicity potential (13, 59, 81–85). The intravenous (IV) administration of the AAV vector allows for transduction of multiple tissues, including liver, and, based on the proposed immune tolerizing effect of the liver expressed transgene protein, may result in a reduced level of anti-TP immunity (11, 13, 81, 86, 87). In contrast, intramuscular (IM) administration, while may produce a persistent expression of TP (88, 89), has been suggested to result in a potentially enhanced anti-TP immune response (59, 81, 84, 85) and impact exposure to the TP. Additionally, IM vector delivery may be associated with a relatively higher risk of anti-TP immunogenicity as compared to an IV route (90). This was attributed to the heightened activation of innate immune cells locally at the site of IM delivery of the AAV vector, as well as activation of memory T-cell response due to the presence of immunogenic epitopes on the vector capsid (91). Mendell *et al.* reported that, after IM administration of an AAV vector encoding mini-dystrophin transgene, T-cell immunity against the TP correlated with the lack of transgene protein expression (22). With both pre-treatment and post-treatment AAV specific T-cell immune responses detected in patients, lack of detectable mini-dystrophin expression in biopsy samples correlated with the presence of anti-TP CTL responses. Interestingly, no dystrophin specific antibodies were detected in any of the tested patients (22).

An option of repeat administration of an AAV vector, including vectors with a different serotype of the AAV virus, remains an area of an active investigation as reduction in the number of AAV transduced cells due to cell division and death is expected (92). At the time when this review is written, repeat administration remains generally restricted to specific cases where AAV vector is injected into immune privileged tissues, like the eye (2). Immune privileged status of the site of administration has been shown to have critical importance on reducing the potential for anti-AAV vector and anti-TP immune response and enabling repeat administration (44, 93–95).

Other routes of administration that deliver AAV vectors to immune privileged sites have been investigated, particularly for neurodegenerative diseases. Direct delivery via various routes has emerged as a promising therapeutic approach for conditions such as Spinal Muscular Atrophy (SMA), Parkinson's Disease, Giant Axonal Neuropathy, Aromatic L-amino acid decarboxylase deficiency, and others. Harkins *et al.* reviewed information on the immune response following the administration of AAV vectors directly into the central nervous system (CNS) that traditionally was considered an immune-protected compartment (96). Recent discussions highlight that CNS-resident immune cells, such as microglia, can mediate immune responses against novel antigens (97). Examples of humoral and cellular immunity, including anti-vector and anti-transgene protein responses, following direct intra-CNS administration (e.g., intraputaminal delivery), have been reported (98, 99)

Immunosuppressive treatment regimens have been applied to mitigate potential impact of anti-TP immune

response (100). Prednisolone and its derivatives have been commonly used to achieve inhibition of immune responses to AAV and TP (100–102). Immunosuppressive comedications may be administered based on a clinical signal, for example observation of elevated liver enzymes (7), or prophylactically (103) with the latter application been most common (100). An array of other immunosuppressants has been tested to reduce the impact of humoral and cellular immune responses on the outcome of AAV based treatment (100).

AAV Context of TP Expression Risk Factors

The fact that transgene products are expressed by AAV transduced cells adds additional parameters that may have an impact on anti-TP immunogenicity risk potential. Factors including AAV vector promoter strength and tissue specificity, the ability of AAV capsid and genome vector to induce local inflammatory environment, the nature of the cell type and tissue transduced by the AAV vector are discussed below.

The continuous nature of transgene expression is an intended and integral part of the mechanism by AAV vector transduction and is one of the key elements of the viral TP delivery. The limited ability to regulate expression levels of TP following vector administration constrains the potential mitigation strategies available in the event that an anti-TP immune response is triggered and proves detrimental. The anti-TP immune response may manifest as an adverse reaction and could also impede application of an alternative rescue treatment, for example ERT. Fortunately, reported levels of ATPA in patients administered AAV vectors are generally low with no associated serious adverse events (104, 105). Nevertheless, given the potential consequences of development of ATPA and the expected continuous nature of TP expression, this risk is important to consider.

The tissue specificity of the promoter used to drive the expression of the transgene has been suggested as an important risk factor for the anti-TP immune response potential (90, 106). Use of promoters that are liver specific or are tandem and allow for liver and target tissue expression was shown to induce immune tolerance to the transgene protein in animal models (106–110) while application of ubiquitous or non-liver tissue specific promoters may result in an induction of anti-transgene protein immunity and precipitous reduction of expression (106). In an infantile Pompe disease mouse model Colella *et al.* demonstrated that use of a monocistronic expression cassette promoter that provides combined hepatic and muscle tissue specificity protects from anti-acid alpha glucosidase (GAA) immune response induction. As a result, high and persistent expression of the TP was observed in non-dividing extra-hepatic tissues of immunocompetent GAA-/- mice (106). In contrast, the use of a muscle specific promoter that is active in both type I and II fibers (cardiac and skeletal muscle tissues), resulted in induction of a significant anti-GAA antibody response. Importantly, anti-GAA immunity negatively correlated with the detected levels of hGAA protein. An example of comparative impact of tissue localization of transgene product expression on anti-TP immunity was assessed by Poupiot *et al.* (81). Based on the data generated in a non-clinical model, the expression of TP in liver tissue demonstrated the ability to induce immune tolerance to the transgene protein while intramuscular vector delivery caused a strong inflammatory response, which could be prevented and reversed by the concurrent expression of the TP in the liver. This suggests that AAV-mediated liver gene transfer induces strong peripheral immune tolerance, likely controlling anti-transgene immune responses systemically. The effect of liver expression induced immune tolerance and its application for hemophilia A and B gene therapies were described and discussed in detail elsewhere (86, 87, 111–114).

The immune privilege status of the tissue where TP is expressed can play a key role in determining whether the anti-TP response, even if mounted, can generate a clinically meaningful impact (93, 94). The significance of localization of TP expression can be illustrated by comparing studies on SMA and Duchenne Muscular Dystrophy (DMD). Both are monogenic disorders leading to progressive muscle dysfunction, caused by mutation in SMN1 gene and Dystrophin gene, respectively (115). While genetic treatment for SMA has been successful (116), three recent DMD gene therapy trials (117–119) were affected by severe adverse events that were attributed to cytotoxic cellular immune responses against the microdystrophin (the transgene protein), leading to the development of myositis (120). It is important to note that the target tissue for gene therapies for the treatment of SMA is the central nervous system (CNS), protected by immune privilege, keeping in mind that the route of administration is IV and the TP expression is driven by a ubiquitous promoter. Healthy skeletal muscle, targeted in DMD therapy, is an immune competent tissue. The difference in the immune privilege status between these two tissues was pointed out as one possible contributing factor to the asymmetric outcome of the SMA and DMD trials (121). Other potential contributing factors may include the indicated age of the patients and presence of a paralogous protein (SMN2) in the case of the SMA condition.

The assumed immune privileged status of a tissue may not completely prevent the late onset of an inflammatory response. As a specific example, subretinal inflammation may still be observed after an AAV vector injection with limited or no increase in clinically relevant binding or neutralizing antibodies to the vector and no impact on the

treatment of the second eye (122, 123). Such cases of retinal inflammation are more apparent in some retinal diseases (e.g., Age-Related Macular Degeneration) where the blood-ocular barrier may be damaged, and the immune privilege of the eye tissue is compromised (124).

The nature of the promoter used in the AAV vector may result in various rates and levels of TP expression (125, 126). The potential for AAV vector potency to impact anti-Factor VIII transgene protein immune response was investigated by Lundgren *et al.* (127). The anti-TP humoral response was evaluated in a mouse study focusing on the relationship between FVIII expression levels and the potential for anti-FVIII neutralizing antibody response (inhibitor) induction. The study evaluated the rate of initial AAV-encoded FVIII product expression kinetics as a key risk factor for inhibitor development. Using vectors with a broad range of potency, it was shown that promoter strength together with the AAV dose were associated with the likelihood of induction of anti-FVIII antibodies and FVIII inhibitors. Reduced levels of the FVIII protein observed in animals dosed with lower doses of a low potency AAV vectors correlated with an absence of detectable anti-TP antibody response. In contrast, the combination of a vector dose and promoter type led to a rapid increase in the FVIII protein levels and was associated with a subsequent decline in the TP activity, partly due to the development of a cytotoxic or humoral anti-TP immune response. These findings suggest that a lower rate of initial transgene expression may help with induction of immune tolerance to TP and prevent development of a significant anti-TP immune response (127).

The potential for pre-existing or AAV administration induced local inflammation to enhance adaptive immune response against the AAV vector and, potentially, the AAV encoded TP has been reviewed by Mays *et al.* (128). Studies designed to demonstrate that inflammation caused by a chemical treatment can impact AAV vector encoded transgene product expression confirmed this phenomenon (129). Similar observations were made for murine models of DMD with known evidence of muscle tissue inflammation (130). Anand *et al.* (131) reported outcome of a mouse study investigating induction of anti-green fluorescent protein (GFP) antibody responses after administration of GFP encoding adenovirus (AdV) and AAV vectors administered via subretinal and intradermal injections. While no significant anti-GFP antibody induction was observed in serum for the AAV vector group of animals, the AdV vector administration induced a significant and similar anti-GFP antibody response in both intradermal and subretinal groups, likely due to induction of a general inflammation response.

Rybniker *et al.* (132) discussed the use of an engineered AAV capsid based vaccine that triggered an anti-AAV vector immune response, thereby enhancing overall immune system activation and anti-TP immunity. The combination of antigen presentation by the viral capsid and overexpression of the TP after cell transduction was shown to significantly increase the antigenic potential of AAV-based vaccines (132). This model represents an extreme case of inducing an anti-TP response if the transgene protein sequence is present in the vector or formulation. Similar to previous examples, it suggests that the design of the viral vector and its ability to induce inflammatory events by activating the immune system are critical immunogenicity risk factors for inducing anti-TP immune response.

Table I lists some of the risk factors discussed here, also illustrated in Fig. 1. The question of whether a specific assessment is recommended in support of clinical and non-clinical investigation is discussed in other sections of the manuscript.

Evaluation of anti-Transgene Product Immune Response

Creating a Context of Use Relevant Testing Strategy

The nature of methods required for assessment of transgene product immunogenicity depends on the mechanism of action of the expressed protein and other factors. The various immunogenicity risk factors discussed in previous sections of the manuscript can help determine an overall immunogenicity risk potential for a transgene product. The diversity and interconnections between various factors make it challenging to design a context of use-based anti-TP immunogenicity assessment strategy for clinical trials. Therefore, we believe that a more streamlined approach can be based on two specific characteristics of AAV therapy application: a) immune privilege status of the site of administration and b) the intended localization of the expressed transgene product, i.e., whether it is circulating systemically, expressed on the cell membrane or localized intracellularly.

As discussed earlier in the manuscript, it has been generally agreed that administration of AAV vector directly to an immune privileged site should significantly reduce the potential for both the induction of and the impact from an anti-TP humoral or cellular response. In these cases, the evaluation of the anti-TP humoral and cellular immune response could be viewed as optional: samples can be collected and stored, and the decision to test can be made based on specific pre-defined clinical events, for example a significant change in the efficacy of treatment or the development of adverse events.

For other routes of administration, including IM and IV, the intended localization of the expressed protein should be considered when developing the anti-TP immunogenicity assessment strategy. The approach to monitor immunogenicity response against soluble transgene products found

Table I Risk Factors Associated with Potential Anti-transgene Product Immunogenicity Induction

Risk Factor	Brief Description
Product related factors	
Sequence liabilities	Human, humanized, truncated, mouse, or combination sequences Presence of T/B cell epitopes
Transgene protein structure liabilities	Misfolding, aggregation, truncations, post-translational modifications
Drug product quality	For example, ratio of empty/full capsids, impurities
Patient related factors	
Patient's genetic status	Residual Protein Expression status Endogenous protein is truncated, mutated or not expressed Mutation *vs.* deletion: null *vs.* spliced
Patient's Immune system competence	Age, prior treatment with immune modulatory regimens
Prior treatments factors	Prior treatment with recombinant ERT Evidence of anti-ERT or anti-endogenous protein immunity Clinical immune response to recombinant ERT or similar gene therapy treatment Prior treatment with a recombinant protein with the goal to induce immune tolerance
Prior sensitization/history of allergic reaction	History of hypersensitivity Precedence for hypersensitivity reaction in general population due to ERT
Concomitant medications	Concomitant immunosuppressive treatment with the goal to reduce cellular or humoral immune response to transgene product
Treatment related factors	
Immune privilege status of the site of administration/target tissue	Immune privileged *vs.* non-immune privileged tissue/organ
Route of administration	IV/IVT/IT/IM/SC
Dose level	Low *vs.* high
Frequency of dosing	Intended repeat administration
AAV context of transgene product expression related factors	
AAV as potential adjuvant	Inflammation due to anti-AAV immune response
Promoter expression potency	Fast/slow, continuous expression
Site of transgene product expression	Intracellular, secreted, membrane bound
Targeted site	Potential for off-target expression e.g., liver *vs.* muscle

in systemic circulation is likely to be different from that for a cell surface or intracellularly localized TP. For TP that is active systemically, a testing paradigm commonly used for protein-based therapeutics could be relevant with separate assays applied to detect binding and/or neutralizing antibody response. In some cases, the site of expression of the TP may be different from the site of its biological activity. As an example, TP that is initially expressed in liver is then trafficked to the muscle tissue, where it is internalized and becomes active intra-cellularly. We propose that for such transgene products, assessment for antibodies that can directly neutralize enzymatic activity of the TP should be viewed as optional. Understanding the potential impact of anti-TP immune response on the cellular uptake of TP by the target cell becomes more critical. As such, evaluating the presence of anti-TP antibodies that can block (neutralize) TP's ability to bind target cell receptors and, consequently, prevent the cellular uptake negatively impacting treatment efficacy, may be more clinically relevant. Similar justification was discussed for enzyme replacement therapeutics and is based on the understanding that ADA-protein complexes have low potential for crossing the cellular membrane or staying intact at the low pH of the intracellular environment (133–136).

Specific considerations should also be given to other factors, such as previous or potential post-AAV treatment application of a replacement protein therapeutic (commonly, ERT). Potential impact of anti-ERT antibodies, developed as a result of a prior ERT administration, and capable of cross-reacting with the TP, should be considered as part of the anti-TP immunogenicity evaluation planning (64). Similarly, the development of anti-TP antibodies that may cross-react with an ERT or endogenous version of the protein may present a considerable risk factor for future treatments should the AAV-based therapy fail. It is therefore important to understand structural and chemical differences and similarities between transgene product, replacement therapeutic, if relevant, and endogenous versions of the protein. We recognize that such characterization of the transgene protein may be challenging and could be limited to information available

Table II Proposed Strategy for Assessment of Anti-transgene Product Immune Response in Clinical Studies

Transgene Product localization	Examples	Humoral anti-transgene product immunogenicity evaluation		Cellular immunity assay for transgene product requirement
		Binding ATPA assay	Neutralizing ATPA assay	
Intracellular with no systemic exposure	Intracellular enzymes (137)	Testing is optional; collect and bank samples [1,2]	Testing is optional; collect and bank samples [1,2]	Testing should be considered [3]
Cell membrane bound	Structural proteins, membrane transporters (138)	Testing is recommended	Testing is optional; collect and bank samples [1,2]	Testing should be considered [3]
Secreted/localized to an immune privileged site	Subretinal delivery of AAV encoding secreted but localized protein (104)	Testing is optional; collect and bank samples [1,2]	Testing is optional; collect and bank samples [1,2]	Testing should be considered [3]
Secreted/systemic	Blood clotting factors, Lysosomal storage enzymes [4] (139–141)	Testing is recommended	Testing is recommended [5]	Testing should be considered [3]

1- The decision to conduct testing is gated by pre-defined events, for example, an AE observation that can be rationally attributed to ATPA

2- The assessment of binding or neutralizing ATPA responses may be recommended if the following conditions are met: (a) site of administration of the AAV vector is immune competent, (b) TP has an endogenous counterpart with no redundancy of function and (c) development of neutralizing ATPA antibody may potentially lead to a serious impact on safety, for example, based on observations made in non-clinical or clinical studies

3- Testing for cellular anti-TP immune response may be considered if development of cellular cytotoxic anti-TP response has potential to result in serious consequences, for example based on observations made in non-clinical or clinical studies, or there is evidence of TP clearance in the absence of detectable ATPA response

4- For example, expressed in liver and trafficked systemically to a target tissue

5 - Lack of the need for neutralizing ATPA testing may be justified based on observed correlation of binding and neutralizing ATPA induction and access to the binding ATPA data. For low immunogenicity risk TPs testing for neutralizing ATPA may be viewed as optional

based on the transgene sequence or through the analysis of an *in vitro* expressed protein.

Table II presents a decision tool that may be used as a guideline to determine the most relevant strategy to assess anti-transgene product immune responses in clinical studies, including testing for binding and neutralizing antibodies as well as cellular immune response assessment. The decision tool emphasizes the intended localization of the expressed transgene product, although other factors should be considered for the overall immunogenicity risk evaluation as already discussed earlier in the manuscript. Proposed categories of TP localization include a) intracellular, for example, intracellular enzymes (137), b) cell membrane bound, for example, structural proteins and membrane transporters (138), c) secreted and localized to an immune privileged site, for example, TP that is delivered and secreted subretinally (104), d) secreted and systemically delivered, for example, blood clotting factors and lysosomal storage enzymes (139–141).

Briefly, assessment of humoral responses for transgene products intended for an intracellular site of action may be viewed as optional, and samples could be collected and stored with an event-gated approach to conduct testing. The overall risk assessment should help with determining timing for the assay development activities related to the study initiation. For cell membrane bound transgene products with an extracellular domain present, binding ATPA assessment is recommended, while neutralizing ATPA activity detection is viewed as optional. For transgene products that are present systemically, binding and neutralizing ATPA testing are generally recommended. The overall risk assessment of a systemically circulating TP may further inform about the criticality of neutralizing ATPA assessment, and, with additional justification for low immunogenicity risk TP molecules, evaluation of the binding ATPA may be sufficient.

Assessment of cellular immune response to transgene products may be needed if there is known evidence of an anti-TP cytotoxic T-lymphocytes response to trigger an adverse event (AE). For example, evidence of liver enzyme elevation has been linked to a CTL response after AAV administration (83). An anti-TP CTL response may be warranted based on this observation. Cellular responses may also need to be monitored if there is evidence of unexpected clearance of TP in the absence of ATPA response. Anti-TP CTL monitoring can inform about the presence of long-term memory T cells with anti-TP cytotoxic activity if a repeat treatment with AAV therapeutic is planned.

Similarly to protein biotherapeutics, the decision to test for humoral and cellular anti-TP immunity should consider multiple patient and treatment specific characteristics, including intended route of administration, presence of endogenous protein counterpart, that may be subject to

neutralization by ATPA, potential for ATPA related serious sequalae, and information obtained in non-clinical studies. For products administered to immune privileged sites, such as subretinal space, collection and banking of samples is likely an acceptable approach with an event, for example AE observation, triggered testing provision. In addition, testing for ATPA and/or the cellular cytotoxic anti-TP response is justified when planning for a repeat administration of the AAV therapeutic. This is particularly important as it helps to assess the potential impact of an anti-TP immunogenicity response due to the presence of memory immune cells. An adaptive approach to testing strategy should be considered based on observations made during early clinical trials. For instance, the decision to conduct a test for the development of anti-TP cellular response might be omitted in later phase clinical studies if there is no evidence linking the cellular response to clinical safety or efficacy outcomes.

Relevance of the Protein Therapeutics Immunogenicity Testing Paradigm to Assessment of ATPA

The ADA testing strategy commonly applied for protein therapeutics was established almost twenty years ago when advanced assay methodologies were not available and serious immunogenicity-related safety concerns were observed (29, 34, 142, 143). The conservative approach to ADA detection for protein-based biotherapeutics has been a multi-tier based and uses stringent cut points with the overall goal to minimize false negative results and ensure patient safety. In recent years, comprehensive immunogenicity risk assessment is commonly conducted prior to study initiation and is a key component in establishing therapeutic, indication specific, and treatment relevant immunogenicity monitoring plans. Consequently, the need for complicated and resource intensive multi-tiered testing has recently been questioned (144, 145).

In addition, current health authority guidelines on immunogenicity assessment for protein-based biotherapeutics (146, 147) do not include specific recommendations for evaluating immunogenicity in gene therapy studies, including anti-TP immunity. Indication or modality-specific regulatory guidelines may recommend anti-TP immunogenicity evaluation. For example, FDA guidance on development of gene therapy based treatments for hemophilia recommends testing for coagulation factor inhibitors, which are inherently anti-coagulation factor neutralizing antibodies (148). In practice, testing for coagulation factor inhibitor activity is initiated based on clinical signs, such as a reduction in enzyme activity, while a patient is considered negative for inhibitors if the activity of coagulation factor remains normal. The inhibitor detection is not directly linked with a typical tiered approach used in immunogenicity assessment of protein biotherapeutics, where samples that are ADA positive are further evaluated for neutralizing antibody activity. Overall, a commonly applied multi-tiered assessment, that is often recommended for evaluation of immunogenicity response to protein-based biotherapeutics, may not be fully relevant when assessing the ATPA response.

When evaluation of anti-TP humoral immunogenicity is identified as required, an alternative single tier strategy that includes detection of either the binding or the neutralizing ATPA response can be applied. In addition, alternative methodologies to those outlined in regulatory guidelines for the protein based biotherapeutics (146, 147), may be considered when monitoring ATPA response, including implementation of instrument signal-to-noise ratio in lieu of the titer tier and lowering the false positive rate (FPR) from 5% to 1%. For products where development of ATPA presents high risk for treatment safety, a more extensive testing paradigm may be appropriate. For other product categories, testing strategy should be based on the immunogenicity risk assessment and could range from collecting and banking samples only to performing a subset of the tiers of assessment used in ADA testing.

Methodologies Designed to Detect Anti-transgene Product Binding Antibodies (binding ATPA)

Assays developed for detection of all anti-TP specific antibodies are referred herein as binding ATPA methods. Similar to the common design of anti-protein biotherapeutic ADA methods, binding ATPA assays may follow direct, antigen-capture or a bridging format. However, binding ATPA methods poses unique challenges, including generating suitable reagents and potentially requiring high sensitivity assays.

While for protein therapeutics the material used to generate ADA assay reagents is the drug itself, the situation is different for the ATPA detecting methods. Challenges may be associated with expressing a sufficient amount of transgene protein via a recombinant process due to complexities of protein folding, ability to express a full-length version of the TP, and the difficulty in expressing a correctly folded protein segments (e.g., extracellular domain) of TP. Differences in post-translational modifications between the recombinant and *in vivo* expressed TP can also impact functionality and stability. Although in many cases these differences may be inconsequential, their potential impact on the ability of the ATPA method to detect anti-TP antibody response should be evaluated. A tagged version of TP may be considered as an assay reagent, in which case the suitability of the tagged TP or its equivalence to non-tagged protein for use in an immunogenicity assay should be evaluated. If a recombinantly expressed TP cannot be efficiently labeled to generate ATPA method reagent, it would be prohibitive to the development of a bridging

immunogenicity assay format, and an antigen-capture design, which bypasses the need for a biotinylated or ruthenylated TP, may be considered. As a potential solution, for difficult to label TPs, the SpyCatcher-SpyTag system may be used to label the protein (149).

Although the generally recommended ADA assay sensitivity set at 100 ng/mL (147) can be viewed as acceptable, it should be recognized that different, potentially more sensitive ATPA assays may be needed, particularly when the concentration of TP in circulation is expected to be low. It is possible that non-clinical investigations may shed some light on the levels of anti-TP antibodies that have the potential to impact circulating TP concentration and the overall efficacy and safety of treatment. If soluble transgene product is expected to be released into the extracellular space at a high concentration, a sample pre-treatment that can reduce interference of the circulating TP in the ATPA method, for example, an acid-capture-elution based protocol or similar (150–152), should be considered.

Validation of the binding ATPA assays can be performed in an alignment with the FDA 2019 guidance on Immunogenicity Testing of Therapeutic Protein Products (147) and include evaluation of several assay performance characteristics, including cut-point, sensitivity, selectivity, specificity, precision, reproducibility, and drug tolerance. As stated earlier, a single tier binding ATPA methods with FPR threshold set at 1% may be appropriate.

Methodologies Designed to Detect Anti-transgene Product Neutralizing Antibodies (neutralizing ATPA)

Neutralizing ATPA detection assays are designed to assess the presence of neutralizing antibodies against TP. These assays may be cell-based, competitive ligand binding plate-based, or enzyme activity-based. Cell-based neutralizing ATPA assays may leverage cellular receptor binding or cellular uptake for the TPs intended for intracellular activity-based mode of action (MOA). Similar to binding ATPA protocols, method validation for neutralizing ATPA assays can be performed in alignment with the FDA 2019 guidance on Immunogenicity Testing of Therapeutic Protein Products (147) and involves evaluation of several assay performance characteristics, including cut-point, sensitivity, selectivity, specificity, precision, reproducibility, and drug tolerance. Targeted delivery of ERT to a specific tissue has been recently explored (134, 153). If a similar approach is considered for the TP MOA, using a neutralizing ATPA activity assay that is based on the antibody's ability to inhibit TP binding to and/or uptake by specific targeted cells may be more appropriate than assays detecting antibody's ability to inhibit TP activity (133).

Methodologies Designed to Detect Anti-transgene Product Cellular Immune Response

Anti-transgene product cellular response assays are designed to detect the presence of transgene product specific cytotoxic T-lymphocytes. Functional assays for the detection of cellular responses including their specificity, magnitude and breadth have been well established and described and are commonly conducted using either ELISpot or intracellular cytokine staining (ICS) followed by flow cytometry. Information about development and qualification or validation of such assays, including specific assays intended to detect anti-AAV cellular immune response, has been provided elsewhere (25, 154, 155). Furthermore, comprehensive guidelines for achieving optimal assay performance and data harmonization are available (156–161). ELISpot and ICS methodologies may be utilized to assess anti-transgene product cellular responses. As with other cellular response assays, selecting appropriate high-quality reagents, including target protein peptides synthesized with capping protection in order to avoid the introduction of neoepitopes that may lead to measuring false positive responses, and relevant assay controls is critically important for generating study relevant information (154, 159, 162). Anti-TP cellular response assay optimization and validation should follow the approach described elsewhere (154, 163, 164). Importantly, it should be appreciated that peripheral blood mononuclear cell (PBMC) material is usually the sample of choice for evaluating cellular immunogenicity due to a relative ease of access and the potential to be replenished in contrast to most other biological tissues. However, responses detected in PBMC preparations might not necessarily inform on the immune responses occurring in other tissues targeted by the AAV-mediated therapy. Anti-TP cellular immune responses may either be induced by the treatment or represent a pre-existing memory response that is boosted after vector administration. Pre-existing T-cell immunity assessment is not commonly performed and samples collected pre-treatment are typically evaluated together with post-dose material. Simultaneous testing of pre-treatment and post-dose samples can reduce inherent analytical variability in methods assessing function of single cells, such as ELISpot and intra-cellular cytokine staining. Furthermore, computational tools are also available to correct for batch effects (e.g., CytoNorm (165)), allowing for pre-treatment samples to be tested in separate experiments (159, 166). The relevance of PBMC detected anti-TP cellular response to the tissue specific response would need to be demonstrated by a correlative analysis together with related PD and safety data obtained in a clinical study. It is also important to note that assessment of cellular responses in clinical trials is operationally

challenging, and the data generated has been found to be uninformative in some cases (167). Although these assessments may likely be requested by regulators, the utility, reliability, and clinical relevance of testing for anti-TP cellular responses will be an ongoing question for sponsors.

Case Studies

Roctavian® (Valoctocogene roxaparvovec)

Roctavian® (valoctocogene roxaparvovec) is an AAV serotype 5 (AAV5)-vectored gene therapy for the treatment of hemophilia A that encodes a B-domain-deleted human factor VIII (FVIII) protein controlled by a hepatocyte-selective promoter (105, 168, 169). Roctavian® was approved in the EU in 2022 and the US in 2023 with multi-year data from a Phase 3 pivotal trial, GENEr8-1. In the phase 3 GENEr8-1 study, 134 adult participants with severe hemophilia A received a single I.V. dose of 6×10^13 vg/kg valoctocogene roxaparvovec (139). Immunogenicity specific enrollment criteria included the exclusion of participants with evidence of AAV5 specific TAb at screening and a requirement that participants had no history of FVIII inhibitors following a minimum of 150 days of exogenous FVIII exposure. The FVIII protein encoded by Roctavian® is secreted from liver hepatocytes and, like other FVIII replacement therapies, may stimulate an immune response. Historically, about one-third of patients with Hemophilia A generated a FVIII-specific neutralizing antibody response, or FVIII inhibitors, following FVIII replacement therapy. Previous studies of FVIII inhibitors identified subsets of Hemophilia A patients and healthy donors with low levels of anti-FVIII binding antibody that appear to be transient and often did not progress to high levels of neutralizing antibodies, or inhibitors (105, 170, 171). To better understand the potential for FVIII specific immune responses, the sponsor elected to evaluate both FVIII inhibitors and FVIII TAb following dose administration with Roctavian®. The results of the immunogenicity monitoring program were recently summarized (105). While no clinical trial participants developed a clinically meaningful inhibitor response, 12 of 134 (9.0%) of participants in GENEr8-1 tested positive for FVIII TAbs, with some of these positives detected at baseline prior to dose administration (despite testing negative for inhibitors). FVIII TAb positivity was transient, was not concordant with FVIII inhibitors or cellular responses to FVIII and was not associated with safety of efficacy outcomes. Sporadic FVIII TAb-positive results were not considered clinically relevant as they are consistent with transient, low-titer FVIII TAbs that do not typically progress to FVIII neutralizing antibodies (FVIII inhibitors). Correspondingly, none of these participants developed a clinically meaningful FVIII neutralizing inhibitor response (170).

Zolgensma® (Onasemnogene abeparvovec; AVXS-101)

Zolgensma® (onasemnogene abeparvovec; AVXS-101) is an intravenously administered AAV serotype 9 (AAV9)-vector based gene therapy for the treatment of patients less than 2 years of age with spinal muscular atrophy (SMA) with bi-allelic mutations in the survival motor neuron 1 (SMN1) gene. SMA patients have low levels of expression of SMN protein from the SMN2 gene. The SMN protein is part of a complex located in the Cajal body of mammalian cell nuclei with an essential role in the assembly of small nuclear ribonucleoproteins (RNP) (172, 173). Zolgensma® contains a transgene that encodes for human survival of motor neuron (hSMN) protein and was approved for marketing in the US in 2019, the EU and Japan in 2020, and in Australia in 2021 (174–177). Based on related immunogenicity risk factors, the overall risk of anti-TP immune response was assessed as low (178). However, out of an abundance of caution, the immune response to SMN protein was assessed in clinical trials with the presence of anti-SMN antibodies being evaluated using an ELISA assay and the cellular immune response to SMN protein being monitored by an ELISpot assay. In Study CL-101 (the primary study on which Zolgensma® was approved for marketing), an intensive sampling schedule was implemented for the detection of anti-AAV9 and anti-SMN antibodies as well as the cellular immune response to AAV9 and SMN. Samples were collected pre-dose and at 1, 2, and 3 weeks and 1, 2, 3, 6, 9, 12, 15, 18, and 24-months post-dose (179). The review documents that are publicly available from the FDA, EMA, TGA, and PMDA contain very little, if any, information regarding the immune response to SMN other than noting that no immune response (either humoral or cellular) to SMN protein was observed (174–177). Additionally, the Product Labels do not mention the immune response to SMN protein. The humoral immune response against SMN was also assessed in other clinical trials that were ongoing at the time of the marketing application for Zolgensma® (180–183). The cellular immune response was assessed in older children but was not assessed in newborn infants due to the volume of blood required for the ELISpot assay. No mention of the immune response to SMN could be found in the results posted to clinicaltrials.gov.

Luxturna® (voretigene neparvovec)

Luxturna® (voretigene neparvovec) is an AAV2-vector based gene therapy for the treatment of patients with inherited retinal dystrophy due to mutations in both copies of the retinal pigment epithelium 65 (RPE65) gene, including Leber congenital amaurosis (94, 101, 184, 185). Luxturna® contains a transgene that encodes human retinoid isomerohydrolase. It was approved in the US in 2017 (94) and in the

EU in 2018 (186). In a randomized, controlled, open-label Phase 1 study with 2 parts (study 101/102), 11 children with Leber congenital amaurosis were injected with 1.5×10^{11} vg/300 µL voretigene neparvovec in one eye (187). After at least 8 weeks the contralateral eye was treated as well (4). A 3-year follow-up study was conducted and shown to be safe and efficacious (188). Twenty (20) children with Leber congenital amaurosis were treated in a randomized, controlled, open-label, phase 3 study with the same dose regimen in each eye (101). The anti-AAV2 immune response was evaluated for anti-AAV2 antibodies and T-cell responses (101, 189). Anti-RPE65 immune responses were assessed using an ELISA, and anti-RPE65 cytotoxic T-cell response by Interferon-γ ELISPOT in PBMC. Immune reactions were generally minimal, even with sequential administration to each eye. Limited cytotoxic T-cell responses to the transgene product RPE65 in a small subset of the subjects were observed with long-term-follow up studies (LTFU) ongoing. The LTFU study will continue until 2030, and annual assessments using both ELISA and ELISpot assays so far have not shown any enhanced immune response (94, 186). It is important to note that oral and topical corticosteroids were administered before and after subretinal injection of Luxturna® to each eye to minimize ocular inflammation (101, 190). Corticosteroids are likely to further decrease the potential immune reaction to either vector capsid or transgene product. In addition, the subretinal administration route has important implications as the subretinal space is characterized by the presence of a blood-retinal barrier which renders it immune privileged (191).

ST-920 (isaralgagene civaparvovec)

ST-920 (isaralgagene civaparvovec) is an investigational AAV serotype 6 (AAV6)-based gene therapy for the treatment of Fabry disease, an X-linked lysosomal storage disease caused by mutations in galactosidase alpha (GLA) gene (192). The AAV6 vector contains a human GLA cDNA under the control of liver-specific enhancer and promoter to selectively express and secrete the alpha-galactosidase A (α- Gal A) enzyme in hepatocytes. ST-920–201 (NCT04046224) is a Phase 1/2, multicenter, open-label, single-dose, dose-ranging study in adult subjects with Fabry disease (141). This study enrolled 33 patients including naïve, ERT-pseudo-naïve and ERT-treated subjects. Patients received ST-920 via intravenous administration with no prophylactic corticosteroid treatment. ERT-experienced patients often develop persistent binding and neutralizing antibodies to the recombinant enzyme drug product (67). These pre-existing ADAs may potentially impact the efficacy and safety of gene therapy. Conversely, in animals with pre-existing antibodies, liver-directed AAV gene therapy has been shown to induce immune tolerance that results in a decrease and eradication of pre-existing antibodies. In naïve animals, hepatic gene transfer was found to prevent antibody formation against the transgene expressed protein (87).

To assess potential immunogenicity of transgene expressed α-Gal A enzyme and investigate the fate of the pre-existing ADAs post ST-920 treatment, serum samples from ST-920–201 and its long-term follow-up study (up to approximately 47 months) (data cut off as of September 12, 2024) were tested in TAb and NAb assays. Gene therapy studies for rare diseases typically have fewer patients and samples compared with biologics studies. Therefore, the first tier testing combined TAb screening, confirmatory and with titer assays to improve efficiency and reduce costs. The confirmed positive samples were then analyzed in the NAb assay (with titer assessment). The enzyme activity based NAb assay incorporated an alkaline sample pre-treatment step that eliminated drug interference (193). Twenty-three of the 33 subjects were negative for anti-α-Gal A TAb at baseline and ST-920 treatment did not induce anti-α-Gal A antibody formation except for one subject who tested TAb positive (with a lowest detectable titer of 1:40 assay cutoff) and NAb negative for only one timepoint of week 16. Ten subjects had measurable titers for TAb and NAb at baseline due to ERT use. After ST-920 treatment, TAb and NAb titers decreased markedly in 9 of these subjects. Seven of the 9 subjects had undetectable TAb and/or NAb starting from week 4 to week 52. Based on favorable safety profile, cellular immune response was not evaluated.

Kebilidi® (US)/Upstaza® (EU and UK) (eladocagene exuparvovec-tneq)

Kebilidi® (US)/Upstaza® (EU and UK) (eladocagene exuparvovec-tneq) is an AAV vector-based gene therapy indicated for the treatment of adult and pediatric patients with aromatic L-amino acid decarboxylase (AADC) deficiency (167, 194). AADC deficiency is an inherited disease that affects the nervous system leading to symptoms such as developmental delays, weak muscle tone and inability to control the movement of the limbs.

Kebilidi® is a non-replicating recombinant adeno-associated virus serotype 2 (AAV2) based vector containing the cDNA of the human dopa decarboxylase (DDC) gene under the control of the cytomegalovirus immediate-early promoter (167). It was approved in 2022 in the EU and in 2024 in the US (167, 194). Patients will receive a total dose of $1.8 \times 10\text{^}11$ vg delivered as four 0.08 mL infusions by bilateral intraputaminal infusion in one surgical session at two sites per putamen.

The efficacy of Kebilidi® was evaluated in one open-label, single arm study (195). The study enrolled pediatric patients with genetically confirmed, severe AADC deficiency who had achieved skull maturity assessed with

neuroimaging. The main efficacy outcome measure was gross motor milestone achievement evaluated at week 48 and assessed using the Peabody Developmental Motor Scale, Second Edition (PDMS-2). Patients treated with Kebilidi® were compared to an external untreated natural history cohort of 43 pediatric patients with severe AADC deficiency who had at least one motor milestone assessment after 2 years of age (167). In Study 1, anti-AAV2 total binding antibodies and anti-AAV2 neutralizing antibodies were assessed from Day 3 up to Week 48 following administration of Kebilidi®. There is no information available regarding transgene product immune response assessment (167).

Immunogenicity response against AAV2 vector was evaluated in clinical trials submitted in support of the MAA to evaluate efficacy and safety (194, 196, 197). A method for detection of anti-AADC antibodies was under development with no data reported. When this manuscript was published, there was no available information regarding the humoral or cellular immune response to the transgene product (194).

Table III presents a summary of several of the above case studies together with the associated immunogenicity risk-based recommendations for the anti-TP immune response analysis and a summary of tests and conclusions made during program specific clinical development.

Evaluation of Anti-TP Immunity in Non-Human Species: Value and Translatability

Regulatory authorities have issued several guidelines that provide agency's position on non-clinical evaluation of gene therapy products, including guidance on specific studies required to support initiation of clinical trials as well as to support a marketing authorization application (198, 199). Other guidelines discuss immunogenicity and immunotoxicity of AAV in connection with other critical factors that require careful assessment during pre-clinical development phase.

Non-human animal models used during AAV therapeutic development are often selected based on the relevance to the expected clinical pharmacology of the transgene product, also on the ability of AAV vector serotype to transduce desired cell or tissue type, tissue tropism and similarity to the human physiology (200, 201). The immune responses to AAV and TP may potentially impact interpretation of non-clinical study data. Critical aspects related to mechanisms of immune response against AAV vectors and transgene proteins in various animal models was previously reviewed by Martino *et al.* (201). It is generally considered that immunogenicity related information collected in non-clinical studies provides limited ability to predict immunogenicity potential and impact in the clinic. Such limited translatability was previously discussed and is well recognized for protein based biotherapeutics (201, 202). Still, some degree of translatability exists, particularly in the ability to predict and interpret toxicity events (203). The capacity to translate non-clinical to clinical immunogenicity signals is acknowledged for some indications, for example hemophilia A (127).

Various questions related to the potential impact of anti-transgene product immunogenicity have been evaluated non-clinically. Ashley *et al.* investigated the ability of viral vector to induce toll-like receptor 9 (TLR9) leading to activation of cytotoxic T-cell response against transgene protein and loss of the TP expression (204). It was concluded that TLR9 mediated inflammation is critical for the development of anti-TP adaptive immunity and may play an important role in altering transgene product expression. Other factors have been identified as important contributors to AAV vector driven inflammation, as was demonstrated in a number of non-clinical investigations (204–206). Similarly, significance of TP expression in the liver and potential use of a tandem promoter to reduce anti-TP immunogenicity response have been discussed earlier in this manuscript. Utilization of immunosuppressive treatments aiming to reduce immune response against AAV encoded transgene product has been actively assessed in various non-clinical studies, including evaluation of tolerogenic nanoparticles encapsulating rapamycin, anti-thymocyte globulin, nondepleting anti-CD4 monoclonal antibody, and other methods (110, 111, 207, 208).

The significant and critical importance of the promoter type, transcriptional control elements and capsid on the expression of transgene product and development of anti-transgene product antibodies was demonstrated in a non-clinical model by Greig *et al.* (61, 209). The study highlights the importance of the AAV vector design and sequence optimization, work that could only be performed in non-clinical settings. The feasibility of using non-clinical models to identify critical biological factors that may lead to induction of anti-transgene immune response was reported by Hordeaux *et al.* (210). In the study, an AAV9 based vector encoding human acid-alpha glucosidase (hGAA), a Pompe disease drug candidate, was tested in a non-human primate (NHP) model. The unusually high variability in anti-hGAA immune response with a potential to trigger severe cytotoxic T-cell mediated myocarditis led to additional investigations. The major histocompatibility complex class I haplotype-based variability between animals was identified as the root cause of the observed phenomenon. Hordeaux *et al.* pointed out that complexities associated with GAA polymorphism in NHP *vs.* humans confound translatability of conclusions made in the NHP study into the clinic. Still, the importance of MHC I haplotype, its potential connection with the anti-TP immunogenicity and associated toxicities could be considered when designing relevant clinical trials.

Table III Immunogenicity Risk-based Analysis of Anti-transgene Product Immune Response and Therapeutic Specific Testing Strategies: Examples

Groups of Risk Factors	Example 1 Roctavian® Hemophilia A (105, 168, 169)	Example 2 Elevidys®, Duchenne Muscular Dystrophy (77, 78)	Example 3 Luxturna®, Retinal Dystrophy (101, 185)	Example 4 ST-920, Fabry Disease (192)
Protein/vector factors	High (lack of redundancy in biology, systemic exposure)	Medium (transgene protein location is cell membrane associated)	Low (intracellular location of transgene protein)	High (lack of redundancy in biology, systemic exposure)
Patient factors	High (likely prior ERT exposure, potential to develop inhibitors)	Medium or High (Lack of functional protein, no ERT available)	Medium (reduced level of RPE may not provide robust immune tolerance; no ERT available)	High (prior ERT exposure with pre-existing antibodies to ERT, potential to develop NAbs)
AAV context factors	Low (liver directed)	High (Muscle specific promoter)	Low (immune privileged site of administration)	Low (liver directed)
Treatment factors	High (systemic administration)	High (systemic administration)	Low (immune privileged site of administration)	High (systemic administration)
Overall risk assessment-based recommendation	ATPA Binding - Test ATPA Nab (inhibitors) - Test ATP CTL - Consider	ATPA Binding - Test ATPA Nab - Optional ATP CTL - Consider	ATPA Binding - Optional ATPA Nab - Optional ATP CTL - Consider	ATPA Binding - Test ATPA Nab (inhibitors) - Test Anti-TP CTL - Consider, trigger based
Testing strategy applied/reported	Anti-TP binding antibody –Tested. Low to mid % incidence with no apparent association with TP activity reported post-dose. Anti-TP antibody development is not discussed in the label	Anti-TP Binding Ab was evaluated with 0% and > 50% prevalence at pre-dose and post-dose, respectively. Lack of consistent pattern of anti-TP Ab development. Lack of association of anti-TP Ab and TP expression in muscle tissues. Anti-TP antibody development is not discussed in the label	Anti-TP Binding Ab was evaluated with minimal or no sustained increase in anti-TP antibody titers reported. Anti-TP antibody development is not mentioned in the label	Anti-TP binding antibody testing combined screening and confirmatory steps. In patients negative for pre-existing anti-ERT antibodies at baseline, ST-920 generally did not induce anti-TP antibody formation post-dose. In subjects with pre-existing antibodies to ERT, titers decreased or became undetectable post treatment in most patients
	Patients were evaluated for the development of anti-TP neutralizing antibody response (inhibitors). No sustained incidence was reported. Monitoring for inhibitor development is discussed in the label	Anti-TP neutralizing antibody activity was not tested for	Anti-TP neutralizing antibody activity was not tested for	Patients confirmed positive for anti-TP binding antibodies were analyzed in NAb assay. NAb titers decreased or became undetectable in most patients at later timepoints
	Anti-TP T-cell response was evaluated for with sporadic (~65%) post-treatment prevalence of response and no apparent correlation with the observed FVIII activity. Anti-TP cellular response is discussed in the label	Anti-TP T-cell response was evaluated. Response in some patients was reported with generally no evidence of impact on the study outcome. Limited number of patients showed a correlation of anti-TP T-cell response with immune-mediated myositis. This observation was used to formulate a patient exclusion criterion. Anti-TP cellular response is discussed in the label	Anti-TP T-cell response was evaluated with limited and clinically insignificant response. Anti-TP cellular response is discussed in the label	A trigger-based cellular immune response monitoring plan was adopted. Based on a favorable safety profile, cellular immunity to TP was not evaluated

Overall, the potential for the anti-TP immunogenicity induction is not expected to be translatable between animal species and humans, particularly in cases with less than 100% homology between non-human and human TP sequences. Additionally, endogenous levels of the TP homologue in target human disease population may differ greatly from the levels observed in healthy animals used during non-clinical safety evaluations. However, evaluation of anti-TP immunogenicity can be informative, particularly if AAV vector administration results in induction of anti-TP antibodies that can cross-react with the endogenous version of the transgene protein, allowing investigation of anti-TP immunogenicity related safety implications. Overall, non-clinical evaluations may generate critical data that will help with the design of a risk-based evaluation of anti-TP immunogenicity in the clinic.

The bioanalytical and immunogenicity testing strategy for non-clinical studies conducted in support of AAV based therapies may include evaluation of humoral and cellular anti-TP immune responses. The extent of this evaluation should be driven by the need to understand how the expected or observed safety and efficacy signals are impacted, including an assessment on the level and durability of TP expression. The approach presented in Table IV can be considered to design a risk-based assessment of non-clinical anti-TP immune response evaluation.

Recommendations for Clinical Protocols

Selection of the appropriate population is a key component of any clinical trial. In some cases, as relevant to AAV therapy development, patients with pre-existing immune responses that could cross react with the transgene product may be excluded. For example, for the approved gene therapies for hemophilia A and B, patients with a history of inhibitor development were excluded from the registrational trials (105, 211). These factors should be specified in the exclusion criteria in the clinical protocol. For hemophilia, assessment of potential inhibitor development is part of standard clinical care and potential study participants may already have this information in their medical records. However, in other instances, a new test may need to be developed to detect TP specific immune responses. If the results of the test are then used for patient management decisions, the test will need to meet regulatory requirements for *in vitro* diagnostics (212–214). These details also need to be specified in the clinical protocol.

One of the key risks for gene therapies is that the drug cannot be easily withdrawn in cases of adverse events, including the development of anti-TP immune responses. Therefore, a study stopping rule may need to be incorporated to address the risk of de novo development of an anti-TP immune response. This is an especially important consideration for high immunogenicity risk transgene products where development of a neutralizing response could raise safety concerns and/or impact application of a rescue treatment, for example with ERT. If development of anti-TP immune response occurs in a pre-defined number of participants (for example, ≥ 2), a study stopping rule could trigger thorough reconsideration of the risk assessment. These details, as well as the rules governing implementation of the relevant investigation, should be specified in the clinical protocol. Because the anti-TP immunogenicity assessment results may have a major impact on the conduct and the outcome of the trial, the data may need to be confirmed using a separate sample. Because these data could inform patient treatment decisions, the *in vitro* diagnostics regulations need to be considered when implementing the tests (212–214).

The scope of anti-TP antibody and cellular response testing should be based on the immunogenicity risk assessment and the context of use specific for the therapy. Clinical study protocol should clearly state the objectives and endpoints of these tests. The risk assessment should also guide whether the anti-TP immunogenicity response testing is viewed and listed in the protocol as a secondary (typical if safety is a concern) or exploratory endpoint. Anti-TP immunogenicity testing may only very rarely be a primary endpoint. The nature of the tests used to assess anti-TP immunogenicity response can be based on the proposed strategy outlined in Table II. As an example, for hemophilia-related AAV-based therapies, assessment of coagulation factor inhibitors is expected. Assessment of anti-TP immune response for other transgene products with a lower immunogenicity risk potential may include evaluation of binding antibody development only. For intracellular transgene products, banking systemic samples for potential assessments, as proposed in Table II, may be adequate. Evaluation of cellular immune response should also be considered based on its potential risk (Table II). If a cytotoxic T cell response against the TP is suspected to impact therapeutic efficacy and safety, as observed in DMD gene therapy trails (23), cellular immunogenicity may be considered a critical component of the study protocol. For lower-risk programs, such as liver-directed AAV gene therapy, which may induce immune tolerance, PBMCs can be collected and banked for future analysis in case safety or efficacy issues arise. The sample collection schedule should balance scientific rationale, practical feasibility and clinical trial practices. If the decision is made that conducting a cellular immune response evaluation is value added, the baseline PBMC sample should always be collected to establish pre-treatment immune status. Early response (weeks 2–4), peak response (weeks 4–8) and follow-up collection should be considered. A reduced schedule is recommended for lower-risk programs and at later timepoints beyond year 1.

As discussed above, the strategic approach to ATPA assessment shares many similarities with that for the assessment of ADA responses to protein therapeutics. Therefore, the number and timing of collections outlined in the clinical protocol should align with the sponsor's general strategy for ADA collection for protein therapeutics. Importantly, even if immunogenicity related sample testing is not planned, the protocol should include the collection of relevant material necessary for assessing antibody responses in the case of AEs. Clinical protocols could incorporate a statement that enables the use of pharmacokinetic or other systemic samples for the immunogenicity assessment in cases where an ATPA sample is not already collected. Because transgene proteins encoded by many AAV gene therapies mimic various endogenous proteins, it is likely that the evaluated TP immunogenicity risk category will be high. Consequently, there may be a need for sample collection starting at timepoints when humoral responses start to develop (e.g., ~ 1–4 weeks), and then relatively frequent for the first year (every 8–12 weeks). Extended sampling up to 2 years is required for long-term follow up gene therapy trials (215). In addition, FDA guidance recommends long-term follow up observations of 5 years for AAV and 15 years for genome editing therapies. These 5 to15 year follow up requirements do not specify collection of samples, although specific observations over time may trigger collection of material required to investigate causes for a reduction in efficacy or other events. Therefore, flexibility should be incorporated in the study protocol to allow for sampling in case of an adverse event or when there is a change in the level of clinical response.

Current Regulatory Agencies Position

Regulatory authorities have acknowledged the potential negative impact of immune responses against transgene products in AAV gene therapy products (198, 215, 216). These responses can pose immediate safety risks, such as inflammation and damage to tissues transduced by the vector carrying the therapeutic transgene, as well as potential efficacy risks, including the loss or diminished levels of transgene expression. The guidances also suggest that prolonged expression of the transgene product may lead to the development of antibodies against self-antigens, potentially resulting in autoimmune-like reactions.

The EMA guidance outlines several parameters that may lead to the generation of an adaptive immune response against the transgene product. These include prior exposure to the transgene product, the immune status of the patient, gene transfer protocols, target tissue, the nature and dose of the viral vector, the type of transgene promoter, and the nature of the transgene product (198).

To mitigate potentially severe impacts, regulatory authorities have provided broad recommendations for drug developers. These include designing AAV GT products and manufacturing processes that minimize product immunogenicity, avoiding subtherapeutic dosing and repeat administration, limiting exposure, and treating clinical trial participants with immune suppressive regimens. Regulatory guidelines also recommend monitoring anti-transgene product immune responses in clinical trial participants, although there is currently no clear consensus on specific assays, sampling points, or approaches.

As a specific case, the FDA guideline for the development of human gene therapies for hemophilia provides

Table IV Proposed Strategy for Assessment of Anti-transgene Product Immune Responses in Non-clinical Studies

Transgene Product localization	Humoral anti- transgene product response assessment		Cellular immune response to transgene product assay requirement
	Binding ATPA assay requirement [1]	Neutralizing ATPA assay requirement [1]	
Intracellular	Testing is optional. Collect and bank samples [3]	Testing generally is not recommended	Testing is optional. Collect and bank samples [3]
Cell Membrane associated	Testing is recommended	Testing is optional. Collect and bank samples [2]	Testing is optional. Collect and bank samples [3]
Secreted/systemic	Testing is recommended	Testing is optional. Collect and bank samples [2]	Testing is optional. Collect and bank samples [3]

1- Single-tier approach in design of binding ATPA response detection is recommended. A 1% false positive rate is recommended when determining assay cut-point value

2- It is suggested that assays are developed and ready for use. The decision to conduct testing should be gated by a pre-defined event, for example, safety related

3- The need to conduct test may be determined based on a safety related observation or an impact on TP levels that cannot be explained otherwise. Assays could be developed prior to the study initiation, or the development can be triggered by a pre-defined study signal

recommendations for assessing anti-transgene product immune responses. The development of neutralizing antibodies against Factors VIII or IX, typically referred to as inhibitors, is considered a significant adverse event and would require alternative therapies to mitigate. Additionally, periodic monitoring and assessment of peripheral blood mononuclear cells for the presence of anti-transgene T-cell reactivity is recommended (148).

Discussion and Recommendations

Similar to exogenously introduced biotherapeutics, transgene products have a potential to active innate and adaptive immune responses leading to the production of TP specific antibodies and cytotoxic T lymphocytes. These immune responses can hinder therapeutic efficacy by targeting secreted TP or eliminating transduced cells. The risks of anti-TP immune responses should be evaluated considering factors like the patient's immune tolerance to the TP, the similarity between the TP and endogenous proteins, prior exposure to similar products, and comparative expression levels of endogenous and transgene-encoded proteins. While other publications have explored anti-AAV immunity to gene therapy products, this manuscript concentrates on evaluating immune responses to TPs, encompassing both humoral and cellular aspects. The emergence of anti-TP antibodies or a cytotoxic cellular response can lead to enhanced TP clearance and destruction of AAV-transduced cells, thereby compromising the treatment's durability and effectiveness. Moreover, anti-TP antibodies may cross-react and neutralize residual endogenous proteins, potentially exacerbating the clinical condition being addressed.

A risk-based approach is widely adopted for evaluating immunogenicity responses to exogenously introduced protein therapeutics. This methodology is also proposed for evaluation of the potential induction of immune responses against transgene proteins encoded by AAV vectors since there are commonalities in immunogenicity risk factors between exogenous biotherapeutics and endogenously generated TPs. However, there are also notable differences between biotherapeutics and TPs that warrant detailed analysis.

The humoral and cellular immune responses to TPs depend on their presentation in the context of MHC Class I and/or Class II. Localization of the expressed TP may further impact the nature of the anti-TP immune response. For example, expression of TP in association with cellular membrane may result in presentation in the context of both MHC Class I and Class II and lead to production of cellular and humoral anti-TP immunity. Alternatively, a systemically circulating TP may be captured by APCs and presented via MHC Class II, potentially inducing humoral immunity. Finally, an intracellularly contained TP, or any protein produced within the cell that could be subject to proteasomal degradation, may be presented via MHC Class I, potentially resulting in cellular CTL immune responses. This dynamic interplay underscores the complexity of immunogenicity risks for TPs encoded by AAV vectors.

To systematically evaluate anti-TP immunogenicity risks, these factors are grouped into four categories:

- Product-Related Risks: Includes TP characteristics, AAV vector characteristics, presence of impurities, expression levels, TP biology and site of action.
- Patient-Related Risks: Involves underlying genetic mutations, residual protein expression status, patient's genetic background, disease-related inflammation, and pre-existing anti-TP immunity.
- Treatment-Related Risks: Considers route of administration, the need for repeat dosing, vector dose level.
- AAV-Context of TP Expression Risks: Addresses promoter specificity and strength, capsid serotype, and continuous TP expression.

These immunogenicity risk factors should be viewed as dynamic and assessed on a data-dependent basis. Changes in product characteristics, patient populations, treatment regimens, and immunogenicity assessment methodologies can alter the overall risk profile.

A key challenge in developing the TP immunogenicity assessment strategy lies in the diverse and interconnected nature of risk factors, which complicates the design of a testing strategy based on the specific context of use. To streamline this process, two primary characteristics of AAV therapy applications are emphasized: the immune privilege status of the site of the AAV vector administration and the cell-context localization of the expressed transgene product.

Administering AAV vectors directly to immune-privileged sites is generally believed to significantly reduce the risk of both humoral and cellular anti-TP immune responses. In such cases, evaluating these immune responses could be considered optional, with samples collected and stored for potential testing based on pre-defined clinical events, such as significant changes in treatment efficacy or the emergence of adverse events.

For other administration routes, such as IM and IV, the localization of the expressed protein is proposed as key for shaping the immunogenicity assessment strategy. It is suggested that the approach for soluble transgene products circulating systemically differs from that for cell surface or intracellularly localized TPs. Systemically active proteins may be assessed using paradigm similar to that for traditional protein-based therapeutics, employing separate assays

to detect binding and neutralizing responses. In scenarios where the protein is initially expressed in one tissue (e.g., liver) and then trafficked to another (e.g., muscle), where it is taken up by specific cell type and becomes active intracellularly, assessing antibodies that neutralize TP activity may be optional. Instead, understanding the impact of the ATPA on the cellular uptake of the TP by target cells may be more critical.

For indications where ERT remedy is available, the potential impact of anti-ERT protein antibodies, that may cross react with the TP, should be considered. Conversely, the development of anti-TP antibodies that may cross-react with the associated ERT or endogenous protein versions presents a potential risk if AAV-based therapy fails. A key distinguishing factor for gene therapies when compared to ERT is the continuous expression of TP. Consequently, the potential for ATPAs to cross-react to the ERT is an important consideration.

Based on the intended localization of the TP (intracellular, membrane-bound, or secreted/systemic), the manuscript presents decision tools, shown in Tables II and Table IV, that guide the determination of the most relevant strategy for assessing of anti-TP immune responses in clinical and non-clinical studies. When immune response assessment is proposed as optional, samples could be collected and stored for event-driven testing. An assessment of cellular anti-TP immune response may be needed when evidence of an anti-TP CTL response triggering adverse events is available. For example, monitoring cellular responses may help with understanding of unexpected TP clearance in the absence of humoral anti-TP immune response.

Understanding the immunogenicity risk factors associated with transgene product is instrumental for developing the most appropriate plan to assess anti-TP immune responses in clinical trials. The plan may include evaluation of pre-existing anti-TP immunity as part of the treatment inclusion criteria and assessment of humoral and cellular anti-TP response post-treatment. The scope of anti-TP antibody and cellular response testing should align with immunogenicity risk assessments and therapy context. Protocols should clearly state test objectives, with risk assessment guiding whether the specific testing is a primary, secondary or exploratory endpoint. As an example, for hemophilia-related AAV therapies, coagulation factor inhibitor (neutralizing anti-TP antibody) assessment is expected, while lower-risk transgene products may only require evaluation of the development of binding anti-TP antibodies. Cellular immune response evaluations should be conducted based on understanding of potential risks, especially if cytotoxic T cell responses could affect therapeutic efficacy and safety. For lower-risk programs like liver-directed AAV gene therapy, which may induce immune tolerance, PBMC samples may be banked for future analysis. Sample collection schedules should balance scientific rationale with practical feasibility, including collection of baseline PBMC samples to establish pre-treatment immune status. The strategic approach to anti-TP antibody assessment shares many similarities with assessing anti-drug antibodies for protein based biotherapeutics. Clinical protocols should align with the general ADA collection strategies, and even if immunogenicity testing isn't planned, relevant material may be collected to assess immune responses if adverse events occur.

Conclusions

The manuscript covers topics related to factors associated with the risk of anti-TP immunogenicity induction, potential impact and implications, the importance of anti-TP immunogenicity response assessment, and associated analytical methodologies. It suggests an approach for determining the scope of anti-TP immunogenicity assessment in clinical and non-clinical studies. The recommendations are broadly based on the risk-based approach used for protein therapeutics, but also consider factors unique for gene therapy modality such as site of administration and intended localization of the TP. Authored by scientists from various industry organizations involved in AAV-based therapeutic development, the manuscript aims to offer recommendations and guidance to industry sponsors, academic labs, and regulatory agencies for a consistent approach to assessing anti-TP immune responses.

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Declarations

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