Molecular Therapy
Methods & Clinical Development
Editorial

Rationale for using centralized transduction inhibition assays in three phase 3 rAAV gene therapy clinical trials

INTRODUCTION

Pre-existing anti-adeno-associated virus (AAV) antibodies can impede successful gene transfer and potentially have safety consequences for patients receiving recombinant AAV (rAAV)-mediated gene therapy (GTx). Hence, assessing patients' anti-AAV antibody status is an important consideration prior to systemic rAAV GTx administration.

Sponsors of rAAV GTx clinical trials typically use either: (1) transduction inhibition (TI) or (2) total antibody (TAb) assays to screen for pre-existing antibodies[1] (Figure 1). TI assays are cell-based functional assays that directly measure the level of TI by antibodies and non-antibody neutralizing factors (e.g., small molecules, innate immune activators, and shed AAV receptors).[2] These assays use the same rAAV capsid as the GTx, with a choice of reporter genes and cell lines.[2] TI assays are complex and can be influenced by multiple parameters (e.g., serum proteins, multiplicity of infection, indicator cell line, reporter gene, and capsid). Therefore, these assays are more difficult to be developed into commercialized test kits than TAb assays.[1] In contrast, TAb assays are biochemical immunoassays that measure all antibodies (both neutralizing [NAbs] and non-neutralizing) that bind to the rAAV vector. These assays can be used to detect specific immunoglobulin isotypes and subclasses, but also measure low-avidity antibodies that may not impede successful gene transfer.[3] Due to a lack of standardization, an accurate comparison of assay performance and clinical utility within and between TI and TAb assays is currently not feasible. Pfizer selected centralized laboratory TI assays to screen patients for pre-existing anti-AAV neutralizing activity in its three phase 3 rAAV GTx clinical development programs, and currently aims to develop these assays into companion diagnostic tests (CDx).[4–6] Here, we provide the scientific and clinical rationale behind this decision.

HISTORICAL PERSPECTIVE

Early studies evaluating the humoral immune response to AAV2 vectors in healthy volunteers noted that, while 80% of study participants carried binding antibodies for AAV2, only 18% had NAbs.[7] Similarly, among cystic fibrosis patients, only 32% were NAb positive, even though nearly all patients (96%) had binding antibodies.[8] Of interest, one study using both TAb and TI assays found that 24% of study participants were negative for TAbs but positive for TI activity, suggesting the presence of non-antibody neutralizing factors.[9] These studies underscore a disconnect between the detection of binding anti-AAV antibodies in patient plasma or serum and the ability of these antibodies to effectively neutralize vectors. Of concern is which assay can best predict the impact of pre-existing humoral immunity on *in vivo* neutralization and safety for a particular rAAV GTx without excluding patients who might benefit from the therapy.

EFFICACY

Previous rAAV GTx pre-clinical[10–13] and clinical studies[14–16] using TI assays have shown that even low levels of pre-existing anti-AAV NAbs can limit or completely impede transduction. For example, inoculating mice with anti-AAV8 NAbs before rAAV GTx[10] led to undetectable factor IX (FIX) levels post-infusion. In another murine study, the presence of low anti-AAV2 NAb titers (1:3.8) resulted in a 96% reduction in FIX expression post-vector infusion compared with NAb-negative animals.[12] Similar findings were reported in non-human primates. Vector administration to macaques with low anti-AAV8 NAbs (<1:10) led to reduced gene transfer[13] and, separately, FIX expression was diminished in macaques with anti-AAV8 NAb titers of 1:5.[11]

In humans, rAAV GTx efficacy was lower in the presence of pre-existing neutralizing activity, as detected by TI assays. FIX expression was attenuated in a study participant with a pre-existing NAb titer of 1:17.[14] Another study reported that a participant with an anti-AAV-Spark100 (AAVrh74var) NAb titer of 1:1 achieved significantly lower FIX activity than participants with NAb titers <1:1.[16] In a hemophilia B clinical trial that did not employ AAV seropositivity as an exclusion criterion, one patient with an anti-AAV5 NAb titer of 1:3,212 failed to respond (i.e., no transgene expression) and the mean FIX activity in the subgroup with pre-existing NAb titers up to 1:678 was numerically lower compared with the subgroup without detectable NAbs.[17]

Despite differences in assay conditions and capsids, these studies underscore the significance of the TI assay in predicting vector transduction.

SAFETY

The impact of pre-existing anti-AAV antibodies on the safety of vector administration is less well studied. While rAAV8 vectors were redirected to the spleen in seropositive macaques, there was no evidence of increased toxicity.[13] Similarly, no increased toxicity or serious adverse events (SAEs) linked to NAb positivity were noted upon rAAV5 vector administration to seropositive animals or human trial participants.[18,19] However, the studies mentioned above did not evaluate high vector doses (i.e., >1E-14 vg/kg), which are more commonly associated with SAEs.[20] Nevertheless, *in vitro* complement activation has been observed when rAAV9 vectors were admixed with serum from NAb-seropositive donors,[21] which suggests a potential risk upon dosing patients with pre-existing neutralizing activity. While complement activation and related adverse events have been observed

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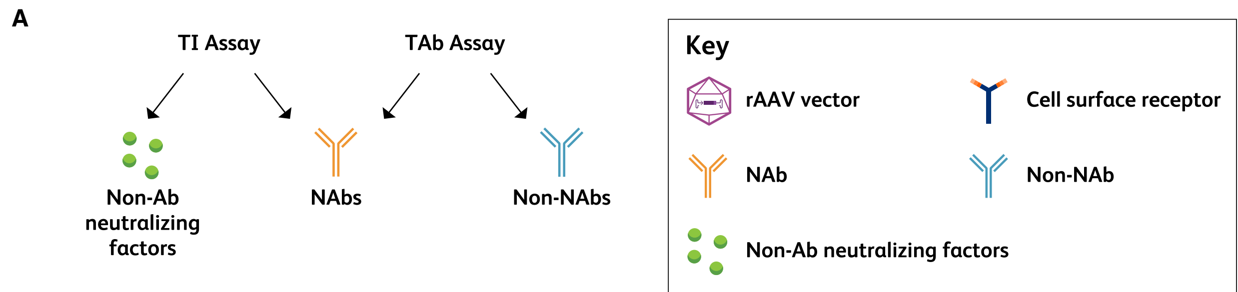
A



B

Potential effects of anti-AAV Abs and non-Ab neutralizing factors	**Inhibition of target cell transduction**	**Redirection and increased clearance of rAAV vectors**	**Increased *uptake* of rAAV vectors in some tissues**	**Complement activation**
Mediated by	NAbs and non-Ab neutralizing factors	NAbs and Non-NAbs	Non-NAbs	NAbs and non-NAbs (pre-existing or developed *de-novo* following rAAV GTx administration)
Potential outcome	Transgene expression ↓	Transgene expression ↓	Currently unclear*	Complement-mediated adverse events
Mechanism				
	1. Prevention of rAAV vector binding to target cell surface receptors[2] 2. Interference with endocytosis[2] 3. Blockage of endosomal escape[2] 4. Blockage of nuclear import[2]	• rAAV vector clearance via opsonization, and redirection to secondary lymphoid organs[23]	• The mechanism remains to be determined; however, *in vitro* and *in vivo* murine studies showed that non-NAbs (but not NAbs) have the potential to enhance the transduction efficiency of rAAV vectors in some cell types (e.g., hepatocytes)[10]	• Formation of immune complexes with rAAV may result in complement-mediated adverse events, such as TMA or aHUS[26] • Of note, the role of pre-existing anti-AAV Abs in such adverse events is currently not well understood. Patients in Pfizer's DMD rAAV GTx clinical studies with complement-mediated adverse events were seronegative at baseline in both the TI and TAb assay[22]

(legend on next page)

in several high-dose rAAV trials (e.g., in spinal muscular atrophy and Duchenne muscular dystrophy [DMD]),[20] in the Pfizer DMD trial this was not associated with pre-existing anti-AAV antibodies as participants who experienced complement-mediated adverse events were Ab negative in both TI and TAb assays.[22] Complement activation here may have been related to an antibody response in participants after rAAV GTx administration. Other theoretical safety concerns when dosing seropositive patients include inflammatory and hypersensitivity reactions, including potential infusion-related reactions.

RATIONALE FOR SELECTING A CENTRALIZED TI ASSAY

The efficacy and safety implications of rAAV GTx administration to seropositive patients described above necessitate the development of an anti-AAV Ab assay. Pfizer's approach involves using validated TI assays to enroll only seronegative patients in their phase 3 rAAV GTx clinical programs.[4–6] The rationale for selecting the TI assay was as follows: (1) existing evidence of a strong correlation between pre-existing anti-AAV neutralizing activity (NAbs and non-Ab neutralizing factors) and reduced efficacy.[10–16] (2) Limited evidence of the impact of non-NAbs. While these antibodies could opsonize and redirect vectors to secondary lymphoid organs,[23] there are also reports of these antibodies *increasing* liver transduction.[10] Hence, the relevance of binding Abs is difficult to gauge without concomitant knowledge of their neutralizing ability. (3) Pfizer's rAAV GTx clinical development programs (fordadistrogene movaparvovec for DMD, giroctocogene fitelparvovec for hemophilia A, and fidanacogene elaparvovec for hemophilia B) were each developed in collaboration with an external partner (Bamboo Therapeutics, Sangamo Therapeutics, and Spark Therapeutics, respectively). Each sponsor had independently selected the TI assay to screen patients for their successful phase 1/2 clinical trial and Pfizer has continued to use TI assays in the three pivotal clinical trials.

Owing to the potential impact of pre-existing anti-AAV antibodies on efficacy and safety of rAAV GTx, regulatory bodies have recommended that sponsors consider the development of diagnostic tests to screen for these antibodies.[24] Several patient advocacy groups, including the World Federation of Hemophilia, European Haemophilia Consortium, and National Hemophilia Foundation, request that appropriate anti-AAV antibody assays should be available with marketing authorization of the respective rAAV GTx.[25]

Consistent with the above recommendations, it is the intention of Pfizer to submit the TI assays for rAAV GTx investigational products to regulatory authorities for approval as CDx. CDx approval will provide confidence that the assay can accurately identify seronegative patients who are likely to benefit from the corresponding rAAV GTx medicinal product.

Furthermore, we plan to use the same screening approach for anti-AAV neutralizing activity after marketing approval as in the pivotal clinical trials (i.e., centralized assays run by the same Clinical Laboratory partner) to ensure protocol adherence and transferability of results from the pre- to post-approval setting. Monogram Biosciences, a Labcorp company specializing in cell-based assays, was selected as Pfizer's diagnostics partner for anti-AAV TI CDx assay development and patient screening.

CONCLUSION

rAAV GTx administration to an NAb-positive patient can have potential efficacy and safety implications. In addition, the strong, durable, and cross-reactive antibody response that manifests after receiving systemic rAAV GTx currently precludes the patient from receiving another rAAV GTx in the future. Hence, optimizing preconditions for successful treatment outcomes with the first rAAV GTx administration is paramount. Consequently, we believe the accurate measurement of anti-AAV neutralizing activity using a validated assay is important before administering systemic rAAV GTx to a patient.

We propose that an anti-AAV antibody bioanalytical testing strategy should be developed for each rAAV GTx individually that considers various factors, including, but not limited to, the capsid, dose, route of administration, patient population, and underlying disease. With patient benefit and safety at the core of the decisions, Pfizer has selected centralized TI assays for three current rAAV GTx investigational products and aims to submit these for approval as CDx.

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Figure 1. Anti-AAV antibody assays and potential effects of anti-AAV Abs and non-Ab neutralizing factors on efficacy and safety

(A) Sponsors of rAAV GTx clinical trials typically use one of two assays to screen for pre-existing Abs: (1) TI assay, a cell-based functional assay that directly measures the level of transduction inhibition by NAbs or non-Ab neutralizing factors (e.g., small molecules, innate immune activators, and shed AAV receptors), or (2) TAb assay, a biochemical immunoassay that measures all Abs that bind to the vector. In general, concordant assay results, particularly for samples with high NAb titers, are expected between TI and TAb assays. (B) Potential effects of anti-AAV Abs and non-Ab neutralizing factors on efficacy and safety.[2,10,22,23,26] *Depending on the tissue type in which the increased rAAV vector uptake occurs there may be a positive or negative effect on the transduction of target tissues. More research is needed to determine the potential impact on target cell transduction due to increased uptake of vector-antibody complexes. AAV, adeno-associated virus; Ab, antibody; aHUS, atypical hemolytic uremic syndrome; DMD, Duchenne muscular dystrophy; GTx, gene therapy; NAb, neutralizing antibody; rAAV, recombinant AAV; TAb, total antibody; TI, transduction inhibition; TMA, thrombotic microangiopathy.

AUTHOR CONTRIBUTIONS

M.S. and S.S. provided conceptualization. M.S., G.B., S.H.C., D.I.L., M.L., L.W., I.W., and S.S. conducted the investigation. M.S. and S.S. wrote the original draft of the manuscript. M.S., G.B., S.H.C., D.I.L., M.L., L.W., I.W., and S.S. contributed to the writing, and review & editing of subsequent drafts. S.S. provided supervision.

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