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REVIEW ARTICLE

Immunogenicity of Biotherapeutics: Current Industry Experience, Challenges and Opportunities

Assessment of Neutralizing Antibody Activity in Clinical Studies: Use of Surrogate Measurements Instead of Stand-alone Assays

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Abstract

Neutralizing antibodies (NAbs) to protein therapeutics have traditionally been assumed to be the most impactful subset of anti-drug-antibodies (ADA). NAbs can block the biotherapeutic from engaging its target impacting efficacy and may also cause serious safety events. Stand-alone NAb assays have been employed to detect neutralizing responses, often with reconfigured versions of other assays. These methods have historically been implemented in registrational trials for all molecules, and in early-stage studies for high risk biotherapeutics. However, data has demonstrated that NAb response and ADA magnitude are highly correlated. Additionally, the use of other markers to identify clinically relevant immunogenicity, such as apparent impact on pharmacokinetics (PK) or pharmacodynamics (PD), has been increasing. This manuscript reviews the available data on clinically meaningful immunogenic responses to biologics and proposes a risk-based strategy to determine if and when to employ a stand-alone NAb assay. For molecules with a high risk of safety consequences of immunogenicity (e.g., biological mimics) a NAb assay is recommended. However, for lower-safety risk molecules a stand-alone NAb assay does not enhance the interpretation of clinical data and is likely not needed. A combination of other assessments including ADA status, magnitude and persistence, PK, and PD (and efficacy) can be used as a surrogate for NAb assay data. Integration of data from all clinical evaluations is recommended by Health Authorities and can provide a more accurate overall assessment of neutralizing activity. This approach identifies clinically impactful downstream readouts of neutralizing activity without the need for a stand-alone NAb assay.

Keywords Anti-drug antibodies (ADA) · Biotherapeutics · Immunogenicity · Neutralizing antibodies (NAb) · Pharmacokinetics/Pharmacodynamics (PK/PD)

Introduction

All biological molecules have the potential to generate an immune response. However, immune responses to protein therapeutics are generally unwanted as anti-drug antibodies (ADA) have the potential to impact safety and

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efficacy of the therapy. Neutralizing antibodies (NAbs) are a subset of ADA that bind to a biotherapeutic and may render the drug biologically inactive *in vivo* (1, 2). Of particular concern is the potential of NAb to neutralize endogenous counterparts, which could result in serious safety consequences. The most prominent example is among erythropoietin treated patients who developed NAbs against both the biotherapeutic and endogenous erythropoietin and suffered from pure red blood cell aplasia that has made them dependent on blood transfusions (3, 4).

Due to these serious safety concerns, the immunogenicity testing paradigm for biotherapeutics has traditionally included NAb assessment with a stand-alone assay. However, over the last two decades immunogenicity testing methodology has dramatically improved and robust assessment strategies for understanding the immunogenicity risk of biotherapeutics have been implemented. Importantly, serious adverse events stemming from immunogenicity have also proved to be rare. As a result, there has recently been a movement to reassess the immunogenicity testing paradigm for biotherapeutics, in particular whether the confirmatory and titer steps of ADA assays add value (5–8). Given the strong correlation between ADA magnitude and NAb response, the benefit of NAb testing for all clinical programs has also recently been challenged (9–13).

This manuscript was authored by a cross-industry group as part of the Neutralizing Antibody Working Group from the AAPS's Bioanalytical Community to examine the value and timing of implementation of NAb assays. The paper reviews the available data on clinically meaningful immunogenic responses to biologics and proposes a risk-based strategy to determine if and when to employ a stand-alone NAb assay. In the event of an immunogenic response, there is often considerable information available from alternative measurements, such as pharmacokinetics (PK), pharmacodynamics (PD) and efficacy, in addition to ADA magnitude and persistence, to understand the impact of ADA and NAb on clinical outcomes. The working group proposed that for most molecules a strategy that incorporates the use of these alternative clinical assessments provides a more valuable evaluation of "neutralizing activity" than a stand-alone NAb assay. Given the well-documented deficiencies of NAb methodologies, these surrogate measurements may actually be superior to a stand-alone assay in many cases. If an assessment of immunogenicity risk for a particular molecule suggests a high level of concern, particularly for safety, then a stand-alone NAb assay may be appropriate. The requirements, format, and the advantages and disadvantages of these methods have been discussed elsewhere (14–16).

Neutralizing Antibodies and Their Effects

Development of NAbs

Humoral immunogenicity to an antigen manifests as a polyclonal response which can change and mature over time in both magnitude and affinity. The kinetics of an ADA response to antigen are characterized by initial transient IgM and IgD responses, followed by the subsequent more persistent development of IgG, IgA or IgE antibodies (17). NAb is typically detected as the immune response to an antigen matures, and among antigen-specific IgG, the IgG4 isotype is often represented at higher levels in neutralizing antibodies when compared to typical serum concentrations (Fig. 1) (18–23).

Impact of ADA and NAbs

Monitoring immunogenicity and the impact of ADA and NAb on the clinical response of a therapeutic is a key parameter of clinical trials. However, the development of ADA does not always compromise the efficacy of a drug. In fact, although most protein therapeutics can elicit an immune response, ADA typically forms in only a subset of patients, and many of these patients develop low-titer ADAs or weak NAbs that are often not clinically relevant (Fig. 1). Second, even if there is a decline in clinical response attributable to immunogenicity, NAbs are not necessarily the sole contributing factor. For example, elevated levels of binding ADA could clear the drug through Fc-mediated interactions and hence be functionally neutralizing (24–26).

Evidence of NAbs

A common misperception is that stand-alone NAb assays are necessarily a superior approach to measure NAbs. In fact, there are other assays that can directly or indirectly detect the presence of NAbs. NAbs are a subset of the polyclonal antibody response and are therefore a component of the antibodies detected in the binding ADA method. Consequently, NAbs contribute to the incidence, magnitude (titer) and duration of response data generated with the ADA method. In addition, the ADA magnitude is generally well correlated with the NAb positivity and/or NAb titer, and in particular elevated ADA responses are routinely NAb positive (Fig. 1). This association has been demonstrated using a variety of methodologies to detect ADA and NAb (including bridging or direct immunoassays for binding ADA, and cell based and competitive ligand binding NAb assays) in participants in clinical trials for various modalities (multiple mAb therapeutics as well as protein hormones and AAV-based

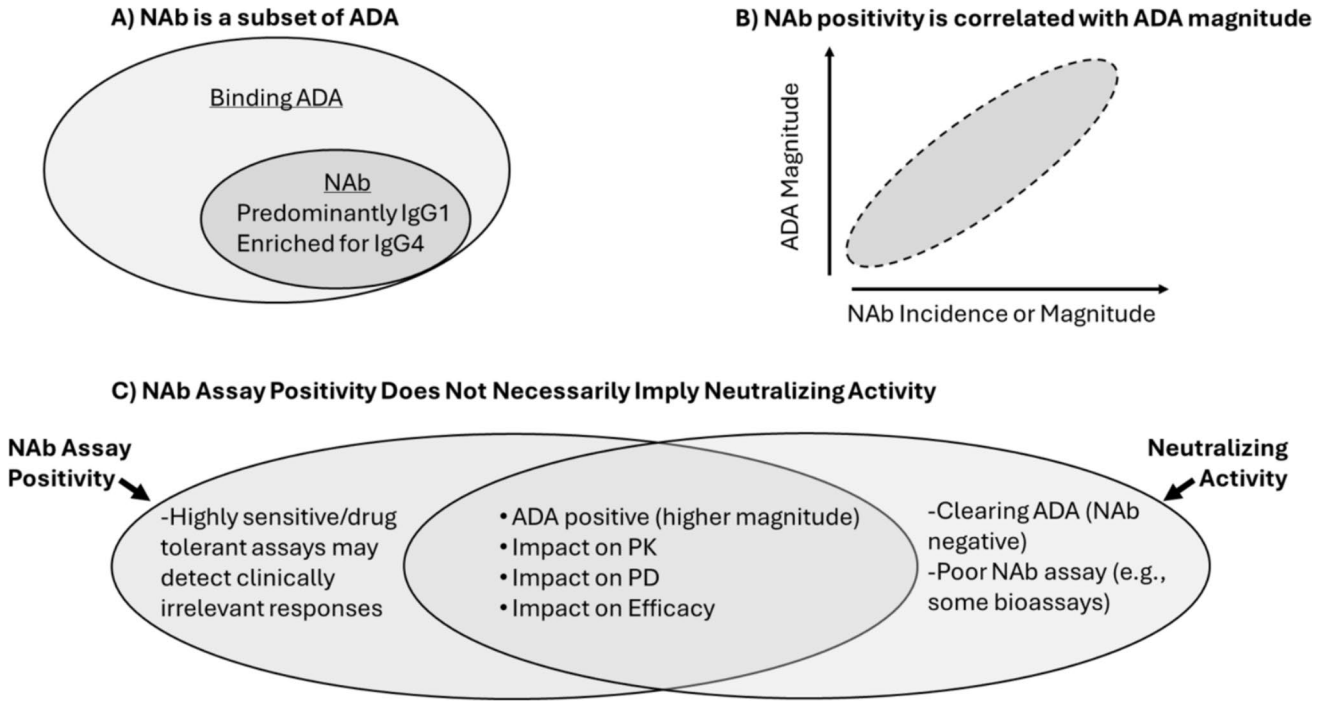

Fig. 1 Neutralizing antibody assays and the relationship to ADAs. **A** Neutralizing antibodies are a subset of ADAs that develop as the immune response matures. In contrast to ADAs which can be a variety of isotypes, NAbs are predominantly IgG1 and IgG4 isotypes. **B** As the immune response to a biotherapeutic progresses, the ADA magnitude and titer increases, which correlates with increased NAb positivity. **C** Due to the development of increasingly sensitive NAb assays, NAb positivity does not always correspond to clinically meaningful neutralizing activity. Due to the surrogate nature of neutralizing antibody assays, it is possible to detect an ADA as neutralizing, but if there is no impact on PK, PD, or efficacy, it can be considered an irrelevant response. In contrast, poorly sensitive NAb assays might miss the detection of clinically relevant neutralizing antibodies

gene therapies) (10–13, 27–29). Notably, high titer, persistent ADA responses have been shown to be correlated with reduced clinical response, either due to the presence of anti-drug neutralizing activity, increased clearance, or a combination of both of these factors (10–13).

In addition to the ADA assay, the drug concentration assay (PK measurement) can also indirectly detect the presence of NAbs. For assays that measure free or active drug (e.g., a target capture immunoassay format), the presence of NAbs could inhibit detection in the method. Similarly, anti-idiotypic antibodies used to capture the drug in some PK methods may also be impacted by NAbs, since NAbs could bind to the same epitope on the drug as the anti-idiotypic antibody. Consequently, the first indication of impactful immunogenicity, including NAbs, may be observed as a reduction in apparent exposure via the drug concentration assay (12, 30). Even when measuring total levels of the therapeutic (both unbound and bound to ADA/NAb), higher titer and clearing ADA responses (which are likely correlated with NAb) will often manifest as reduced drug exposure.

Another indirect measurement of neutralizing activity can be from the PD assessment. PD assays measure the bioactivity of the drug and can take a wide range of formats, from *in vivo* measurements like blood pressure to complicated ex vivo stimulation assays, all with the intention of assessing the effect of the drug on the patient. The presence of neutralizing antibodies could serve to block the drug mechanism of action, resulting in decreased signal in the PD assay. Therefore, PD assays are another alternative means of assessing the impact of NAbs.

Case Studies of PD Assays used in Lieu of Stand-alone NAb

An excellent example of indirect assessment of neutralizing activity through PD assays is with Factor VIII and Factor IX replacement products to treat hemophilia. In clinical practice, production of neutralizing antibodies (referred to as "inhibitors" in the hemophilia field) is monitored through the use of functional assays that measure clotting activity in patient samples (31). When reduced clotting activity is observed, these samples may subsequently be tested in a dedicated assay to detect inhibitors.

Another example is with clesrovimab, a prophylactic mAb designed to protect infants from respiratory syncytial virus (RSV) (32). The PD assay for this product

measures functional clesrovimab activity in an RSV-infection assay. The sensitivity of this assay to detect neutralizing activity against clesrovimab was shown to be around the threshold of the clinically meaningful efficacy change. Thus, the sensitivity of the PD assay to detect NAb at a 1:1 ratio of drug is sufficient to monitor clinically relevant efficacy change. These two examples highlight that PD readouts can be used successfully to determine neutralizing activity of protein therapeutics, including in clinical practice.

Stand-alone NAb Assays: Reformatted Versions of Other Methods

As mentioned above, while other indirect methods can indicate the presence of neutralizing activity, traditionally stand-alone NAb assays have often been employed. There are two main assay formats for stand-alone NAb detection: cell-based (CBA) and competitive ligand binding (CLB). Cell-based NAb assays, frequently cited as the gold standard, measure downstream functional changes due to presence of drug (e.g., increase or decrease in analyte downstream of signaling pathway) (14). These assays are often based on potency assays that measure the functional effect of the drug, therefore mimicking the *in vivo* response. Examples include IL-2 induction after treatment with anti-CTLA-4, antibody-dependent cell-mediated cytotoxicity (ADCC), and proliferation assays, as well as assays that expand beyond the functional response of the drug such as cell-based binding assays (33–36). Any change in the drug-induced response can serve as a biomarker of the presence of NAb within the context of the assay.

Similarly, competitive ligand binding NAb assays are based on detecting competition from NAb to block the target from binding to the drug. Often these assays are reformatted versions of the target capture drug concentration assay described previously (13, 21, 37).

Both of these types of common stand-alone NAb assays can therefore be considered as surrogate NAb readouts, since they are indirect measures of neutralizing activity against the drug.

CBA and CLB NAb assays typically have perceived methodological deficiencies, such as poor sensitivity or interference from drug product. More recently, the application of sample pretreatment to these methods through steps that remove drug or extract NAb from the sample have resulted in NAb assays with increased drug tolerance and increased sensitivity (38–40). However, the increased assay sensitivity due to these sample pretreatment methods frequently result in the detection of responses that are not clinically relevant (21).

Implementation of an *In Vitro* NAb Assay: Immunogenicity Risk Impact

The decision of whether and when to implement a stand-alone NAb assay in clinical testing is part of the assessment of immunogenicity risk of a biotherapeutic. Immunogenicity risk is determined through a variety of means, including but not limited to *in silico* and *in vitro* immunogenicity assessments as well as through analysis of other factors such as the structure of the biotherapeutic itself, its drug product quality characteristics, similarity to other molecules already tested in the clinic, and the intended patient population (41, 42). Protein therapeutics classified as low or medium risk may not need a stand-alone NAb assay, or the assay may be expected by regulators in later stages of drug development. In contrast, for molecules designated as high-risk, neutralizing antibodies would need to be assessed in a stand-alone assay earlier in the clinical trial phases (9).

Low Immunogenicity Risk: Potential ADA Impact on Efficacy Only

Examples of low-risk molecules include many standard mAbs and oligonucleotide therapies with a low-risk of inducing ADA and/or a low likelihood of safety impact if ADA were to develop. Therefore, for this risk class it may be possible to forgo a stand-alone NAb assay throughout clinical development, and instead use an integrated data approach by combining together the PK/ADA/PD/efficacy results to determine if there is any impact from NAb.

Bivalent Monoclonal Antibody (mAb) Therapeutics

Standard bivalent mAb biotherapeutics specific for a single target have been in clinical studies for over 30 years and the impact of immunogenicity upon patient outcomes has been well-documented. Overall, the historical examples of immunogenicity of mAb biotherapeutics impacting safety are rare, making the need for a stand-alone NAb assay in early clinical phases unnecessary.

Two recent reviews of publications for approved biological products have questioned the value of NAb assessment of biotherapeutics, particularly for low-risk molecules such as human mAbs. In the review of the immunogenicity data from 84 recently approved therapeutic mAbs for treatment of 9 major disease indications, Hassanein *et al.* (2020) noted that the majority of mAbs either had no reported NAbs, or NAbs were observed but there was no clinical impact (24). The authors question whether assessing NAb for mAb therapeutics has become a "box-checking exercise" as this was historically a regulatory expectation for all new products but does not add value for low-risk monoclonal antibody therapeutics.

Wang *et al.*, reviewed the prescribing information of 121 biotherapeutics approved before February 2015 (majority approved over the past 2 decades), which included 43 mAbs, 26 enzyme products, 11 cytokines, 12 growth factors and hormones, and peptides, proteins, and toxins for the remaining 29 biotherapeutics (26). It was noted that all cases observing impactful ADA resulted in more rapid drug clearance, and none observed any immunogenicity-related safety events. Therefore, NAb assessment for mAb therapeutics and other drugs with low immunogenicity risk for the purpose of safety evaluation may not provide any significant value.

For low-risk biotherapeutics, utilizing other surrogate markers such as ADA magnitude and duration of response, serum drug levels or PD biomarker data may be even more informative than a stand-alone NAb assessment to detect the presence and impact of neutralizing activity, and is a recognized alternative in the FDA 2019 guidance (43). Having a proximal PD marker available for a mAb biotherapeutic can de-risk the decision to not develop a stand-alone NAb assay. In fact, an integrated analysis of PK/PD/ADA data to interpret neutralizing activity of low-risk biotherapeutics has been proposed by the industry as an alternative approach for a stand-alone NAb assay (44). However, the availability of a PD assay is not a prerequisite for the decision to forgo implementing a stand-alone NAb assay. As discussed below, even in the absence of both a robust PD assessment and a stand-alone NAb assay, sufficient data may be obtained from ADA status and magnitude, drug concentration measurements and efficacy evaluations to assess neutralizing activity.

Oligonucleotide Therapeutics

Oligonucleotide therapeutics (ONTs) have molecular features common with both small and large molecules. They consist of nucleotide strands between 15–25 residues in length that are synthetically engineered like small molecules. However, they also have the potential to be presented by antigen-presenting cells (APCs), inducing an immune response to foreign epitopes like large molecule biotherapeutics (45). Given the small number of potential epitopes, the reduced propensity for DNA to induce an immune response, and the mechanism of action, the overall risk of ONTs is considered low. In addition, the site of action for these molecules is intracellular in either the nucleus or endoplasmic reticulum, spaces where the anti-drug antibodies are unlikely to be found (46). Therefore, if PD assays are available, it is recommended to use the integrated data analysis to support assessment of neutralizing activity for ONTs.

Moderate Immunogenicity Risk: Potential Impact on Safety

For biotherapeutics that have a potential to induce immunogenicity resulting in risk to safety, the decision of when to employ a stand-alone NAb assay becomes more nuanced. In the absence of safety risk, historically it was acceptable to wait to implement a stand-alone NAb assay until later clinical stages (Phase 2 and beyond). However, more recently there have been successful submissions for drug approvals where NAb data was not included, indicating that Health Authority's stance may have evolved on the requirement for NAb assessments (5).

Monoclonal Antibody Therapeutics with Moderate to High Magnitude ADA

A drug product with a higher risk of immunogenicity may result in an elevated ADA incidence as well as higher magnitude ADA titers which is commonly associated with an elevated NAb positivity (10–13). High magnitude ADA and NAb would increase the chance of drug clearance, formation of drug: ADA immunocomplexes, and neutralization of drug function, leading to a higher likelihood of impact on drug efficacy and patient safety.

Under most circumstances, assessment of all available data, including PK, PD and binding ADA, is still sufficient to understand the impact of immunogenicity on clinical response (e.g., high magnitude, persistent ADA response is more likely to clear the drug). There are some limited circumstances, however, where a stand-alone NAb assay may be scientifically necessary, and can actually help to interpret the data from the other bioanalytical assays.

In some cases measurement of "total" drug concentrations (drug bound or unbound to target or ADA/NAb) may indicate adequate systemic drug levels are present, but NAbs bound to drug may render the drug biologically inactive (47). If the measured drug concentration does not reflect the observed efficacy (either PD or other clinical readouts), use of a stand-alone NAb assay could explain the adequate PK but poor clinical response. Alternatively, the sponsor could develop a free or active drug concentration assay that would provide data on the level of active drug without the need for a NAb assay.

Multidomain Biotherapeutics

The more a mAb diverges from the native endogenous sequence or format, the higher the propensity for recognition by the immune system as foreign (48). Therefore, multidomain protein therapeutics, for example antibody–drug conjugates (ADCs), bi- or tri-specific T-cell engagers, may have an increased risk of inducing immunogenicity compared to standard mAb therapeutics. Multidomain antibodies likely have increased protein engineering, with differing Fv/Fc regions in the CDR, and may also have mutations in the Fc framework. All of these variations can enhance the risk of immunogenicity, and also the potential for neutralizing

antibodies. While the enhanced potential for NAbs do not necessarily reflect an increased safety risk, the impact of NAbs upon PK and efficacy can be important to monitor. Dependent upon the mechanism of action of the multimeric antibody, multiple NAb assays may be desired to elucidate neutralizing antibodies to each domain of the biotherapeutic. Alternatively, there may be a need to develop a NAb assay for only one domain, or to monitor one component of the mechanism of action. These decisions should be made in conjunction with a thorough risk assessment. As stated previously, these mechanism-based NAb assays rely on indirect measurements of drug function, typically based on inhibition of activity. The deployment of assays that directly measure active drug levels could provide the relevant information without the requirement of a NAb assay (49).

Protein Therapeutics with a Narrow Therapeutic Index

If a drug product has a narrow therapeutic index due to the potential for off-target toxicity (for example, ADCs or bispecifics that bind to a target expressed on both healthy and disease tissues), then the implementation of a NAb assessment to detect the presence and impact of neutralizing activity might be needed during early phases of drug development. If the concentration of the drug is limited due to possible toxicity, then the potential for a NAb to block the mechanism of action resulting in a lack of efficacy is greater. In this case, a NAb assay could help the sponsor understand whether the lack of efficacy is due to the NAb or a deficiency in the drug's mechanism of action.

It is important to restate that the examples above (e.g., where total PK doesn't detect neutralizing impact, products with a narrow therapeutic index) are only relevant where NAb is present at levels sufficient to impact the drug's biological effect but not at the magnitude where the drug is rapidly cleared. If NAb is at low levels there may be no impact on PK, PD or efficacy, and at high levels binding ADA and NAb present in the polyclonal response would likely clear the drug and be functionally neutralizing. Therefore, these examples are confined to a relatively limited set of circumstances and for most moderate-risk molecules the impact of immunogenicity on clinical response can be assessed without implementing a stand-alone NAb assay.

Biotherapeutics with Pre-existing ADA

Advanced therapy medicinal products (ATMPs) that utilize delivery vehicles such as viral vectors or lipid-based nanoparticles have the potential to enroll drug-naïve patients with pre-existing ADAs, some of which may be associated with previous exposure to particular epitopes within the ATMP (50). In some cases, the specificity and neutralizing potential of these ADA may need to be assessed. The elevated prevalence of pre-existing ADA in these therapeutics has prompted a shift in the standard protein therapeutics immunogenicity testing tiers. When pre-existing ADA is anticipated, samples may not need to be tested following the traditional three-tiered paradigm.

One example of this is gene therapy programs that use adeno-associated virus (AAV) as the delivery vector. AAV-based gene therapies may implement NAb assays as early as non-clinical biodistribution studies, where there may be a need to screen animals that are either negative or low titer for anti-AAV antibodies as some published non-clinical data suggest the presence of pre-existing antibodies may limit transduction resulting in reduced transgene expression (51). However, observed nonclinical immune responses do not necessarily translate to the clinic and clinical data demonstrating this is limited and ambiguous (52).

As with ADA to protein therapeutics, it is possible that a clear relationship between anti-AAV antibodies and (lack of) transgene expression (and efficacy) may only be observed in patients with elevated responses. Regardless, the need for an assay to assess humoral immunogenicity earlier may be an essential tool for understanding and interpreting non-clinical programs. These assays may be used to exclude animals from the study or to stratify animals (based on positive status or titer) across all arms of a non-clinical study to allow for better interpretation of the study and balance of anti-AAV levels in animals per dose level tested. It should also be noted although it is typical for animals to be screened for anti-AAV antibodies using a NAb assay, a binding or total antibody assay may provide very similar data (29). Historically, the neutralizing antibody assay was considered the preferred method, with the logic that only the pre-existing ADAs that actively blocked the mechanism of AAV uptake would be of relevance. However recent work comparing the pre-existing immunogenicity of AAV vectors have shown great concordance between the NAb and total antibody assay (27, 29, 53).

As more AAV-based therapies enter the clinic, NAbs to AAV become an increasing concern given that a high incidence rate also occurs in the human population, leading some AAV-gene therapy programs to exclude any individuals with anti-AAV antibodies (binding or neutralizing). Over time, as we learn more about the impact of pre-existing antibodies on safety and efficacy of gene therapy programs, the need for a NAb assay may become less of a priority as sponsors favor an operationally simpler total antibody (TAb) assessment given the high correlation between the two types of assays. The various factors to be considered in implementing a screening immunogenicity assay (NAb or TAb) and regulation required are outside of the scope of this manuscript (54).

High Immunogenicity Risk: Known Impact on Safety

Therapeutic Proteins with Endogenous Counterpart

Unlike low-risk mAbs, or even biologics with elevated levels of immunogenicity, if a therapeutic protein has an endogenous counterpart this presents an important immunogenicity risk factor that may require early development of a stand-alone NAb assay. NAb against a biotherapeutic product can have severe consequences if it cross-reacts to and inhibits the function of a nonredundant endogenous counterpart. Although these events have only been observed very rarely (3), it is important to include evaluation of the potential consequence of neutralization of an endogenous counterpart in the consideration of when and how to implement the most relevant neutralizing antibody assay.

Biotherapeutics where neutralization of the endogenous counterpart and/or other biotherapeutic treatment options is known to be critical, will call for implementation of a NAb assay from Phase 1 (Table I). Dependent upon the degree of difference between the biotherapeutic and the endogenous counterpart, two separate NAb assays may need to be developed; one that uses the drug product, and a second that uses a recombinant version of the endogenous counterpart. If a sample is detected as NAb positive toward the drug and cross-reactive toward the endogenous counterpart in the binding antibody assay, the sample is categorized as positive for NAb to the endogenous counterpart. In these cases, it is recommended to implement regular and/or on demand analysis of ADA including NAb during the treatment period to be able to detect early signs of neutralizing effects. Furthermore, suspicion of lack of treatment effect (e.g. based on clinical or lab parameters) can result in expedited ADA analysis to detect if this is caused by ADAs with neutralizing effects.

Knowledge about immunogenicity consequences of therapeutic proteins in the same class is very valuable when evaluating the potential consequence and risk of neutralization of an endogenous counterpart. For products that would theoretically be high-risk, there may be substantial clinical data for other molecules in that class indicating the risk is significantly lower.

For example, antibodies to insulin products are developed in a high number of subjects (40% in Type 2 Diabetes (T2D), 70% in Type 1 Diabetes (T1D) and it would be a significant risk for diabetic patients if these ADAs also neutralized the endogenous insulin and/or other insulins treatments, potentially leaving the patient with no treatment options. However, anti-insulin antibodies have been shown in several clinical trials to not affect the efficacy and safety of the insulin treatment. This has been demonstrated by lack of correlation of binding antibody incidence/titer to validated efficacy parameters such as HbA1c and blood sugar and safety and NAb assays have historically not been part of the development program for insulin drugs (55, 56).

The GLP-1 therapeutic proteins are another drug class where the presence of an endogenous counterpart has warranted measurement of NAb in a stand-alone NAb assay, while the clinical data have shown that the NAbs detected were not associated with an impact on efficacy or safety (57). Although it is likely an expectation from health authorities to test for NAb (against the therapeutic and the endogenous counterpart), for compounds such as GLP-1 therapeutics, it is important to assess whether the data from a stand-alone NAb assay provides value in addition to other clinical efficacy and safety data. If the NAb data is not adding to the interpretation of clinical response, it may be appropriate to consult with health authorities and potentially stop testing for NAbs.

Enzyme Replacement Therapy and Gene Therapy Transgene Products

Enzyme replacement therapies (ERT) and transgene products from gene therapies have some similarities in their immunogenicity risk profiles. In most cases these products are intended to mimic biological counterparts and as such typically have a higher risk profile. NAb to enzyme

Table I Decision Algorithm to Assess the Need for Implementation of a Stand-alone NAb Assay Based on the Immunogenicity Risk Assessment and Results From Other Clinical Tests

Immunogenicity Risk Category	ADA Assay	PK Assay	PD Marker	Stand-Alone NAb Needed?	Implementation Stage
Low	Yes	Free or total	N/A	No	N/A
Moderate	Yes	Free or total	Yes	No	N/A
Moderate	Yes	Free	No	No	N/A
Moderate	Yes	Total	No	Yes/No	Safety data driven. May be needed for later phase studies
High Safety Risk (Biological Mimic)	Yes	N/A	N/A	Yes	Phase 1

replacement therapies for Pompe's disease, Gaucher's disease, or factor VIII have impaired clinical efficacy and resulted in disease progression and complexity of treatment options (58–60). Because the loss of efficacy for ERT in these cases may be life-threatening, an appropriate dedicated NAb assay will likely be recommended.

ERTs are often administered and are active systemically, although some recent products have tissue targeting motifs (61, 62). In contrast, transgene products can have either systemic or intracellular localization. For systemic products such as FVII and FIX, NAb (inhibitor) assays are recommended in guidance documents (63). However, for cytosolic, nuclear or non-secreted transgene products, a neutralizing antibody assessment is likely not needed.

For some products there may be a distinction between neutralizing antibodies that can block the functional activity of the ERT or gene therapy product and those that block uptake into the target cell. Although the standard NAb assay will detect impact on functional activity, these responses may not be clinically relevant as functional blockade in serum may not reflect impact at the site of action or blockade of targeting to these tissues (61). Therefore, the format for these NAb assays are two-fold, one assay is specific to the mechanism of action of the enzyme, and are often enzymatic activity assays, designed to detect the inhibition of the enzymatic response (64, 65). The other format detects inhibition of the uptake of the ERT to the tissues, measuring the internalization of the therapeutic (66). Thus, an in-depth characterization of the nature of impactful NAb activity becomes an important aspect of the immunogenicity risk assessment. Similar to the situation with multi-domain therapeutics, sponsors should discuss with regulators their intention to evaluate the NAb responses that have an impact on drug efficacy.

If antibodies to the transgene product are detected in early phase clinical development, a strategy to detect neutralizing activity should be developed. This strategy can include evaluation of pharmacodynamic endpoints to detect changes potentially due to neutralizing activity. As described above, standard of care for treating hemophilia is to monitor PD (clotting), and this can serve as a surrogate for the development of inhibitors (NAbs). If there are no suitable PD assays available, a specific neutralizing antibody assay may need to be developed and used to test banked samples.

If it is necessary to develop a NAb assay for the transgene product, a mechanism of action approach to selecting assay format should be used (analogous to activity testing for inhibitors to FVIII or FIX ERT (31)). Implementation of this assay may not be needed at enrollment. However, in circumstances where pre-existing antibodies may be present, it could drive a greater urgency for a NAb assay to the transgene. For example, in cases where the patient may be on ERT and is transitioning to a gene therapy-based replacement, they may have pre-existing Abs that could impact efficacy of the gene therapy product. Under these circumstances, it is likely that the NAb assay for the replacement therapy will be able to serve the same purpose for measuring NAbs to the gene therapy transgene product.

Is NAb Necessary in Absence of a Pharmacodynamic Marker?

For low-risk molecules where ADA, PK and efficacy endpoints are available, a stand-alone NAb may not be necessary at any stage of clinical development. For moderate-risk molecules, the situation is more nuanced. In a patient that is ADA positive a surrogate assessment of clinical neutralization may be inferred from the PD or efficacy data. However, proximal PD assays are not always available or are not implemented at later stages of clinical development. For example, in oncology indications the primary PD assessment is often a receptor occupancy assay that is used in early dose escalation studies. Once the optimal dosing is determined, receptor occupancy is typically not assessed in later stage studies, and PD cannot be used to infer NAb. However, in many cases sufficient data to interpret neutralizing activity may still be obtained from ADA status, magnitude and peristence, drug concentration measurements and efficacy evaluations. NAb activity can also be inferred using data from both total PK and a free/active PK assays. When free drug concentrations are lower than total drug level (assuming no soluble target interference) then NAb is likely interfering in detection in the free drug assay. However, it's rare that both total and free PK assays are available for the same drug candidate.

Outlined in Table I. is an example of a decision algorithm to assess whether a stand-alone NAb assay is needed and when it should be implemented. Different scenarios are presented for the immunogenicity risk category, the availability of different assay types for drug concentration measurement (free/active or total PK) and the availability of a PD marker. Although every clinical study presents different challenges, some broad generalizations can be made. For example, low immunogenicity risk molecules likely do not need a NAb assessment at any stage of the program, while high risk molecules likely need a NAb assessment implemented at early phases. For the moderate immunogenicity risk molecules an understanding of the suite of assays that are available and their characteristics will inform the decision of whether or not to implement a stand-alone NAb assay.

Regulatory Considerations

Both the EMA and FDA immunogenicity guidance documents mention a requirement to perform NAb assessment (43, 67). However, the FDA allows that for cases where

there is a PD marker or appropriately designed PK assay (or both), it may be possible to use these assessments instead of a NAb assay. The EMA recommends providing strong justification when deviating from the requirement for NAb testing. Both health authorities recommend seeking regulatory advice before implementing an immunogenicity strategy without testing for NAb. However, as mentioned previously, Health Authority's stance may have evolved on the requirement for NAb assessments as more recent submissions have been accepted where NAb data was not included, indicating that regulators are also recognizing that alternate data sets are able to inform the presence and impact of neutralizing activity (5).

In general Health Authority guidances recommend the use of cell-based assays but also indicate that competitive ligand binding assays may be used for protein therapeutics with antagonistic mechanism of action. Details on the application of different assay formats are available elsewhere (16). However, numerous sponsors have indicated that when NAb data was included in a submission, competitive ligand binding assays were often acceptable regardless of the mechanism of action, especially for low-risk molecules (12, 68, 69).

If NAb testing is not going to be included in a submission, especially for higher risk molecules, it is highly recommended to have early scientific engagement with regulatory agencies to align on assessment strategy. Regulators want to know that the sponsor has thoroughly assessed the immunogenicity risk. Providing Health Authorities with a clear description of the risk assessment along with the immunogenicity testing strategy at the time of IND filing and at subsequent interactions during product development (e.g., end of Phase 2 meeting) can allow alignment on the planned strategy, which will be reinforced or revised as data emerge. The case of a moderate-risk therapeutic where a PD assessment is not available or not performed is a good example. For early studies there may not be a need for NAb, but as PK, ADA and efficacy data emerge, and especially as the safety profile matures, that strategy can be revised as needed.

Most Health Authorities have separate reviewers to assess the suitability of the ADA/NAb testing methods and the relationship of the data to any clinical response, including ADA study data. Therefore, it is critical for the sponsor to communicate to all the reviewers how the data from these different assessments (e.g., binding ADA, drug concentration and PD) are related, and, for example, how NAb activity can be assessed without a stand-alone assay. This should be discussed in the Integrated Summary of Immunogenicity where the relationship between the different clinical assessments in the clinical dossier can be explained. The sponsor should not assume that each reviewer will be familiar with the contents of all the modules in the submission, and the reviewer should not be expected to perform the integration of the datasets from different locations in the dossier.

Finally, for ATMPs NAb testing is not currently specified in Health Authority documents, with the exception of the FDA's gene therapies for hemophilia guidance where inhibitor testing is recommended (63). Of note, for currently approved ATMP therapies, sponsors have taken varied approaches to immunogenicity testing. The different approaches include: following the standard paradigm for protein therapeutics and testing for both binding ADA and NAb, testing for NAb (inhibitors) only, testing binding ADA only, or testing for neither (70–74). The lack of guidance for immunogenicity assessment for newer modalities represents an opportunity to implement a science-driven approach that prioritizes the generation of data that informs clinical impact rather than following the standard immunogenicity strategy for protein therapeutics where NAb testing is more routine.

Reporting Nab Activity With or Without a Stand-Alone Assay

In the EMA guideline for immunogenicity assessment, the Health Authority specifically requested that sponsors integrate general immunogenicity data with multiple sources of clinical data (67). Therefore, even when a stand-alone NAb assay is deployed, NAb results should be interpreted in context of all available data sets. With or without stand-alone NAb assay, a clear articulation of how neutralizing activity will be described is critical. As described above, even stand-alone NAb assays measure an indirect effect of neutralizing activity, whether measuring the downstream effect of the drug in a cell-based assay or the binding of drug to target in a CLB assay.

Stakeholder management is a critical aspect of cross-functional drug development, and it is important to note that many clinical teams may expect to have a stand-alone NAb assay. There may be a misconception that the interpretation of the impact of immunogenicity on clinical response requires data from the assay that specifically detects antibodies that "neutralize the biological effect of the drug". In other words, it may not be understood by the clinical teams that other assessments of clinical response (ADA, PK, PD etc.) may provide a suitable (or even superior) understanding NAb activity. To mitigate these concerns, establishing an internal data interpretation plan prior to initiation of sample testing is recommended.

As shown in Table II, in the case where the ADA assay has acceptable sensitivity and drug tolerance and a PK assay that detects 'free' or active drug (unbound to NAb or soluble target), integration of PK, ADA and clinical data can be performed to draw conclusions about the impact of neutralizing activity as follows:

Table II Example of Data Interpretation and Reporting without a Stand-alone NAb Assay

ADA Status (magnitude)	PK Impact	PD/Efficacy Impact	Clinical NAb Interpretation
Neg	No	No	Neg
Pos (Low)	No	No	Non-neutralizing
Pos (Mid)	Yes	No	Neutralizing - No clinical impact
Pos (High)	Yes	Yes	Neutralizing – Clinically impactful

- ADA(+) with no impact on exposure or clinical efficacy or PD change will be interpreted as non-neutralizing
- ADA(+) with reduced exposure, but no impact on clinical efficacy or PD change, will be interpreted as presence of neutralizing activity that is not clinically meaningful.
- ADA(+) with reduced exposure and reduction or loss of clinical efficacy or PD change will be interpreted as presence of clinically meaningful neutralizing activity

Similarly, integration is also necessary when a stand-alone NAb assay has been deployed as there are important challenges with interpreting NAb assay results. Historically, NAb assays, particularly cell-based assays, have had poor sensitivity and drug tolerance and therefore failed to detect some positive responses (14). More recently sample pretreatment strategies have dramatically improved assay performance characteristics (38–40). However, these approaches have introduced a different problem where very sensitive NAb assays detect responses that are not clinically relevant (21). Examples of data interpretation and reporting with a stand-alone NAb are outlined as follows and in Table III:

- A positive result in the NAb assay with evidence of clinical impact on exposure and clinical efficacy and safety will be interpreted as positive for neutralizing activity.
- A negative result in the NAb assay (but ADA positive) with no impact on PK/PD/efficacy will be interpreted as a lack of neutralizing activity
- A negative result in the NAb assay (but ADA positive) with observed impact on PK/PD/efficacy is more complicated and requires further examination.

  A high magnitude, persistent ADA response can generally be considered positive for neutralizing activity (e.g., due to accelerated clearance from binding ADA - functionally neutralizing).

  A low/moderate magnitude ADA response requires additional investigation in consultation with clinicians, clinical pharmacologists and bioanalytical scientists. For example, a drug which achieves therapeutic effect at relatively low concentrations (or a 15 participant with lower exposure due to high body weight) may be impacted by low/moderate magnitude ADA either through increased clearance (functionally neutralizing) or NAb that may not be detected due to poor NAb assay sensitivity. Alternatively, even in ADA positive participants, a range of other covariates can impact clinical readouts in response to therapy (baseline disease characteristics, body weight etc) and these should be assessed as a potential cause of impact on PK, PD or efficacy.

Table III Example of data interpretation and reporting with a stand-alone NAb assay

NAb Assay Result[1]	Impact on Clinical Response (Efficacy, PK, PD)	Clinical NAb Interpretation
Positive	Yes	Neutralizing—Clinically impactful
Positive	No	Neutralizing—No clinical impact
Negative	No	Non-neutralizing
Negative	Yes	High ADA: Neutralizing—Clinically impactful Low/Moderate ADA: Additional investigation required[2]

[1]*Participants are ADA positive;*[2]*See text above*

An integrated approach to assessment of neutralizing ADA activity should be deployed throughout clinical development, with additional assays being introduced and deployed (or discontinued) based upon emergent data and learnings that indicate which data sets are most informative. Setting a strategy early, critically evaluating the strategy in an iterative fashion as clinical development progresses and course correcting as needed will enable a more robust understanding of the presence and impact of neutralizing ADAs throughout the course of the development cycle. Ultimately, the decision of whether or when to implement a stand-alone NAb assay will be based upon its immunogenic risk level, the disease indication, and whether the stand-alone assay will provide meaningful insight into neutralizing activity of these responses (75, 76).

Discussion and Conclusion

The major focus of this manuscript is to clearly distinguish between the assessment of clinical neutralizing activity and the implementation of a traditional stand-alone NAb assay. The assessment of neutralizing activity does not necessarily require a stand-alone assay, and the implementation of an assay does not guarantee a superior assessment of the

neutralization of clinical response. In support of this perspective, the EMA guideline on immunogenicity assessment of therapeutic proteins states: "The goal of immunogenicity studies is to investigate presence of an immune response to the therapeutic protein and its clinical impact. Thus, the evaluation of immunogenicity should be based on integrated analysis of immunological, pharmacokinetic, pharmacodynamic, as well as clinical efficacy and safety data" (67).

The critical question of whether and when to implement a stand-alone NAb assay in a drug development program depends first on the relative immunogenic safety risk of the biotherapeutic, second on the availability and quality of other relevant assessments, such as ADA, PK and PD, and thirdly on the phase of the clinical trial. In most cases, other types of study data inform efficacy as effectively (or more so) than data from a NAb assay, and generally NAb assay data is most useful for safety evaluation. Importantly, most protein therapeutics do not induce ADA that impacts safety, and therefore in many cases NAb data may not be needed nor add value to the overall study interpretation.

As discussed, low-risk molecules can leverage other bioanalytical readouts such as pharmacokinetic and pharmacodynamic assessments to determine neutralizing activity. This approach is applicable to a range of therapeutics, including oligonucleotide and standard monoclonal antibodies, utilizing combined datasets, where PK, ADA, and PD assays results collectively serve as an indicator of neutralizing antibody activity. Stand-alone neutralizing antibody assays have limitations, including key parameters such as drug tolerance and sensitivity, which can impact the interpretability of the data on clinical impact. For moderate-risk biotherapeutics, such as those with pre-existing immunogenicity or multimeric structures, if a stand-alone Nab is needed implementation can often be deferred until later phases of the clinical program. As these moderate-risk biotherapeutics progress to late phase studies where dosing is continued for an extended period, the eventual impact of NAb may only become evident over time. In these cases, implementation of a stand-alone NAb assay may provide an earlier readout of NAbs whose clinical impact won't be observable in PD or efficacy data until later in the study.

High-risk molecules, including biotherapeutics with non-redundant endogenous counterparts, such as enzyme replacement therapies and some transgene proteins expressed from genetic medicines, often warrant development of a stand-alone NAb assay for clinical studies, sometimes as early as Phase I. Notably, the experience with epoetin, where numerous patients developed pure red-cell aplasia due to neutralizing antibodies, underscored the important of early immunogenicity risk assessment and detection strategy throughout the pre-clinical and clinical phases of drug development (3). Importantly, in the intervening decades since the epoetin cases were first observed, ADA assays have evolved significantly, becoming more sensitive and able to detect responses that inform immunogenicity-related adverse events during early clinical phases of research. In addition, *in vitro* methods of predicting potential immunogenicity have been developed, helping to select drug candidates with reduced immunogenic risk (77). It is essential to monitor all the clinical readouts and adapting the immunogenicity strategy according to the emerging data.

Therefore, the decision to deploy a stand-alone NAb assay and/or utilize data from other informative assays that indicate NAb impact, should take into consideration the particular therapeutic molecule, its MOA, the suite of assays available to interrogate its physiological impact, and the quality of those assays (Table I). This assessment can be included in the immunogenicity risk assessment which is conducted for each therapeutic molecule. The assay or assays that can best assess NAb impact should be deployed. This holistic approach takes into account the full spectrum of available data and has become more widely accepted. A clear plan of how different datasets will be collectively interpreted to report neutralizing activity should be described. This approach focuses on scientifically robust methods to understand the impact of neutralizing activity irrespective of whether a dedicated stand-alone NAb assay implemented.

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Declarations

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References

1. Rosenberg AS. Immunogenicity of biological therapeutics: a hierarchy of concerns. Dev Biol (Basel). 2003;112:15–21.
2. Schellekens H, Casadevall N. Immunogenicity of recombinant human proteins: causes and consequences. J Neurol. 2004;251(Suppl 2):II4-9.
3. Casadevall N, Nataf J, Viron B, Kolta A, Kiladjian JJ, Martin-Dupont P, et al. Pure red-cell aplasia and antierythropoietin antibodies in patients treated with recombinant erythropoietin. N Engl J Med. 2002;346(7):469–75.
4. Pollock C, Johnson DW, Horl WH, Rossert J, Casadevall N, Schellekens H, et al. Pure red cell aplasia induced by erythropoiesis-stimulating agents. Clin J Am Soc Nephrol. 2008;3(1):193–9.
5. Goodman J, Cowan KJ, Golob M, Nelson R, Baltrukonis D, Bloem K, et al. Re-thinking the current paradigm for clinical immunogenicity assessment: an update from the discussion in the European Bioanalysis Forum. Bioanalysis. 2024;16(17–18):905–13.
6. Kubiak RJ, Zhang L, Zhang J, Zhu Y, Lee N, Weichold FF, et al. Correlation of screening and confirmatory results in tiered immunogenicity testing by solution-phase bridging assays. J Pharm Biomed Anal. 2013;74:235–45.
7. Lai CH, Chen M, Fraser S, Wang J, McAfee S, Speaks E, et al. Challenging the Standard Immunogenicity Assessment Approach: 1-Tiered ADA Testing Strategy in Clinical Trials. AAPS J. 2024;27(1):11.
8. Starcevic Manning M, Hassanein M, Partridge MA, Jawa V, Mora J, Ryman J, et al. Comparison of Titer and Signal to Noise (S/N) for Determination of Anti-drug Antibody Magnitude Using Clinical Data from an Industry Consortium. AAPS J. 2022;24(4):81.
9. Grudzinska-Goebel J, Benstein K, Bloem K, Cowan KJ, Gorovits B, Jadhav M, et al. Immunogenicity risk assessment for tailored mitigation and monitoring of biotherapeutics during development: recommendations from the European Immunogenicity Platform. Front Immunol. 2025;16:1581153.
10. Chan JL, Koda J, Heilig JS, Cochran EK, Gorden P, Oral EA, et al. Immunogenicity associated with metreleptin treatment in patients with obesity or lipodystrophy. Clin Endocrinol (Oxf). 2016;85(1):137–49.
11. Ding X, Xue L, Wang M, Zhu S, Zhu K, Jiang S, et al. Dynamics and implications of anti-drug antibodies against adalimumab using ultra-sensitive and highly drug-tolerant assays. Front Immunol. 2024;15:1429544.
12. Kamal MA, Kosloski MP, Lai CH, Partridge MA, Rajadhyaksha M, Kanamaluru V, et al. Immunogenicity of dupilumab in adult and pediatric patients with atopic dermatitis. Front Immunol. 2024;15:1466372.
13. Wu Y, Akhgar A, Li JJ, Yu B, Chen C, Lee N, et al. Selection of a Ligand-Binding Neutralizing Antibody Assay for Benralizumab: Comparison with an Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC) Cell-Based Assay. AAPS J. 2018;20(3):49.
14. Finco D, Baltrukonis D, Clements-Egan A, Delaria K, Gunn GR 3rd, Lowe J, et al. Comparison of competitive ligand-binding assay and bioassay formats for the measurement of neutralizing antibodies to protein therapeutics. J Pharm Biomed Anal. 2011;54(2):351–8.
15. Gupta S, Devanarayan V, Finco D, Gunn GR 3rd, Kirshner S, Richards S, et al. Recommendations for the validation of cell-based assays used for the detection of neutralizing antibody immune responses elicited against biological therapeutics. J Pharm Biomed Anal. 2011;55(5):878–88.
16. Wu B, Chung S, Jiang XR, McNally J, Pedras-Vasconcelos J, Pillutla R, et al. Strategies to Determine Assay Format for the Assessment of Neutralizing Antibody Responses to Biotherapeutics. AAPS J. 2016;18(6):1335–50.
17. Schutz K, Hughes RG, Parker A, Quinti I, Thon V, Cavaliere M, et al. Kinetics of IgM and IgA antibody response to 23-valent pneumococcal polysaccharide vaccination in healthy subjects. J Clin Immunol. 2013;33(1):288–96.
18. Barger TE, Wrona D, Goletz TJ, Mytych DT. A detailed examination of the antibody prevalence and characteristics of anti-ESA antibodies. Nephrol Dial Transplant. 2012;27(10):3892–9.
19. Hofbauer CJ, Whelan SF, Hirschler M, Allacher P, Horling FM, Lawo JP, et al. Affinity of FVIII-specific antibodies reveals major differences between neutralizing and nonneutralizing antibodies in humans. Blood. 2015;125(7):1180–8.
20. Montalvao SA, Tucunduva AC, Siqueira LH, Sambo AL, Medina SS, Ozelo MC. A longitudinal evaluation of anti-FVIII antibodies demonstrated IgG4 subclass is mainly correlated with high-titre inhibitor in haemophilia A patients. Haemophilia. 2015;21(5):686–92.
21. Partridge MA, Karayusuf EK, Shyu G, Georgaros C, Torri A, Sumner G. Drug Removal Strategies in Competitive Ligand Binding Neutralizing Antibody (NAb) Assays: Highly Drug-Tolerant Methods and Interpreting Immunogenicity Data. AAPS J. 2020;22(5):112.
22. van Schouwenburg PA, Krieckaert CL, Nurmohamed M, Hart M, Rispens T, Aarden L, et al. IgG4 production against adalimumab during long term treatment of RA patients. J Clin Immunol. 2012;32(5):1000–6.
23. Wu B, Sternheim N, Agarwal P, Suchomel J, Vadhavkar S, Bruno R, et al. Evaluation of atezolizumab immunogenicity: clinical pharmacology (part 1). Clin Transl Sci. 2022;15(1):130–40.
24. Hassanein M, Partridge MA, Shao W, Torri A. Assessment of clinically relevant immunogenicity for mAbs; are we over reporting ADA? Bioanalysis. 2020;12(18):1325–36.
25. Opolka-Hoffmann E, Jordan G, Otteneder M, Kieferle R, Lechmann M, Winter G, et al. The impact of immunogenicity on therapeutic antibody pharmacokinetics: A preclinical evaluation of the effect of immune complex formation and antibody effector function on clearance. MAbs. 2021;13(1):1995929.
26. Wang YM, Wang J, Hon YY, Zhou L, Fang L, Ahn HY. Evaluating and reporting the immunogenicity impacts for biological products–a clinical pharmacology perspective. AAPS J. 2016;18(2):395–403.
27. Dai Y, Dong H, Gleason C, Mora J, Kolaitis G, Balasubramanian N, et al. Comparison of Pre-existing Anti-AAV8 Total Antibody Screening and Confirmatory Assays with a Cell-Based Neutralizing Assay in Normal Human Serum. AAPS J. 2023;25(3):35.
28. Falese L, Sandza K, Yates B, Triffault S, Gangar S, Long B, et al. Strategy to detect pre-existing immunity to AAV gene therapy. Gene Ther. 2017;24(12):768–78.
29. Gardner MR, Mendes DE, Muniz CP, Martinez-Navio JM, Fuchs SP, Gao G, et al. High concordance of ELISA and neutralization assays allows for the detection of antibodies to individual AAV serotypes. Mol Ther Methods Clin Dev. 2022;24:199–206.
30. Bartelds GM, Krieckaert CL, Nurmohamed MT, van Schouwenburg PA, Lems WF, Twisk JW, et al. Development of antidrug antibodies against adalimumab and association with disease activity and treatment failure during long-term follow-up. JAMA. 2011;305(14):1460–8.
31. Miller CH. Laboratory testing for factor VIII and IX inhibitors in haemophilia: A review. Haemophilia. 2018;24(2):186–97.
32. Xu W, Maas B, Roadcap B, Swarup A, Steinmetz T, Luo L, et al. Neutralization Activity of Anti-drug Antibodies Against a Biotherapeutic Can Be Predicted from a Comprehensive Pharmacokinetics, Pharmacodynamics, and Anti-drug Antibody Data Analysis. AAPS J. 2022;24(6):102.

33. Wei X, Swanson SJ, Gupta S. Development and validation of a cell-based bioassay for the detection of neutralizing antibodies against recombinant human erythropoietin in clinical studies. J Immunol Methods. 2004;293(1–2):115–26.
34. Wu Y, Li JJ, Kim HJ, Liu X, Liu W, Akhgar A, et al. A Neutralizing Antibody Assay Based on a Reporter of Antibody-Dependent Cell-Mediated Cytotoxicity. AAPS J. 2015;17(6):1417–26.
35. Xu W, Bano N, Guzman-Valdes O, Amberman J, Bandlamudi E, Khanna P, et al. Development and validation of a cell-based binding neutralizing antibody assay for an antibody-drug conjugate. AAPS J. 2024;26(3):37.
36. Xu W, Cummings J, Sank M, Juhel M, Li X, Gleason C, et al. Development and validation of a functional cell-based neutralizing antibody assay for ipilimumab. Bioanalysis. 2018;10(16):1273–87.
37. Hammer C, Ruppel J, Kamen L, Hunkapiller J, Mellman I, Quarmby V. Allelic variation in HLA-DRB1 is associated with development of antidrug antibodies in cancer patients treated with atezolizumab that are neutralizing *in vitro*. Clin Transl Sci. 2022;15(6):1393–9.
38. Rathi A, Rinker S, Niu H, Carter C, Kumar S, Cowan K. Assay development considerations to improve drug tolerance in direct competitive ligand binding neutralizing antibody assays, pretreatment strategies. J Immunol Methods. 2023;517: 113484.
39. Wickramarachchi D, Wagner J, Woo T, Ferrari F, Steinmetz T, Helmy R, et al. A novel neutralization antibody assay method to overcome drug interference with better compatibility with acid-sensitive neutralizing antibodies. AAPS J. 2023;25(1):18.
40. Wu B, Schnarr M, Devlin JL, Brown S, Yang TY. Approaches to improve drug tolerance and target tolerance in the assessment of neutralizing anti-drug antibodies. Bioanalysis. 2019;11(22):2061–74.
41. Mora JR, Richards SM. The AAPS Journal Theme Issue: Compendium of Immunogenicity Risk Assessments: an Industry Guidance Built on Experience and Published Work. AAPS J. 2023;25(3):43.
42. Cohen S, Chung S. In vitro immunogenicity prediction: bridging between innate and adaptive immunity. Bioanalysis. 2021;13(13):1071–81.
43. FDA. Guidance for Industry: Immunogenicity Testing of Therapeutic Protein Products —Developing and Validating Assays for Anti-Drug Antibody Detection. 2019.
44. Gupta S, Richards S, Amaravadi L, Piccoli S, Desilva B, Pillutla R, et al. 2017 White Paper on recent issues in bioanalysis: a global perspective on immunogenicity guidelines & biomarker assay performance (Part 3 - LBA: immunogenicity, biomarkers and PK assays). Bioanalysis. 2017;9(24):1967–96.
45. Stebbins CC, Petrillo M, Stevenson LF. Immunogenicity for antisense oligonucleotides: a risk-based assessment. Bioanalysis. 2019;11(21):1913–6.
46. Henry SP, Arfvidsson C, Arrington J, Canadi J, Crowe D, Gupta S, et al. Assessment of the immunogenicity potential for oligonucleotide-based drugs. Nucleic Acid Ther. 2022;32(5):369–77.
47. Wang SJ, Wu ST, Gokemeijer J, Fura A, Krishna M, Morin P, et al. Attribution of the discrepancy between ELISA and LC-MS/MS assay results of a PEGylated scaffold protein in post-dose monkey plasma samples due to the presence of anti-drug antibodies. Anal Bioanal Chem. 2012;402(3):1229–39.
48. Zhou Y, Penny HL, Kroenke MA, Bautista B, Hainline K, Chea LS, et al. Immunogenicity assessment of bispecific antibody-based immunotherapy in oncology. J Immunother Cancer. 2022.
49. Kroenke MA, Milton MN, Kumar S, Bame E, White JT. Immunogenicity Risk Assessment for Multi-specific Therapeutics. AAPS J. 2021;23(6):115.
50. Gorovits B, Clements-Egan A, Birchler M, Liang M, Myler H, Peng K, et al. Pre-existing Antibody: Biotherapeutic Modality-Based Review. AAPS J. 2016;18(2):311–20.
51. Long BR, Sandza K, Holcomb J, Crockett L, Hayes GM, Arens J, et al. The Impact of Pre-existing Immunity on the Non-clinical Pharmacodynamics of AAV5-Based Gene Therapy. Mol Ther Methods Clin Dev. 2019;13:440–52.
52. Manno CS, Pierce GF, Arruda VR, Glader B, Ragni M, Rasko JJ, et al. Successful transduction of liver in hemophilia by AAV-factor IX and limitations imposed by the host immune response. Nat Med. 2006;12(3):342–7.
53. Butala-Flores E, Nguyen T, Selvan N, Armstrong L, Miller M, Kamen L, et al. Validation of Anti-Adeno Associated Virus Serotype rh10 (AAVrh.10) Total and Neutralizing Antibody Immunogenicity Assays. Pharm Res. 2023;40(10):2383–97.
54. Gorovits B, Azadeh M, Buchlis G, Harrison T, Havert M, Jawa V, et al. Evaluation of the Humoral Response to Adeno-Associated Virus-Based Gene Therapy Modalities Using Total Antibody Assays. AAPS J. 2021;23(6):108.
55. FDA. Guidance for Industry: Clinical Immunogenicity Considerations for Biosimilar and Interchangeable Insulin Products. 2019.
56. Fineberg SE, Kawabata TT, Finco-Kent D, Fountaine RJ, Finch GL, Krasner AS. Immunological responses to exogenous insulin. Endocr Rev. 2007;28(6):625–52.
57. Buse JB, Garber A, Rosenstock J, Schmidt WE, Brett JH, Videbaek N, et al. Liraglutide treatment is associated with a low frequency and magnitude of antibody formation with no apparent impact on glycemic response or increased frequency of adverse events: results from the Liraglutide Effect and Action in Diabetes (LEAD) trials. J Clin Endocrinol Metab. 2011;96(6):1695–702.
58. Banugaria SG, Prater SN, Patel TT, Dearmey SM, Milleson C, Sheets KB, et al. Algorithm for the early diagnosis and treatment of patients with cross reactive immunologic material-negative classic infantile pompe disease: a step towards improving the efficacy of ERT. PLoS One. 2013;8(6): e67052.
59. Eckhardt CL, Loomans JI, van Velzen AS, Peters M, Mauser-Bunschoten EP, Schwaab R, et al. Inhibitor development and mortality in non-severe hemophilia A. J Thromb Haemost. 2015;13(7):1217–25.
60. Richards SM, Olson TA, McPherson JM. Antibody response in patients with Gaucher disease after repeated infusion with macrophage-targeted glucocerebrosidase. Blood. 1993;82(5):1402–9.
61. Diaz-Manera J, Kishnani PS, Kushlaf H, Ladha S, Mozaffar T, Straub V, et al. Safety and efficacy of avalglucosidase alfa versus alglucosidase alfa in patients with late-onset Pompe disease (COMET): a phase 3, randomised, multicentre trial. Lancet Neurol. 2021;20(12):1012–26.
62. Yamamoto Y, Sanwald Ducray P, Bjornsson M, Smart K, Grimsey P, Vatakuti S, et al. Development of a population pharmacokinetic model to characterize the pharmacokinetics of intrathecally administered tominersen in cerebrospinal fluid and plasma. CPT Pharmacometrics Syst Pharmacol. 2023;12(9):1213–26.
63. FDA. Guidance for Industry: Human Gene Therapy for Hemophilia. 2020.
64. de Vries JM, Kuperus E, Hoogeveen-Westerveld M, Kroos MA, Wens SC, Stok M, et al. Pompe disease in adulthood: effects of antibody formation on enzyme replacement therapy. Genet Med. 2017;19(1):90–7.
65. Rombach SM, Aerts JM, Poorthuis BJ, Groener JE, Donker-Koopman W, Hendriks E, et al. Long-term effect of antibodies against infused alpha-galactosidase A in Fabry disease on plasma and urinary (lyso)Gb3 reduction and treatment outcome. PLoS ONE. 2012;7(10): e47805.
66. Wang J, Lozier J, Johnson G, Kirshner S, Verthelyi D, Pariser A, et al. Neutralizing antibodies to therapeutic enzymes:

considerations for testing, prevention and treatment. Nat Biotechnol. 2008;26(8):901–8.
67. EMA. Guideline on Immunogenicity assessment of therapeutic proteins. 2017.
68. Valentine JL, Dengler A, Zhao A, Truong T, McAfee S, Hassanein M, et al. Immunogenicity of cemiplimab: low incidence of antidrug antibodies and cut-point suitability across tumor types. J Clin Pharmacol. 2024;64(1):125–36.
69. Corsaro B, Yang TY, Murphy R, Sonderegger I, Exley A, Bertholet S, et al. 2020 White Paper on Recent Issues in Bioanalysis: Vaccine Assay Validation, qPCR Assay Validation, QC for CAR-T Flow Cytometry, NAb Assay Harmonization and ELISpot Validation (Part 3 - Recommendations on Immunogenicity Assay Strategies, NAb Assays, Biosimilars and FDA/EMA Immunogenicity Guidance/Guideline, Gene & Cell Therapy and Vaccine Assays). Bioanalysis. 2021;13(6):415–63.
70. Spark Therapeutics, Inc., Luxturna (voretigene neparvovec-rzyl) [package insert]. 2017.
71. Kite Pharma Inc., Yescarta (axicabtagene ciloleucel) [package insert]. 2017.
72. CSL Behring LLC, Henegenix (etranacogene dezaparvovec-drlb) [package insert]. 2022.
73. Bano N, Ehlinger C, Yang TY, Swanson M, Allen S. Considerations in the immunogenicity assessment strategy for Oligonucleotide therapeutics (ONTs). AAPS J. 2022;24(5): 93.
74. BioMarin Pharmaceutical Inc., Roctavian (valoctocogene roxaparvovec-rvox) [package insert]. 2023.
75. Hagman C, Chasseigne G, Nelson R, Anlauff F, Kagan M, Goldfine AB, et al. Immunogenicity assessment strategy for a chemically modified therapeutic protein in clinical development. Front Immunol. 2024;15:1438251.
76. Koren E, Smith HW, Shores E, Shankar G, Finco-Kent D, Rup B, et al. Recommendations on risk-based strategies for detection and characterization of antibodies against biotechnology products. J Immunol Methods. 2008;333(1–2):1–9.
77. Carter PJ, Quarmby V. Immunogenicity risk assessment and mitigation for engineered antibody and protein therapeutics. Nat Rev Drug Discov. 2024;23(12):898–913.