From the bench to clinical practice: understanding the challenges and uncertainties in immunogenicity testing for biopharmaceuticals

G. R. Gunn III,* D. C. F. Sealey,† F. Jamali,‡ B. Meibohm,§ S. Ghosh¶ and G. Shankar*

*Bioanalytical Sciences, Janssen R&D, LLC (Johnson & Johnson), Spring House, PA, USA, †Regulatory Affairs, Janssen Inc., Toronto, ON, Canada, ‡Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, AB, Canada, §College of Pharmacy, University of Tennessee Health Science Center, Memphis, TN, USA, and ¶Department of Medicine, University of Calgary and Alberta Health Services, Calgary, AB, Canada

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Correspondence: Gopi Shankar, Bioanalytical Sciences, Janssen R&D, LLC (Johnson & Johnson), Spring House, PA, USA.
E-mail: Gshanka3@its.jnj.com

Summary

Unlike conventional chemical drugs where immunogenicity typically does not occur, the development of anti-drug antibodies following treatment with biologics has led to concerns about their impact on clinical safety and efficacy. Hence the elucidation of the immunogenicity of biologics is required for drug approval by health regulatory authorities worldwide. Published ADA ‘incidence’ rates can vary greatly between same-class products and different patient populations. Such differences are due to disparate bioanalytical methods and interpretation approaches, as well as a plethora of product-specific and patient-specific factors that are not fully understood. Therefore, the incidence of ADA and their association with clinical consequences cannot be generalized across products. In this context, the intent of this review article is to discuss the complex nature of ADA and key nuances of the methodologies used for immunogenicity assessments, and to dispel some fallacies and myths.

Keywords: anti-drug, antibodies, biologic, biomedicine, immunogenicity

Introduction

Biopharmaceuticals (also called biological drugs or biologics) are prescribed increasingly for the treatment of autoimmune, haematological, oncological, neurological, metabolic and other diseases. These drugs are primarily proteins (monoclonal antibodies, cytokines, growth factors/hormones, enzymes and fusion proteins) and peptides that are specific to certain targets known to modulate disease mechanisms. Although most biologics offer a favourable benefit–risk profile in a population of patients overall, particularly for the treatment of severe and chronic indications where conventional (chemical, ‘small molecule’) drugs are often inadequate, one key hurdle to the maintenance of clinical response with biologicals has been the development of immunogenicity.

Immunogenicity occurs when the immune system perceives ‘foreignness/non-self’ [1] or ‘danger signals/stressed self’ [2] in the biological product, and launches specific immune responses against it. The development of a drug-specific immune response is assessed by detecting the presence of anti-drug antibodies (ADA) that bind specifically to the biological and neutralize it, eliminate it from the body, or both. Most biologicals are immunogenic, and the incidence of ADA can reach more than 90% [3–6]. While ADA have often been found to be clinically benign, a subset of ADA-positive patients can experience adverse impacts on safety and efficacy. Importantly, the incidence of ADA can vary greatly between same-class products and different patient populations, thus hindering predictions of immunogenicity and necessitating clinical testing. Such differences may reflect disparate bioanalytical methods and interpretation approaches [7,8], as well as a plethora of product-specific and patient-specific factors that are not fully understood. Further compounding this issue is a lack of standardization in the terminology and approaches used for the collection, analysis and presentation of immunogenicity results, and only recently has an attempt been made to achieve global harmonization [11]. In the early years of treating patients with foreign proteins, such as purified preparations of bovine or porcine insulin, the development of ADAs was common and expected [12]. Subsequently, when murine monoclonal antibodies (mAbs) were used in the clinic, human anti-mouse antibodies (HAMA) were expected and usually observed [13]. Currently, despite the fact that most biotechnology-derived therapeutics are derived from recombinant human protein libraries, immunogenicity continues to occur confirming that multiple factors other than ‘non-humanness’ influence immunogenicity.
Unlike conventional (chemical, ‘small molecule’) drugs where immunogenicity typically does not occur, ADA development due to treatment with biologicals has led to concerns about their impact on safety and efficacy. The clinical consequences of ADA can range from clinically asymptomatic to limited therapeutic efficacy of the drug, to more devastating conditions such as anaphylaxis and specific antibody-mediated immunodeficiency diseases [14–19]. Thus, with the widespread use of biologicals, therapeutic drug monitoring, including ADA testing, is becoming a reality in clinical settings [20,21]. Contrary to the monitoring approaches for small molecule drugs, which are limited to the measurement of the drug in reference specimens, both the drug and ADA are often assessed for biologicals. This difference in approaches also applies to the drug development process, whereby elucidation of the immunogenicity of biologicals is required for drug approval by health regulatory authorities worldwide.

The published ADA ‘incidence rate’ for any biological is dependent upon the comparative strengths and caveats of the laboratory method(s) used to detect, confirm and characterize ADA. Therefore, the incidence of ADA and their association with clinical consequences cannot be generalized across products. In this context, the intent of this review paper is to discuss the complex nature of ADA and key nuances of the methodologies used for immunogenicity assessments, and to dispel some fallacies and myths. When choosing treatments for their patients, physicians can consider these clinical and pharmacological factors rather than only the incidence of ADA reported in drug labels or other publications.

The nature of ADA complicates their measurement

Whereas the biological substance itself is a unique molecule manufactured by a defined process and comprises a single protein analyte for therapeutic drug monitoring (or pharmacokinetic) methods, ADA represent a very complex set of analytes. Note that the word ‘analytes’, not ‘analyte’, was used for ADA, as they are usually polyclonal, may include different isotypes [immunoglobulin (Ig)G, IgA, IgM or IgE], bind to different regions (‘domains’) of the drug molecule, vary in affinity (binding strength) and can differ between patients. Laboratory methods for the assessment of ADA, therefore, should be: (a) able to selectively detect antibodies of all isotypes that may have formed against the numerous epitopes (antigenic sites that antibodies can bind) on the drug molecule and (b) sufficiently sensitive for detecting low- and high-affinity antibodies alike.

ADA test methods are not quantitative (neither definitive nor relative quantitative) [22]; the use of calibrator ADA (animal- or human-derived) to quantify ADA is inappropriate, because a standard preparation of ADA that represents ADA in all subjects cannot be established [23]. ADA methods should be performed in a qualitative format, because the lack of similarity (parallelism) between a sample and the calibrator, or between different samples, can lead to inaccurate analytical results when determined by the use of a calibrator (interpolation of ADA concentration values from the standard curve) [24]. Instead, titre is recommended for an estimate of ADA concentration [23,25], whereby a fourfold change in titre values between samples can be considered a real difference in ADA levels [11]. Nevertheless, some commercial laboratories apply ADA calibration curves using proprietary reagents in their laboratory-developed tests and risk reporting biased and inaccurate ADA ‘concentration’ results. Because such tests are not regulated by the Food and Drug Administration (FDA) nor the European Medicines Agency (EMA), physicians are advised to gain an understanding of the assay format and its caveats before utilizing test results in clinical practice. A cross-validation of commercial assays to the drug maker’s original methods may provide some assurance and a reference for interpretation of results in light of the original method results.

Neutralizing and non-neutralizing antibodies are both clinically relevant

Neutralizing antibodies (NABs) are a subset of binding ADA that bind to the drug and inhibit its pharmacological function by preventing target binding [11]. Accordingly, non-neutralizing antibodies (non-NAB) are ADA that bind to sites on the drug molecule that do not affect target binding and thereby do not impact the drug’s pharmacodynamic activity. Non-NABs are often referred to as ‘binding antibodies’, but that is an incorrect term because all ADA (including NAB) are inherently binding antibodies (because they bind drug). Once the screening test method detects ADA (‘binding’ ADA), it is useful to determine their neutralizing ability, particularly for drugs with short half-lives (minutes to a few days) or those with an identical, endogenous counterpart.

NABs can inhibit drug activity soon after the drug is administered, but non-NAB do not inhibit the pharmacodynamic activity of the drug; however, the latter can lower the drug’s systemic exposure just as well by increasing the rate of drug clearance, resulting in a clinically similar outcome to that of Nabs – reduced clinical efficacy. Both types of ADA can form immune complexes upon binding drug, which are cleared by the reticuloendothelial system leading to complete elimination within days [26]. In the case of biologicals with long half-lives (e.g. human mAb drugs), it is a fallacy that only NABs are clinically relevant; both NABs and non-NABs are likely to have similar effects on efficacy due to the relatively rapid clearance of antigen–antibody complexes [27]. For biologicals with relatively short half-lives, NABs can impede efficacy even while they circulate within immune complexes before they are cleared. Additionally, rare drug-sustaining ADA have been reported.
that are non-neutralizing but extend the biological half-life of the drug significantly, up to the half-life of human IgG. By delaying clearance of an active agonist therapeutic, such drug-sustaining ADA can generate clinical effects substantially different from those elicited by ADA that neutralize or accelerate clearance of a biological.

Every ADA laboratory method is unique

Several laboratory assay platforms have been used for ADA detection. Up to the mid-1990s immunogenicity methods used radioimmunoassay (RIA) technology. This approach was adequate for ADA detection, but time-consuming and harmful if the associated radioactive chemicals were mishandled. Thereafter, the development of safer and higher-throughput immunoassay technologies with similar or better sensitivity (lower limit of detection) led to a preference for the use of enzyme-linked immunosorbent assay (ELISA) and electrochemiluminescence immunoassay (ECLIA) methods. Other technologies, such as surface plasmon resonance (SPR) and bio-layer interferometry (BLI), have also been used. SPR and BLI excel in the detection of lower-affinity ADA; however, they typically offer several-fold lower sensitivity (31,32) (thus prone to under-reporting the incidence of immunogenicity) and lower-throughput compared to ELISA and ECLIA methods. High-pressure liquid chromatography (HPLC)-based methods are also lower-throughput, and are used rarely (33,34).

Immunoassay approaches typically employ either a ‘direct’ or ‘bridging’ immunoassay format. In a direct immunoassay, the drug molecule is adhered either primarily to a plate’s plastic surface (Fig. 1a) or secondarily to a streptavidin-coated plastic surface via a biotin linker (Fig. 1b). Thus anchored, the drug molecule can capture ADA when exposed to a test sample. The captured ADA are then detected by binding to a ‘detector molecule’ – an enzyme-
conjugated antibody that reacts specifically with human antibodies – to form a molecular link complex. To improve the specificity of signal detection, the immunoassay plates are washed between the assay steps with a chemical buffer that eliminates non-specific, sticky antibodies. In the end, the addition of a chemical substrate (appropriate for the enzyme) leads to the production of a coloured, fluorescent or luminescent signal proportional to the amount of ADA in the test sample. Similarly, a bridging immunoassay wherein enzyme-conjugated drug is used as the detector molecule also leads to the formation of a molecular complex involving plastic surface-coated drug (Fig. 1c) or drug coated via a biotin-linker (Fig. 1d). Bridging assay formats may be designed as homogeneous methods, wherein capture and detection reagents are combined with sample matrix simultaneously, eliminating wash steps to improve detection of lower-affinity ADA or those with rapid off-rates. Alternatively, bridging assay methods may incorporate wash steps between capture and detection steps to improve assay specificity and reduce background signals (heterogeneous format). Selection of a homogeneous or heterogeneous method format is largely dependent upon the desired assay and reagent performance characteristics. The bridging assay format relies upon the identical dual binding sites of antibodies to generate a molecular bridge between the capture and detection drug molecules. In direct and bridging immunoassay formats, test samples with signals greater than or equal to a defined method cut-point are classified as ‘ADA-positive’. The ADA concentration is then estimated by titre as assessed by testing serial dilutions of the ADA-positive sample and determining the highest dilution of the sample that still produces a positive signal in the assay. Direct immunoassays which utilize an isotype-specific enzyme-conjugated detector antibody can have limited usefulness, because not all the circulating human antibody isotypes (IgG, IgM, IgA, IgE) can be detected together. Typically, such methods are designed to detect IgG alone, whereas other isotypes in peripheral blood – IgM and IgE – can also have significance for anti-drug immunogenicity. Because early or intermittent immune responses, such as those against polyethylene glycol (PEG) domains of certain drugs or carbohydrate chains of glycoprotein drugs, are often comprised of IgM or IgE, it is vital to investigate these types of ADA. Although direct immunoassays [35] and ImmunoCAP-based methods [36–38] have been used specifically for detection of IgE, the bridging immunoassay format is generally preferred for the detection of ADA, as it offers excellent assay sensitivity and can detect antibodies of all isotypes because the drug molecule is used both for ADA capture and detection.

Higher-affinity ADA that bind more tightly in immunoassays than lower-affinity ADAs are less susceptible to inadvertent release from the molecular complex during wash steps. In fact, the greater the number of wash steps the more antibodies can be washed away before detection. Thus, ELISA and ECLIA platforms usually provide adequate sensitivity for the detection of higher affinity ADA, but not for lower-affinity ADA. Because ELISA platforms typically involve more washing cycles, they are generally more prone to this limitation than ECLIA platforms [23].

Irrespective of the technology used, a general limitation to method development is that human ADA are not usually available during method development (or not feasible to use due to the limited quantity available). It is, therefore, necessary that surrogate ‘positive’ controls be substituted, either generated or procured, to approximate the characteristics of human ADA and assure consistent method performance. To ensure detection of ADA across a range of affinities that are likely to be encountered in a patient population, one might expect immunoassays to be developed with monoclonal ADA ‘positive control’ reagents representing at least a high and a low affinity. However, positive controls are used uncommonly, due to the practical challenges associated with the procurement of such reagents. Instead, an animal anti-serum or a polyclonal ADA preparation purified therefrom, usually of very high affinity due to adjuvant-based hyperimmunization, is usually applied as a positive control reagent. Alternatively, a drug-reactive mouse or rat monoclonal antibody is used. Individual monoclonal or polyclonal reagents often have unique binding characteristics due to the incredible diversity inherent in the generation and maturation of antibodies. While these unique characteristics (e.g. epitope, binding affinity, antibody isotype, pH tolerance) of reagent antibodies (‘surrogate’ ADAs) enable sensitive and robust method development and validation, they may be suboptimal for detecting clinically meaningful ADA. Because human proteins are foreign antigens in other species, animal-derived ADA generally bind to different epitopes on the same antigen than human antibodies. ADA method sensitivity, specificity and susceptibility to interference from matrix components, including excess drug or excess target, are all directly dependent upon the reagent antibody or antibodies used to develop and validate the methods. In fact, if a panel of antibodies is used to assess the sensitivity of an individual method, the sensitivity of each antibody is different even using the same method. Additionally, secondary reagents used in the method are conjugated typically with binding agents (such as biotin or streptavidin), enzymes (such as alkaline phosphatase or horseradish peroxidase), fluorescent dyes (such as Alexa Fluor) or a chemiluminescent tracer [such as Ruthenium (II) tris-bipyridine-(4-methylsulphone)], whose conjugation ratios often differ between laboratories. Not only do such caveats imply that a well-developed laboratory method, suitable for detecting a positive control antibody, can under-report clinically relevant ADA, but also underscore that the results of one ADA method cannot be compared to the results of another.
Fig. 2. Binding anti-drug antibodies (ADA) can be classified as neutralizing or non-neutralizing by evaluating impact on pharmacological drug function. (a) Drug ‘D’ and target ‘T’ interact at a specific site on the drug called the ‘active site’. (b) In the presence of binding, non-neutralizing ADA (Y-shaped grey symbol), drug may be bound at sites that do not interfere with the pharmacological function of the drug, but binding NAbs (red, Y-shaped symbol) engage drug in a manner that inhibits the pharmacological activity of drug.

When different laboratories develop ADA methods for the same biological using disparate, often proprietary, positive controls and reagents, their respective test results must not be considered equivalent without extensive cross-validation.

In most immunogenicity assessment schemes the detection of binding ADA within a sample is followed typically by an additional test for drug neutralization. In this in-vitro test method the sample is evaluated for its ability to diminish the drug’s pharmacological activity, or potency (Fig. 2). ADA-positive samples that impact drug potency adversely are inferred to contain NAbs. Conversely, ADA-positive samples that do not reduce drug potency are inferred to contain non-NAbs. For the evaluation of neutralizing activity, two types of methods are used typically: bioassays (i.e. cell culture-based test methods) or competitive ligand binding immunoassays. Bioassays are frequently preferred for the detection of NAbs against biologicals that pose a higher risk of ADA-mediated clinical consequences [7]. Such methods generally use a cell line that produces a measurable response (e.g. proliferation, generation of signalling molecules or activation of a reporter gene) when exposed to the drug. As shown in Fig. 3 a, b, when binding, non-neutralizing ADA interact with the biological drug function is retained, and the cellular response to drug is detectable. In contrast, NAbs will block target binding and inhibit the cellular response partially or fully. Competitive ligand-binding immunoassays are prone to interference from matrix components including excess drug or excess target.

Given the polyclonal nature of ADA in a test sample, only a subset of ADA may be able to neutralize the drug’s activity; yet when a binding ADA-positive sample is found to produce neutralization activity in a NAb assay, the sample is deemed NAb-positive. The NAb-positive sample may contain NAbs alone or in a mixture with non-NAbs, but currently there is no way to distinguish whether all the ADA are neutralizing. However, this all-or-none inference serves the purpose of identifying patients who develop NAbs adequately. It is important to realize that if the NAb method is substantially less sensitive than the associated binding ADA method, samples with low ADA titres are likely to be classified as non-NAbs (false-negative) in the NAb assay method – an inaccurate outcome. The classification of binding ADA as NAbs or non-NAbs is thus dependent upon the level of ADA present in the test sample and the relative sensitivities of the binding ADA and NAb methods.

Interference by drug and target in ADA detection methods impacts the accuracy of results

Immunoassays and bioassays are prone to interference from drug within test samples. In a drug-bound state binding ADA and NAB can go undetected leading to false-negative results, or underestimation of titre. Therefore, the
reported immunogenicity of most biologicals approved before ca. 2010 was probably underestimated.

In recent years it has been recognized that to overcome drug interference it is necessary to disrupt existing drug-ADA immune complexes in order to shift the equilibrium in favour of binding immunoassay reagents. This molecular disruption is accomplished by including an acid pretreatment step in ADA and NAb immunoassays (Fig. 4). Such ‘drug-tolerant’ ADA detection methods are developed to reduce the pH to a range in which ADA–drug complexes dissociate but ADA are not denatured. The acidified sample is exposed to capture and/or detection reagents to enable ADA binding and detection. By dissociating the ADA–drug complexes through pH manipulation, equilibrium is shifted in the presence of assay capture and detection reagents, and ADA previously bound and undetectable without acid dissociation become readily identifiable. Through this approach, most drug-tolerant ADA detection methods can measure ADA in samples containing drug in excess of concentrations commonly expected in test samples. Thus, guidance documents from regulatory health authorities recommend that ADA detection methods are developed with intent to address drug interference [25,39]. Additionally, health authorities have required many drug companies to replace older ADA detection methods with newer, drug-tolerant methods. The application of a new drug-tolerant method (which may also have improved sensitivity compared to an original method due to
technological advancements) to retest clinical trial samples (cross-validation) can result in the identification of previously undetected ADA-positive samples, thereby increasing the reported rate of immunogenicity. It is critical that the differences in detection between the older and improved methods be evaluated by cross-validation with historical clinical samples. If the improved method (e.g. with increased sensitivity or an ability to detect weaker responses masked previously by drug) was applied only on a new clinical trial, an unexpectedly high immunogenicity may be interpreted incorrectly as the drug having increased immunogenicity.

Apart from interference by drug, another interferent has been identified more recently as a major problem for ADA detection methods – the drug’s soluble target. Antagonistic biologicals (e.g. mAbs) that bind small protein targets (such as cytokines) usually retain greater quantities of ‘bound target’ compared to the active, unbound target [40,41]. The target, which is in a bound state after the drug is administered to the patient, typically did not cause assay interference in older ADA detection methods where acid dissociation was not involved (i.e. where drug interference had been a problem). However, the ‘new and improved’ drug-tolerant ADA detection methods can be prone to target interference because the acidification step disrupts not only the drug–ADA complex but also the drug–target complex, potentially releasing supraphysiological levels of drug target. This can lead to false-positive or -negative results depending on whether the target is monomeric with a single epitope or comprised of two or more identical subunits (homodimers, multimers or drug molecules bearing multiple identical epitopes) [42]. As shown in Fig. 5, multi-subunit drug targets can form molecular bridges in the assay method, resulting in a false-positive assay result. Similarly, monomeric targets can bind in the assay leading to a false-negative result. Thus, the acid dissociation solution to the original problem of drug interference led to a new problem of target interference, which frequently goes undetected and unremediated. A recent publication demonstrated the removal of target from test samples to mitigate this phenomenon and improve accuracy. As described in that paper [43], the interference by nerve growth factor (NGF) was recognized only after healthy volunteers and osteoarthritis subjects treated with fulranumab (an anti-NGF antagonist mAb) exhibited vastly different ADA incidence results using an acid dissociation-based method (3%
in healthy volunteers versus 72% in osteoarthritis patients). Dimeric NGF, which had accumulated to supraphysiological levels in a drug-bound state in osteoarthritis patients, but obviously not in healthy volunteers, had produced false-positive ADA results. By incorporating an additional target capture step to mitigate NGF interference, treated patients were found to have a similar ADA incidence to that of healthy volunteers [43]. Thus, drug and target interferences, if unaddressed, can result in inaccurate immunogenicity results and inaccurate interpretations of the impact of ADA on drug safety and efficacy.

**Comparisons of ADA methods or immunogenicity results of different biological products is inappropriate**

Although it may be tempting to compare the incidence of immunogenicity and characteristics of two biologicals, these comparisons can be misleading. As cautioned earlier, methodological differences make cross-method comparisons of immunogenicity data inappropriate. Additionally, wide-ranging semantics and variable ADA sampling strategies, analytical and reporting approaches have muddled public information. Different laboratory methods may have applied different statistical approaches to assay cut-point calculations (based on 1.65, 2 or 3 standard deviations; note that 1.65 is better, as the others pose a higher risk of false-negative results), or one may have used a titre-based approach while another used a quantitative calibrator curve-based approach. Last, but not least, clinical trials of two same-class biologicals may have employed different ADA sampling schedules, one with appropriately chosen time-points for the detection of ADA and the other without. In the absence of suitably designed time-points, studies are likely to under-report the incidence of ADA. There is no regulatory standard for ADA sampling time-points, although recent publications have provided some guidance on risk-based approaches [7,8]. The use of a risk-based approach can result in wide variation in the frequency and timing of immunogenicity assessments. Sampling time-points should be frequent enough to provide an understanding of ADA response kinetics. For example, taking samples within only 2–3 weeks after biological administration is often inadequate for a robust IgG-based immune response to develop, and hence for the ADA to be detected, whereas a few samples taken at equally spaced points up to 3 months after administration is usually adequate. For biologicals with higher immunogenicity risk, sampling may need to continue through a follow-up period after the study (which may be longer for a drug that is administered on a chronic basis) to ensure that clinical sequelae of immunogenicity are captured adequately. Hence, even if two methods were cross-validated and deemed equivalent, differences between study designs and sampling schemes can produce different results that could be mistaken as true differences in immunogenicity.

Other differences are worth pointing out. ADA responses may be termed ‘transient’ or ‘persistent’ but, historically, these terms have not been defined consistently. Recently, a White Paper was published by experts across industry and health authorities that recommended harmonized approaches for assessing and reporting clinical immunogenicity to biologicals [11]. It was recommended that the words ‘transient’ and ‘persistent’ be avoided, and instead the duration of ADA be described in simple statistical terms: median and interquartile ranges. Similarly, titres should be described with median titre and interquartile ranges rather than the arbitrary descriptors ‘high’, ‘medium’ or ‘low’ [11].

A higher reported incidence of ADA, therefore, should not be taken automatically as an indication that one drug has an inferior immunogenicity profile than another drug. Other considerations, such as the reported incidence of potential clinical manifestations of ADA such as infusion or hypersensitivity reactions, or loss of efficacy over time, can also guide the selection of a drug treatment. It is important to keep in mind that many biologicals, despite relatively high ADA incidence rates, have proved to be safe and effective.

**Conclusion**

All biologicals are capable of eliciting immune responses in treated subjects, leading to the development of ADA. A single, universal ADA detection method cannot exist, and neither does any such NAb method. These methods are unique for each drug and test matrix (serum, plasma, etc.) and often utilize a variety of methodological approaches and technology platforms, each having inherent strengths and caveats. Thus, although it can be tempting to compare the reported incidences of immunogenicity or neutralization among different drugs, it is inappropriate and can be very misleading. Method sensitivities and tolerances to various interfering substances are unique for each method and define the limits whereby individual methods can detect ADA in test samples given the study design and sampling time-points. Additionally, the reporting and presentation of immunogenicity results has been historically inconsistent, even for biologicals in the same class. Therefore, it is critical to understand analytical methods and study details in order to comprehend accurately the impact of immunogenicity on safety and efficacy during the treatment of patients with biologicals.

**Disclosure**

The authors have no competing interests or disclosures to declare.
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